Integration strategies of cortical neurons in the early postnatal neocortex

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by

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Abstract

Over the course of the first three postnatal weeks the cells of the neocortex undergo considerable maturation. My aim was to track some of the changes that occur, to create a detailed electrophysiological study of the postnatal neocortex. This would allow me to better understand the developmental journeys taken by discrete neuronal populations within the maturing network. To this end, I used glutamate uncaging and whole-cell patch-clamp electrophysiology to record from neocortical pyramidal cells and Nkx2-1 derived interneurons located in different cortical layers and regions of the cortex between postnatal days (P) 5-21. In so doing I was able to track the maturation of their intrinsic electrophysiology and synaptic connectivity.

The electrophysiological properties of cortical neurons develop markedly over the course of development and there is a gradual emergence of distinct electrophysiological firing types amongst both pyramidal cells and interneurons. In terms of their synaptic inputs pyramidal neurons and interneurons possess unique methods of synaptic integration. Pyramidal neurons show a stereotyped pattern of input maturation, whereby there is a gradual emergence of the mature, canonical pattern of input. This is predicted by translaminar, NMDA receptor mediated inputs at early ages, that likely act as the substrate for the formation of mature AMPA receptor containing synapses. In contrast Nkx2-1 derived interneurons showed no shift in laminar input organization over the course of development, with prominent inputs observed from the earliest ages of the study. However, there seems to be a significant degree of heterogeneity in the organization of these inputs and I provide some insight into this variation. Taken together these findings provide a picture of the connectivity of the early network and using these data I am able to produce a final hypothesis, which shows how interneurons and pyramidal cells may interact to produce the canonical pattern of cortical connectivity.
Statement of originality

All the work reported in this thesis is my own with the following exceptions:

The figures in chapter 1 are taken from a number of reviews on themes relevant to this thesis. All figures are used with permission from the publishers and the papers from which they have been taken are cited in the respective figure legends. The permission documents are enclosed in the appendix.
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## List of abbreviations

**A-** anterior

**A1-** primary auditory cortex

**ACSF-** artificial cerebrospinal fluid

**AHP-** action potential afterhyperpolarization

**AHP amp.-** peak amplitude of action potential afterhyperpolarization

**AHP delay-** delay to peak of action potential afterhyperpolarization

**AMPA-** 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid

**AMPA R-** AMPA receptor

**ANOVA-** analysis of variance

**ANR-** anterior neural ridge

**AP-** action potential

**Avrg. ISI-** average interspike interval

**BMPs-** bone morphogenic proteins

**CaMKII-** calcium calmodulin dependant protein kinase type II

**cENOs-** cortical early network oscillations

**CFuPNs-** corticofugal projection neurons

**cGDPs-** cortical giant depolarizing potentials

**CGE-** caudal ganglionic eminence

**CH-** cortical hem

**ChR2-** channel rhodopsin

**CNS-** central nervous system

**CP-** cortical plate

**CPNs-** cortical projection neurons

**CR-** calretinin

**ddH2O-** double distilled water

**depol >200ms-** number of depolarizations lasting longer than 200ms

**DIV-** days *in vitro*

**d (NFS/FS) -** delayed (NFS/FS)

**E-** embryonic day

**E\textsubscript{GABA}-** GABA reversal potential

**EGFP-** enhanced green fluorescent protein

**EPSC-** excitatory postsynaptic current

**Final Freq.-** maximum frequency in last 100ms of current pulse

**F/M-** frontal/motor cortex

**Fgfs-** fibroblast growth factors

**FS-** fast spiking

**FSstut-** stuttering FS

**GABA-** gamma-aminobutyric acid

**HDC-** high divalent cation

**IB-** intrinsic bursting

**ID-** initial double

**iIB-** initial intrinsic bursting

**Initial Freq.-** maximum frequency in first 100ms of current pulse

**ISI-** inter spike interval

**IZ-** intermediate zone

**L-** lateral

**LGE-** lateral ganglionic eminence
LSPS- laser scanning photostimulation
LTD- long-term depression
LTP- long-term potentiation
LTS- low threshold spiking
LY- Lucifer yellow
M- medial
Max. ISI- maximum interspike interval
Max. Freq.- maximum firing frequency
Mctx- motor cortex
MGE- medial ganglionic eminence
MNI- Methyl 1-[5-(4-amino-4-carboxybutanoyl)]-7-nitroindoline-5-acetate hydrate
MZ- marginal zone
Narp- neuronal activity–regulated pentraxin
Ncx- neocortex
NFS- non-fast spiking
NGS- normal goat serum
NMDA- N-Methyl-D-aspartic acid
NMDAR- NMDA receptor
p- posterior
P- postnatal day
PBS- phosphate buffered saline
PBS-T- PBS with 1% tween-20
PSC- postsynaptic current
PKA- protein kinase A
POA- preoptic area
PP- preplate
PSD- postsynaptic density
PV- parvalbumin
rIB- rebound intrinsic bursting
R_{in}- input resistance
RMP- resting membrane potential
RS- regular spiking
S1- primary somatosensory cortex
SEM- standard error of the mean
Shh- sonic hedgehog;
SP- subplate
SSC- somatosensory cortex
SST- somatostatin
STDP- spike time dependant plasticity
Sub T’hold >200ms- subthreshold depolarizations lasting longer than 200ms
SVZ- subventricular zone
TH- thalamus
UV- ultraviolet
V1- primary visual cortex
VIP- vasoactive intestinal peptide
VZ- ventricular zone
WT- wild type
0Mg- zero magnesium
Chapter 1- General introduction

1.1 Understanding Sherrington’s enchanted loom

One of the critical problems facing complex, multi-cellular life forms is to process proprioceptive information and coordinate the actions of disparately located body parts. In order for these actions to be of maximal benefit it is also essential to combine this with sensory information from the exterior and the intrinsic state of the organism, in order to produce sensory modulatable behaviour. The role of the brain in integrating inputs derived from individual sensory modalities led the English Nobel laureate Sir Charles Sherrington to describe it as an “enchanted loom” weaving together these multiple strands of information (Sherrington, 1942). However the brain does not simply function in the here and now, it carries within it memories of past events that allow us to learn and respond to the world in an experience dependant manner. This duality of function within the brain is an essential component of our individual personality, and was so beautifully captured in the verse of Emily Dickinson, who wrote:

“The Brain is wider than the sky
For put them side by side
The one the universe will contain
With ease and you beside.”

The area of the brain that encodes our conscious perception of sensory information and stores our memories is the cerebral cortex. Understanding the function of this interconnected mass of cells is one of the central questions of modern neuroscience. An important part of the role of the cortex is to compare novel information with stored information, particularly as we learn over the course of development. In fact, the protracted period of both cortical development and human childhood, and the associated increases in cortical complexity and the ability to learn, are thought to be important factors in the success of our species (Bogin, 1997; Rakic, 2009). Central to the question of how the brain stores information as we mature are the relative roles of intrinsic genetic mechanisms (nature) and externally derived sensory experience (nurture). The processing of sensory information occurs within different regions of the
cortex known to possess unique functional architectures. Although these cortical domains are specified during early embryogenesis (O’Leary & Sahara, 2008), their development and ultimate function is dependant upon sensory inputs at later ages (Espinosa & Stryker, 2012).

The purpose of this thesis is to shed light on the emergence of mammalian cortical networks. This study will encompass a period of development where sensory information is beginning to be received and stored by the nascent cortex. As such it will be possible to track changes in the properties of the network that occur as this process takes place. To perform these studies I will use a number of molecular genetic, electrophysiological, pharmacological, and optical tools to dissect cortical connectivity throughout postnatal development and track the developmental trajectories of discrete neuronal subtypes, specified by unique genetic programs, as they integrate into the nascent network. This thesis will also provide information on the mature structure of the cortical circuit, which is essential if ultimately we are to understand its functional role in the processes that underlie behaviour.

1.2 Cortical structure

‘A man without a cortex is almost a vegetable, speechless, sightless [and] senseless’

(Hubel & Wiesel, 1979)

The cerebral cortex of the brain can be broadly subdivided into two discrete structures. These are the neocortex, or new cortex, which is structurally and functionally distinct from the archicortex, or hippocampus (O’Keefe & Dostrovsky, 1971; Paxinos et al., 2007; Vann & Albasser, 2011). Despite this there are many similarities in the properties of cells located within these two regions. Throughout this thesis cortical shall be used to broadly define both regions, whilst neocortex and hippocampus shall be used when describing features that are specific to one or other structure. Discussion of the structure of the cortex shall be primarily limited to the neocortex, due to the distinct architectures of these two regions. However molecular data, primarily in later sections on synapse formation, shall be provided from both structures.
The neocortex makes up over three-quarters of the mass of the human brain (Azevedo et al., 2009). It is therefore easy to see why the Nobel laureates David Hubel and Torsten Wiesel felt that it was of such importance to human cognition. Despite the huge energetic demand placed on the bearer of an enlarged neocortex (Mink et al., 1981), it has drastically increased in size throughout human evolution with a surface area 1000 times that of a mouse and 10 times that of a macaque monkey (Clowry et al., 2010). This enlargement is thought to be a key factor in the enhanced mental abilities of Homo sapiens and therefore understanding the nature of the neocortical network is of immense significance. The complexity of the cortex is vast, with each of its roughly 10-20 billion constituent neurons possessing roughly a thousand individual synapses (Kerchner & Nicoll, 2008). This interconnectivity facilitates the integration of information as it is transmitted throughout the brain and as such it is important to study the individual components of this network, and how they are connected to each other, in order to better dissect their roles in cortical function.

1.2.1 Cellular components of the neocortex

The components of the cortex can be separated into two classes of cells, known as neurons and glia, which are present in a roughly 1:1 relationship in the human brain (although within the cortex itself this ratio is slightly elevated with around 1.5 times as many glia, Mountcastle, 1997; Azevedo et al., 2009). Neurons act as the conduits for the electrical impulses that characterise the nervous system and glia are traditionally thought of as being “support cells”, acting to maintain an optimal environment for neurons to function (see however Kang et al., 1998; Gourine et al., 2010 for examples of astrocytic signalling influencing neuronal activity). The neuronal component of the cortex can be broadly divided into excitatory neurons, which signal via the release of the neurotransmitter glutamate, and inhibitory interneurons that release gamma-aminobutyric acid (GABA). Excitatory pyramidal neurons are the major neuronal type in cortical networks, with inhibitory interneurons interspersed amongst them in a roughly 4:1 ratio (Micheva & Beialeau, 1995; Rudy et al., 2011). Pyramidal neurons are so named because their morphology displays a stereotypical long apical dendrite with fairly dense basal dendrites that all together resembles a pyramid-like structure. Whilst fairly physiologically homogeneous, pyramidal neurons show diversity in their efferent projections, with cells that display
similar intrinsic properties projecting to different locations in the brain (Alcamo et al., 2008; Britanova et al., 2008). The morphology and intrinsic physiology of GABAergic interneurons is far more diverse than that of pyramidal neurons (Markram et al., 2004; Ascoli et al., 2008). They are primarily thought of as local circuit neurons, with the vast majority of their projections maintained within the adjacent network. However there is some evidence for long-range interneuron connectivity, particularly in the hippocampus (Sik et al., 1994; Ascoli et al., 2008). The role of interneurons within the cortex is in phasing and organizing neighbouring ensembles of pyramidal cells, and as such the diversity of this population likely underlies their numerous computational roles (Cobb et al., 1995; Moore et al., 2010). Thus it would appear that locally projecting interneurons act to organize the local network activity of pyramidal cells, whose axons provide long-range connections for information transfer throughout the central nervous system (CNS).

1.2.2 Layers, columns and the canonical circuit

The laminar organization of the neocortex was initially proposed by an 18th century neuroanatomist called Francesco Gennari (1782), who observed a white line in sections of human cortex (now known as Gennari’s line). Further work using microscopes confirmed this idea (Baillarger, 1840), however it was not until Santiago Ramon y Cajal applied Camillo Golgi’s staining technique that it was possible to observe the heterogeneous neuronal composition of the individual cortical layers (fig. 1.1 A). Cajal’s studies showed that the mammalian neocortex could be subdivided into six broadly defined layers, based upon their underlying cytoarchitecture. Individual layers can be discerned based upon distinct patterns of cell density and the segregation of morphologically distinct neuronal subtypes; for example stellate cells in layer 4 and large subcortical projection neurons in deep layers (Koester & O’Leary, 1992; Staiger et al., 2004; Brenhouse & Andersen, 2011). There is however some degree of variation in the laminar organization of the neocortex, with layers found to be absent (e.g. layer 4 from primary motor cortex, Shepherd, 2009), or displaying increased complexity causing them to be further subdivided- for example the common distinction between layer 5a and 5b in most cortical regions and the subdivisions of layer 4 in the primary visual cortex of higher mammals (Gilbert, 1983; Shepherd, 2009; Anderson et al., 2010).
Figure 1.1

Figure 1.1 Laminar organization of the neocortex and the canonical circuit. A drawing of Nissl (left) and Golgi stained (right) neurons by Santiago Ramon y Cajal showing laminar variation in the cytoarchitecture of the neocortex. B organization of the canonical cortical circuit, green arrows represent primary canonical connections, red arrows represent intra-cortical connectivity. Panel A adapted from Brenhouse & Andersen, 2011. Panel B taken from Lubke & Feldmeyer, 2007. Roman numerals indicate layers 1-6 with layer 5 subdivided into 5a and 5b in panel B, TH=thalamus.

The main neuronal population throughout the cortical layers is pyramidal neurons. They can be subdivided into one of three main types based upon their axonal projections: 1 cortical projection neurons (CPNs) whose axons travel across the corpus callosum to the opposing hemisphere of the brain. 2 corticofugal projection neurons (CFuPNs) that project subcortically to regions such as the thalamus and to the spinal cord and 3 local circuit neurons that target other neurons located within the same cortical hemisphere (Fame et al., 2011). These cells display subtype dependent lamination patterns, with CFuPNs primarily inhabiting deep layers (V & VI), whilst CPNs are principally located in layer II/III, with another significant population in layer V (around 20% of total, Fame et al., 2011). The outputs of the neocortex are primarily mediated by pyramidal neurons because, as we have seen, the axonal
projections of interneurons are mostly restricted to the local cortical domain (section 1.2.1; Ascoli et al., 2008). Therefore, the deep layers of the cortex can be seen as the primary subcortical output layer, whilst layer 2/3 primary mediates intracortical connectivity. Layer 4 functions as the primary input layer of the neocortex and, as we shall see, this input dictates the form of sensory information to which that region of the cortex responds.

In addition to layers, the neocortex can also be segregated into individual processing units known as columns. Columns are radial clusters of neurons within the neocortex whose constituent neurons possess similar receptive fields (Mountcastle et al., 1957, Mountcastle, 1957). Evidence for the column as the basic computational unit of the neocortex stems from observations of evolutionary biology, which show that as brains expand in size, and therefore computational power, there is an expansion in the number of columns rather than an increase in overall columnar size (Rakic, 1995). One of the most studied columnar circuits is that of the rodent barrel cortex, where individual cortical columns (termed barrels) form a somatotopic map of the mystacial vibrissae (Woolsey & Van der Loos, 1970). In the barrel cortex each barrel receives thalamic inputs from an individual vibrissae, or whisker (Woolsey & Van der Loos, 1970; Fox et al., 2003). The whisker input is transmitted, via the thalamus, to layer 4, which forms the primary thalamorecipient layer and as such is the main target for subcortical sensory inputs to the neocortex (Chmielowska et al., 1989; Bruno & Sakmann, 2006).

Once sensory input has reached the cortex it is transmitted between neurons located in the individual layers before exiting to other cortical and subcortical regions. A number of studies have revealed that there is a stereotyped pattern of interlaminar connectivity within columns of the neocortex. These connections are also biased towards one direction, such that there is a feed-forward flow of information between the individual cortical layers (Reyes & Sakmann, 1999; Shepherd & Svoboda, 2005; Lubke & Feldmeyer, 2007; Thomson & Lamy, 2007). In contrast local intralaminar connectivity has a much higher instance of reciprocal connection (Markram et al., 1997; Feldmeyer et al., 1999; Atzori et al., 2001). The pattern of feed-forward, interlaminar connectivity has been found to occur across different cortical regions; giving rise to the concept of a canonical cortical circuit whereby information flows
into the cortex, through the various layers in a sequential fashion, before exiting to other parts of the brain (fig. 1.1 B; Gilbert & Wiesel, 1983; Douglas & Martin, 1991, 2004). The canonical circuit begins in layer 4, which functions as the primary input layer of the cortex. From layer 4 it is transmitted to layer 2/3, which can be thought of as an intracortical layer; either targeting local neurons within the column, or those located in the opposing hemisphere via the corpus callosum. From layer 2/3 it passes to deep layer neurons, which are the primary output layer of the cortex and target other regions of the CNS. An overview of the primary connections that make up the canonical circuit can be seen in figure 1.1 (B).

1.2.3 Cortical arealization

In addition to whiskers, the rest of the body can also be mapped to a corresponding region of the cortex. Wilder Penfield performed one of the classical studies that revealed this highly reproducible pattern of sensory and motor organization (Penfield, 1959). During neurosurgery, when the cortex of the brain was exposed, he would provide stimulation to different regions of the cortex and record any effects. This revealed that there was a strong correlation between the region of the brain that was stimulated and the corresponding body part where the response occurred (Penfield & Jasper, 1954). The somatotopic map of the body can be seen represented in the sensory homunculus in figure 1.2. The compartmentalization of the brain can also be observed using numerous histological (Brodman, 1909; Economo & Koskinas, 1925), genetic (O’Leary & Sahara, 2008), or functional assays (Penfield, 1959; Kwong et al., 1992; Tank et al., 1992), which allow us to distinguish between distinct regions of the cortex.

This parcellation of the brain into separate modules allows it to perform multiple functions in parallel, as well as being computationally optimal, by reducing the requirement for excessive long-range wiring to connect cells involved in similar neural representations (Mitchison, 1991). The organization of discrete cortical areas, in addition to many of the cortical structures described in this section, can be best understood by looking at the developmental processes that underlie their formation.
Figure 1.2

The sensory homunculus. Map of the cortex showing the localization of cortical representations of sensory information derived from individual body parts. The labeled regions of the cortex correspond to the body parts that preferentially project to that region of the brain. Figure taken from Ramachandran & Altschuler, 2009.

The focused patterns of thalamic input to different regions of the cortex not only dictates the sensory modality that is processed by that region of the cortex, but has been shown to influence the functional architecture of individual brain regions (Roe et al., 1990; O’Leary et al., 1994). Furthermore, the functional segregation of different cell types, their organization into discrete layers and the formation of columns all occur due to ongoing developmental processes as the brain matures.

1.3 The development of cortical structure

Over the past twenty years two competing points of view have attempted to explain the emergence of the observed variation between cortical regions; these are the protomap and protocortex theories devised by Pasko Rakic and David O’Leary respectively (Rakic, 1988; O’Leary, 1989). The protomap hypothesis states that progenitor cells of the cortex are aware of their three dimensional location in the
embryonic brain through their relative proximity to various patterning centres, which guide the arealization of the nascent telencephalon (Rakic, 1988). Thus before they begin to integrate into the nascent cortical network, neurons are already programmed towards being a part of the visual, somatosensory, auditory or motor cortices. The protocortex hypothesis proposes the contrary, that the cortex is produced as a *tabula rasa*, or blank slate, which is then programmed via extrinsic factors such as thalamic input (O’Leary, 1989). It would appear that there is some degree of merit to both of these theories, however the large body of evidence shows that there is an early specification of cortical location prior to the arrival of sensory input.

1.3.1 Morphogenic patterning of the telencephalon

Morphogens are important molecules that are involved in the genetic patterning of the developing embryo, including the telencephalon. Morphogens function in a concentration dependant fashion to drive downstream gene expression. During early cortical development morphogenic gradients occur along rostral caudal, medial lateral and dorsal ventral axes (fig. 1.3). This spatial organization is due to the secretion and diffusion of morphogens away from the main cortical patterning centres, which are each distinguished by their expression of distinct classes of morphogens: bone morphogenetic proteins (BMPs) and Wnts from the cortical hem, fibroblast growth factors (Fgfs) from the anterior neural ridge (which becomes the commissural plate), sonic hedgehog (Shh) from the ventral mesoderm, and a further selection of different morphogens arising from the antihem (fig. 1.3; O’Leary & Sahara, 2008). These morphogens induce the expression of downstream transcription factors within progenitors of the neuroepithelium, who pass on this patterning to their neural progeny.

Morphogenic gradients are essential for the dorsal ventral separation of the forebrain into pallial and subpallial regions. The formation of these two structures is primarily driven by the ventral expression of Shh in the underlying mesoderm of the ventral telencephalon. Shh promotes the expression of *Nkx2-1* followed closely by *Gsx2* (formerly known as *Gsh2*) in the subpallium, which prevents the encroachment of *Pax6* from the dorsal pallium (Corbin *et al.*, 2003; Sousa & Fishell, 2010). This division is particularly important as it creates an anatomical and functional
segregation, giving rise to the pallium that will go onto form the cortex, and the subpallium that will form the basal ganglia. In addition it produces two discrete neurogenic regions that are the sources of cortical pyramidal neurons and GABAergic interneurons (Anderson et al., 2002; Gorski et al., 2002).

**Figure 1.3**

*Figure 1.3 Patterning centers of the telencephalon.* The panel to the left shows the four main patterning centers of the telencephalon and the axes along which they occur. The graded expression pattern of the various morphogens gives rise to the graded expression of transcription factors such as *Pax6*, *Sp8*, *Emx2* and *COP-1* in the ventricular zone. This graded expression is converted to gene expression patterns with sharp boundaries, which facilitates the formation of cortical layers and the functional areas of the cortex. ANR, anterior neural ridge; Shh, sonic hedgehog; A, anterior; p, posterior; M, medial; L, lateral; CP, cortical plate; VZ, ventricular zone; F/M, frontal/motor cortex; S1, primary somatosensory cortex; A1, primary auditory cortex; V1, primary visual cortex. Figure taken from O’Leary & Sahara, 2008.

1.3.2 Development of cortical arealization

In addition to promoting the formation of two distinct neurogenic regions, morphogenic gradients influence the graded expression of genes within the developing cortical plate that will give rise to discrete functional areas. In terms of cortical arealization four key transcription factors are known to be necessary for segregating the cortex into the four primary cortical regions (somatosensory, auditory, visual and motor). These genes are the homeodomain transcription factor *Emx2*, the paired box domain transcription factor *Pax6*, the zinc-finger domain transcription factor *Sp8* and the orphan nuclear receptor *COUP TF1* (O’Leary & Sahara, 2008). The individual genes show distinct patterns of expression along similar lines to the morphogenic gradients that gave rise to them (figs. 1.3 & 1.4).
Studies of mutant mice with deficits in these transcription factor pathways reveal alterations to the area patterning of the cortex (Bishop et al., 2000; Mallamaci et al., 2000; Armentano et al., 2007; Sahara et al., 2007; Zembrzycki et al., 2007). The severity of the deficit depends upon the type of mutation; for example complete removal of *Emx2* has drastic effects leading to a significant depletion of caudal structures (Bishop et al., 2000; Mallamaci et al., 2000), however this is partially rescued in the heterozygote knock-out, which displays a much less severe phenotype (Leingartner et al., 2007). It is also possible that more subtle changes in expression amongst the healthy population may have similar yet less drastic effects, with slight variations in the relative representation of different cortical regions influencing the ability to process information relating to that modality (Leingartner et al., 2007). In inbred mouse strains such changes are not observed, suggesting that subtle genetic differences can have broad reaching effects on the cognitive processing of sensory information (Airey et al., 2005).

1.3.3 Pyramidal cell neurogenesis

Pyramidal neurons of the cortex are specified from the region adjacent to the ventricular lumen of the pallium. As we have seen the cortex is distinguished from subcortical regions by the expression of genes *Pax6* and *Emx2*, which prevent the encroachment of genes that specify a ventral fate (Muzio et al., 2002; Corbin et al., 2003; Kimura et al., 2005). Further segregation occurs within the cortex as *Lhx2* and *Foxg1* expression prevent the expansion of midline structures such as the
hippocampus (Monuki et al., 2001; Muzio & Mallamaci, 2005). From within this region, bordered medially by the archicortex, or hippocampus, and ventrolaterally by the subpallium, the layers of the neocortex emerge. The laminar organization of this structure could easily be imagined to occur as layer upon layer of new neurons slowly displace those that preceded them and take up a position bordering the region of neurogenesis. Somewhat paradoxically it was in fact found that the reverse occurs, with early born neurons populating the deeper cortical layers and more recently produced cells residing in the superficial domain (fig. 1.5; Angevine & Sidman, 1961; Rakic, 1974). This “inside out” laminar formation occurs as later born neurons migrate through the developing cortical plate before reaching the marginal zone adjacent to the pial surface, where they cease migrating and form the next layer of the nascent cortex.

The progenitor cells that give rise to excitatory neurons of the rodent cortex are located in the ventricular zone (VZ) and subventricular zone (SVZ) directly below the emerging cortical plate (fig. 1.5). Within the VZ, radial glia comprise the major precursor pool (Noctor et al., 2001), as well as providing a scaffold for migrating postmitotic neurons (Rakic, 1971; Hatten, 1999). In contrast the SVZ is primarily made up of intermediate progenitor cells (Noctor et al., 2004). This division of origin has functional consequences, with cells of the SVZ giving rise to neurons that mostly acquire a superficial layer fate (Miyata et al., 2004), whilst deep layer cells arise primarily from the VZ (Haubensak et al., 2004; Noctor et al., 2004). The inside out formation of cortical layers occurs due to a stepwise restriction of cell fate with early neural progenitors able to produce neurons of all cortical layers, whilst those at later ages preferentially adopt superficial layer fates (McConnell & Kaznowski, 1991). The transcriptional code that underlies this sequential limitation of eventual fate is beginning to be uncovered. It appears that a temporal genetic cascade produces cells that are eventually restricted to the superficial most layers (Frantz & McConnell, 1996; Hanashima et al., 2004; see review by Molyneaux et al., 2007).

To better understand the genetic specification and diversity of individual layers, a number of studies have taken advantage of the relative ease and affordability of comparative gene chip analysis to begin to dissect the genetic expression patterns observed across cortical layers. These studies have revealed that there are large
numbers of genes that are expressed in distinct laminar patterns (Arlotta et al., 2005; Belgard et al., 2011).

**Figure 1.5**

*Inside out formation of the neocortex.* Top panel shows the organization of the developing cortical plate across development. From left to right the formation of the preplate (PP), which is then split by the developing cortical plate (CP) to form the marginal zone (MZ) and subplate (SP). The individual layers of the cortex then emerge, with deep layers formed first and superficial layers forming at later ages. The bottom panel shows the approximate ages of each neurogenic wave that give rise to the individual layers in the mouse. CH, cortical hem; E, embryonic day; Ncx, neocortex; IZ, intermediate zone; LGE lateral ganglionic eminence; MGE, medial ganglionic eminence; SVZ, subventricular zone; VZ, ventricular zone. Figure taken from Molyneaux et al., 2007

Whilst many of these genes will likely preside over the mature intrinsic and functional properties of specific subpopulations of neurons that reside in each layer, some are also expressed during cortical neurogenesis. For example cut-like 2 (*Cux2*) and subventricular-expressed transcript 1 (*Svet1*), which are expressed in upper layer neurons, as well as the SVZ that is known to give rise to cells that populate superficial
layers (Tarabykin et al., 2001; Nieto et al., 2004; Wu et al., 2005). Other genes such as Fezf2 (also known as Fezl), Ctip2 and Satb2 have been identified as markers that regulate the maturation of pyramidal neuron subtypes with discrete patterns of axonal targeting (Molyneaux et al., 2005; Alcamo et al., 2008; Britanova et al., 2008). Some of these genes are able to induce a fate switch in non-cortical neurons (Rouaux & Arlotta, 2010), or promote the adoption of a given fate in neuronal stem cells (Alcamo et al., 2008), indicating their cell autonomous roles in the maturation of given pyramidal neuronal subtypes within the neocortex.

1.3.4 Interneuron neurogenesis

Unlike pyramidal neurons that are specified in the VZ of the pallium and migrate a short distance into the developing cortical plate, interneuron neurogenesis occurs in the subpallium before they undertake a more circuitous tangential migration to reach the nascent cortex (Anderson et al., 1997, 1999). Despite these distinct sites of neurogenesis the mechanisms that underlie the subtype specific variation between neurons, be they excitatory or inhibitory, are essentially the same; relying upon the spatial and temporal segregation of the point of final cell division that gives rise to the postmitotic neuron (McConnell & Kaznowski, 1991; Valcanis & Tan, 2003; Butt et al., 2005).

The segregation of the sites of excitatory and inhibitory neurogenesis is initiated by Shh, which induces the expression of Nkx2-1 and Gsx2 in the ventral telencephalon (Sussel et al., 1999; Corbin et al., 2000; Sousa & Fishell, 2010). Nkx2-1 and Gsx2 function to restrict the expression of Pax6 in ventral regions of the developing telencephalon, therefore promoting the formation of the pallial subpallial boundary (fig. 1.6; Inoue et al., 2000; Corbin et al., 2003). The primary neurogenic anlages of the subpallium, or ventral forebrain, can be subdivided into three main anatomical structures. These are the medial, lateral and caudal ganglionic eminences (MGE, LGE and CGE respectively), which have been shown to give rise to populations of neurons that go on to populate numerous brain regions, including the olfactory bulb (Anderson et al., 1997; Wichterle et al., 2001), basal ganglia (Deacon et al., 1994; Marin et al., 2000; Wichterle et al., 2001) and cortex (Wichterle et al., 2001; Butt et al., 2005; Miyoshi et al., 2007, 2010; reviewed in Wonders & Anderson, 2006). The majority of
cortical interneurons emerge from the MGE (~70% Rudy et al., 2011), with a significant proportion also arising from the CGE (~30%, Miyoshi et al., 2010; Rudy et al., 2011) and a smaller population from the embryonic preoptic area (POA, Gelman et al., 2009, 2011). The role of the LGE in the production of cortical neurons has been much debated, the current consensus is that the majority of interneurons derived from this region go on to occupy the olfactory bulb and basal ganglia. However if cortical populations do exist they likely only constitute a small minority of the overall population (Wichterle et al., 2001; Wonders & Anderson, 2006). The formation and spatial localization of the ganglionic eminences is shown in figure 1.6.

**Figure 1.6**

*Figure 1.6 Subpallial progenitor domains*. The emergence of the main sites of interneuron neurogenesis. Left panel from top to bottom, Nkx2-1 expression is induced by Shh at around embryonic day (E) 9.5; this is followed by the expression of Gsx2 at around E10. By E12.5 the distinct neurogenic regions are more easily defined with the MGE and LGE visible in the coronal sections to the left. The CGE is located more caudally, and the relative organization of the three eminences can be seen more clearly in the sagittal section in the panel to the right. The neuronal populations that emerge from each region are colour coded with the key at bottom right. Figure adapted from Sousa & Fishell, 2010.
Mature interneuron populations can be defined based upon the anatomical structure from which they arose, with a number of early studies using anatomical dissection and transplantation to gain insight into the spatial organization of interneuron precursor pools (Nery et al., 2002; Xu et al., 2004; Butt et al., 2005). However complications can occur when using this method, as some MGE cells migrate through the CGE therefore contaminating the dissected population (Butt et al., 2005). It is therefore preferable to define neurons based upon the expression of genetic factors rather than their spatial localization, even though in most cases there is a significant degree of overlap between the two. A number of recent genetic fate mapping studies have used Cre recombinase techniques to provide detailed spatial and temporal profiles of interneuron neurogenesis (Fogarty et al., 2007; Miyoshi et al., 2007, 2010; Gelman et al., 2009; Sousa et al., 2009). The lines used in these experiments, and others, have recently been published in a detailed overview of the range of Cre driver lines available to label interneuron populations (Taniguchi et al., 2011).

1.3.5 Genetic determinants of interneuron populations

The first interneuron fate determinant to be expressed in the subpallium is Nkx2-1, which demarcates the MGE, embryonic POA and extends into the region adjacent to the sulcus between the dorsal MGE and LGE (Fogarty et al., 2007; Butt et al., 2008; Gelman et al., 2009, 2011). The emergence of Nkx2-1 derived cells from the VZ of the MGE initiates the expression of downstream elements, including the LIM-homeodomain transcription factors Lhx6 and Lhx8 (also known as Lhx7)(Du et al., 2008; Flandin et al., 2011). These genes cause an up-regulation of Shh expression in postmitotic Nkx2-1 derived neurons in the mantle, which induces the expression of Nkx6.2 in the region adjacent to the sulcus (Flandin et al., 2011) and gives rise to a further discrete region of interneuron neurogenesis (Fogarty et al., 2007; Sousa et al., 2009). Nkx6.2 is also expressed in the embryonic POA along with Dbx1 (Gelman et al., 2009), however expression in this region appears to be less effected by loss of Shh from the mantle and is more likely due to the close proximity of the POA to the initial ventral source of Shh (Shimamura et al., 1995; Sousa & Fishell, 2010; Flandin et al., 2011).
The factors involved in the specification of CGE derived cortical interneurons remain more elusive. Amongst possible candidates are members of the orphan nuclear receptor CoupTF transcription factor family, which become increasingly restricted to the CGE and caudal extremity of the sulcal region around E13.5 (Lodato et al., 2011b), the same time as cortical interneurons begin to arise from this domain (Miyoshi et al., 2010). The restriction of CoupTF transcription factors to more dorsal and caudal aspects appears to be mediated via Nkx2-1, as conditional loss of function mutants reveal increased expression of CoupTF2 in the ventral MGE and a fate switch to CGE derived interneuron subtypes (Butt et al., 2008). This suggests that these two genetic programs are mutually exclusive, however the sulcal region appears to express both Nkx2-1 and CoupTF1 (Fogarty et al. 2007; Lodato et al., 2011b). This could be due to the expression of Nkx6.2, which may act to partially soften this repressive interaction, allowing both genes to be expressed to some degree in the sulcus. In support of this hypothesis is the observation that Nkx6 proteins in the spinal cord act to diminish the expression of other transcription factors involved in fate specification, but not totally eliminate them (Vallsstedt et al., 2001). Whatever the reason, interneurons fate mapped from the Nkx6.2 expressing region possess phenotypes that have been reported amongst both CGE and MGE derived populations (Sousa et al., 2009).

In addition to the spatial domain from which they emerge, it has also been shown that an interneurons fate is dictated by the stage of embryogenesis at which it exits the VZ and begins its migratory journey towards the cortex (Valcanis & Tan, 2003; Miyoshi et al., 2007). The pattern of gene expression in the developing subpallium is not constant through development, but shift as different genes are turned on, or their expression becomes restricted towards more defined domains (Sousa & Fishell, 2010; Flandin et al., 2011; Lodato et al., 2011b). These changing patterns of expression may underlie the temporal differences in the specification of mature interneuron subtypes (Butt et al., 2005; Miyoshi et al., 2007). They may also be important in creating the diversity of subtypes that emerge from the ganglionic eminences, not just the diversity of cortical interneurons but olfactory bulb and striatal populations as well, from within a limited number of cardinal progenitor domains.
1.3.6 Discrimination of mature interneuron subtypes

Genetic fate mapping studies are able to label discrete populations of interneurons, allowing researchers to determine the mature interneuron subtypes that arise from a single neurogenic niche. However in order to accurately assign a given cell to one of the twenty or so categories of known interneuron it is necessary to utilize a number of different experimental criteria (Cauli et al., 1997, 2000; Markram et al., 2004). Each individual subtype can be broadly determined based upon its morphology, intrinsic electrophysiology, immunohistochemistry and gene expression profile (Cauli et al., 2000; Ascoli et al., 2008). This is due to the considerable diversity displayed amongst interneurons, such that no technique is able to delineate a single subtype (with the possible exception of axoaxonic chandelier cells, Somogyi, 1977).

The most abundant GABAergic interneurons in the neocortex are the fast spiking parvalbumin (PV) positive multipolar basket cells. These emerge from the Nkx2-1 positive domain, and particularly from more ventral aspects of this region (Miyoshi et al., 2007; Wonders et al., 2008; Inan et al., 2012). The axons of PV interneurons target perisomatic regions and proximal dendrites, producing “baskets” of inhibitory terminals that surround the target cell soma (Martin et al., 1983; Kawaguchi & Kubota, 1993). A further population of PV positive neurons are chandelier cells, which are unique in that their synaptic target is the axon initial segment, around which they form characteristic “cartridge” axonal terminals (Somogyi, 1977; Inan et al., 2012). The remaining Nkx2-1 derived interneurons are labelled by the neuropeptide somatostatin (SST), forming a non-overlapping cluster discrete from PV cells and possessing diverse electrophysiological profiles (Gonchar et al., 2007; Miyoshi et al., 2007). SST positive interneurons have been fate mapped using a number of different genetic strategies (Fogarty et al., 2007; Miyoshi et al., 2007; Sousa et al., 2009) and have been shown to emerge from both the Nkx2-1 positive MGE and the Nkx2-1/Nkx6.2 positive region adjacent to the sulcus. These cells comprise non-fast spiking (NFS), delayed (d) NFS and intrinsic bursting (IB) subtypes with Martinotti and bitufted morphologies that target distal dendrites, and along with SST these cells can show immunoreactivity for calretinin (CR) (Markram et al., 2004; Fogarty et al., 2007; Miyoshi et al., 2007; Sousa et al., 2009). A further CR positive population arises from the CGE, which has been fate mapped using a Mash1CreERTm mouse line.
that shows restricted recombination in non-\textit{Nkx2-1} positive regions (Miyoshi \textit{et al.}, 2010). This population also contains a number of vasoactive intestinal peptide (VIP) positive neurons and cells that express the 5-HT\textsubscript{3} ionotropic serotonin receptor (Lee \textit{et al.}, 2010). CGE cells primarily possess neurogliaform and bi-/tripolar morphologies and typically target other interneurons (Gulyas \textit{et al.}, 1996; Freund & Gulyas, 1997; Miyoshi \textit{et al.}, 2010). CGE interneurons are preferentially located in superficial cortical layers, which is in contrast to \textit{Nkx2-1} derived populations whose lamination pattern is similar to pyramidal neurons- with early born cells populating deep layers and late born cells located more superficially (Miyoshi \textit{et al.}, 2007, 2010; Miyoshi & Fishell, 2011). A summary of the mature interneuron subtypes derived from each of the main regions of interneuron neurogenesis is shown in figure 1.7.

\textbf{Figure 1.7}

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\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Overview of the correlation between progenitor domain and mature intrinsic properties of cortical interneurons. Top, left panels indicate the transcription factors (coloured bars) expressed in the ventricular zone (VZ) and subventricular zone (SVZ) that have been shown to correlate with the mature properties that are used to categorize interneuron subtypes, which are shown in the panels to the right (grey bars). Below, the regions shaded in grey indicate the main progenitor domains that give rise to cortical interneurons. POA, Preoptic area; MGE medial ganglionic eminence; CGE, caudal ganglionic eminence. References: (1) Batista-Brito \textit{et al.}, 2009, (2) Butt \textit{et al.}, 2005, (3) Fogarty \textit{et al.}, 2007, (4) Fuentemilla \textit{et al.}, 2010, (5) Gelman \textit{et al.}, 2009, (6) Kanatani \textit{et al.}, 2008, (7) Karagiannis \textit{et al.}, 2009, (8) Kawaguchi, 1995, (9) Lee \textit{et al.}, 2010, (10) Lodato \textit{et al.}, 2011b, (11) McGarry \textit{et al.}, 2010, (12) Miyoshi \textit{et al.}, 2007, (13) Sousa \textit{et al.}, 2009. Figure taken from Anastasiades & Butt, 2011.}
\end{figure}
1.3.7 What is the purpose of interneuron diversity?

The diversity of interneuron subtypes in the adult cortex has yet to be fully understood and there is much that remains to be uncovered regarding both the true extent of interneuron subtype heterogeneity and its purpose in cortical function. The diversity of interneuron subtypes manifests itself in their individual biophysical properties (Butt et al., 2005; Miyoshi et al., 2007), morphologies (Ascoli et al., 2008; Defilipe et al., 2013), sub-cellular targeting along the somato-dendritic axis (Somogyi, 1977; Kawaguchi & Kubota, 1993; Markram et al., 2004) and the types of neuron that are targeted (Freund & Gulyas, 1997; Markram et al., 2004). In addition it has been shown that individual interneuron subtypes show distinct, but in many cases overlapping, expression of certain immunohistochemical markers, such as calcium binding proteins, as well as both metabotropic and ionotropic receptor isoforms (Cauli et al., 2000; Butt et al., 2005; Picardo et al., 2011; Rudy et al., 2011).

Evidence suggests that individual interneuron subtypes play specific roles in the functioning of the cortical network (Klausberger & Somogyi, 2008; Cardin et al., 2009; Lee et al., 2012; Wilson et al., 2012). The primary role of interneurons in the mature cortex is to inhibit the firing of other neurons by gating an inhibitory chloride conductance (discussed in greater detail in section 1.4.2). However, the diversity of this population means that some interneurons provide state dependent depolarizing input (Woodruff et al., 2011), and the properties of inhibitory outputs in the cortex are also known to be subtype specific (Klausberger et al., 2002). Furthermore, some interneurons typically target other interneurons (Gulyas et al., 1996; Freund & Gulyas, 1997). As such their recruitment by the network will lead to a decrease in overall inhibitory tone, through disinhibition of the local circuit (Letzkus et al., 2011; Xu et al., 2013). All this means that the selective recruitment of individual interneuron subtypes can have drastically different effects on the overall excitatory/inhibitory balance within the cortical network. Maintaining this balance seems to be an important function of interneurons within the cortex, as the emergence of an epileptic phenotype is frequently observed amongst mutations that give rise to interneuron deficits (Cobos et al., 2005; Batista-Brito et al., 2009).
In addition to preventing over excitation, interneurons regulate spike timing (Gabernet et al., 2005; Cruikshank et al., 2007) and the phasing of cortical oscillations (Cobb et al., 1995; Klausberger & Somogyi, 2008; Moore et al., 2010). By synchronizing the firing of local pyramidal neurons (Cobb et al., 1995) interneurons play an important role in the efficiency and precision of cortical circuits (Cardin et al., 2009; Sohal et al., 2009). Individual subtypes of interneuron drive distinct frequencies of oscillation (Blatow et al., 2003; Cardin et al., 2009) and fire at different phases within such oscillations (Klausberger & Somogyi, 2008). The importance of these oscillations to sensory perception is indicated by their increased power in areas associated with the specific processing of a sensory modality upon performing tasks associated with that modality (Fries et al., 2001).

Although the functions of individual interneuron subtypes are not fully understood, we have a broad understanding of their roles within the cortex. We can also see how their different properties allow them to influence the surrounding network in unique ways, to produce subtype specific effects (Blatow et al., 2003; Cardin et al., 2009; Berger et al., 2010). Recent work has shown that individual interneuron subtypes take part in distinct computational processes within the cortex- even if the role played by individual subtypes is disputed (Lee et al., 2012; Wilson et al., 2012). The hope is that as we continue to uncover the genetic code that gives rise to the diversity of interneuron subtypes in the cortex (see section 1.3.5), we will be better able to label and manipulate individual interneuron populations and provide greater insight into the role played by specific subtypes in the cortical network.

1.4 Circuit integration of cortical neurons

Upon their specification in the embryonic VZ, neurons begin a migratory journey towards their final location in the emerging cortical plate. In the case of pyramidal neurons this occurs in a radial fashion, along processes that stretch up to the marginal zone, adjacent to the pial surface. These processes can either be projections from the migrating neuron, leading to migration via translocation, or derived from radial glial cells, which act as guides for neurons migrating via locomotion (Nadarajah et al., 2001; reviewed in Kriegstein & Noctor, 2004). Interneurons on the other hand undertake a much longer route, migrating in a tangential fashion from the ganglionic
eminences up towards the pallium before entering the marginal zone or intermediate zone/SVZ, where they continue their journey to their final location within the cortex (Anderson et al., 2001; Corbin et al., 2001; Métin et al., 2006). Once they have reached the cortex both neuronal populations begin to form connections with other neurons and integrate into the nascent cortical network.

1.4.1 Early cortical activity patterns

Spontaneous activity occurs throughout much of embryonic and early postnatal development and is found in a wide range of neuronal structures such as the retina, spinal cord (Landmesser & Donovan, 1984; Sernagor & Mehta, 2001; Firth et al., 2005), hippocampus and neocortex (Garaschuk et al., 2000; Dupont et al., 2006; Allene et al., 2008; Golshani et al., 2009; see review by Blankenship & Feller, 2010). The ubiquitous nature of spontaneous activity suggests that it is essential for the correct maturation of neuronal systems. Indeed its importance has been demonstrated in numerous key developmental stages, including neuronal proliferation, migration and synapse formation (Komuro & Rakic, 1996; Weismann et al., 2004; Wang & Kriegstein, 2008). Within the cortical network it has been difficult to elucidate the true nature of this activity, as the reported physiological properties seem to vary (Garaschuk et al., 2000; Allene et al., 2008; Golshani et al., 2009; Yang et al., 2009). This diversity likely stems from the various experimental techniques employed by these researchers (e.g. calcium imaging versus extracellular recording, Golshani et al., 2009; Yang et al., 2009), as well as the intactness of the cortical preparation (e.g. in vitro versus in vivo, Garaschuk et al., 2000; Golshani et al., 2009 and Allene et al., 2008; Yang et al., 2009) and the age at which the study took place. There may also be multiple activity patterns present during each developmental stage that are elicited through different mechanisms and subserve distinct developmental roles (see Yang et al., 2009).

The mechanisms that underlie these early network activity patterns have been primarily studied in vitro and therefore the following section will focus on these in vitro studies. It remains to be seen if the activity patterns elicited in vitro truly mimic those found in vivo, however there does appear to be at least some similarity between the types of activity observed in the two systems (Allene & Cossart, 2010). Initially
activity is generated and propagated via a variety of non-synaptic mechanisms such as intrinsically generated spontaneous events, volume transmission of various excitatory agents and direct coupling of adjacent neurons through gap junctions (Yuste et al., 1992; Kandler & Katz, 1998; Weismann et al., 2004). In mice the organization of cortical network activity begins to show increased synchrony around the time of birth (Allene & Cossart, 2010), which leads to the emergence of local calcium domains and cortical early network oscillations (cENOs), driven by gap junction coupling and extracellular glutamate (Yuste et al., 1992; Garaschuk et al., 2000; Allene et al., 2008). As the cortex matures and cells begin to form synaptic connections within the network, there is a transition from gap junction coupled activity patterns towards synaptically driven oscillations (Dupont et al., 2006; Allene et al., 2008, 2012; Yang et al., 2009). These activity patterns are termed cortical giant depolarizing potentials (cGDPs) and appear towards the end of the first postnatal week (Allene et al., 2008, 2012). In contrast to the earlier patterns of activity, cGDPs are dependant upon synaptic GABAergic signalling (Allene et al., 2008) and likely function to further facilitate the maturation and integration of cells in the nascent network (Wang & Kriegstein, 2008). Within the hippocampus these activity patterns occur due to the presence of highly interconnected “hub” cells that primarily comprise early born GABAergic interneurons and are capable of influencing the activity of the local network (Bonifazi et al., 2009; Picardo et al., 2011). However, to date cells of this type have not been reported in the neocortex. cGDPs and cENOs exhibit distinct spatiotemporal profiles suggesting they may serve different functions. cGDPS occur at higher frequency, but each event recruits a lower percentage of the overall cortical network than cENOS (Allene et al., 2008). It is therefore possible that the shift in activity underlies a transition towards the formation of discrete synaptically coupled sub-networks within the cortical circuit.

1.4.2 Changes in GABAergic signalling through development

Neuronal signalling relies upon the flow of charged ions through gated channels inserted across the neuronal membrane, resulting in local fluctuations in membrane voltage. The flow of ions through an open channel in the membrane of a given neuron depends upon the electrochemical driving force, which itself is dependent on the membrane voltage and the relative concentrations of the conducting ion inside and
outside the cell (Hodgkin & Huxley, 1952). GABAergic signalling functions primarily via the flow of anionic chloride currents through the GABA<sub>A</sub> receptor and is therefore dependent upon the chloride gradient across the neuronal membrane. The ability of GABA to promote network oscillations during early postnatal development is due to an altered chloride gradient, which produces a depolarized GABA reversal potential (E<sub>GABA</sub>) (Ben-Ari et al., 1989). This gradient is maintained through the action of two ion transport proteins each with opposing roles, NKCC1 and KCC2, whose relative expression has been shown to vary during development (Rivera et al., 1999, 2004; Ben-Ari, 2002). A change in the relative levels of these two proteins over the second postnatal week influences the internal chloride concentration of the cell; such that GABA elicits a net efflux of chloride ions in the immature brain, but by postnatal day (P) 9-12 it either causes an influx of chloride ions that hyperpolarizes the cell, or inhibits excitation through shunting mechanisms (fig. 1.8 A-B; Ben-Ari, 2002; Rheims et al., 2008).

**Figure 1.8**

Figure 1.8 The shifting role of GABAergic signaling during development. A the mechanism of chloride regulation during early stages of development. Higher expression of NKCC1 over KCC2 produces increased intracellular chloride concentrations such that GABAergic signaling causes a depolarizing efflux of chloride ions. B the developmental upregulation of KCC2 causes a shift in intracellular chloride levels, such that at mature ages intracellular chloride concentrations decrease and GABA is hyperpolarizing. Figure adapted from Ben-Ari, 2002.

The true function of GABA in development is controversial as it would appear that whilst it depolarizes cells in many, but not all, cases, the reversal potential is still below the firing threshold of cortical neurons (Rheims et al., 2008). Thus whilst GABA is depolarizing it may not be truly excitatory and may function to serve multiple functions in the brain; creating synchronous depolarizations within the
network, but remaining below the firing threshold to prevent the deleterious expression of runaway excitation and associated excitotoxicity.

1.4.3 Synapse formation

The term synapse, derived from the Greek “to bind together”, was originally coined by Charles Sherrington to describe the connections that were observed to occur between individual neurons (Sherrington, 1897; however see Bennett, 1999). Synapses can occur at sites where axonal and dendritic arbors intersect, such that a synapse is able to form between them (Kalisman et al., 2005). The majority of excitatory synapses onto pyramidal neurons of the neocortex occur at sites known as spines (Gray et al., 1959). In contrast interneurons often lack spines, or where they are present they are typically sparsely expressed (Markram et al., 2004; Keck et al., 2011). Spines are small protrusions that occur from the dendritic membrane and possess dense clusters of synaptic proteins and glutamate receptors (Yuste, 2011). Spines can increase the effective area with which a neuron can form connections by “reaching out” to adjacent axons, preventing the necessity for large-scale shifts in dendritic or axonal morphology (Yuste, 2011). Once formed, spines provide the cell with the ability to compartmentalize its inputs and easily modify individual synapses without producing global changes to dendritic structure or function (Koester & Sakmann, 1998; Araya et al., 2007; Bloodgood et al., 2009).

1.4.4 Synapse receptor composition

The two main receptor subtypes at excitatory glutamatergic synapses are named after their selective affinity for 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) respectively. These two receptor classes each show distinct functional properties; AMPA receptors (AMPARs) are low affinity and possess rapid kinetics, whereas NMDA receptors (NMDARs) have slower kinetics and higher affinity of glutamate binding (Patneau et al., 1990). NMDARs also possess a magnesium block, which prevents the flow of ions through the channel pore at hyperpolarized membrane potentials (Mayer et al., 1984). A major difference in synaptic structure throughout early development is the dominance of NMDAR mediated currents and their influence of the ongoing activity.
of the network (Garaschuk et al., 2000; Hall et al., 2007; Allene et al., 2008; Hall & Ghosh, 2008). In its most extreme form this manifests itself as so-called silent synapses, that possess a functional NMDA current but lack synaptic AMPARs (see fig. 1.9).

Figure 1.9

![Figure 1.9 Receptor composition of silent and mature synapses](image)

These silent synapses occur at higher frequency in the early brain (Isaac et al., 1997; Rumpel et al., 1998, 2004) and appear to represent a key stage in synapse maturation (Hall et al., 2007; Adesnik et al., 2008). Due to the voltage-dependant magnesium block on NMDAR conductivity, silent synapses do not contribute to changes in the cells membrane potential and firing unless the cell is already depolarized. This has led to their being thought of as coincidence detectors, or Boolean “AND gates”, being able to detect both presynaptic glutamate release and postsynaptic depolarization (Koch, 1987). Repetitive stimulation at silent synapses has been shown to integrate AMPARs into the postsynaptic membrane in an activity dependant fashion (Isaac et al., 1995; Liao et al., 1995). Thus silent synapses are ideally placed to promote functional connectivity between cells that are connected by the same presynaptic locally projecting neuron, or “hub” cell (Bonifazi et al., 2009) and therefore part of the same local network. A process of this sort would not only provide a mechanism for synaptic integration, but could also explain the preferential connectivity amongst
local cells observed in the mature cortical network (Markram, 1997; Song et al., 2005).

1.4.5 Synaptic plasticity

Ever since Hebb it has been thought that activity may be important in sculpting the connectivity of neuronal networks. His now famous postulate on the role of activity in the promotion of connectivity is often paraphrased to “cells that fire together wire together” and was given experimental grounding in the classical studies into long-term potentiation (LTP) (Hebb, 1949; Bliss & Lomo, 1973). This revealed that it was possible to modify synapse strength, and that these changes could bring about functional changes to pathways that underlie behaviour (Antonov et al., 2003). However this posed a problem, as a constantly potentiated network would quickly become saturated and therefore unable to function correctly (Sejnowski, 1977; Barnes et al., 1994). Thankfully this was resolved by the discovery of long-term depression (LTD) (Lynch et al., 1977) and spike time dependant plasticity (STDP), where bidirectional plasticity occurs such that the sign of the change depends on the order of the firing of the postsynaptic cell relative to any synaptic inputs (Markram et al., 1997; Sjostrom et al., 2001).

NMDA receptors have been widely implemented in cortical plasticity, with their ability to function as coincidence detectors providing an ideal substrate for Hebbian plasticity (Malenka & Nicoll, 1999; Nicoll & Malenka, 1999). In addition to their role in synaptic plasticity at more mature ages, the increased prevalence of NMDA receptors and silent synapses at early ages (section 1.4.4) and the ubiquitous presence of synchronous activity in developing neuronal systems (section 1.4.1), suggests that a similar mechanism may underlie the formation and potentiation of synapses during development; driving the integration of cells into the nascent network (Katz & Shatz, 1996; Wang & Kriegstein, 2008; Wang et al., 2011).

1.4.6 The emergence of cortical connectivity

At present much less is known about the synaptic connectivity of the early cortical network compared with the mature. A few studies have looked at the emergence of
specific features of the network, such as the integration of the thalamocortical pathway (Daw et al., 2007; Ashby & Isaac, 2011) and feed-forward connectivity in the barrel cortex (Bureau et al., 2004). However in order to fully understand the emergence of the canonical circuit it is necessary to probe connectivity across multiple layers and cell types. Classical approaches such as recordings of mini-excitatory postsynaptic currents (mini-EPSCs) (Wang & Kriegstein, 2008), responses elicited by stimulating electrodes (Daw et al., 2007) and paired recordings (Pangratz-Fuehrer & Hestrin, 2011), have been able to provide some information about how, when and with whom individual neuronal subtypes form synaptic connections during development. However these techniques lack the high-throughput spatial mapping ability of other methods, such as laser scanning photostimulation (LSPS), and therefore do not yield complete maps of interlaminar connectivity. Producing such maps will allow us to track the development of translaminar connectivity in the neocortex and as a result the emergence of the canonical circuit.

1.5 Thesis overview

This chapter has discussed the mature structure of the neocortex and a number of the genetic and physiological processes that contribute to the development of this network. During postnatal maturation, in between the cessation of migration and maturity, we know that the canonical pattern of connectivity emerges amongst the individual components of the cortex. However, at present less is known about the organization of the network at the earliest points of circuit formation, or how and when the mature pattern of input emerges. There is a particular paucity of knowledge into the organization of synaptic inputs onto cortical GABAergic interneurons during development. Our current understanding of network maturation indicates an important role for GABAergic signalling in promoting the early activity patterns within the network (Allene et al., 2008; Bonifazi et al., 2009), which are important for the synaptic integration of excitatory pyramidal cells (Kasyanov et al., 2004; Wang & Kriegstein, 2008). Therefore an understanding of the synaptic properties of the early network, and particularly the GABAergic interneurons contained within it, will be of significant benefit in dissecting their role in this activity, and consequently the synaptic maturation of the cortex.
LSPS allows researchers to produce maps of synaptic connectivity onto neurons across a whole cortical column. Recording from cells of different subtypes, located within different layers of the neocortex, will allow me to compile a detailed study of the organization and maturation of this circuit. Initially I will focus on mapping the synaptic inputs onto cortical pyramidal cells. Studies of pyramidal connectivity will record both AMPAR and NMDAR mediated inputs, as this will allow me to determine the spatial localization of these inputs at different points in development and the role that silent synapses may play in the formation of the canonical circuit. I will also examine the influence that rhythmic activity has on the integration of cells into the network. As we have seen, synchronous network activity is highly prevalent within the developing cortex. However, how this activity will impact the integration of cells at different points in development is less certain.

After assessing the integration of pyramidal cells I will next map the inputs onto Nkx2-1 derived cortical interneurons. The period of this study will begin around the time of the transition from cENOs to cGDPs, so I will be able to uncover the relative integration and organization of inputs onto pyramidal cells and interneurons during the phase of development where GABAergic signalling is important for promoting synaptic integration (Kasyanov et al., 2004; Wang & Kriegstein, 2008). The study will continue to follow the integration of these cells up until they reach their mature organization of synaptic inputs, at the end of the third postnatal week (Xu & Callaway, 2009). Therefore I will also uncover the mature organization of synaptic inputs onto Nkx2-1 derived interneurons. After having studied the integration of pyramidal cells and interneurons in isolation, the final section of this thesis shall compare the synaptic integration of these distinct neuronal populations and search for any differences that may indicate their roles in the development of the cortical network.

Before describing the results obtained during my studies, I will firstly provide a detailed account of the experimental protocols used to obtain this data (chapter 2). Of particular importance in LSPS experiments is the calibration of the system, which allows accurate, spatially defined maps to be produced. Chapter 3 will detail the steps that were taken to maximise the accuracy of this technique and allow precise mapping of translaminar connectivity throughout postnatal development.
Chapter 2- Materials and methods

2.1 Slice preparation

All of the recordings reported in this study were performed on mice aged between postnatal days (P) 5-21. For pyramidal cell recordings I used a CD1 wild type (WT) mouse strain. To selectively target Nkx2-1 derived cortical interneurons I recorded from the Nkx2-1iCre line (Kessaris et al., 2006; Fogarty et al., 2007) on an outbred C57 black six background, crossed with a Z/EG reporter line (Novak et al., 2000) on an outbred CD1 background. The resultant progeny were bred within our facility on this mixed background. Prior to slice preparation mice of either genotype were anaesthetised using isoflourane inhalation anaesthetic until unconscious. The mice were removed from the anaesthetic chamber and quickly decapitated using surgical scissors. The head was immediately placed in ice-cold artificial cerebrospinal fluid (ACSF) of composition (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂ and 20 Glucose. The ACSF had been previously infused with 95% O₂ /5% CO₂ for a period of ten minutes. The brain was isolated, within the ice-cold ACSF, by removing the cutaneous tissue and by making an incision along the midline of the skull. The skull was then separated, allowing the brain to be extracted. The cerebellum was removed with a razor blade, providing a flat surface for attaching the brain to the platform of the Vibrotome used for slicing the tissue. This was performed by gluing the brain such that the posterior of the brain was attached to the slicing platform and the dorsal aspect of the brain orientated towards the blade of the Vibratome 3000 tissue sectioning device (Vibratome St. Louis MO, USA), such that slicing would produce coronal slices. The platform was placed into the slicing chamber of the Vibratome 3000, which had been previously filled with ACSF cooled to 3°C via a Vibratome 900R refrigeration unit (Vibratome St. Louis MO, USA) and infused with 95% O₂ /5% CO₂ for a period of at least ten minutes. The brain was sliced using a sapphire blade with the thickness of the slice set at 375µm on the Vibratome 3000. Upon slicing, the individual slices were transferred to a storage chamber (Harvard Apparatus, UK) containing ACSF at room temperature also infused with 95% O₂ /5% CO₂. Slices were maintained in ACSF, which was continually oxygenated, to equilibrate at room temperature for a period of at least 45-60 minutes prior to recording.
2.2 Whole-cell patch-clamp recordings

For whole-cell patch-clamp recordings, individual slices were transferred to the recording chamber and continually perfused with room temperature ACSF infused with 95% O₂ /5% CO₂. To prevent movement of the tissue, a slice hold-down (Harvard Apparatus, UK) was placed on top of the slice and positioned so that the individual strands of the hold-down did not overlap with the region of the brain that I intended to record from. Intracellular recordings were obtained using individually pulled borosilicate glass microelectrodes (Harvard Apparatus, UK), of resistance between 6-9MΩ. These were produced immediately prior to recording using a Narishige PC-10 electrode puller (Narishige, Japan). The microelectrodes were then filled, using a syringe, with intracellular solution of composition (in mM) 128 K-gluconate, 4 NaCl, 0.3 GTP, 5 ATP, 0.0001 CaCl₂, 10 HEPES, and 1 glucose, with the pH altered to pH 7.3 using 1M KOH. Approximately 0.2% w/v Lucifer yellow (Sigma, UK) was also added to the intracellular solution to facilitate morphological reconstruction of the cell post-recording. The tip of the microelectrode was backfilled with intracellular solution lacking Lucifer yellow, to prevent it leaching out and staining the tissue prior to formation of a tight seal on the soma of the cell to be recorded. For NMDA mapping an alternative intracellular solution was used to ensure stable recordings at depolarized membrane potentials (Rumpel et al., 2004). The composition of this solution was (in mM) 135 CsCl, 20 TEA-Cl, 2 MgCl₂, 10 HEPES, 10 EGTA, 0.3 GTP, 5 ATP with the pH adjusted using 1M CsOH until pH 7.3. Cells were visualized using an Axiocam Mrm camera (Carl Zeiss Ltd., UK) attached to a Zeiss axioskop FS-2 plus microscope (Carl Zeiss Ltd., UK) with a LUPlanFL N 40x objective (Olympus UK Ltd.), IR-DIC filter block (Carl Zeiss Ltd., UK) and axiovision software (v4.6). Target neurons were randomly selected amongst cells located >50µm below the surface of the slice (typically 50-70µm below the slice surface), and located in layers 2/3 to 5 of the somatosensory (S1BF, S1Tr, S1HL) and motor cortices (M1, M2, MPta; Paxinos et al., 2007). Pyramidal cells were distinguished by their characteristic morphology with apical dendrites ascending towards the pial surface. Nkx2-1 derived interneurons were distinguished by their fluorescence, caused by their expression of enhanced green fluorescent protein (EGFP) and, unlike pyramidal cells, were recorded solely from the somatosensory cortex.
The micropipette was lowered onto selected neurons using a motorised manipulator (Luigs & Neumann, Germany) with constant positive pressure applied via a mouthpiece, connected to the pipette holder by silicone tubing. As the electrode was lowered towards the target neuron a 25mV test pulse at a frequency of 20Hz was applied in voltage clamp mode (Multiclamp 700B, Molecular Devices, CA). The positive pressure used for lowering the electrode was changed to negative pressure once a dip in the test pulse was observed, indicating the electrode was in proximity to the target cell. Negative pressure was applied until a tight-seal was achieved- this was determined by a flat test pulse on the axioclamp software, indicative that a gigaohm seal had been reached. Once the seal was established a short pulse of negative pressure was applied in order to rupture the membrane and obtain whole-cell access to the recorded cell.

2.3 Determining intrinsic membrane properties of recorded neurons

Upon patch rupture, intracellular recordings were obtained in current clamp mode (Multiclamp 700B, Molecular devices, CA). To assess the intrinsic membrane properties of the cell, 500ms square current pulses occurring at 0.5 Hz were programmed into Clampex software (Molecular devices, CA), both depolarizing and hyperpolarizing current steps were used, with the magnitude of the pulse tailored to the intrinsic excitability of the cell. Intrinsic membrane properties were also assessed at random points throughout recordings to determine the health of the cell and quality of the patch; cells that showed deterioration were discarded from future analysis. All cells were analysed offline using clampfit (version 10.1, Molecular Devices, CA).

The use of whole-cell patch-clamp allows an accurate determination of both passive (membrane dynamic) and active (spike dynamic) properties and offers unrivaled access and control of the cell. In order to determine the values of the various parameters for each recorded cell, different current input protocols were implemented (fig. 2.1 A-D). These were based around a 500ms square current impulse occurring at a rate of 0.5 Hz, which was of sufficient duration for cells to exhibit steady state firing responses and allowed the determination of any frequency adaptation that occurred over the course of the pulse. The rate of pulse delivery was also slow enough to
ensure that cells were able to return to rest prior to being subjected to the next current input.

Figure 2.1

**Figure 2.1 Current input protocols used to calculate electrophysiological parameters.** A-D response of cortical neurons to current input protocols. A a ramp to spike, to determine the approximate threshold of the cell and determine the presence of a threshold oscillation. B small current steps to elicit a threshold spike and calculate the delay to threshold spike. C the response to hyperpolarizing current steps. D the maximum firing frequency. The red trace in C shows the trace used to calculate input resistance, which was ¼ of the current needed to reach -100mV. E how the various single spike properties were calculated, each property in E was calculated from the threshold spike of the recorded cell. Current pulses in B-D are of 500ms duration, the current input that elicited each response is shown next to the trace in panels A-D.

The magnitude of the pulse was tailored to the intrinsic maturity of each recorded cell, which itself was determined by a ramped supra-threshold current injection upon initial patch rupture (fig. 2.1 A). This initial current response also allowed the health of the
cell and quality of the patch to be determined. A stable baseline, spike height above +20mV and a normal spike threshold distinguished good quality patches; where as poor access typically resulted in stunted spikes and atypical spike threshold values. The properties observed at initial patch rupture were also used to compare with later responses to look for signs of patch deterioration. If the initial patch was deemed to be of poor quality, or if it deteriorated significantly over the course of recording, the cell was discarded. For the remaining healthy cells that showed good access, multiple current input protocols were performed. In each case the current pulse was adjusted to produce: a threshold spike (fig. 2.1 B), hyperpolarizing responses to -100mv to determine voltage sag and the presence, or not, of rebound spikes (fig. 2.1 C), hyperpolarizing responses of <10mV to determine input resistance (fig. 2.1 C), and the maximum firing frequency of the cell prior to spike failure (fig. 2.1 D). From within these recordings a number of additional parameters could also be determined (fig. 2.1 B-E).

2.4 UV laser scanning photo uncaging of Glutamate

Once the intracellular profile of the recorded cell had been obtained, the perfusing media was switched to a high divalent cation (HDC) ACSF (4mM Mg$^{2+}$, 4mM Ca$^{2+}$) with 100mM Methyl 1-[5-(4-amino-4-carboxybutanoyl)]-7-nitroindoline-5-acetate hydrate (MNI) caged glutamate (Sigma, UK)- again infused with 95% O2/5% CO2. The HDC ACSF + caged glutamate was allowed to wash onto the slice for a period of 6 minutes, which assured that the slice was saturated with caged glutamate prior to uncaging. After passing over the slice, the solution was recycled by collecting in clean glassware and periodically adding it back to the initial mixture.

Uncaging was performed with a 355nm ultraviolet (UV) laser (DPSL-355/30), targeted using a UGA-40 targeting module (both from Rapp Optoelectronic GmbH, Hamburg, Germany). The laser was coupled to the microscope via a fiber-optic cable and focused through the microscope using a 10x UPLFLN objective (Olympus UK Ltd.), with UV filter block (Carl Zeiss Ltd., UK) to facilitate efficient transmission of the UV laser. Initial experiments were made using an alternative 10x objective lens (10x Achromat, Carl Zeiss Ltd., UK), however the UV transmission of this lens was significantly less than the Olympus lens, so the lens was upgraded to increase the
efficiency of the system. The maximum laser power obtainable at the slice (using the Olympus lens) was measured using a field max II laser power meter (Coherent, CA) and found to be just over 1mW.

The organization of the laser targeting grid was programmed manually into custom software provided by Rapp Optoelectronic (Hamburg, Germany). This controlled the galvanometers in the UGA-40 laser targeting module, which directed the position of the laser beam at the slice. Prior to initial recordings, and periodically throughout the course of these experiments, the system was calibrated to ensure accurate targeting of the laser beam. The mapping grid was arranged with 50µm between individual uncaging sites, in a 9x13 or 9x17 rectangular grid pattern. For mapping experiments the grid was aligned perpendicular to the surface of the slice and such that the tip of the recording electrode was below a specific uncaging site, which was noted at the time of recording. This allowed the responses from each uncaging site to be accurately allotted relative to either the recovered morphology, or a photomicrograph of the location of the recording pipette that was taken through the 10x objective for each cell prior to initiating the mapping protocol.

Before mapping synaptic inputs in voltage clamp, 1-2 runs of the uncaging grid were performed in current clamp mode to ascertain the response of the recorded cell to glutamate uncaging. Once the laser intensity had been set to produce restricted excitation in close proximity to the soma of the recorded cell (discussed in more detail in chapter 3), cells were switched to voltage clamp mode to record synaptic inputs. Voltage clamp recordings were made with a high pass filter set at 0.05Hz at a holding potential of -60mV for pyramidal neurons, -70mV for interneurons and at both -70mV and +40mV for NMDA mapping. Multiple repeats of each map (typically between 3-6) were performed for each cell. The trace was separated into one second recording bins, triggered from a master 8 pulse stimulator (A.M.P.I., Israel). The laser was triggered to fire from a separate channel on the master 8 at a delay of 100ms from the start of each one second recording epoch. Laser pulses were typically of 100ms duration, however during initial experiments, where the alternative 10x objective with lower UV transmission was used, a pulse of 200ms was required to drive some cells to spike.
2.5 Considerations given the shifting nature of GABAergic signaling

The purpose of this study was to map the excitatory inputs onto cortical neurons, from the point at which they first integrate into the network until maturity. Whilst GABA is traditionally viewed as an inhibitory neurotransmitter, it is generally believed to play a depolarizing role in early development (chapter 1, section 1.4.2; Ben-Ari et al., 1989; Rivera et al., 1999; Ben-Ari, 2002). GABA depolarization occurs over the first postnatal week and, in the mouse neocortex, slowly shifts towards its normal mature state by P9-12 (Rheims et al., 2008). As this period encompasses part of my study, early synaptic events could be due to depolarizing GABA as opposed to AMPA mediated glutamate responses. In order to solely map responses arising from AMPA receptors, I used a mature intracellular solution with a low chloride concentration (4mM) throughout the duration of this study. Due to the vastly superior volume of the recording pipette relative to the cell, the chloride concentration inside the cell should dialyze to that of the pipette solution and therefore could be considered constant at all recording ages. Using the Nernst equation and the values for chloride concentration determined from the intracellular and extracellular solutions used in these experiments (Cl\textsuperscript{out} 133.5mM, Cl\textsuperscript{in} 4mM), and taking room temperature as 20°C, I calculated that the reversal potential for chloride was -88.6mV, close to the baseline at which the cells were voltage clamped (-60/70mV) and meaning GABAergic responses would not contributed to the excitatory inputs observed at these holding potentials.

2.6 Eliciting network activity in the acute in vitro slice preparation

Two approaches were implemented to induce activity within the acute in vitro slice preparation. Both involved altering the ionic concentrations of the ACSF with which the slice was perfused, thus altering the excitability of the cells in the slice. The first approach involved elevating the concentration of potassium ions to a concentration of 4mM, which has been used by others to induce cGDPs (Allene et al., 2008). The second involved removing Mg\textsuperscript{2+} ions from the bath ACSF to produce a nominally zero magnesium ACSF as used by other laboratories (Silva et al., 1991). With the exception of these modifications, the ionic content of these ACSF solutions (both elevated potassium and zero magnesium) was the same as described previously in section 2.1.
2.7 Staining to recover morphologies of recorded neurons

The addition of Lucifer yellow to the intracellular solution meant that after recording, the morphology of the cell could be determined using fluorescent microscopy. At this time addition notes were made on the location of the cell, both its predicted layer and cortical region (motor or somatosensory cortex). In order to recover the morphology of the cell a number of brief current pulses were applied via the electrode, utilizing the “buzz” function on the Multiclamp 700B. This helped to dislodge the neuronal membrane from the electrode tip. A brief pulse of positive pressure was then applied via the mouthpiece whilst simultaneously retracting the microelectrode at high velocity. This method was able to recover the morphology of the cell in many cases; however particularly at younger ages, when the morphology of the cell was less developed and had fewer neurites in place to anchor it to the tissue, cells were pulled from the slice along with the retracted electrode. If the soma of the cell remained within the slice it was transferred to an 8 well plate and placed in ice cold 4% paraformaldehyde. The slice was covered with fine gauze and held with a slice hold-down to ensure there was no distortion of the tissue. The plate containing the slices was covered in aluminium foil and stored at 4°C overnight. The slices were then transferred to 1x phosphate buffered saline solution (PBS, Sigma, UK), where they were stored at 4°C until developed.

The following protocol was used to develop the morphologies of recorded cells. Throughout this protocol the slices were kept covered in aluminium foil wherever possible. All wash stages were performed on a shaker. Firstly the PBS was removed using a pipette and replaced with a fresh wash of 1x PBS (Sigma, UK). After 5 minutes this was removed and the slices were flash frozen over dry ice. The slices were then washed in fresh 1x PBS at room temperature until thawed. The PBS was removed and replaced with 3.3% v/v hydrogen peroxide (Sigma, UK), made up in a solution of 75% methanol (Fisher scientific UK Ltd.) and 25% 1x PBS; slices were incubated in the final mixture for 20 minutes. This was then removed and the slices were washed twice in 1x PBS for at least 5 minutes each. The slices were then washed once more in 1x PBS with 1% tween-20 (PBS-T). After removing this final wash, slices were blocked for one hour in 5% v/v normal goat serum (NGS) made up in PBS-T. Blocking occurred at room temperature and the slices were kept on a shaker.
The blocking media was removed and the primary antibody was then added. This was a rabbit anti-Lucifer yellow biotin conjugated antibody (Invitogen, UK) at a concentration of 1:500 made up in 5% v/v NGS in PBS-T. Slices were incubated overnight at 4°C on a shaker. After removing the antibody slices were washed six times for a minimum of 5 minutes in 1x PBS at room temperature. Using the peroxidase standard PK-4000 kit (Vector, Burlingame, CA) I mixed 3 drops of solution A with 3 drops of solution B and made this up in 9.5ml PBS-T. This was left for 15 minutes before being added to each slice, after first removing the PBS wash. Slices were incubated overnight at 4°C on a shaker.

After removing the previous mixture, slices were washed 6x in 1x PBS for a minimum of 5 minutes, they were then washed once more in double distilled water (ddH2O). Using the peroxidase substrate kit (Vector, Burlingame, CA) I mixed 4 drops of buffer, 8 drops of DAB, 4 drops of H2O2 and 4 drops of Nickel and added this to 10ml ddH2O, this solution was kept in the dark. I removed the wash and added 800µm of the DAB solution to each slice; incubating on a shaker for 20 minutes, or until slices were well stained. I washed once in ddH2O, once in 25%, 50% and 75% glycerol in ddH2O and once in 100% glycerol (Sigma, UK), each wash lasted a duration of 5 minutes. The slices were then stored in 100% Glycerol at 4°C until mounting on microscope slides for imaging.

For Ctip2 staining in conjunction with morphological development, Ctip2 rat monoclonal antibody (Abcam, UK) was used at a concentration of 1:400 with a goat anti-rat Cy3 or Cy5 conjugated secondary antibody, both used at 1:200 (Millipore, UK). Both were incubated overnight at 4°C, and the rest of the protocol was as above.

2.8 Statistical analysis and clustering

All statistical analysis to compare between different populations was performed using a Student’s t test. For all statistical tests the threshold for significance was taken as p=0.05, with data reported as falling within either the 95% (p<0.05), 99% (p<0.01), or 99.9% (p<0.001) significance level. In chapters 4 and 5 multiple comparisons are made between the intrinsic electrophysiological properties of cortical neurons relative to cells recorded at immature ages. This approach may lead to an increase in the
observance of type 1 statistical errors (false positives) and as such it is important to note that a small proportion of weakly significant findings, particularly those that do not fall into clear trends, may in part be an artifact of this approach. For all population data the average value used is the mean average, with the data reported as the mean ± the standard error of the mean (SEM). To determine the significance of input organization for individual input maps, one-way analysis of variance (ANOVA) was performed in Matlab using the anova1 function (Matlab R2010b, Mathworks, USA). I also used Matlab to perform both k means and hierarchical cluster analysis. In each case I examined a range of distance metrics for the analysis, to find which was the best fit for the data. For k means clustering of the intrinsic membrane properties in chapter 5, the success of the clustering was determined by the agreement with the subtypes assigned at mature ages. For hierarchical clustering, of both intrinsic membrane properties and excitatory inputs, it was determined by using the cophenetic correlation of the cluster data (cophenet function in Matlab). The distance metric that produced the highest cophenetic correlation value was used on each occasion. For all cases throughout this thesis the distance metric used for both k means and hierarchical clustering was Euclidean distance squared.
Chapter 3- Calibration of glutamate uncaging

3.1 Introduction

The primary objective of my thesis was to understand the postnatal maturation of neocortical networks, specifically focusing on synaptic integration and the formation of the canonical circuit. Work from other laboratories has begun to shed some light on this topic, with particular emphasis on afferent and efferent connectivity of layer 4 neurons in the somatosensory barrel cortex (Bureau et al., 2004; Daw et al., 2007; Ashby & Isaac, 2011). My aim was to complement these studies by recording the maturation of synaptic inputs onto both pyramidal cells and GABAergic interneurons, located in deep and superficial layers of the neocortex, and thus provide a more complete overview of the development of the cortical circuit. In addition, this approach allowed me to observe any similarities or differences that occur in the integration strategies employed by individual components of the cortical network. In order to record the excitatory inputs onto these neurons, I used laser scanning photo uncaging of glutamate, the use of which has been reported by numerous others (Callaway & Katz, 1993; Dalva & Katz, 1994; Dantzker & Callaway, 2000; Schubert et al., 2001; Shepherd et al., 2003; Bureau et al., 2004; Anderson et al., 2010; Hooks et al., 2011). However, this technique requires significant calibration to optimize both presynaptic excitation and postsynaptic response detection. This chapter details the experimental measures that were taken to maximize the accuracy of this technique, and thus support the data reported in subsequent chapters.

3.2 Assaying connectivity in neocortical networks

Electrophysiology provides an ideal tool to probe the synaptic connectivity that occurs between neurons in cortical circuits. Whilst such connectivity can be resolved through other means (Binzegger et al., 2004; Wickersham et al., 2007), electrophysiology not only confirms the presence of a connection, but also allows functional properties of the synapse to be determined; such as strength, kinetics, degree of facilitation/depression and failure rates. Electrophysiological methods have been employed to study the synaptic coupling that occurs between individual
components of the cortical network. In these experiments presynaptic activity can be directly evoked using dual/multiple whole cell recordings, stimulating electrodes, or the release of excitatory agent (Reyes et al., 1998; Feldmeyer et al., 2006; Bannister & Thomson, 2007; Le Bé et al., 2007; Silberberg & Markram, 2007; Otsuka & Kawaguchi, 2009; reviewed in Thomson & Lamy, 2007). Many of these techniques allow for fine-scale manipulation of presynaptic stimulation, and are extremely useful for studying synaptic interactions between small groups of cells (Song et al., 2005; Perin et al., 2011). However they are somewhat limited in terms of their ability to rapidly and repeatedly map synaptic inputs emanating from multiple locations across large regions of the cortical network (Ashby & Isaac, 2011). This can be overcome by the use of optical methods to excite neurons; either by glutamate uncaging, or stimulation of cells that express exogenous light sensitive ion channels and pumps (Callaway & Katz, 1993; Dantzker & Callaway 2000; Shepherd et al., 2003; Petreanu et al., 2007, 2009; Katzel et al., 2011).

3.2.1 An introduction to LSPS

Over the past twenty or so years laser scanning photostimulation (LSPS) has become a commonly used method for probing connectivity in cortical circuits (Dalva & Katz, 1994; Hooks et al., 2011). It works in a similar manner to the local administration of pharmacological agents via pressure pulse (Otsuka & Kawaguchi, 2009), however LSPS utilizes a finely calibrated laser pulse that can be targeted to alternative locations with greater rapidity (around 1ms) than if manually moving the micropipette used to administer the compounds. This facilitates studies across multiple sites, allowing researchers to repeatedly map hundreds of responses elicited across large regions of the cortical network. Light can be used to uncage a wide range of molecules (Kaplan et al., 1978; Ellis-Davies, 2008; Jerome & Heck, 2011), including excitatory molecules such as glutamate (Dalva & Katz, 1994) and inhibitory molecules such as GABA or glycine (Ueno et al., 1995; Rial Verde et al., 2008). The study of synaptic connectivity typically relies upon promoting action potentials in a presynaptic neuron, which induces a postsynaptic response in recorded downstream cells if a synapse exists between them (see however Silberberg & Markram, 2007). Therefore initial application of LSPS to the cortex focused on the focal release of
glutamate, due to its role as the main excitatory neurotransmitter in the brain (Dalva & Katz 1994; Dantzker & Callaway, 2000).

In such experiments glutamate is rendered physiologically inert through coupling to a caging molecule via a photo-scissile bond. Upon photo-excitation, and cleavage of this bond, glutamate becomes released from the caging compound and is able to act upon cells in the vicinity as it would if released from an endogenous source (Callaway & Katz, 1993). The uncaged glutamate is free to bind ionotropic glutamate receptors, which are expressed by cortical neurons at their somas and dendrites. This causes the neurons to depolarize and, if sufficient glutamate is released, will drive them to fire action potentials that can be observed in synaptically coupled cells as postsynaptic currents (PSCs) (Callaway & Katz 1993; Shepherd et al., 2003). In order to use glutamate uncaging to produce accurate maps of synaptic connectivity, it is necessary to carefully calibrate the region of presynaptic excitation in addition to addressing a number of other technical considerations outlined below:

1) Ensure the reliable excitation of presynaptic cells, but only in close proximity to the soma- to produce a high degree of inter-trial consistency and maximal map resolution.

2) Calculate the relative excitability of cells located in different cortical layers- to accurately represent the inputs that arise from each layer.

3) Account for any changes in the excitability of cells that may be caused by the ongoing maturation of the network over the course of the study.

4) Reliably detect synaptic responses, which must also be monosynaptic in nature and caused by the direct stimulation of a presynaptic neuron.

5) Collate the individual responses and assign them to a location within the cortex to produce the final synaptic input map.
3.3 The spatial resolution of LPS mapping experiments

The use of lasers to release glutamate allows the region of excitation, and therefore the resolution of the maps created, to be tightly controlled (Dal Maschio et al., 2010). The optimal resolution for uncaging studies depends upon the scientific question that is being addressed. Whilst 2-photon uncaging can achieve resolutions at the level of individual cells, or even single spines (Fino et al., 2009; Zito et al. 2009; Fino & Yuste, 2011; Jerome & Heck, 2011), mapping an entire cortical column in this way is experimentally challenging due to time constraints that occur when recording from neurons using whole-cell patch-clamp electrophysiology. Single photon UV laser uncaging can rapidly map large areas of the cortex, such as columns, whilst still providing sublaminar resolution (Dantzker & Callaway, 2000; Hooks et al., 2011). It is therefore well suited to answer the questions posed in this study about the formation of translaminar connectivity and the emergence of the canonical circuit.

The resolution of LSPS is dependant upon a number of variables. The area where glutamate will be uncaged is related to the diameter of the UV laser beam at the slice, however the area is typically expanded as UV light is scattered upon impact with the tissue. Furthermore the resolution is not calculated from the area of glutamate release, but from the area in which action potentials are elicited. For LPS experiments it is preferable that the level of presynaptic excitation at each uncaging site is robust- to ensure the majority of cells located within the region of excitation are driven to spike, and limiting the impact of any inter-trial variation in the degree of excitation. However excessive stimulation can be deleterious, producing surplus glutamate that diffuses away from the initial site of uncaging and decreases the effective resolution of experiments by activating cells across a greater area. In addition, enhanced stimulation at distal dendrites may be able to produce a sufficient somatic current to drive cells to spike, confounding attempts to correlate the site of glutamate uncaging with the somatic location of the presynaptic cell. The laser intensity at the slice must therefore be tuned to balance the requirements of robust excitation and maximal resolution. This ensures reliable activation, whilst preventing the negative effects of over stimulation. Typically this results in an actual experimental resolution of 50-100µm for most single photon uncaging studies (Callaway & Katz, 1993; Jerome & Heck, 2011).
3.3.1 Calculating the resolution of LSPS

To calculate the working resolution of the particular experimental set-up used in our laboratory, I modeled the response of the other cells in the network on individually recorded cortical neurons. This method for calibrating the spatial resolution of LSPS experiments has been previously reported by others (Dantzker & Callaway, 2000), but does assume a certain degree of homogeneity amongst other cells in the network (discussed further in section 3.4). To implement this approach I recorded from cortical pyramidal neurons in current clamp mode and fired the laser at the slice using a 50µm spaced grid pattern to target the laser. This value was chosen after a literature survey of previous uncaging experiments and corresponds to the lower bound of effective resolution for this technique (Jerome & Heck, 2011). The uncaging grid had a width of 450µm and a depth of 650-850µm.

3.3.2 Presynaptic and postsynaptic responses to glutamate uncaging

Because glutamate receptors are expressed on all cortical neurons (Hall & Ghosh, 2008), uncaging glutamate across the laser targeting grid will produce responses in both presynaptic neurons and the recorded postsynaptic cell. Therefore, glutamate uncaging gives rise to synaptic inputs, caused by stimulation of connected presynaptic neurons, and direct responses, where glutamate is released close to the soma or dendritic arbor of the recorded cell- producing a depolarization independent of synaptic input (Dantzker & Callaway, 2000; Shepherd et al., 2003). To calculate the maximum resolution of uncaging, the goal was to find the laser intensity that produced action potentials at the minimum number of uncaging sites. Essentially limiting the number of direct responses that are large enough to become suprathreshold. However to assist in recording an accurate picture of network connectivity, it was reasoned that multiple action potentials (2 or more) would be desirable at each uncaging site. This aids the detection of connectivity in multiple ways: 1 if there is any inter-trial variation in the degree of excitation, then at least one action potential will be elicited and connectivity can still be observed, 2 having multiple presynaptic action potentials facilitates the detection of weak connections (Dantzker & Callaway, 2000), which it was reasoned would be more likely to exist in the immature cortex (Bureau et al., 2004; Pengratz-Fuehrer & Hestrin, 2011), 3 if
there is any slight disparity in the excitability of pyramidal neurons that comprise the network, a higher degree of excitation helps ensure that the majority of cells would be recruited at the set laser intensity- allowing a high percentage of connections in the slice to be assayed, and building a more reliable picture of network connectivity. Therefore when tuning the laser intensity my aim was to elicit at least two action potentials at the soma, whilst limiting spike activity at other uncaging sites.

3.3.3 Maximum resolution of LPS mapping

When mapping cells in current clamp, as outlined above, I found that the minimum resolution obtainable was 50µm; so action potentials were observed when firing directly at the soma but not at other uncaging sites (fig. 3.1 A-B). In addition to action potentials at the soma, other uncaging sites showed laser-evoked depolarizations, either subthreshold direct responses, synaptic inputs, or a combination of both (fig. 3.1 B-C). In many cells the large direct responses that were observed adjacent to the soma (fig. 3.1 B-C points 1 and 3) also gave rise to action potentials (fig. 3.1 D). Often it was not possible to minimize the spatial resolution further, as altering the laser power to reduce the number of suprathreshold responses at adjacent sites was unable to maintain robust excitation at the soma (two or more action potentials).

The most consistent resolution obtainable, that fulfilled all the necessary criteria for robust presynaptic excitation (section 3.3.2), was to have action potentials elicited within 50µm adjacent to the soma, i.e. one uncaging site either side (boxed region fig. 3.1 D). This level of excitation did not produce action potentials at distal dendrites (such as in fig. 3.1 E), which would negatively influence the spatial accuracy of the maps and the allocation of synaptic inputs to individual layers. It also limited the impact of large direct responses caused by excessive stimulation adjacent to the soma (fig. 3.1 D-E). These responses can dwarf synchronously occurring synaptic responses, such that a greater number of large magnitude direct responses effectively reduces the number of useful uncaging sites and therefore the information obtainable from each map (fig. 3.1 E). A resolution of one uncaging site either side of the soma (fig. 3.1 D) was able to produce maps with sublaminar resolution and was in agreement to values produced by other laboratories using this technique (Dantzker & Callaway, 2000; Shepherd et al., 2003). For each mapped pyramidal cell the process
outlined above was repeated prior to recording synaptic inputs. This ensured that the laser power was set to provide appropriate spatial resolution and reliable presynaptic activation.

Figure 3.1

Figure 3.1 Spatial resolution of LSPS input maps. **A** extended trace showing the responses of a layer 2/3 motor cortex pyramidal neuron to LSPS mapping when recorded in current clamp mode. The soma is located at the sight where action potentials (AP) are elicited, with various subthreshold responses also highlighted (points 1-3). **B** the resulting map produced from the trace in **A**. The soma of the recorded cell is indicated by a white triangle and the site of subthreshold responses 1-3, shown in **A** and **C**, by white squares. The laminar boundaries are indicated by dashed lines. **C** expanded current clamp traces taken from the trace in **A**. The top trace shows the suprathreshold somatic response, the three traces below represent: 1 a subthreshold direct response with synaptic responses nested on top of it, 2 synaptic inputs, 3 a subthreshold direct response. The blue line represents the 100ms UV laser pulse. **D** current clamp map of a layer 5 somatosensory pyramidal cell with the laser intensity set such that action potentials are only elicited in close proximity to the soma. The location of the soma is indicated by a white triangle with laminar boundaries indicated by dashed lines. The boxed region surrounding the soma indicates the region to which action potentials were confined to ensure well-focused maps. It corresponds to one pixel (50µm) either side of the uncaging site where the soma of the recorded cell was located. **E** current clamp map of a layer 5 somatosensory cortex pyramidal neuron with the laser intensity set such that action potentials are elicited at distal sites. In this case action potentials occur outside the boxed region described in **D** and the laser settings would be of little use for accurate mapping. Soma location laminar boundaries and scale as in **D**. The number of action potentials elicited at each site in panels **B**, **D** and **E** are indicated by the colour of the pixel; sub threshold depolarizations are shown in greyscale (see scales below panels **D** and **E**).
3.4 Accounting for laminar differences in glutamate mediated excitability

Whilst the method for setting the laser intensity outlined in the previous section takes into consideration a degree of heterogeneity in the relative excitability of cells in the network, it does not account for a consistent laminar bias in the response to uncaging. Were such a discrepancy to exist, any layer with a higher threshold of excitability in response to glutamate uncaging would be underrepresented when looking at the laminar make up of inputs onto recorded postsynaptic neurons. Particularly given the inside-out formation of the cortex, with deeper layers forming earlier and therefore possibly being more mature (Angevine & Sidman, 1961; Rakic, 1974), it is necessary to ascertain the relative laser intensities required to effectively fire action potentials in cells located across layers.

3.4.1 Calculating the laser power recorded at the slice

The laser power was set by a dial on the laser module that moved a graduated neutral density filter across the path of the laser to adjust its intensity. To determine how this influenced the laser power recorded at the slice, I used a field max II laser power meter (Coherent, CA) placed under the microscope in line with the laser beam. This allowed me to plot a calibration curve for the laser power corresponding to each dial position. The dial that regulated laser intensity had five notches, with the first indicating the lowest laser power and the fifth the highest. I recorded the laser intensity when the dial was set to each of the five dial positions; in addition I estimated the position midway between each notch and made a further measurement at these points, giving a total of nine independent dial positions. At each dial position three measurements of laser power were taken and the mean average value calculated. Plotting these values revealed there was a sigmoidal pattern of laser power relative to dial position, as expected larger dial numbers correlated with greater laser power (fig. 3.2 A). For each mapped cell I recorded the dial position used to produce maps with good spatial resolution and robust excitation at the soma (see section 3.3.3 & fig. 3.1 D), the dial position was estimated down to the nearest tenth of a notch and I used this value to calculate the laser power at the slice from the calibration curve (fig. 3.2 A). This allowed me to calculate the relative laser intensity used to excite cells located in
different layers of the cortex and determine if there was any variation in their relative excitability.

**Figure 3.2**

![Graphs showing laser intensity calibration and laminar excitability](image)

**Figure 3.2 Laser intensity calibration and laminar excitability.** A plot of the laser intensity recorded at slice corresponding to different dial positions on the laser module. B plot indicating the laser power used to produce restricted excitation of layer 2/3 pyramidal cells during development. In all cases action potentials were restricted to sites within 50µm proximity to the soma. C as B but for pyramidal neurons recorded in layer 5.

3.4.2 Relative excitability of layer 2/3 and layer 5 pyramidal cells

To determine if there was any difference in the excitability of cells recorded in different layers, I recorded from 55 cells in both deep (layer 5, n=30) and superficial (layer 2/3, n=25) layers of the cortex and calculated the laser intensity used to produce finely tuned maps (such as those in fig. 3.1 D). This revealed that deep and superficial neurons showed similar excitability across the developmental period of this study (fig. 3.2 B-C). Previous studies have found that cells of layer 4 are slightly less excitable than those located in layer 2/3 (Bureau et al., 2004). To determine if this was also the case using the experimental set-up outlined herein, my supervisor, Dr Simon Butt made recordings of layer 4 neurons after I had left the lab. This showed that using our LSPS set-up layer 4 cells of the somatosensory barrel cortex are similarly excitable to those from other layers (data reported in Anastasiades & Butt, 2012). Therefore there is no laminar bias in excitability to be accounted for in this study. Because of this, laser settings calibrated to cells recorded from one layer should produce an analogous response in other pyramidal cells. By tuning the laser intensity to the excitability of the recorded neuron I could ensure that I stimulated the majority of cells in the network to a similar degree- producing restricted regions of excitation at each individual uncaging site, across all layers of the cortex.
3.4.3 Determining temporal difference in glutamate mediated excitability

The excitability of recorded neurons was found to undergo a significant shift over the course of development (fig. 3.2 B-C). It is essential to correct for this when undertaking uncaging studies, to maintain the spatial accuracy of the maps and allow for direct comparisons to be made between cells recorded at different ages. This can occur in one of two ways: either by adjusting the degree of excitation to the intrinsic properties of the cells, or using a constant laser setting and adjusting the maps according to the changing degree of presynaptic excitation (Bureau et al., 2004). The laser intensity that produced excitation profiles with appropriate spatial resolution at each age fell within a fairly narrow range, while the developmental shift in laser intensity required to reliably excite cells across development was considerable (fig. 3.2 B-C). Therefore employing the latter strategy would either give rise to maps with poor spatial resolution at immature ages (i.e. similar to those in fig. 3.1 E), or produce insufficient excitation to fire action potentials in mature cells. It was therefore necessary to set the laser intensity so that it was tuned to the intrinsic state of the recorded cells at each age.

Tuning the laser intensity to the intrinsic excitability of the recorded cell (to produce maps similar to those in fig. 3.1 D), and showing that this represents a fairly accurate picture of pyramidal cells across both deep and superficial layers (fig. 3.2 B-C and section 3.4.2), will ensure that in each individual experiment cells are excited to a similar degree and the level of presynaptic activity elicited will be the same across all uncaging sites. To confirm the consistent recruitment of cells at different stages of development, I calculated the average number of action potentials elicited when mapping pyramidal cells in current clamp at four age ranges, P5-8, P9-12, P13-16 and P17-21. This revealed that there was no significant increase in the number of action potentials elicited ($p>0.05$, P5-8 6.3±0.8, P9-12 5.7±1.5, P13-16 4.7±1.4, P17-21 8.4±1.2). Therefore any differences between the individual maps can be attributed to the synaptic inputs of each recorded cell. Calibrating the maps in this manner will allow me to make a reliable assessment of network connectivity at each stage in development, and compare the relative integration of cells located in distinct layers of the neocortex.
3.5 Analysis of postsynaptic responses

The previous sections of this chapter (sections 3.3 & 3.4) defined the spatial domain in which presynaptic excitation was elicited and how I accounted for the first two of the 5 points listed in section 3.2.1. I have also begun to look at the third point pertaining to the developmental differences in glutamate excitability (section 3.4.3). After careful calibration of the presynaptic excitation, it was next possible to look at the postsynaptic responses elicited by this activity and specifically the synaptic inputs; which must be isolated to compile the input maps for individual cells. This section will focus on how I ensured that I was recording laser evoked monosynaptic excitatory postsynaptic currents (EPSCs) at each point in development and how I separated these responses to produce the final input maps.

3.5.1 Separating direct and synaptic responses

When calibrating the resolution of the LSPS set-up (section 3.3), I observed both direct and synaptic currents in response to glutamate uncaging (see section 3.3.2 and fig. 3.1 A-C). For synaptic input mapping it is necessary to parse apart these two types of events, which is possible due to their different temporal profiles (Shepherd et al., 2003; Bureau et al., 2004). Glutamate release occurs rapidly upon laser excitation (Callaway & Katz, 1993) and is immediately able to act upon cells in the vicinity. Because of this, direct responses initiate at the start of the laser pulse and grow in magnitude over its duration (fig. 3.3 A), whilst synaptic responses occur at a delay to the initial firing of the laser; due to the requirement of first stimulating the presynaptic cell (fig. 3.3 B). Calculating the temporal domain in which this presynaptic excitation occurs allowed me to separate out and exclude direct responses, thus biasing my analyses towards laser evoked EPSCs.

3.5.2 Defining the analysis window for EPSC detection

Whilst I was unable to uncover the timing of every presynaptic action potential elicited by each laser pulse, I have shown that the response of pyramidal cells to LSPS is fairly homogeneous across the developmental period of this study (sections 3.4.2 & 3.4.3). Because of this I reasoned that the timing of presynaptic activity should be
similar to the spike times observed in each recorded neuron. As described in section 3.3.1, I fired the laser at the soma of the recorded cell to calibrate the laser intensity required to produce spatially restricted maps. I could therefore use the timing of the individual spikes in the recorded cell to predict the period during which presynaptic activity would most likely occur.

**Figure 3.3**

![Figure 3.3](image)

**Figure 3.3 Separating direct and synaptic responses based upon time of onset.** A examples of direct responses as a result of glutamate uncaging in proximity to the soma. The top and bottom traces show multiple trials from the same cell recorded at two different uncaging sites. Traces are recorded in voltage clamp at -60mV. B examples of synaptic EPSCs in response to glutamate uncaging. Each trace shows multiple trials recorded from the same cell and from the same uncaging site. The bottom four traces show consistent laser evoked EPSCs across trials. The top trace shows an uncaging site where no laser evoked EPSCs are observed, however there is a spontaneous EPSC that occurs in one of the traces indicated by the star (*) above the EPSC. The blue bar indicates the duration of the 100ms laser pulse. Each trace covers an entire one second recording epoch. The black dashed line indicates the point of initiation of direct responses closely after initiation of the laser pulse. The grey dashed line indicates the point of initiation of the earliest synaptic responses, which is delayed from the start of the direct responses.
Given that laser evoked EPSCs should be coincident with this activity, I defined the EPSC detection window as the period between the first spike and 100ms after I observed the last spike in the recorded cell (fig. 3.4 B). The addition of 100ms to the period of spike activity allows me to take into account any variability that may occur in the activation of cells across the slice, and is based upon the value used by other laboratories (Dantzker & Callaway, 2000; Bureau et al., 2004; Xu & Callaway, 2009).

**Figure 3.4**

**Figure 3.4** Postsynaptic responses to LSPS and calculating the EPSC detection window. A timing of the first and last laser evoked action potentials elicited at the soma in response to glutamate uncaging for pyramidal neurons recorded at P5-8. Data is divided into ten millisecond bins. The blue bar indicates the laser pulse. B current clamp trace of a recorded pyramidal neuron in response to the laser being fired at the soma. The EPSC detection window (highlighted in red) is calculated from the first spike, until 100ms after the last. The blue bar indicates the laser pulse. C as A but for cells recorded at P17-21. D delay profile for EPSCs recorded in response to glutamate uncaging at P5-8. EPSCs that fall within the average detection window at that age are shown in green, all others are in black. The laser pulse corresponds to the blue bar. Data is pooled into ten millisecond bins. E cumulative plot of data shown in D. F as D but for data recorded at P17-21. G Cumulative plot of data shown in F.
To support the validity of the assumptions made above, I observe that there is a marked increase in EPSC frequency during the EPSC detection window, and that this occurs in pyramidal cells recorded at both early (P5-8, n=4, fig. 3.4 D-E) and mature ages (P17-21, n=4, fig. 3.4 F-G). In total the majority of recorded EPSCs fall within the detection window (fig. 3.4 D-G, 73% of total recorded EPSCs at P17-21 and 67% at P5-8), with EPSCs occurring outside this window likely produced by factors other than the direct presynaptic activation of a connected pyramidal cell (discussed in section 3.5.3). Calculating the EPSC detection window in this way also ensures that it is based upon the intrinsic excitability of cortical neurons at the specific age the recording is made. This allows any temporal changes that occur in the response to glutamate uncaging to be taken into account. This is necessary, as I have already shown that pyramidal cells show shifts in their response to glutamate uncaging over the period of this study (fig. 3.2 B-C), and it is known that there is considerable maturation in the intrinsic membrane properties of cortical pyramidal neurons during this time (Kasper et al., 1994; Oswald & Reyes, 2008).

When comparing the spike times in response to glutamate uncaging directly at the soma, for cells recorded at the earliest stage of this study (P5-8, n=18, fig. 3.4 A) and at mature ages (P17-21, n=12, fig. 3.4 C), I find that mature cells show slightly earlier firing onset than cells recorded at immature ages (fig. 3.4 A, C). If I had not taken into account the more rapid onset of activity at mature ages, I would have missed around 38% of the laser evoked EPSCs that occurred prior to the average initial spike recorded at P5-8 (fig. 3.4 D-G). By basing the analysis window on the firing onset of each individual cell, I could take into account these developmental changes and ensure that the majority of laser evoked EPSCs are effectively recorded during the EPSC detection window.

3.5.3 Minimizing the impact of erroneous responses

Observed synaptic EPSCs were highly reproducible, and time locked to the firing of the laser (figs. 3.3 B & 3.4 D, F). This makes it highly likely that they are induced by glutamate mediated presynaptic excitation of cortical neuron, as opposed to spontaneous synaptic release. The fact that they are elicited over a similar time scale to the EPSC detection window (fig. 3.4 A-G) also supports the assumption made
earlier in this section, that presynaptic activity in the remaining cells of the cortex occurs synchronously with that of the recorded neuron. However there were also low levels of EPSCs observed throughout the duration of the recording epoch (fig. 3.4 D, F), which could be caused by spontaneous release at synaptic sites (fig. 3.3 B).

To maintain the accuracy of the maps it was important that I limit the impact of any events that might occur due to random release at non-stimulated synapses (so called spontaneous EPSCs), or through polysynaptic excitation. These responses are undesirable as they negatively influence the spatial allocation of synaptic inputs and produce an overestimation of synaptic connectivity in the recorded cell. To prevent glutamate uncaging leading to polysynaptic events, I used a high divalent cation (HDC) ACSF (4mM Mg\(^{2+}\), 4mM Ca\(^{2+}\)) that has been shown to have a dampening effect on network activity and prevent disynaptic excitation (Shepherd et al., 2003; Jin et al., 2006). Although I did not directly assay the influence of disynaptic inputs, when mapping in current clamp the region in which action potentials were observed was restricted to the immediate layer. This does not negate the potential influence of disynaptic input emanating from the host layer of the recorded cell, however it does rule out a polysynaptic influence from evoked translaminar inputs. This is because despite the strong excitatory connection known to exist between layers 2/3 and 5 (Thomson & Bannister, 1998; Hooks et al., 2011), or equally layers 4 and 2/3 (Dantzker & Callaway, 2000; Hooks et al, 2011), uncaging in either layer 2/3 or 4 did not produce suprathreshold activity in pyramidal cells that receive prominent translaminar input from these layers (see Dantzker & Callaway, 2000).

The use of the EPSC detection window (fig. 3.4 B) minimized the impact of spontaneous EPSCs on the synaptic input maps, as it excluded those events that occurred prior to action potentials being elicited in presynaptic cells, or at such a delay that they were unlikely to be caused by glutamate uncaging. Whilst this accounted for the majority of non-laser evoked EPSCs that were not coincident with the firing of the laser, any spontaneous events that fell within the detection window would end up contributing to the input maps (see starred EPSC fig. 3.3 B). To limit the impact of such events I recorded multiple trials for each cell, and averaged the sum amplitude of recorded EPSCs to produce the final input maps. Therefore
consistently evoked synaptic EPSCs would maintain their representation within the maps, whilst the influence of spontaneous events would be diminished.

3.5.4 Measuring the degree of synaptic connectivity

After recording multiple sweeps of the mapping grid for each recorded neuron, and determining the window in which EPSCs were to be expected, it was possible to analyze the results and measure the synaptic inputs. Construction of the synaptic input maps occurred off line, post recording, and was performed using minianalysis software (version 6.0.3, Synaptosoft) and custom Matlab script (Matlab R2010b, Mathworks, USA). The first step when compiling the input maps was to ensure all direct responses were excluded. Direct responses were classed as any responses that initiated at, or closely after, the start of the laser firing (fig. 3.3 A). The cut off value was calculated from the spiking activity of the recorded cell in response to direct firing of the laser at the soma (see fig. 3.4 B). Any response that initiated prior to the start of the EPSC detection for the recorded cell was rejected (typically all events initiating <60ms after the start of the laser were excluded).

In cases where direct responses were observed at the same uncaging site as synaptic inputs, attempts were made to record the synaptic responses only. However in some instances, particularly at sites directly adjacent to the soma of the recorded neuron, direct responses were sufficiently large to prevent the discrimination of individual synaptic inputs. Given there is a strong local component to synaptic input onto cortical neurons (Song et al., 2005; Perin et al., 2011), there is likely a certain underestimation of local connectivity due to it being masked by large magnitude direct responses.

To calculate the connectivity that was observed at the remaining sites, the amplitude of any observed EPSCs was measured, as this is a good indicator of synaptic strength and therefore connectivity (Shepherd et al., 2003). EPSC amplitudes were analyzed using minianalysis software (version 6.0.3, Synaptosoft), after first calibrating the search parameters so that the EPSC amplitudes calculated by the software were the same as those measured manually in clampfit (Molecular Devices, CA). Unitary EPSCs (such as that shown in the schematic response of fig. 3.5 A) could then be
reliably measured to provide the peak amplitude of evoked EPSCs. However in compound events, which were frequently observed (fig. 3.3 B), simply calculating the peak amplitude of the compound EPSC masks the actual size of each individual EPSC contained within it (fig. 3.5 B). For events of this type the double peak function in minianalysis was used to extrapolate the size of any EPSCs that occurred after the first peak, producing a more accurate representation of total input (fig. 3.5 C). To increase the accuracy of the maps, multiple files were recorded for each cell. Once all the EPSCs had been measured for each file, their properties were entered into Matlab. The custom script I developed firstly excluded all EPSCs that fell outside the predefined EPSC detection window (fig. 3.4 B). The EPSCs were then allocated to the uncaging site from which they were recorded and the results combined and averaged across trials to give the final input map for each cell.

Figure 3.5

Figure 3.5 Calculating EPSC amplitude in unitary and compound EPSCs. A example of a unitary EPSC where amplitude is measured relative to baseline. B example of a compound EPSC where amplitude is measured relative to baseline. C compound EPSC where individual amplitudes are extrapolated to yield a more accurate measure of total input. In A-C the grey dashed line indicates the point the EPSC baseline is measured from and the black dashed line the peak of the EPSC, the EPSC amplitude that would be recorded in each instance is shown to the left of each panel.

3.6 Producing the final input maps

The preceding sections of this chapter have covered how I evoked, isolated and measured the synaptic inputs onto cortical neurons elicited using LSPS. They also detail how I accounted for any changes in the excitability and temporal responses of cortical neurons as they developed. Having fulfilled each of the first four points outlined in section 3.2.1, all that remains is to explain how I organized the individual responses into the synaptic inputs maps for each cell. Firstly I will show how I ensured that the maps captured an accurate image of local columnar connectivity and then how the inputs from each uncaging site were associated with a defined laminar
coordinate, allowing the relative contribution of inputs from each layer to be determined.

3.6.1 Grid arrangement

Two grid arrangements were used in the experiments described in the following chapters. Initial mapping experiments used 117 uncaging sites arranged in a 9x13 grid, however for the vast majority of experiments an expanded grid was used with 153 spots arranged in a 9x17 grid pattern (fig. 3.6 A).

Figure 3.6

Figure 3.6 Morphological reconstructions and assigning uncaging sites to layers. A the uncaging grid used in mapping experiments with the sequence in which sites were uncaged indicated by the colour of each pixel. Sites 1-153 are represented by colours going from dark blue (1) to dark red (153). B photomicrograph of a morphologically reconstructed pyramidal cell. C photomicrograph of the recording pipette with the mapping grid overlaid. D schematic indicating how the morphological reconstruction in B and image of the mapping grid and recording pipette in C can be combined to assign morphological locations to individual uncaging sites. E and I show photomicrographs of recovered pyramidal cell morphologies at x4 magnification where the location of the cell relative to the midline and laminar location can be determined. F and G show x10 magnifications of the cells in E and I respectively. H image of a layer 5 Lucifer yellow (LY) filled pyramidal neuron taken post recording after staining with Ctip2 antibody. The LY filled cell can be seen surrounded by Ctip2 stained pyramidal cells. Scale bars in B and C correspond to 100µm in E and I they correspond to 300µm and in F, G and H they correspond to 60µm.
The enlarged grid was used as it gave a more complete coverage of the cortex. The size of the 9x17 grid was limited by the targeting area available to the galvanometers of the UGA-40 at the magnification produced by the objective lens of the microscope (10x). When using both grid sizes, if it was felt that a large portion of the map was missing attempts were made to move the grid and map the remaining region. However on occasion a portion of layer 6 input is missing from the maps for some cells. Mapping in layer 6 typically only produced a weak contribution to the inputs of recorded cells, in agreement with other studies (Hooks et al., 2011).

In addition to the size of the grid, the sequence in which individual uncaging sites are stimulated is also important. Repetitive stimulation of the same uncaging site, or those adjacent to it, could lead to the local accumulation of uncaged glutamate, which could produce receptor desensitization (Shepherd et al., 2003). To prevent this occurring, a pseudo random grid pattern was used to prevent adjacent sites being targeted in quick succession (fig. 3.6 A). Using this pattern, adjacent sites were not stimulated within 4 seconds of one another. The firing frequency of the laser was set at one Hertz to ensure that the trace from the recorded cell had returned to baseline prior to subsequent stimulation aiding reliable detection of EPSCs at each individual uncaging site.

3.6.2 Grid alignment

As discussed in chapter 1, the cortex is organized into functional units known as columns. A columnar width in rodent somatosensory cortex is approximately 300µm (Woolsey & Van der Loos, 1970; Contreras & Llinas, 2001; Shepherd et al., 2003). To ensure that I would collect an accurate picture of local columnar connectivity for all cells, I recorded from an area with a width of 450µm and situated the recorded neuron at the middle of the grid (fig 3.6 C-D). When aligning the grid consideration was made to assure that the orientation of the grid was parallel to that of the column, such that inputs were not lost by any misalignment. The organization of both columns and the neurons within them is radial (Woolsey & Van der Loos, 1970; Lefort et al., 2009; Oberlaender et al., 2011; and see fig 3.6 B, E-G), thus by aligning the grid perpendicular to the pial surface I would be able to obtain an accurate representation of columnar input across all layers (fig. 3.6 C-D).
3.6.3 Maximizing connectivity within the slice

In acute \textit{in vitro} preparation the loss of a certain degree of synaptic connectivity occurs, due to the severing of both axonal and dendritic processes during the slicing procedure (Thomson & Lamy, 2007). To minimize its effect on the assay of connectivity I ensured that the cortical region from which recordings were made had good preservation of the local columnar architecture. This was determined by choosing slices that had been cut in parallel to the orientation of the cells contained within them, such that apical processes were maintained from layer 5 neurons up towards the pial surface (fig. 3.6 B, F-G). It is known that the majority of axonal and dendritic processes are arranged in this plane (Schaefer \textit{et al.}, 2003; Oberlaender \textit{et al.}, 2011, also see fig. 3.6 B, E-G); therefore this is a good way of preserving connectivity between local neocortical neurons. Cells were also recorded a minimum of 50µm below the surface of the slice to assist the preservation of their morphology and therefore synaptic inputs. In instances where morphology could be recovered I often observed cells with intact morphologies, possessing apical dendrites extending from layer 5 up to the pial surface (fig. 3.6 B, F-G). This confirms that this approach was able to preserve translaminar connectivity within the slice.

3.6.4 Morphological reconstruction and assigning uncaging sites to layers

To correctly assign the inputs recorded at each uncaging site to a given layer, it was essential that I knew the location of the recorded cell (both its layer and cortical region), as well as its position relative to other layers. In order to calculate the relationship between the recorded cell and the individual laminar boundaries, Lucifer yellow was added to the intracellular solution so that it would be possible to create a morphological reconstruction of the cell post-recording and correlate the position of the cell with each layer of the cortex (fig. 3.6 B-H). In case the cells morphology could not be recovered photomicrographs of the location of the patch electrode were taken relative to the pial surface, such that the depth of the cell could be noted in the slice (fig. 3.6 C). For each map a single uncaging site was aligned above the soma of the cell and the grid number of this site noted. Once the relationship between the soma of the recorded cell and the laminar boundaries had been determined, sites located
deeper or more superficially to the soma could be assigned to the correct layer, as there was a set distance of 50µm between each uncaging site (fig. 3.6 C-D).

For cells where the morphology of the recorded cell could not be recovered, notes were taken at the time of recording on the estimated location of the cell; both the predicted laminar location and anatomical region, somatosensory cortex being located lateral to motor cortex, which is adjacent to the midline (Paxinos et al., 2007). The depth of the cell could be calculated from the photomicrographs taken of the location of the recording pipette relative to the pial surface (fig. 3.6 C-D). In many cases it was also possible to visualize the laminar boundaries as dark and light bands that ran along the cortex, and thus assign the soma position and individual uncaging sites to the correct layer. To further assist with this process, Ctip2 staining was performed on fixed tissue to measure the relative contribution of individual layers to the overall cortical thickness at early and late developmental time points (fig. 3.7 A-B).

Figure 3.7

**Figure 3.7 Contribution of deep layers to cortical depth at P7 and P17.** A-B fixed cortical tissue stained with Ctip2 in red and DAPI in blue. Dashed lines indicate the pial surface and border with the white matter as well as layer 5 (L5). In each case the left image shows an overlay of both blue and red channels whilst the image to the right has the DAPI channel removed to more clearly show the region of Ctip2 staining. Tissue in A is taken at P7 whilst B is taken at P17. Scale bars at bottom left in both A and B correspond to 100µm.
Ctip2 is selectively expressed in pyramidal cells of deep cortical layers, particularly those in layer 5 (Molyneaux et al., 2007; Lickiss et al., 2012). This meant that it would be possible to calculate the relative contribution of deep (layers 5/6) and superficial (layers 2/3 and 4) layers to the overall cortical thickness. In some cases Ctip2 staining was also performed on slices post recording, allowing the location of the recorded cell to be directly determined relative to layer 5 (fig. 3.6 H).

For cells whose morphology could not be recovered it was possible to assign both the layer of the cell and the laminar boundaries based upon the photomicrograph of the location of the recording pipette (fig. 3.6 C), the calculated contribution of each layer at the age the recording was made (fig. 3.7 A-B) and data acquired from other cells whose morphology had been recovered, and were mapped from the same cortical region, at the same age.

### 3.7 Chapter summary

The accurate application of LSPS requires that certain calibration steps are taken and technical considerations made. To this end I have outlined the steps undertaken to ensure the robust excitation of cortical pyramidal neurons, with restricted spatial localization of action potentials to sites directly adjacent to the soma. By showing that pyramidal neurons located across deep and superficial layers of the cortex respond to glutamate uncaging in a similar manner, it is highly likely that by calibrating the laser to the recorded cell I will produce spatially restricted excitation at the remaining uncaging sites. Calibrating to the recorded cell also allowed me to take into account the changes that occur in the excitability of cortical neurons over development, and thus ensure that I stimulate cells to a similar degree regardless of age. This lets me make direct comparisons between the connectivity observed in immature and mature neurons in the following chapters.

By employing a window for EPSC detection I minimize the contribution of spontaneous and disynaptic EPSCs, the later being further prevented through the use of high divalent cation ACSF. The start and finish time of this window were calculated from the response of the recorded cell to direct glutamate uncaging at its soma. This was also based on the idea that other cells in the network would respond in
a similar manner to the recorded cell. Support for this was found in the time locked nature of recorded EPSCs, with the vast majority falling within the allocated detection window- indicating that presynaptic activity was coincident with that observed in the recorded cell. Once synaptic EPSCs could be isolated, I outlined how I accurately recorded their amplitudes and combined them to create the synaptic input maps.

To produce a laminar profile of the inputs recorded onto each cell it was necessary to correlate each uncaging site with an individual cortical layer. This was made possible by both intracellular filling of the recorded cells, to allow visualization post-recording, taking photomicrographs of the location of the recording pipette relative to laminar boundaries, and by using laminar specific antibodies to build up a picture of the relative contribution from deep and superficial layers of the neocortex at different ages. Having taken these necessary steps, it was possible to directly compare the synaptic inputs onto cortical neurons of different subtypes, in different layer of the cortex, across postnatal development. This will form the focus of the remaining chapters of this thesis, where I shall discuss the subtype specific integration of cortical neurons and the maturation of individual components of the canonical circuit.
Chapter 4- Postnatal network maturation of neocortical pyramidal neurons

4.1 Introduction

The neocortex is a six-layered structure, where each individual layer possesses distinct patterns of intracortical and subcortical connectivity (chapter 1, section 1.2; Gilbert & Wiesel, 1983; Douglas & Martin, 1991; Hooks et al., 2011). The development of the cytoarchitecture of the neocortex is relatively well understood (Angevine & Sidman, 1961; Rakic, 1971; Rakic, 1974; Hatten, 1999) and a number of studies have highlighted physiological processes that are important for the synaptic integration of cells into the developing network (Adesnik et al., 2008; Wang & Kriegstein, 2008; Gray et al., 2011; Kwon & Sabatini, 2011). However what is lacking at present is a global understanding of the organization of synaptic inputs within the neocortex as it matures. This would not only allow us to track the emergence of the canonical pattern of network connectivity (Gilbert & Wiesel, 1983; Douglas & Martin, 1991), but also provide greater insight into the circuitry that underlies the early activity patterns of the neocortex (chapter 1, section 1.4.1; Allene et al., 2008; Golshani et al., 2009; Yang et al., 2009).

Pyramidal cells are the predominant neuronal component of the cortical network, contributing around 80% to the total complement and populating every layer of the cortex with the exception of layer 1 (Micheva & Beaileau, 1995). Their prominent position within the network makes them an ideal subject for studies attempting to gain a broad understanding of network maturation. Furthermore, by uncovering the way pyramidal cells integrate into the network, it will provide a benchmark against which other cell types can be compared, such as interneurons in the following chapters. To produce this developmental overview of the cortical network I chose to look at the electrophysiology and synaptic connectivity of pyramidal cells located in different layers and regions of the neocortex. To perform these studies I used whole-cell patch-clamp recordings, in conjunction with laser scanning uncaging of glutamate (LSPS). Previous developmental studies have primarily focused on pyramidal cells located in individual layers and regions (Kasper et al., 1994; Oswald & Reyes, 2008), or on specific pathways; such as the maturation of feed-forward connectivity from layer 4...
of barrel cortex (Bureau et al., 2004), or the integration of the subplate (Viswanathan et al., 2012). By extending these studies to look at the concurrent maturation of both deep and superficial layers, in addition to the development of somatosensory and motor cortex circuits, I can consolidate these previous studies into a more global overview of cortical maturation.

The second part of this chapter will focus on two factors that are important for the synaptic integration of cortical pyramidal neurons—activity and NMDA receptors (Katz & Shatz, 1996; Adesnik et al., 2008; Wang & Kriegstein, 2008; Gray et al., 2011). Over the course of development there is a gradual transition from synapses that are primarily dominated by NMDA receptor (NMDAR) mediated currents, to synapses whose composition includes a prominent AMPA receptor (AMPAR) component (Isaac et al., 1997; Hsia et al., 1998; Rumpel et al., 2004; Ashby & Isaac, 2011). The AMPAification of synapses can be driven by activity (Liao et al., 1995, 1999, 2001; Durand et al., 1996; Isaac et al., 1997), such that the ongoing activity of the cortical network during early development (Allene et al., 2008; Golshani et al., 2009; Yang et al., 2009) likely drives the synaptic insertion of AMPA receptors (Wang & Kriegstein, 2008). Although the prevalence of NMDAR mediated inputs and silent synapses at early stages of development is well known (Isaac et al., 1995; Rumpel et al., 1998, 2004), little is understood about the laminar source of silent inputs outside of layer 4 (Ashby & Isaac, 2011). Furthermore, whilst synchronous network activity abounds within the cortex during the period where silent synapses are prevalent (Rumpel et al., 2004; Allene et al., 2008; Golshani et al., 2009), the impact of such activity on the synaptic integration of pyramidal neurons and its role at later ages is less certain, as the network remains plastic once NMDAR mediated silent synapses have disappeared from the cortex (Markram et al., 1997; Sjostrom et al., 2001; Rumpel et al., 2004), and there are multiple types of plasticity that are preferentially observed at different ages within developing cortical networks (Yasuda et al., 2003; Groc et al., 2006).

The experiments described in the following sections of this chapter will firstly track the maturation of pyramidal cell intrinsic membrane properties and the timescale over which glutamatergic inputs emerge in these cells. I will then report on the role NMDA
Chapter 4- Postnatal network maturation of neocortical pyramidal neurons

receptors and activity play in the ongoing maturation of the network during the early postnatal period.

4.2 Electrophysiological maturation of neocortical pyramidal neurons

To determine the changes that occur to the intrinsic electrophysiology of cortical pyramidal neurons during postnatal development, I performed whole-cell patch-clamp recordings from neocortical pyramidal cells located in layers 2/3 and 5 between postnatal days (P) 5-21. By calculating the relative maturity of cells in individual layers of the cortex, I could assess if there is any difference that occurs due to the late specification of superficial layers relative to deep layers (Angevine & Sidman, 1961; Rakic, 1974). By recording from both somatosensory and motor cortices I could also determine if the development of intrinsic membrane properties differed between pyramidal cells located in distinct cortical regions.

In total 124 pyramidal neurons were recorded, which were spread between layer 5 (n=64) and layer 2/3 (n=60) of motor (n=64) and adjacent somatosensory cortices (n=60). Cells were recorded between P5-21 with the recording period arbitrarily divided into four equal bins (P5-8, P9-12, P13-16, P17-21). Figure 4.1 (A-D) shows four pyramidal cells recorded from the somatosensory cortex during each of the four developmental windows of this study. At first glance it may appear that the traces appear somewhat similar. However, upon closer inspection it is possible to discern that there are changes that occur over the course of development. At more mature ages the individual spikes become taller and narrower and the current input required to elicit each spike train becomes larger (fig. 4.1 A-D). The differences in the individual spikes are more clearly illustrated by the expanded traces (fig. 4.1 E), where spike height and spike half-width can be more easily compared. In order to look in more detail at the changes that occurred over the course of development, a number of commonly used electrophysiological parameters were calculated from each of the current injection protocols outlined in the materials and methods (chapter 2, section 2.3). This allowed me to compare these values at different ages, for cells recorded in different layers and regions.
4.2.1 Postnatal development in the intrinsic membrane properties of motor cortex pyramidal neurons

Within layer 2/3 of the motor cortex (Mctx), pyramidal cells possessed a number of parameters that exhibit significant maturation between P5-8 and P17-21 (table 4.1). The resting membrane potential of recorded neurons becomes more hyperpolarized ($p<0.01$), as does the spike threshold ($p<0.05$). Individual action potentials are taller ($p<0.05$), and narrower ($p<0.01$), and the initial firing frequency within the first 100ms becomes faster ($p<0.05$), which brings about a significant increase in the percentage adaptation ($p<0.05$).
### Table 4.1

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<td>26.2 ±2.0</td>
<td>30.6 ±2.6</td>
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<td>27.3 ±2.0</td>
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<td>30.8 ±3.5</td>
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<td>49.4 ±6.6</td>
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<td><strong>Tau (ms)</strong></td>
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<td>84.1 ±9.3</td>
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<td></td>
<td>57.7 ±7.3</td>
<td>30.1 ±7.6</td>
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<tr>
<td><strong>AHP time (ms)</strong></td>
<td>100.3 ±11.9</td>
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**Table 4.1 Electrophysiological development of motor cortex pyramidal cells.** Average values of commonly used electrophysiological parameters for motor cortex pyramidal cells recorded in layer 2/3 and 5. Cells are divided into four age ranges, postnatal day 5-8, 9-12, 13-16 and 17-21. The number of cells recorded at each age is indicated in brackets. RMP resting membrane potential, R_in input resistance, Max. Freq. maximum firing frequency, Initial Freq. maximum frequency in first 100ms of current pulse, Final Freq. maximum frequency in last 100ms of current pulse, AHP delay, delay to peak of action potential afterhyperpolarization, AHP amp, peak amplitude of action potential afterhyperpolarization. Values indicate the mean average ± SEM. Greyscale shading indicates degree of significance for cells recorded in the same layer relative to P5-8. Light grey p<0.05, darker grey p<0.01, darkest grey p<0.001. Boxes with no colour show no significant difference relative to P5-8 (p>0.05). Red boxes indicate parameters that are significantly distinct when compared between layer 2/3 and layer 5 motor cortex pyramidal cells at a given age (p<0.05).
The membrane time constant (tau) also decreases significantly over this period ($p<0.05$). The remaining properties are varied in their development. Some appear to be stable across the entire period, whilst others show developmental trends that are not statistically significant (table 4.1). The average spike delay is fairly consistent across development, whilst input resistance shows a trend towards lower values relative to P5-8 at both P13-16 and P17-21 ($p=0.06$ for both). Maximum firing frequency across the entire 500ms pulse shows a trend towards higher values at larger ages, but the values within the last 100ms are almost identical (table 4.1). The action potential afterhyperpolarization (AHP) of the cells has a relatively constant time to peak over the course of development, but the amplitude decreases as cells mature, being significantly smaller relative to P5-8 at P9-12 ($p<0.05$) and P17-21 ($p<0.01$).

Layer 5 motor cortex pyramidal cells also show considerable maturation in their intrinsic membrane properties (table 4.1). The spike threshold becomes significantly hyperpolarized ($p<0.01$), whilst spike height increases ($p<0.01$) and spike half-width values become narrower ($p<0.001$). In contrast to layer 2/3 neurons, the maximum firing frequency during the initial 100ms and final 100ms are both enhanced at later ages ($p<0.001$ and $<0.05$ respectively). This brings about a significant increase in overall maximum firing frequency ($p<0.01$), but as both initial and final maximum firing frequencies mature to a similar degree, there is no significant shift in percentage adaptation (which is the ratio of the two values). In addition to shifts in the spike dynamics of the cells, the passive properties also undergo significant maturation, with both input resistance and tau decreasing over the course of development ($p<0.001$ and $<0.05$ respectively). The resting membrane potential shows a significant shift relative to P5-8 at P13-16, but not at other ages. There appears to be a trend towards more hyperpolarized potentials up until this point, with a jump to a more depolarized value occurring at P17-21. As found in layer 2/3 cells, spike delay is relatively constant through development ($p>0.1$ across all ages), with a slight, non-significant drop in delay in the latest age group. Also in agreement with layer 2/3 pyramidal cells, the time taken to reach the trough of the action potential afterhyperpolarization shows no significant enhancement over development, and there is a significant decrease in the amplitude of the spike AHP (table 4.1, $p<0.05$ at P17-21 and $p<0.01$ at P13-16).
4.2.2 Postnatal development in the intrinsic membrane properties of somatosensory cortex pyramidal neurons

Layer 2/3 somatosensory cortex (SSC) pyramidal neurons show a similar pattern of development to their counterparts in the motor cortex (table 4.2). Again there is considerable maturation in the resting membrane potential, which becomes more hyperpolarized between P5-8 and P17-21 \((p<0.01)\). Over the same period action potentials become taller and narrower \((p<0.01\) and \(<0.05\) respectively) and are fired at higher rates across the duration of the 500ms current pulse \((p<0.01)\). As was found in layer 2/3 cells of the motor cortex, the initial firing frequency is significantly enhanced over the course of development \((p<0.01)\), whilst the final firing frequency is not (although there is a trend towards faster final firing frequencies between P5-8 and P17-21, \(p=0.07\)). Adaptation also shows a trend towards greater percentage adaptation at later ages (table 4.2, \(p=0.07\)). Passive properties tau and input resistance change over development, with values for both parameters becoming significantly lower \((p<0.05)\). AHP amplitude and the time to peak were not significantly altered over the course of development, neither was the average spike delay \((p>0.05)\).

The intrinsic membrane properties of layer 5 pyramidal cells of the somatosensory cortex also develop considerably between P5-8 and P17-21 (table 4.2). The action potential properties of the cells undergo strong enhancement, becoming taller \((p<0.01)\), narrower \((p<0.05)\) and firing at faster rates across both the initial 100ms, final 100ms and entire duration of the 500ms current pulse \((p<0.01, \text{table 4.2})\). There is also a significant increase in adaptation \((p<0.05)\) and the spike threshold becomes more hyperpolarized \((p<0.01)\). Input resistance and tau both show maturation, becoming significantly lower at P17-21 when compared to those cells recorded at P5-8 \((p<0.01, \text{table 4.2})\). Resting membrane potential shows a trend towards more hyperpolarized values, but this is not found to be significant \((p>0.1)\). The AHP time to peak shows a non-significant trend towards slower values at older ages and the AHP amplitude is found to diminish significantly between P5-8 and P17-21 \((p<0.001)\). As with all pyramidal cells located in other layers or regions, there is no developmental change in spike delay.
Table 4.2: Electrophysiological development of somatosensory cortex pyramidal cells. Average values of commonly used electrophysiological parameters for somatosensory cortex pyramidal cells recorded in layer 2/3 and 5. Cells are divided into four age ranges, postnatal day 5-8, 9-12, 13-16 and 17-21. The number of cells recorded at each age is indicated in brackets. RMP resting membrane potential, $R_{in}$ input resistance, Max. Freq. maximum firing frequency, Initial Freq. maximum frequency in first 100ms of current pulse, Final Freq. maximum frequency in last 100ms of current pulse, AHP delay, delay to peak of action potential afterhyperpolarization, AHP amp, peak amplitude of action potential afterhyperpolarization. Values shown are mean average ± SEM. Greyscale shading indicates degree of significance for cells recorded in the same layer relative to P5-8. Light grey $p<0.05$, darker grey $p<0.01$, darkest grey $p<0.001$. Boxes with no colour show no significant difference relative to P5-8 ($p>0.05$). Red boxes indicate parameters that are significantly distinct when compared between layer 2/3 and layer 5 somatosensory cortex pyramidal cells at a given age ($p<0.05$).
4.2.3 Comparison of the intrinsic membrane properties of layer 2/3 and 5 motor cortex pyramidal cells

The previous sections showed that the properties of pyramidal cells undergo maturation regardless of their location, however I wanted to assess how their properties might be different based upon where they were recorded. Firstly, I compared the parameters mentioned in the previous section (see tables 4.1 & 4.2), for cells recorded in the same region but in different layers. Within the Mctx the properties of layer 2/3 and layer 5 pyramidal cells were broadly comparable. There was however a significant difference at P9-12 in spike height, input resistance and initial firing frequency (table 4.1, all $p<0.05$). There was also a significant difference in spike height at P17-21; although this seems to be driven by an unexplained dip in spike height in layer 5 neurons recorded at this time (tables 4.1), which may in part be due to the small sample size at this age ($n=4$). The AHP properties of the cells were similar across development, the only exception being P5-8, where the AHP time to peak was significantly faster in layer 5 cells than layer 2/3 ($p<0.05$). Tau appears to show a gradual divergence between the layers, with layer 5 values below layer 2/3 from P9-12 onwards before becoming significantly distinct at P17-21 ($p<0.05$). This appears to be the only noticeable distinction in the maturation of the layers as none of the other differences fall into any clear developmental trend, and the remaining parameters showing no significant separation based on the layer cells were recorded from ($p>0.1$).

4.2.4 Comparison of the intrinsic membrane properties of layer 2/3 and 5 somatosensory cortex pyramidal cells

In the SSC there was a greater degree of interlaminar variation in the intrinsic membrane properties of recorded pyramidal cells compared to Mctx (table 4.1 & 4.2). Differences were found in spike threshold and spike height at P5-8 ($p<0.05$), spike half-width, input resistance and maximum firing frequency at P9-12 ($p<0.05$) and resting membrane potential, spike threshold, spike height, input resistance and adaptation at P13-16 ($p<0.01$ for resting membrane potential and spike height, all others $p<0.05$). There was also a strongly significant difference in AHP amplitude at early ages, with layer 5 possessing much larger AHPs at P5-8 and P9-12 ($p<0.001$).
However the ongoing maturation of the cells brought this value in line with those of layer 2/3 by P13-16 (table 4.2). As found in Mctx the majority of these differences were not part of any observable trend, with each parameter showing little variation between layers at the remaining ages (tables 4.1 & 4.2). However, spike height, input resistance, AHP amplitude and spike threshold were significantly different at two of the four developmental windows, suggesting they may trend towards a consistent developmental divergence. The resting membrane potential was also more hyperpolarized in layer 2/3 post P5-8, although only significantly so at P13-16. Layer 5 trended towards taller spikes, more hyperpolarized spike threshold and up until P17-21 lower input resistance (table 4.2). Overall it seems that there was an increase in the number of parameters that were significantly different at P9-12 and P13-16 relative to other ages. This is suggestive of some developmental divergence between the layers at this time and all in all it appears that the somatosensory cortex has a greater degree of variation in the intrinsic electrophysiology of deep and superficial layers than the motor cortex.

4.2.5 Comparison between the intrinsic membrane properties of pyramidal cells recorded in motor and somatosensory cortex

In order to determine if there were any differences caused by recording from distinct cortical regions, I compared the values for each of the properties in table 4.1 and 4.2 for cells recorded in motor and somatosensory cortex. Comparing age matched samples recorded from the same layer revealed a strong similarity in the intrinsic membrane properties of cortical pyramidal neurons across all values and over the entire developmental period. The vast majority of parameters showed no statistical difference ($p>0.1$). When comparing between layer 2/3 cells of SSC and Mctx, the only exceptions were spike width at P9-12, tau at P17-21 and AHP amplitude at P5-8 and P17-21 (all $p<0.05$). In layer 5 the only significant differences were spike threshold, spike height and AHP amplitude at P5-8 (spike threshold $p<0.01$, spike height and AHP amplitude $p<0.05$). The values reported at other ages for these parameters were similar across development (table 4.1 and 4.2).
4.2.6 Variation in voltage sag in response to hyperpolarizing currents

One characteristic that showed both a regional and laminar difference was the presence of a rebound spike in response to hyperpolarizing current (fig. 4.2 A-B). Rebound spikes were found in cells recorded from both layer 2/3 and 5 and only ever produced a single spike (fig. 4.2 A). Amongst those cells that exhibited a spike, there was a bias towards cells recorded from deep layers, with 2/60 (3.3%) layer 2/3 pyramidal cells possessing a rebound spike, compared to 11/64 (17.2%) layer 5 cells. Also within layer 5 there was a greater prevalence of rebound spikes in the SSC relative to Mctx (layer 5 SSC 8/35 or 22.9%, versus 3/29 or 10.3% in layer 5 of Mctx). This was unlikely due to any significant difference in the resting membrane potentials of cells recorded from the two regions, because as we have seen their properties appear similar across development (see previous section and tables 4.1 and 4.2). Nor was it due to any difference in the degree of hyperpolarizing current injected into the cells, with the average minimum of the hyperpolarizing deflection reaching within 1mV of -100mV for all age ranges in both Mctx and SSC ($p>0.05$ across all age ranges).

The presence of rebound spikes is in part due to the $I_h$ current, which can activate low threshold currents ($I_T$) upon release from hyperpolarized potentials (Huguenard & McCormick, 1992; Mitra & Miller, 2007). The $I_h$ current contributes to the voltage sag in response to hyperpolarizing current steps (see fig. 4.2 A). I found that across all layer 5 cells those that possessed a rebound spike ($n=11$) had a significantly enhanced voltage sag in response to hyperpolarizing currents compared to non-rebound spiking cells ($n=53$) (fig. 4.2 A-B, $p<0.001$, rebounding cells 12.6mV±1.1, non-rebounding cells 6.9mV±0.6). Given that rebound spikes were biased towards layer 5 of the SSC, I compared the magnitude of this voltage sag for cells recorded in each layer and cortical region. In the somatosensory cortex the amplitude was significantly enhanced in deep layer cells relative to layer 2/3 pyraminals at the first three age ranges (fig. 4.2 C, $p<0.05$ for P5-8 and P9-12, $p<0.01$ at P13-16. SSC layer 2/3 voltage sag P5-8 3mV±1, P9-12 3mV±1, P13-16 2mV±0.5. SSC layer 5 voltage sag P5-8 11mV±1, P9-12 9mV±1, P13-16 9mV±1). At P17-21 there was a non-significant trend towards a larger voltage sag in layer 5 (fig. 4.2 C $p=0.18$, SSC layer 2/3 voltage sag P17-21 0.5mV±0.1, SSC layer 5 voltage sag P17-21 5mV±3).
In the motor cortex the only significant difference was at P5-8 where layer 2/3 cells showed enhanced sag amplitude relative to layer 5 (fig. 4.2 D, \( p < 0.05 \), Mctx layer 2/3 voltage sag P5-8 12mV±2, Mctx layer 5 voltage sag P5-8 6mV±2). At later ages the value for layer 5 pyramidal cells was greater than layer 2/3 cells, although this was not found to be significant (\( p > 0.05 \) across all ages, Mctx layer 2/3 voltage sag P9-12 5mV±2, P13-16 3mV±1, P17-21 3mV±2, Mctx layer 5 voltage sag P9-12 8mV±2, P13-16 5mV±2, P17-21 6mV±1). The amplitude of the voltage sag in response to hyperpolarizing current in layer 5 neurons of the SSC was larger than that of layer 5 neurons in the Mctx across the whole developmental period of this study; although the values were only significantly enhanced at P5-8, and P13-16 (\( p < 0.05 \)).

**Figure 4.2**

A-B Current clamp responses of layer 5 somatosensory cortex and motor cortex pyramidal cells in response to hyperpolarizing and depolarizing current steps. The cortical region the cell was recorded from is indicated above the trace and the current input that elicited each response is shown to the right of each panel. The current pulses in each trace were of 500ms duration. A somatosensory cortex pyramidal cell showing a pronounced voltage sag in response to hyperpolarizing current steps taken to -100mV. In some cells this gave rise to a rebound spike upon release from the hyperpolarized potential (indicated by arrow). B motor cortex pyramidal cell showing a lack of pronounced voltage sag in response to hyperpolarizing current steps to -100mV. C-D show the average voltage sag amplitude for cells recorded across development for cells recorded in layer 2/3 and 5 of the somatosensory and motor cortex respectively. Error bars are ± SEM, *\( p < 0.05 \) **\( p < 0.01 \).
These differences seem to explain the increased prevalence of rebound spiking amongst layer 5 neurons of the SSC, which is likely brought about by a greater prevalence of a voltage sag in response to hyperpolarizing current (and presumably a larger $I_{h}$) in cells recorded from layer 5 of the SSC.

4.2.7 Diversity amongst pyramidal neurons, emergence of cells with an initial double spike couplet

Amongst pyramidal cells of the cortex there are a number of distinct electrophysiological firing types (Chagnac-Amitai et al., 1990; Connors and Gutnick, 1990; Schubert et al., 2001). These are categorized as either regular spiking (RS), intrinsic bursting (IB) or those cells with an initial doublet (ID) (Schubert et al., 2001). This electrophysiological diversity is known to emerge at the later developmental ages of this study (Kasper et al., 1994; Flint et al., 1997). In this study I only recorded one IB cell at P19, but the data from this cell was not included in that reported above. I did however record a number of ID cells, which had a spike couplet at the beginning of a train of action potentials (fig. 4.3 A). These cells were observed in all layers and regions examined and showed increased prevalence at later developmental ages. Amongst cells recorded at P5-8 no ID cells were observed (0/35 cells), by P9-12 there were 3 cells (3/41 or 7.7%) and by the later two ages initial double cells made up 25% of the recorded population (8/32 at P13-16 and 4/16 at P17-21). The remaining cells recorded all showed regular spiking firing properties (fig. 4.3 B).

Figure 4.3

Figure 4.3 Electrophysiological diversity of recorded pyramidal cells. A-B current clamp recordings of cortical pyramidal cells showing an initial double and a regular spiking firing pattern respectively. The current pulse in each trace was of 500ms duration. The current input that elicited each response is shown to the right of each panel.
4.3 Maturation of excitatory inputs onto cortical pyramidal neurons

The regions of the cortex recorded from in the previous section are known to possess distinct patterns of synaptic connectivity at mature ages (Hooks et al., 2011). However, less is known about the relative timescales over which these networks develop. To study this phenomenon in more detail I made use of LSPS to uncage glutamate and map excitatory connections onto cortical pyramidal cells (Dantzker & Callaway, 2000; Shepherd et al., 2003; Bureau et al., 2004). This allowed me to assay connectivity within the local cortical network with sublaminar resolution (see chapter 3, section 3.3) and therefore build up a picture of how pyramidal cells located within different layers and regions of the cortex connect to the network throughout postnatal development. LSPS was applied to map excitatory inputs onto pyramidal cells located in deep (layer 5) and superficial (layer 2/3) cortical layers, in both the motor and adjacent somatosensory cortices. By recording from cells located in both these cortical regions, throughout postnatal development, it was possible to compare the spatial organization of the inputs onto cells at different ages, and the developmental trajectories they take as they integrate into the surrounding network.

55 pyramidal neurons were recorded, of which 28 were located in deep (layer 5) and 27 in superficial (layer 2/3) cortical layers. The cells were also fairly evenly distributed between somatosensory (29 cells) and adjacent motor cortex (26 cells). Cells were recorded over the same timeframe as those reported in the preceding section (P5-21), and likewise were subdivided into four equal groups based upon the age the recordings were made (P5-8, P9-12, P13-16, P17-21). Recorded neurons were also separated based upon the layer and region in which their soma was located. To breakdown the organization of the input maps I calculated the percentage of total input and the pA/pixel values for inputs arising from each layer (pA/pixel being the total input from each layer divided by the number of pixels that were located within that layer). I also counted the total number of EPSCs recorded for each cell and then calculated the average EPSC amplitude by dividing the sum input across all layers (in pA) by the number of EPSCs.
4.3.1 Laminar organization of excitatory inputs onto somatosensory cortex pyramidal neurons over development

Layer 2/3 somatosensory cortex pyramidal neurons at P5-8 (n=4) receive the highest percentage of their inputs from within layer 2/3 (fig. 4.4 A, C, layer 2/3 input 47%±5, layer 4 14%±4, layer 5/6 38%±4). The inputs from each layer are of similar magnitude (fig. 4.4 C, layer 2/3 input 3±1 pA/pixel, layer 4 3±1 pA/pixel, layer 5/6 3±2 pA/pixel) and are typically of small amplitude (fig. 4.4 B).

**Figure 4.4**

*Figure 4.4 LSPS maps of excitatory inputs onto layer 2/3 somatosensory pyramidal neurons pre P12. A and D show example input maps of layer 2/3 somatosensory cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A the maximum input on the scale bar corresponds to 20pA and for D 30pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. White circles indicate large magnitude direct responses where synaptic input could not be reliably discerned. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P5-8. F shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P9-12. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.*
At P9-12 (n=3) the main source of input is again from within layer 2/3, however there is a slight increase in the amount of layer 4 input (fig. 4.4 F, layer 2/3 input 40%±8, layer 4 22%±9, layer 5/6 39%±17). In some cells there is also an elevation in the pA/pixel values for inputs emanating from layer 4 (fig. 4.4 F, layer 2/3 input 4±2 pA/pixel, layer 4 7±4 pA/pixel, layer 5/6 3±1 pA/pixel). Cells still receive inputs from all layers of the cortex and as was found at P5-8, appear to be fairly diffusely integrated (fig. 4.4 A, B, D, E).

By P13-16 (n=3) layer 2/3 remains the main source of input, although there is a continued maturation in the percentage of input from layer 4 (fig. 4.5 A-C, layer 2/3 input 45%±10, layer 4 25%±8, layer 5/6 30%±5). This enhancement in input from layer 4 is also seen in the increase in pA/pixel values from that layer, such that these are the strongest inputs onto the majority of layer 2/3 pyramidal cells recorded at P13-16 (fig. 4.5 C, layer 2/3 input 8±0.5 pA/pixel, layer 4 12±5 pA/pixel, layer 5/6 6±3 pA/pixel). This trend continues into the P17-21 window (n=3), and by this stage layer 4 is now the primary input layer to layer 2/3 pyramidal cells; both in terms of the percentage input (fig. 4.5 D-F, layer 2/3 input 30%±3, layer 4 44%±1, layer 5/6 26%±3) and strength of inputs per pixel (fig. 4.5 E-F, layer 2/3 input 6±1 pA/pixel, layer 4 15±6 pA/pixel, layer 5/6 4±1 pA/pixel).

These data suggest that over the course of development there is a gradual increase in the influence of layer 4 inputs onto layer 2/3 pyramidal cells. This is confirmed by statistical analysis, which shows the percentage of total input received from layer 4 at P17-21 is significantly enhanced relative to all other ages (p<0.05 relative to P13-16 and P9-12 and p<0.01 compared to P5-8). There is also a significant decrease in the percentage of total inputs from layer 2/3 and 5/6 at P17-21 relative to P5-8 (p<0.05 for both). The pA/pixel values for input from layer 4 are also significantly enhanced at P17-21 relative to P5-8 (p<0.01), with a trend towards greater values at P13-16 relative to P5-8 (p=0.056). The pA/pixel values from layer 2/3 also trends towards greater values at older ages (layer 2/3 pA/pixel between P9-12 and P13-16 p=0.053 and between P13-16 and P17-21 p=0.071), with a significant enhancement between P13-16 and P5-8 (p<0.05).
Figure 4.5

Figure 4.5 LSPS maps of excitatory inputs onto layer 2/3 somatosensory pyramidal neurons post P12. A and D show example input maps of layer 2/3 somatosensory cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A and D the maximum input on the scale bar corresponds to 50pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. White circles indicate large magnitude direct responses where synaptic input could not be reliably discerned. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P13-16. F shows the percentage of total input and pA/pixel values for input from layers 2/3, 4 and 5/6 received by all cells recorded at P17-21. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

Looking at the inputs onto layer 5 pyramidal neurons of the somatosensory cortex, at P5-8 (n=5) I find that the primary source of input is from within layer 5/6 (fig. 4.6 A-C, layer 2/3 input 40%±8, layer 4 14%±3, layer 5/6 46%±11). The inputs onto layer 5 cells at this age are typically of small magnitude and diffusely spread across the mapping area, with their strength evenly matched across layers (fig. 4.6 B-C, layer 2/3 input 3±1 pA/pixel, layer 4 3±1 pA/pixel, layer 5/6 2±0.5 pA/pixel). At P9-12 (n=4) the total input is primarily shared between 5/6 and layer 2/3, with a lesser contribution from layer 4 (fig. 4.6 F, layer 2/3 input 40%±7, layer 4 20%±3, layer 5/6 40%±4). At P9-12 the pA/pixel values across all layers trend towards stronger inputs relative to
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P5-8 as cells begin to integrate into the network (fig. 4.6 F, layer 2/3 input 6±2 pA/pixel, layer 4 10±4 pA/pixel, layer 5/6 6±2 pA/pixel).

Figure 4.6

Figure 4.6 LSPS maps of excitatory inputs onto layer 5 somatosensory pyramidal neurons pre P12. A and D show example input maps of layer 5 somatosensory cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A the maximum input on the scale bar corresponds to 20pA and for D 25pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the laser targeting grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms in B and 200ms in E. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P5-8. F shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P9-12. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

By P13-16 (n=3) there has been a change in the primary source of input, which has shifted from layer 5/6 towards layer 2/3 (fig. 4.7 A-C, layer 2/3 input 51%±3, layer 4 17%±3, layer 5/6 32%±2). There is also a continued trend towards larger inputs from within superficial layers (fig. 4.7 B-C, layer 2/3 input 11±3 pA/pixel, layer 4 9±3 pA/pixel, layer 5/6 8±3 pA/pixel), with layer 2/3 providing the strongest inputs into layer 5 pyramidal cells recorded at P13-16. In the final age group (P17-21, n=4) the
percentage of total input from each layer has not undergone any further change. The values are similar to P13-16, whereby layer 2/3 provides the main source of input (fig. 4.7 D-F, layer 2/3 input 47%±7, layer 4 17%±3, layer 5/6 35%±3). However, the inputs from within layer 2/3 and 4 have become stronger, with increased pA/pixel values (fig. 4.7 F, layer 2/3 input 12±4 pA/pixel, layer 4 11±2 pA/pixel, layer 5/6 5±1 pA/pixel).

Figure 4.7

As was found for layer 2/3 SSC pyramidal cells, in layer 5 it seems there is a gradual emergence of input from superficial layers. In both layer 2/3 and layer 5 SSC
pyramidal cells the mature pattern of input conforms to that predicted by the canonical circuit (Gilbert & Wiesel, 1983; Douglas & Martin, 1991). However, in layer 5 pyramidal cells, although there was an increase of around 10% in input from layer 2/3 (figs. 4.6 & 4.7 C, F), there was no statistical difference observed in the laminar organization of inputs when comparing between each of the four age groups ($p>0.1$). There was however a significant increase in the pA/pixel values across all layers, which were significantly elevated relative to P5-8 at both P13-16 and P17-21 ($p<0.05$ for all, except layer 4 at P17-21 where $p<0.01$).

4.3.2 Laminar organization of excitatory inputs onto motor cortex pyramidal neurons over development

The motor cortex lacks a pronounced thalamorecipient layer (Shepherd, 2009; Anderson et al., 2010; Hooks et al., 2011), so the first obvious difference between cells recorded in motor cortex and somatosensory cortex was that there was no layer 4 input. In the somatosensory cortex I found that cells of layer 2/3 showed an enhancement in inputs from layer 4 over the course of development. In layer 2/3 of the motor cortex there was no developmental shift in the laminar organization of inputs. The percentage of total input remained relatively constant between P5-8 ($n=4$, fig. 4.8 A-C, layer 2/3 input 69±6%, layer 5/6 30±6%), P9-12 ($n=4$, fig. 4.8 D-F, layer 2/3 input 61±5, layer 5/6 39±5), P13-16 ($n=3$, fig. 4.9 A-C, layer 2/3 input 70±8, layer 5/6 30±8) and P17-21 ($n=2$, fig. 4.9 D-F, layer 2/3 input 67%, layer 5/6 33%), and there was no significant difference in the organization of the inputs between any age group ($p>0.1$). Despite this lack of shift in input organization, the inputs onto layer 2/3 Mctx pyramidal neurons still underwent changes, with the inputs appearing to become larger over the course of development (figs. 4.8 & 4.9 A-F). The input maps also became increasingly organized, with initial inputs weak and spread throughout the mapping area, before becoming focused into defined hotspots of input at later ages (figs. 4.8 & 4.9 A, D).
Figure 4.8

Figure 4.8 LSPS maps of excitatory inputs onto layer 2/3 motor cortex pyramidal neurons pre P12. A and D show example input maps of layer 2/3 motor cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A and D the maximum input on the scale bar corresponds to 20pA and 30pA respectively. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P5-8. F shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P9-12. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

The pA/pixel values for layer 2/3 Mctx pyramidal cells were similar for inputs from layer 2/3 and 5/6 at both P5-8 (fig. 4.8 C, layer 2/3 input 6±2 pA/pixel, layer 5/6 5±1 pA/pixel), and P9-12 (fig. 4.8 F, layer 2/3 input 6±1 pA/pixel, layer 5/6 4±2 pA/pixel). By P13-16 the pA/pixel values for input from layer 2/3 had started to increase slightly (fig. 4.9 C, layer 2/3 input 7±1 pA/pixel, layer 5/6 4±2 pA/pixel) and by P17-21 the pA/pixel value for input from layer 2/3 was significantly enhanced relative to earlier ages (fig. 4.9 F, $p<0.01$. Layer 2/3 input 17 pA/pixel, layer 5/6 6 pA/pixel, n=2). The pA/pixel values for inputs from layer 5 did not increase significantly over development ($p>0.1$).
When recording from layer 5 Mctx pyramidal cells I find that initially, the inputs at P5-8 (n=3) are diffusely spread across the mapping grid and slightly biased towards layer 5/6 (fig. 4.10 A-C, layer 2/3 input 45%±6, layer 5/6 55%±6). Over the course of development the main source of input undergoes a transition away from layer 5/6, towards layer 2/3. This shift is most pronounced at P9-12 (n=3, fig. 4.10 D-F, layer 2/3 input 57%±12, layer 5/6 43%±12), with the percentage of total input from layer 2/3 also increasing at P13-16 (n=3, fig. 4.11 A-C. layer 2/3 input 61%±13, layer 5/6 39%±13) and P17-21 (n=3, fig. 4.11 D-F, layer 2/3 input 67%±9, layer 5/6 33%±9).
Figure 4.10 LSPS maps of excitatory inputs onto layer 5 motor cortex pyramidal neurons pre P12. A and D show example input maps of layer 5 motor cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A and D the maximum input on the scale bar corresponds to 20pA and 30pA respectively. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P5-8. F shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P9-12. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

I also observed a trend towards stronger inputs onto layer 5 motor cortex pyramidal cells from layer 2/3. At P5-8 the inputs from each layer were fairly equal (fig. 4.10 B-C, layer 2/3 input 4±2 pA/pixel, layer 5/6 4±2 pA/pixel). But over the course of development the pA/pixel values from layer 2/3 trended towards greater values (fig. 4.10 & 4.11 C, F, P9-12 layer 2/3 input 6±2 pA/pixel, P13-16 layer 2/3 11±7 pA/pixel, P17-21 layer 2/3 input 9±2 pA/pixel), whilst the values for layer 5/6 remained fairly constant (fig. 4.10 & 4.11 C, F, P9-12 layer 5/6 input 5±0.4 pA/pixel, P13-16 layer 5/6 4±1 pA/pixel, P17-21 layer 5/6 input 5±1 pA/pixel). Whilst there was no significant increase in the pA/pixel values onto layer 5 motor cortex pyramidal
cells ($p>0.1$ for all ages), the increased contribution of inputs from layer 2/3 brought about a significant increase in the percentage input from layer 2/3 and a concordant significant decrease in input from layer 5/6 at P17-21 relative to P5-8 ($p<0.05$ for both).

**Figure 4.11**

![Figure 4.11 LSPS maps of excitatory inputs onto layer 5 motor cortex pyramidal neurons post P12. A and D show example input maps of layer 5 motor cortex pyramidal neurons with the age the cell was recorded indicated at the bottom left of the panel. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For the cell in A and D the maximum input on the scale bar corresponds to 90pA and 40pA respectively. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of the cortex. Individual numbered uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. B and E show two traces from each numbered uncaging site shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input and pA/pixel values for inputs from layers 2/3, 4 and 5/6 received by all cells recorded at P13-16. F shows the percentage of total input and pA/pixel values for input from layers 2/3, 4 and 5/6 received by all cells recorded at P17-21. The red lines for input from each layer in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.]

The findings of preceding sections show that recorded pyramidal cells undergo a gradual transition towards the layers predicted by the canonical circuit to form the main source of input at mature ages (Gilbert & Wiesel, 1983; Douglas & Martin, 1991). There also seems to be an increase in input strength, as indicated by the
developmental enhancement in the pA/pixel values. These values are typically increased at later ages, particularly amongst the layers that form the primary input source at P17-21. This suggests that pyramidal cells also undergo a developmental increase in synaptic input. The next section of this chapter shall therefore examine in more detail any developmental changes in the amount of synaptic input received by the pyramidal cell populations reported thus far.

4.3.3 Development in the total input recorded from pyramidal cells in layers 2/3 and 5 of the somatosensory cortex

Looking at the total input onto pyramidal neurons recorded from layer 2/3 and 5 of the somatosensory cortex it appears there is a trend towards greater input at more mature ages (fig. 4.12 A-D). In layer 2/3 of the SSC, although there is a degree of variation in the amount of input received by cells recorded in each age group, overall the total input increases between P5-8 and P17-21 (fig. 4.12 B, total input at P5-8 393pA±144, P9-12 424pA±130, P13-16 663pA±168, P17-21 948pA±110). There is a significant increase in input between P5-8 and both P13-16 and P17-21 (p<0.05), in addition to increases at the 90% confidence level for the same ages relative to P9-12 (p=0.07 at P13-16 and p=0.055 for P17-21). Breaking this input down, I find that the average number of LSPS evoked EPSCs also trends towards greater values over the course of development (P5-8 77±28 EPSCs, P9-12 85±16 EPSCs, P13-17 156±23 EPSCs, P17-21 129±8 EPSCs). There is a significant increase in the number of LSPS evoked EPSCs between P9-12 and both P13-16 and P17-21 (p<0.05), in addition to an increase at the 90% confidence level between P5-8 and P13-16 (p=0.06). The average size of LSPS evoked EPSCs also trended towards greater values at more mature ages (P5-8 5.1pA±0.5, P9-12 6.8pA±1.3, P13-16 6.4pA±0.4) and was significantly enhanced relative to P5-8 at both P13-16 and P17-21 (p<0.05 and <0.01 respectively).

In layer 5 I also see an increase in total input between P5-8 and P17-21 (fig. 4.12 D, total input at P5-8 329pA±78, P9-12 811pA±227, P13-16 685pA±407, P17-21 1217pA±338). These increases were significant relative to P5-8 at P9-12 and P17-21 (p<0.05), with a trend towards greater input at P13-16 (p=0.059). The total number of LSPS evoked EPSCs also increased over the course of development (P5-8 58±15 EPSCs, P9-12 100±18 EPSCs, P13-16 140±57 EPSCs, P17-21 170±41 EPSCs).
There was a significant increase relative to P5-8 at both P9-12 and P17-21 ($p<0.05$) and again a trend towards a greater number of EPSCs at P13-16 ($p=0.076$). The average EPSC size also trended towards greater values at older ages (P5-8 6.0pA±0.8, P9-12 7.3pA±1.2, P13-16 7.0pA±1.3, P17-21 7.0pA±0.2) but this was not found to be significant ($p>0.1$ between all ages).

**Figure 4.12**

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**Figure 4.12 Maturation of excitatory inputs onto somatosensory cortex pyramidal neurons.** A and C show the organization of excitatory inputs onto layer 2/3 and 5 pyramidal cells respectively. The input map for each cell is divided along the y-axis into individual rows. Each pixel represents the input received from a single row of the mapping grid, such that the data in one column represents the total input for an individual cell. Cells are organized in ascending order of age from P5 to the left to P21 to the right. The boundary pre and post P12 is marked by a dashed line. The laminar boundaries and edge of the mapping area are represented by white lines and the location of the soma by white triangles. The strength of the input in each pixel is denoted by the colour of the pixel and is indicated on the scale bar at bottom left. The maximum value corresponds to 200pA for A and 250pA for C. B and D represent the total input of cells recorded at the four developmental ages of this study, P5-8, P9-12, P13-16 and P17-21. Each circle represents an individual cell, the red bars are the mean average for each data set and the fainter red blocks indicate the SEM ± from the mean. * = $p<0.05$, ** = $p<0.01$.

The data reported above shows that both layer 2/3 and 5 pyramidal cells of the SSC show significant synaptic integration over the period of this study. To visualize the developmental emergence of the canonical pattern of input, I split the total input...
recorded onto each cell into the values received from each row of the mapping grid (fig. 4.12 A, C). This shows that for layer 2/3 cells there is a prominent developmental enhancement in inputs emanating from layer 2/3 and 4 (fig. 4.12 A), with cells in layer 5 receiving inputs of increasing strength from layer 2/3 (fig. 4.12 C). It also seems that in both layer 2/3 and layer 5 pyramidal cells, these patterns of translaminar input emerge fairly rapidly post P12 (fig. 4.12 A, C).

4.3.4 Development in the total input recorded from pyramidal cells in layers 2/3 and 5 of the motor cortex

As was found in the somatosensory cortex, the motor cortex shows a degree of variation in the total input received by cells at each age (fig. 4.13 A-D). Overall there is also a trend towards greater integration at mature ages in both layer 2/3 and 5 (fig. 4.13 A-D). The total input onto layer 2/3 cells trends towards greater input over the course of development, with a more prominent jump in input at later ages (fig. 4.13 B, total input at P5-8 645pA±166, P9-12 585pA±134, P13-16 684pA±97, P17-21 1315pA, n=2). However, there is no significant increase in input between cells recorded at any age (p>0.05). The average number of LSPS evoked EPSCs in layer 2/3 Mctx pyramidal cells also showed trends towards greater values at older ages (P5-8 109±17 EPSCs, P9-12 124±42 EPSCs, P13-16 121±11 EPSCs, P17-21 170 EPSCs, n=2). Again there was no significant increase in the data, with a rise in EPSC number at the 90% confidence level observed at P17-21 relative to P5-8 (p=0.08). The average size of EPSCs also seemed to become larger at older ages (P5-8 5.7pA±0.8, P9-12 5.4pA±0.7, P13-16 6.6pA±1.2, P17-21 7.6pA, n=2), however again there is no significant increase observed between any age (p>0.1).

A similar pattern is observed in layer 5 motor cortex pyramidal cells (fig. 4.13 D, total input at P5-8 503pA±225, P9-12 653pA±115, P13-16 1097pA±567, P17-21 1030pA±211). Although there is a trend towards increased input, it is not found to be significant between any time point (p>0.1). This is also found to be the case for the total number of LSPS evoked EPSCs (P5-8 88±25 EPSCs, P9-12 89±10 EPSCs, P13-16 127±37 EPSCs, P17-21 116±11 EPSCs) and the average EPSC size (P5-8 5.4pA±1.1, P9-12 7.4pA±1.2, P13-16 8.0pA±1.9, P17-21 8.8pA±1.3). Neither of these parameters showed a significant increase over the course of development (p>0.1)
for all except number of EPSCs between P9-12 and P17-21 when \( p = 0.9 \) and size of EPSCs between P5-8 and P17-21 where \( p = 0.07 \).

**Figure 4.13**

Figure 4.13 Maturation of excitatory inputs onto motor cortex pyramidal neurons. A and C show the organization of inputs onto layer 2/3 and 5 pyramidal cells respectively. The input map for each cell is divided along the y-axis into individual rows. Each pixel represents the input received from a single row of the mapping grid, such that the data in one column represents the total input for an individual cell. Cells are organized in ascending order of age from P5 to the left to P21 to the right. The boundary pre and post P12 is marked by a dashed line. The laminar boundaries and the edge of the mapping area are represented by white lines and the location of the soma by white triangles. The strength of the input in each pixel is denoted by the colour of the pixel and is indicated on the scale bar at bottom left. The maximum value corresponds to 250pA for A and 300pA for C. B and D represent the total input of cells recorded at the four developmental ages of this study, P5-8, P9-12, P13-16 and P17-21. Each circle represents an individual cell, the red bars are the mean average for each data set and the fainter red blocks indicate the SEM \( \pm \) from the mean.

The findings of this section indicate that pyramidal cells undergo a gradual increase in input over the course of development. As cells integrate into the network it acts to strengthen the input from the layers that will go on to become the primary input layer at mature ages. Bringing about a gradual shift towards their mature input organization over the course of development (figs. 4.12 & 4.13 A-D). In layer 5 cells, regardless of cortical region, this seems to happen most rapidly during earlier periods of development (between P5-8 and P13-16), whilst in layer 2/3 of the somatosensory
cortex there is continued enhancement up until P17-21, with a significant increase in layer 4 input occurring between P13-16 and P17-21 (fig. 4.5 A-F). Overall it appears that the total input increases most rapidly at the later two ages of this study (figs. 4.12 & 4.13 B, D), however this may not be the case in layer 5 of the SSC which shows a pronounced, significant enhancement in input between P5-8 and P9-12 (fig. 4.12 D).

4.3.5 ANOVA analysis of pyramidal cell synaptic input maps

The findings of this section (section 4.3) track the developmental emergence of the canonical pattern of connectivity in neocortical pyramidal cells (figs. 4.12 & 4.13 A-D; Gilbert & Wiesel, 1983; Douglas & Martin, 1991, 2004). Over development the inputs onto pyramidal cells seem to become more structured, forming defined hotspots of input, after initially appearing diffuse and poorly organized. To quantify this, I applied one-way ANOVA to the synaptic input maps of all cells reported in the preceding sections. One-way ANOVA was used to test the null hypothesis that the input onto a cell was evenly distributed across the laminar axis of the mapping grid, which I have shown undergoes a transition towards the canonical pattern of input over development (figs. 4.12 & 4.13 A-D). When assessed with one-way ANOVA, cells with diffuse, more evenly distributed inputs produce larger p values, whilst those whose inputs are localized to defined hotspots, and therefore better organized, will give rise to lower p values. Any cell whose inputs produce a p value of <0.05 rejects the null hypothesis, and can therefore be said to receive a focused pattern of synaptic input that is significantly organized along the laminar/vertical axis.

Figure 4.14 shows the one-way ANOVA p values produced from the input maps of both motor and somatosensory cortex pyramidal neurons, recorded at each of the four age ranges used previously in this chapter. At P5-8 a large number of cells have p values that are outside the 95% confidence level, with 57% of Mctx and 66% of SSC pyramidal cells possessing p>0.05. Over the course of development the proportion of cells that fall outside the 95% confidence level decreases, with 31% at P9-12, 25% at P13-16 and only 10% of all cells by P17-21 (fig. 4.14). Therefore it seems that the excitatory synaptic inputs onto cortical pyramidal cells become significantly more focused as they mature and that this increased organization coincides with the
emergence of the canonical pattern of network connectivity in these cells (sections 4.3.1-4).

Figure 4.14

Figure 4.14 One-way ANOVA analysis of synaptic inputs over development. One-way ANOVA p values calculated from the excitatory input maps of cortical pyramidal cells recorded at P5-8, P9-12, P13-16 and P17-21. All input maps were recorded in voltage clamp at -60mV. Data is pooled based on the cortical region cells were recorded from. Somatosensory cortex pyramidal cells are indicated by red circles and motor cortex pyramidal cells by blue circles. The significance threshold of $p=0.05$ is marked by the dashed line.

4.4 Mapping of NMDA inputs onto cortical pyramidal cells through development

In addition to AMPA receptors, many synapses in the neocortex contain NMDA receptors that contribute to excitatory synaptic currents (Hall & Ghosh, 2008). Immature synapses at early developmental ages typically possess a larger proportion of NMDA receptors than mature synapses; with some synapses displaying such a strong bias in this respect that they lack AMPA receptor mediated currents completely (Rumpel et al., 1998, 2004; Hall & Ghosh, 2008). These so called “silent synapses” are known to occur at far greater frequency in the immature neocortex than the mature, reaching a peak between P7-11 and becoming almost totally absent after P14 (Rumpel et al., 2004). To understand the developmental contribution of NMDA receptors, and consequently silent synapses, to the synaptic input maps recorded over development, I chose two time points; one where silent synapses would be prevalent (P7-8) and one where they should be observed at low frequency, if at all (P14-16). Whilst previous studies have looked at the predominance of silent inputs within
individual layers of the cortex (Isaac et al., 1997; Rumpel et al., 1998, 2004), outside of layer 4 there is little known about the spatial location of the source of these inputs during development (see Ashby & Isaac, 2011). The use of LSPS allows me to map the spatial localization of inputs mediated via silent synapses and study the relative contribution of NMDAR mediated inputs at different ages.

I chose to study the formation of the layer 2/3 to 5 pathway, as this underwent a significant enhancement over the course of development in both motor and somatosensory cortex (figs. 4.12 & 4.13 D; sections 4.3.1-4), and has been shown to be one of the most consistent intracortical pathways across numerous cortical areas (Hooks et al., 2011). Given that silent synapses are thought to act as immature precursors to the formation of mature synapses (Rumpel et al., 1998, 2004), one could imagine that there is a gradual transition from silent to AMPA containing synapses, as appears to be the case in layer 4 (Ashby & Isaac, 2011), and that this drives the developmental enhancement in input observed in section 4.3.

4.4.1 Technical considerations for NMDA mapping

Silent synapses transmit in a voltage dependant manner, because NMDARs only conduct in cases where the postsynaptic cell is already depolarized (Mayer et al., 1984; Rumpel et al., 1998). This provided me with a method to separate AMPA responses from those containing NMDA currents, by manipulating the holding potential of the recorded cell between -70mV, where NMDARs should not contribute to laser evoked EPSCs, and +40mV, where they would. This has the advantage of working without necessitating pharmacological isolation of the currents, which, in the case of glutamate uncaging experiments, would also influence the degree of presynaptic excitation. This approach allowed me to determine the relative contribution to synaptic inputs of the individual receptor types at different points in development (Rumpel et al., 1998, 2004).

This study required the use of a Cesium replacement electrode solution, to facilitate stable whole-cell recordings at depolarized membrane potentials (Rumpel et al., 1998, 2004). Using this method it is not possible to accurately record action potential responses in current clamp mode, which I had used previously to calibrate the laser
intensity onto each recorded cell (described in chapter 3 section 3.3). However as numerous pyramidal cells had been mapped at both the ages where NMDA mapping would be employed (see section 4.3), it was possible to use the average settings from pyramidal cells recorded in earlier experiments to calculate both the required laser intensity to produce tightly focused maps, and the temporal window where monosynaptic EPSCs would be expected (see chapter 3 sections 3.3 & 3.5).

The cesium replacement electrode solution also had an alternative intracellular chloride concentration compared to that used previously. This gave a GABA reversal potential of 0 mV (based upon a recording temperature of 20°C), therefore GABAergic inputs would contribute to observed responses at both -70mV and +40mV. The method for determining silent synapses is based around searching for sites with no response at -70mV but an observed response when stimulating at the same site at +40mV (Rumpel et al., 2004). Given GABAergic inputs would be present at both potentials, they would not produce any false positive “silent” responses. They would however hinder the ability to observe silent inputs emanating from the immediate vicinity of the recorded cell, due to the dense inhibitory output from cells located in the surrounding layer (Fino & Yuste, 2011; Katzel et al., 2011; Pengratz-Fuehrer & Hestrin, 2011).

4.4.2 Localization of NMDA receptor mediated inputs at P7-8 and P14-16

When recording from early (P7-8) somatosensory cortex pyramidal cells (n=3), input maps recorded at -70mV showed a similar pattern to that found when recording using normal electrode solution and discussed in section 4.3.1. In both cases the primary source of input emanated from within the host layer (fig. 4.15 A-C, average layer 5/6 input 66%±2), however the value reported here is slightly higher than the percentage of layer 5 input recorded using normal electrode solution (P5-8, input onto layer 5 SSC pyramidal from layer 5/6 46%±11, n=5), most likely due to an additional contribution from GABAergic inputs within layer 5 (see section 4.4.1; Katzel et al., 2011). As was found in previous experiments (section 4.3.1) there was relatively little synaptic input from layer 2/3 (fig. 4.15 A-C, layer 2/3 28%±1).
Figure 4.15

Figure 4.15 LSPS input maps for P7-8 layer 5 pyramidal neurons recorded at -70mV and +40mV. A shows the input onto a P8 example layer 5 somatosensory cortex pyramidal neuron recorded at -70mV. D shows the input onto the same cell recorded at +40mV. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For recordings at -70mV the maximum input on the scale bar corresponds to 180pA and at +40mV it is 80pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid, blue shows -70mV and red +40mV. Each pixel represents a 50x50µm region of the cortex. Individual uncaging sites are indicated by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each numbered uncaging site shown in A and E. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C and F show the percentage of total input from layers 2/3, 4 and 5/6 received by all cells recorded at -70mV and +40mV respectively. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

When shifting the holding potential to +40mV, and therefore removing the voltage sensitive magnesium block of the NMDA receptors (Mayer et al., 1984; Rumpel et al. 2004), the distribution of evoked synaptic inputs showed a considerable shift in laminar organization (fig. 4.15 D-F). The degree of input emanating from layer 2/3 increased significantly from 28%±1 of total input at -70mV, to 45%±4 of total input.
at +40mV ($p<0.01$), with a concordant decrease in the amount of input from layer 5 (fig. 4.15 F, layer 5 66%±2 at -70mV, 47%±3 at +40mV). The amount of layer 4 input remained roughly the same at both holding potentials (fig. 4.15 C, F, layer 4 6%±3 of total at -70mV, 8%±2 at +40mV, $p>0.1$).

To confirm the identity of the inputs recorded at +40mV I looked at their kinetics, specifically their 10-90% rise time as this value is known to distinguish between AMPA and NMDA receptors; with the former possessing a far more rapid rise time than the later (Ye et al., 2005). At -70mV the recorded rise time values displayed a unimodal distribution, with a single peak at 12ms (fig. 4.16). When plotting the 10-90% rise time values for events recorded at +40mV a second peak emerged with a slower 10-90% rise time (fig. 4.16, peak 10-90% rise time 26ms). This shows that at +40mV I am able to elicit novel voltage sensitive currents with slower kinetics, characteristic of NMDA receptors (Ye et al., 2005).

Figure 4.16

**Figure 4.16 10-90% rise times for inputs recorded at -70mV and +40mV.** 10-90% rise times for LSPS evoked inputs recorded at -70mV and +40mV. Inputs were binned into 2ms bins and the number of inputs observed in each bin summed. Novel peak at +40mV indicated by arrow.

When mapping at older ages (P14-16, n=3) the primary source of input at this stage was from layer 2/3 (fig. 4.17 A-C). Unlike when mapping at P7-8, this was found to be the case regardless of holding potential, with the percentage of total input recorded at both -70mV (fig. 4.17 A-C, layer 2/3 46%±9, layer 4 21%±1, layer 5/6 33%±9) and
+40mV (fig. 4.17 D-F, layer 2/3 47%±6, layer 4 19%±2, layer 5/6 34%±8) biased towards layer 2/3.

Figure 4.17

Figure 4.17 LSPS input maps for P14-16 layer 5 pyramidal neurons recorded at -70mV and +40mV. A shows the input onto a P16 example layer 5 somatosensory cortex pyramidal neuron recorded at -70mV. D shows the input onto the same cell recorded at +40mV. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. For recordings at -70mV the maximum input on the scale bar corresponds to 1000pA and at +40mV it is 500pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid, blue shows -70mV and red +40mV. Each pixel represents a 50x50µm region of the cortex. Individual uncaging sites are indicated by white squares, with the corresponding traces shown in B and E. White circles indicate large magnitude direct responses where synaptic input could not be reliably discerned. B and E show two traces from the numbered uncaging sites shown in A and D. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C and F show the percentage of total input from layers 2/3, 4 and 5/6 received by all cells recorded at -70mV and +40mV respectively. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

These findings show that by switching the holding potential to +40mV, and therefore removing the Mg$^{2+}$ block from NMDARs (Mayer et al., 1984; Rumpel et al. 2004), I
produce a novel source of input from layer 2/3 onto layer 5 pyramidal cells recorded at P7-8. This suggests that there are “silent” NMDAR mediated inputs occurring from that layer. To support this, I find that at P7-8 a number of uncaging sites were found that possessed responses solely at +40mV (fig. 4.15 B, E), and therefore fulfill the criteria for silent synapse mediated synaptic input (Rumpel et al., 2004; Kerchner & Nicholl, 2008). These novel responses fell into one of two types. The first occurred in time with the firing of the laser in a similar fashion to a direct response and were likely caused by the increased sensitivity to glutamate of NMDARs (Patneau & Mayer, 1990). By unblocking the NMDAR and releasing glutamate in the vicinity of a dendrite it may have produced a glutamate response that was detected by the high affinity NMDARs at +40mV, but not by the lower affinity AMPARs at -70mV. These responses could be distinguished in a similar fashion to direct responses recorded at hyperpolarized potentials (see chapter 3 section 3.5.1) in that they initiated at the onset of the laser, grew in a fairly linear fashion over the course of the pulse and peaked upon its termination. Any responses of this type were excluded from subsequent analysis.

The second type of input displayed properties that would be expected of a synaptic response. They occurred at a delay from the initial firing of the laser (fig. 4.15 B, E), which must precede the observed initiation of synaptic responses due to the delay that occurs in the initial firing of presynaptic cells (see chapter 3 section 3.5.1). These responses were put down to the presence of putative silent synapses between the stimulated presynaptic cells and the recorded cell and shall be henceforth referred to as NMDA only inputs. When comparing the number of these responses it became apparent that they occurred at a much higher frequency early in development (P7-8) than at P14-16. Of the uncaging sites mapped at P7-8 that showed LSPS evoked inputs at +40mV, 27±7% had no reciprocal input at -70mV, fulfilling the criteria for NMDA only inputs. When mapping at P14-16 significantly fewer NMDA only inputs were observed, with only 4±1% of uncaging sites recorded at +40mV showing no response at -70mV ($p<0.05$). At P7-8 the magnitude of NMDA only input from each layer was strongly biased towards layer 2/3 (sum NMDA only input from layer 2/3, 440pA±118, layer 4 107pA±31, layer 5/6 231pA±83), explaining the transition towards layer 2/3 input observed when shifting the holding potential from -70mV to +40mV (fig. 4.15 A-F).
The conventional method for classifying silent synapses is based upon a response that shows no AMPAR mediated current at +70mV but a NMDAR mediated current at +40mV (Rumpel et al., 1998, 2004). Typically these experiments rely upon a stimulating electrode to excite presynaptic neurons, allowing the stimulus to be tuned to produce a unitary response. However the use of LSPS as the source of excitation means that multiple presynaptic neurons fire action potentials upon the focal release of glutamate (Shepherd et al., 2003). Therefore the postsynaptic responses observed in the recorded cell can comprise inputs from more than one presynaptic neuron. If such inputs contained cells coupled via conventional AMPAR containing synapses, and those coupled via NMDAR only synapses, simply searching for sites that only possessed a +40mV response would underestimate the NMDAR mediated contribution to synaptic input. Furthermore it is possible that at a single synapse there may be variation in the AMPA/NMDA ratio, such that although a response is observed at -70mV, the response at +40mV is relatively much larger.

In keeping with this idea a number of sites were observed that, whilst not totally AMPA silent, had a pronounced NMDA bias when comparing the responses between -70mV and +40mV (figure 4.15 A, B, D, E). Plotting the localization of these inputs revealed that, in addition to NMDA only inputs, NMDA biased inputs were also present in superficial layers at P7-8 (fig. 4.18 A). At P14-16 I observed a lesser contribution from these NMDA biased synapses (fig. 4.18 B). Taken together, the data reported in this section suggests an AMPAification of previously NMDA only, or NMDA biased, synapses throughout development. This was confirmed by calculating the overall ratio of inputs at -70mV/+40mV for cells recorded at P7-8 (n=3) and at P14-16 (n=3). This revealed that at early ages there was a significantly larger proportion of total input recorded at +40mV than at mature ages (p<0.01. -70mV/+40mV average ratio at P7-8 0.64±0.07, at P14-16 1.32±0.11). This is indicative of a larger NMDAR component to the total input received by layer 5 SSC pyramidal cells at P7-8.

These results show that there is a significant early (P7-8) contribution to synaptic inputs from NMDA receptors, that is far greater than that observed at P14-16. This NMDA only/biased input alters the spatial distribution of synaptic inputs onto layer 5 pyramidal cells- shifting it away from local inputs derived from the host layer (fig.
4.15 A, C), towards the superficial layers that are known to form the primary mature input onto mature layer 5 pyramidal neurons (fig. 4.15 D, F and section 4.3.1). This effect is not found at P14-16, with no shift in the spatial distribution of inputs observed when switching between -70mV and +40mV (fig. 4.17 A, C, D, F), and very few silent or NMDA biased inputs observed at this point (fig. 4.18 B). This is in keeping with the findings of section 4.3.1, which show that the transition from local input to canonical input appears to have occurred by P13-16. The next section of this chapter will examine the role of activity in the formation of the network, as this may provide a method by which the immature NMDAR mediated inputs are converted to mature AMPAR containing synapses over the course of development (Durand et al., 1996; Isaac et al., 1997; Liao et al., 2001).

**Figure 4.18**

**Figure 4.18 Difference maps from P8 and P16 pyramidal cells recorded at -70mV and +40mV.** A and B show the difference plots for inputs onto the P8 cell shown in figure 4.15 and the P16 pyramidal cell in figure 4.17 respectively. Each pixel shows the input at +40mV minus that recorded at +70mV and highlights sites that received a greater contribution from NMDAR mediated inputs. Inputs were normalized relative to the total input received at +40mV for each cell. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Starred pixels (*) indicate uncaging sites that received NMDA only inputs (where responses were observed at +40mV but not at -70mV). Each pixel represents a 50x50µm region of the cortex. Colours represent increasing levels of input according to the scale bar at bottom left. The maximum input on the scale bar corresponds to 50pA.
4.5 Influence of zero magnesium ACSF induced network activity on the integration of pyramidal neurons during development

During network development cortical pyramidal cells are exposed to a number of synchronous network activity patterns (Golshani et al., 2009; Yang et al., 2009). This activity may play an important role in the recruitment of silent synapses (Liao et al., 2001), and therefore the emergence of canonical connectivity in the neocortex (section 4.4). To study this in more detail I sought a method to induce activity within the in vitro slice preparation used for the mapping experiments described previously. By mapping the cells both before and after promoting activity in the slice, it would be possible to determine any changes to the organization of the synaptic inputs that were brought about by this manipulation. By repeating this experiment at different ages, I would also be able to see if the influence of the activity changed over development.

The method for the promotion of activity in the slice was required to overcome certain experimental constraints imposed upon it by the protocol for LSPS mapping of synaptic inputs. Firstly it had to be reversible- to enable the mapping of synaptic inputs after activity had been induced in the slice, which calls for a stable baseline. Secondly, it required a rapid onset upon application, as well as the quick cessation of activity upon removal, as the amount of time that neurons could be stably recorded from is limited. Thirdly it was preferable that it reliably and consistently elicited activity in the slice, so that every time a cell was exposed to the manipulation there would be a high probability of observing a response and therefore determining the effect of the evoked activity on the LSPS input maps.

4.5.1 Evaluating methods for the induction of activity in neocortical slices

A simple method for enhancing the amount of activity observed within the slice is to create a tonic depolarization, which increases the overall excitability of the cells in the network. An initial attempt was made to use an ACSF solution with elevated levels of potassium ions (4mM), which has been used in other developmental studies to facilitate the study of rhythmic activity in vitro (Allene et al., 2008; Bonifazi et al., 2009). I applied this manipulation to cells recorded at early stages in development (P6-8, n=5), when it has been shown to induce activity in acute in vitro slices (Allene
et al., 2008; Bonifazi et al., 2009; Picardo et al., 2011). I observed that upon a 20 minute exposure to elevated potassium ACSF, there were typically few depolarizations observed in the recorded cells (fig. 4.19 A, average 13±6 depolarizations of >200ms per 20 minute exposure) and many of these were subthreshold (average 5±3 suprathreshold depolarizations per 20 minute exposure). In addition there were occasions where no suprathreshold activity was initiated in the slice over the course of a 20 minute exposure (fig. 4.19 B). To confirm the limited efficacy of this approach, cells that were exposed to 20 minutes of elevated potassium ACSF (4mM K⁺), and then mapped again post exposure (n=3), showed little changes in synaptic input relative to control maps (post exposure input 99%±10 of pre-exposure input). Previous use of elevated potassium to evoke large-scale activity in vitro is typically based around longer periods of slice incubation under elevated potassium conditions (at least 1 hour, Allene et al., 2008), which would not be of use for this experiment given the constraints outlined above. Given the limited success of this approach in reliably inducing activity during briefer periods of exposure (fig. 4.19 A-B), an alternative method to elicit activity was necessary.

As mentioned in section 4.4.1 the pore of the NMDA receptor is restrained from allowing ions to flow across the membrane at hyperpolarized membrane potentials by the presence of a magnesium ion block (Mayer et al., 1984). By using an ACSF solution with a nominally zero concentration of magnesium ions, the block is removed from the NMDA receptor, producing an increase in network activity. This method has been used previously to induce activity in vitro (Silva et al., 1991; Flint et al., 1997) and therefore offers an alternative to the elevated potassium approach mentioned above. However this method has also been used to produce long duration epileptiform like activity (fast run-like potentials, Kawaguchi, 2001). As the purpose of this study was to model the effect of the ongoing activity patterns of the nascent cortex, as opposed to the effect of epileptic activity, it was necessary to ensure that the incubation period of the slice in zero magnesium (0Mg) ACSF was limited to prevent the observance of epileptiform like events. To confirm that brief exposure to 0Mg ACSF would not induce ictal activity, pyramidal cells (n=11) were recorded from neocortical slices and exposed to 0Mg ACSF for between 20-25 minutes (fig. 4.19 C, E).
Figure 4.19

Figure 4.19 Activity elicited in neocortical slices. A-F current clamp traces of activity elicited in vitro by elevated potassium or zero magnesium ACSF. A successful attempt to elicit activity using 4mM K⁺ ACSF. The expanded trace to the right shows the corresponding numbered suprathreshold events from the trace to the left. B a twenty minute exposure to 4mM K⁺ ACSF that did not yield any suprathreshold responses. C response of a P8 pyramidal neuron to twenty minute exposure to zero magnesium ACSF. D expanded traces showing the suprathreshold responses corresponding to those numbered in C. E response of a P17 pyramidal neuron to twenty minute exposure to zero magnesium ACSF. F expanded traces showing the suprathreshold responses corresponding to those numbered in E. The traces in D and E represent, a single spike, a single spike associated with a depolarization of >200ms, and bursts with multiple spikes contained within a depolarization of >200ms. The light and dark grey arrows underneath traces B, C and E indicate the point of wash on and wash off respectively.
In none of these cases did I observe the long duration bursts characteristic of ictal activity (fig. 4.19 C-F). Furthermore in all cases spiking activity was induced within the period of exposure and in the majority of cases gave rise to multiple large bursts (fig. 4.19 C-F).

A period of twenty minutes was chosen as an appropriate period of time to expose the cells to activity as it has been shown no ictal like activity should occur within this window and there was also typically a large amount of activity that occurred over the duration of this period. In addition it was found that cells were capable of surviving the duration of this exposure and maintaining good access throughout, increasing the probability of recording the post-activity maps. During the wash-off phase cells were maintained in normal ACSF but in the majority of cases began to deteriorate around 20 minutes after the cessation of activity. Therefore longer periods of incubation were undesirable, as they would decrease the probability of obtaining accurate data from the post-activity maps. Upon termination of the exposure to 0Mg ACSF, and restoration to a normal ACSF solution, activity was rapidly reversed and restored to baseline levels (fig. 4.19 C, E, wash off time of less than 3 minutes for all cells). Thus 0Mg ACSF fulfills all of the requirements outlined in the previous section (section 4.5.1) making it a suitable method for inducing activity in vitro to study the effect on synaptic integration.

4.5.2 The effect of exposure to zero magnesium ACSF on cortical activity recorded through development

To determine if the response to 0Mg ACSF varied over the course of development, I analyzed the responses of 42 pyramidal cells to a twenty minute exposure of 0Mg ACSF. These cells were recorded between the ages of P5-21. To characterize the response to 0Mg ACSF I looked at a number of parameters related to both supra and subthreshold responses of the cells. To discriminate between activity contained within longer duration depolarizations, I set an arbitrary cut off point of >200ms. Any such depolarizations that contained two or more action potentials were classed as bursts. The activity parameters that were analyzed were, average inter spike interval (ISI) across all spikes, the burst ISI which was the ISI across all action potentials contained within bursts, the maximum ISI, the total number of action potentials elicited, the total
number of action potentials contained within bursts, the percentage of action potentials within bursts, the total number of depolarizations lasting over 200ms, the number of bursts, and the number of subthreshold depolarizations (those over 200ms but without spikes). The values calculated for cells recorded across development can be seen in table 4.3.

Table 4.3

<table>
<thead>
<tr>
<th></th>
<th>P5-8 (n=8)</th>
<th>P9-12 (n=14)</th>
<th>P13-16 (n=7)</th>
<th>P17-21 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avrg. ISI (Hz)</td>
<td>11±2</td>
<td>12±2</td>
<td>15±1</td>
<td>9±2</td>
</tr>
<tr>
<td>Burst ISI (Hz)</td>
<td>16±1</td>
<td>20±2</td>
<td>25±2</td>
<td>20±4</td>
</tr>
<tr>
<td>Max. ISI (Hz)</td>
<td>42±7</td>
<td>44±6</td>
<td>61±8</td>
<td>48±10</td>
</tr>
<tr>
<td>Total APs</td>
<td>57±15</td>
<td>77±19</td>
<td>43±15</td>
<td>102±50</td>
</tr>
<tr>
<td>Total APs in Burst</td>
<td>55±13</td>
<td>78±22</td>
<td>25±9</td>
<td>71±39</td>
</tr>
<tr>
<td>% APs in Burst</td>
<td>82±5</td>
<td>76±4</td>
<td>62±4</td>
<td>62±7</td>
</tr>
<tr>
<td>Depol. &gt;200ms</td>
<td>35±10</td>
<td>31±5</td>
<td>37±6</td>
<td>49±17</td>
</tr>
<tr>
<td>Bursts</td>
<td>5±9</td>
<td>13±3</td>
<td>8±3</td>
<td>23±12</td>
</tr>
<tr>
<td>Sub T’hold &gt;200ms</td>
<td>26±9</td>
<td>18±5</td>
<td>29±5</td>
<td>27±9</td>
</tr>
</tbody>
</table>

Table 4.3 Response of cortical pyramidal cells to zero magnesium ACSF across development. Super and subthreshold properties of activity elicited in response to a twenty minute exposure to zero magnesium ACSF for pyramidal cells recorded at P5-8, P9-12, P13-16 and P17-21. The number of cells recorded in each age group is indicated in brackets below each heading. Avrg. ISI, average interspike interval (ISI); burst ISI, ISI of spikes contained in bursts; max. ISI, maximum ISI; total APs, total action potentials (APs); depol >200ms, number of depolarizations lasting longer than 200ms; sub T’hold >200ms, subthreshold depolarizations lasting longer than 200ms. All values shown are the mean average ± SEM.

Statistical analysis performed for each parameter revealed that in the majority of cases there was no significant change in the activity elicited across development (p>0.05). There were however a couple of instances where differences where observed. The average ISI at P17-21 was significantly decreased relative to P13-16 (p<0.05), and the Burst ISI was significantly increased at P13-16 relative to P5-8 (p<0.05). The percentage of action potentials in bursts was also significantly decreased relative to P5-8 at P13-16 and P17-21 (p<0.05 and <0.01 respectively). Apart from this the activity elicited upon twenty minute exposure to 0Mg ACSF was on average fairly similar across development, even if there was a degree of variation in the responses of individual cells (compare fig. 4.19 traces C and E).
4.5.3 The effect of exposure to 0Mg ACSF induced activity on synaptic integration

To determine the effect of activity on the synaptic inputs of neocortical pyramidal cells, I recorded from 27 cells distributed across different layers (layer 2/3, n=14 and layer 5, n=13) and regions of the cortex (SSC, n=15 and Mctx, n=12). These cells were recorded between P5-21 to search for any changes in the response of cells to activity over development. The influence of activity was distinguished by comparing the maps recorded prior to exposure to 0Mg ACSF for a period of twenty minutes, with those recorded from the same cell after this manipulation (fig. 4.20 A, C).

Figure 4.20

Figure 4.20 Cell showing augmented synaptic inputs after exposure to zero magnesium ACSF. A and C show input maps of the same layer 5 motor cortex pyramidal neuron recorded at P8. The control map is shown in A and the post activity map in C. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. The maximum input on the scale bar corresponds to 80pA. Each pixel represents a 50x50µm region of the cortex. The histograms adjacent to the individual map show the percentage of total input emanating from each row of the LSPS mapping grid. The control histogram is shown in both A and C, with the values post activity shown in red in C. B and D show two traces from the numbered uncaging sites shown in A and C respectively. The black lines at the top of B and D indicate the firing of the laser pulse, which lasted 200ms.
When contrasting the organization of synaptic inputs and the total input between control maps (prior to activity), and post-0Mg maps, it was observed that a number of cells displayed alterations to their inputs after exposure to activity (fig. 4.20 A-D). In some instances novel responses were formed de novo at sites that had previously shown no input (fig. 4.20 A-D), whilst at other sites there was an enhancement of the observed responses (fig. 4.20 A-D). Although activity was able to induce synaptic potentiation at a number of sites, in many cases the responses from uncaging sites were almost identical before and after activity (fig. 4.20 A-D). It was also observed that some cells showed little alteration in their inputs post activity (fig. 4.21 A-D).

**Figure 4.21**

*Figure 4.21 Cell showing stable input after exposure to zero magnesium ACSF.* A and C show input maps of the same layer 2/3 somatosensory cortex pyramidal neuron recorded at P16. The control map is shown in A and the post activity map in C. White triangles indicate the location of the soma of the recorded cell and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. The maximum input on the scale bar corresponds to 50pA. Each pixel represents a 50x50µm region of the cortex. The histograms adjacent to the individual map show the percentage of total input emanating from each row of the LSPS mapping grid. The control histogram is shown in both A and C with the values post activity shown in red in C. B and D show two traces from the numbered uncaging sites shown in A and C respectively. The black lines at the top of B and D indicate the firing of the laser pulse, which lasted 100ms.
Control experiments revealed that the organization of LSPS input maps were consistent across multiple trials. I mapped cells recorded at P11-12 repeatedly over a period of 60 minutes (n=3) and found that the input maps produced were similar across the entire period (fig. 4.22 A-F).

**Figure 4.22**

*Figure 4.22 Stability of LSPS input maps across multiple trials. A-F Show input maps from the same P11 layer 5 pyramidal neuron, recorded across multiple trials. Each individual map shows the input onto the cell calculated after recording an increasing number of trials. A trial corresponded to two sweeps of the mapping grid and there was a gap of 10 minutes between each trial. A-F show the maps generated after 1-6 trials respectively. White triangles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Colours represent increasing levels of input according to the scale bar on the bottom left. The maximum input on the scale bar corresponds to 25pA. Each pixel represents a 50x50µm region of the cortex. White circles indicate the site of large magnitude direct responses that prevented the observation of synaptic inputs.*
I also recorded cells after a twenty minute exposure to normal ACSF, where no activity was induced (n=3 at P5-8 and n=1 at P15) and found limited change in total input, with an average relative shift of 103±4%. This shows that under control conditions potentiation does not occur, and it is not a consequence of repeated mapping. Cells that showed a shift in excess of 110% of control values after exposure to activity were therefore deemed to possess an enhancement in synaptic connectivity. Of the 27 cells that were recorded, 13 showed shifts in synaptic input that were greater than 110% (average change in shifting cells 137.3±6, versus non-shifting cells 106.4±9). Shifting cells were evenly distributed amongst layer 2/3 (n=6) and layer 5 (n=7), as well as between SSC (n=7) and Mctx (n=6). However when comparing the temporal distribution of these cells it was found that there was a prominent bias towards the first two developmental time points (P5-8 7/10 cells, P9-12 4/7 cells, P13-16 1/6 cells, P17-21 1/4 cells). Thus it appeared that activity preferentially influenced the synaptic integration of pyramidal neurons, located across cortical layers and regions, prior to P12 (fig. 4.23 A-D).

**Figure 4.23**

**Figure 4.23** Average shift in response to 0Mg induced activity across development. A-D the average normalized shift in synaptic input between control maps and post activity maps in response to twenty minute exposure to 0Mg ACSF for cells recorded at P5-8, P9-12, P13-16 and P17-21. A shows the pooled data for all cells recorded in motor cortex, B shows the pooled data for somatosensory cortex, C shows cells from all cortical regions located in layer 2/3 and D shows cells from all regions recorded from layer 5. Error bars ± are SEM.

4.5.4 Changes to input maps in response to activity

To better understand how these changes in synaptic connectivity were brought about, I focused on the properties of the maps before and after the initiation of activity in the slice, to determine what was different in the post-activity maps. Given I had
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distinguished there was an increase in synaptic input onto these cells, it seemed logical to see if this change was preferentially localized within an individual layer. Amongst cells that displayed shifts in synaptic input of >110% (n=13) it appeared that there was a slight trend towards larger shifts localized within layer 2/3 over other layers (140%±7 layer 2/3, 131%±17 layer 4 and 132%±7 layer 5), however this trend was not-significant (p>0.1). Given the findings of the previous section (section 4.4.2) one could envision that the silent and NMDA biased synapses were recruited by the activity elicited in the slice, driving the activity dependant AMPAification of these synapses (Durand et al., 1996; Isaac et al., 1997; Liao et al., 1999). In layer 5 pyramidal cells of the somatosensory cortex it was found that the localization of these silent synapses was biased towards the layer that would go on to become the primary input to these cells at mature ages (section 4.4). If this were a global rule for the maturation of the canonical circuit, and if activity were preferentially recruiting these silent synapses, then one could expect to find preferential upregulation within the layers that form the primary input at mature ages. Seven out of the thirteen cells that showed a shift of >110% in their input had the greatest percentage up regulation in the layer that would go on to become their primary input at mature ages (P17-21). However when comparing the overall magnitude of these shifts with those from the remaining layers of the cortex, it was found that there was only a trend towards a preferential increase in the layer that would become the primary input layer at mature ages (p>0.1, average shift in future primary focus 141%±7, average shift in remaining layers 132%±7). Activity was therefore able to influence the integration of inputs emanating from all layers of the cortical network, regardless of where the recorded cell was located.

The observed changes in synaptic input could be brought about in a number of ways; either strengthening of existing inputs, or by increases in the number of inputs. Amongst cells that showed an increase in input of >110% there was a trend towards enhancement in both the number of laser evoked EPSCs (average number of EPSCs before 178.3±35.6, average number of EPSCs after 213±38.4), as well as the average amplitude of these events (average amplitude before 6.4pA±0.5, average amplitude after 6.8pA±0.6) however neither of these differences were significant (p>0.1 for both). Due to the complex nature of inputs at early ages, which could contain classical AMPAR containing synapses, NMDA only silent synapses, or a mixture of the two, it
is possible that multiple forms of plasticity occur. Looking at the example traces in figure 4.20 (A-D) it seems this is the case with some inputs produced *de novo*, consistent with the recruitment of silent synapses or indeed spinogenesis (Durand *et al.*, 1996; Isaac *et al.*, 1997; Kwon & Sabatini, 2011), whilst other sites show existing inputs that become stronger and more numerous after exposure to zero magnesium ACSF.

4.5.5 Discounting the influence of increased presynaptic excitation

To ensure that the potentiation of responses was not caused by an increased sensitivity of presynaptic neurons to glutamate release during the uncaging procedure, I compared the excitability of each recorded pyramidal cell to the laser firing directly at the soma, which was recorded in current clamp mode before and after exposure to zero magnesium. I noticed no increase in the number of action potentials elicited after exposure to activity (average spikes elicited before activity 3.1±0.3 and after 2.8±0.3), nor was there any enhancement when looking specifically at those cells that had exhibited a shift of >110% (average spikes elicited before activity 2.9±0.2 and after 2.4±0.5). I also observed no increase in the magnitude of subthreshold responses, both those adjacent to the soma (average amplitude before 11mV±0.6, after 8mV±0.6), and those elicited at distal sites (average amplitude before 4mV±0.4, after 3mV±0.3). Assuming that the remaining cells in the network respond in a similar fashion to each of the recorded cells, it is unlikely that the augmented input is due to increased presynaptic activity elicited by glutamate uncaging in the post-activity maps.

4.5.6 Differences between shifting and non-shifting cells prior to P12

Whilst it was found that activity preferentially augmented the inputs onto cells during the first two age ranges of this study (fig. 4.23 A-D), not all cells recorded during this period exhibited an increase in input after twenty minutes exposure to 0Mg ACSF. Comparison of the total synaptic input received by cells recorded prior to P12 revealed that there was no difference in the degree of synaptic input between those cells that showed no shift, and those that had a shift of >110% (fig. 4.24 A-B). Therefore it is unlikely that there is some form of threshold that the non-shifting cells
had reached, preventing them from being further integrated. This is further discredited by the observation that some of the cells that showed the largest overall enhancement of input, were amongst those that received the greatest amount of input prior to activity exposure (fig. 4.24 A-B). Given the findings of section 4.4 it is possible that the layer that forms the primary input at mature ages, and therefore contains the main source of silent inputs to be recruited by activity, may itself reach some form of threshold for integration, potentially brought about by the prior integration of all its silent inputs. However, there was no difference observed in the amount of input received from the future primary input layer between shifting and non-shifting cells, with a similar distribution found across these two populations (fig. 4.24 C-D). Furthermore I have shown that there was no significant bias towards greater enhancement of input from any one layer (section 4.5.4).

**Figure 4.24**

<table>
<thead>
<tr>
<th></th>
<th>Shift&gt;110%</th>
<th>Shift&lt;110%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Graph A" /></td>
<td><img src="image2.png" alt="Graph B" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image3.png" alt="Graph C" /></td>
<td><img src="image4.png" alt="Graph D" /></td>
</tr>
</tbody>
</table>

**Figure 4.24 Comparison of the inputs received by shifting and non-shifting cells.** A-D inputs of individual cells pre and post twenty minute exposure to 0Mg ACSF. Cells are colour coded with coloured markers representing shifting cells and greyscale cells markers non-shifting cells. A and B show the total LSPS evoked input pre and post activity for shifting and non-shifting cells respectively. C and D show the total LSPS evoked input specifically occurring from the layer of the cortex that will form the primary input layer at P17-21, inputs are shown pre-and post-activity for shifting and non-shifting cells respectively. The cells labelled by individual colours coding in A and B correspond to the same cells labelled by each colour in C and D.
I next compared the intrinsic membrane properties of those cells recorded prior to P12 that showed a shift of >110% and those that did not (table 4.4). Again I found no difference between the populations for any of the electrophysiological parameters used previously in the study of pyramidal cell development (section 4.2). Each of the parameters was alike, with no statistical difference between the two populations ($p>0.1$, table 4.4). Thus it would appear that neither the inputs onto the cell, nor its intrinsic membrane properties, influenced the magnitude of the input augmentation in response to activity.

**Table 4.4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Shifting</th>
<th>Non-shifting</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>64±1</td>
<td>65±2</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>36±1</td>
<td>39±2</td>
</tr>
<tr>
<td>Spike height (mV)</td>
<td>70±1</td>
<td>68±2</td>
</tr>
<tr>
<td>Spike half-width (ms)</td>
<td>4.0±0.1</td>
<td>4.5±0.4</td>
</tr>
<tr>
<td>Spike delay (ms)</td>
<td>217±11</td>
<td>223±21</td>
</tr>
<tr>
<td>$R_{in}$ (MΩ)</td>
<td>713±31</td>
<td>1001±119</td>
</tr>
<tr>
<td>Max. Freq (Hz)</td>
<td>23±2</td>
<td>28±3</td>
</tr>
<tr>
<td>Initial Freq. (Hz)</td>
<td>37±2</td>
<td>40±3</td>
</tr>
<tr>
<td>Final Freq. (Hz)</td>
<td>18±1</td>
<td>27±4</td>
</tr>
<tr>
<td>Adaptation (%)</td>
<td>47±5</td>
<td>63±13</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>79±3</td>
<td>83±7</td>
</tr>
<tr>
<td>AHP time (ms)</td>
<td>77±4</td>
<td>72±11</td>
</tr>
<tr>
<td>AHP amp. (mV)</td>
<td>19±1</td>
<td>20±1</td>
</tr>
</tbody>
</table>

**Table 4.4** Intrinsic electrophysiological properties of shifting and non-shifting cells. Average values of commonly used electrophysiological parameters for shifting and non-shifting cells recorded at P12 or earlier. $R_{in}$ input resistance, Max. Freq. maximum firing frequency, Initial Freq. maximum frequency in first 100ms of current pulse, Final Freq. maximum frequency in last 100ms of current pulse, AHP delay, delay to peak of action potential afterhyperpolarization, AHP amp, peak amplitude of action potential afterhyperpolarization. Values shown are mean average ± SEM.
The activity elicited in response to exposure to zero magnesium ACSF was somewhat variable (fig. 4.19 C, E). To determine if this may have contributed to the alternate responses of shifting and non-shifting cells, I compared the parameters used to characterize the response to zero magnesium ACSF over development for shifting and non-shifting cells recorded prior to P12 (table 4.5). The only significant differences were in the total amount of action potentials elicited in the cells over the course of the exposure to zero magnesium ACSF ($p<0.05$, table 4.5), and the total number of depolarizations lasting longer than 200ms ($p<0.05$, table 4.5). There was no significant difference in either the number of subthreshold depolarizations or those associated with spikes, although both showed trends towards being more prominent amongst the activity experienced by shifting cells.

Table 4.5

<table>
<thead>
<tr>
<th></th>
<th>Shifting</th>
<th>Non-shifting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avrg. ISI (Hz)</td>
<td>12±2</td>
<td>14±5</td>
</tr>
<tr>
<td>Burst ISI (Hz)</td>
<td>17±1</td>
<td>22±3</td>
</tr>
<tr>
<td>Max. ISI (Hz)</td>
<td>48±5</td>
<td>33±14</td>
</tr>
<tr>
<td>Total APs</td>
<td>94±15</td>
<td>39±18</td>
</tr>
<tr>
<td>Total APs in Burst</td>
<td>99±21</td>
<td>49±16</td>
</tr>
<tr>
<td>% APs in Burst</td>
<td>80±3</td>
<td>68±22</td>
</tr>
<tr>
<td>Depol. &gt;200ms</td>
<td>41±6</td>
<td>18±7</td>
</tr>
<tr>
<td>Bursts</td>
<td>12±2</td>
<td>8±4</td>
</tr>
<tr>
<td>Sub T’hold &gt;200ms</td>
<td>29±7</td>
<td>11±4</td>
</tr>
</tbody>
</table>

Table 4.5 Response of shifting and non-shifting cells upon twenty minute exposure to zero magnesium ACSF. Super and subthreshold properties of shifting and non-shifting cells recorded at P12 or earlier. Avrg. ISI, average interspike interval (interspike interval); burst ISI, ISI of spikes contained in bursts; max. ISI, maximum ISI; total APs, total action potentials (APs); depol >200ms, number of depolarizations lasting longer than 200ms; sub T’hold >200ms, sub threshold depolarizations lasting longer than 200ms. All values are mean average ± SEM. Grey boxes indicate properties that are significantly different between the two populations $p<0.05$. 
It therefore seems that the reason for some cells not shifting prior to P12 is simply due to the variability inherent within the method for eliciting activity. This could be due to less overall activity elicited within the slice in these instances, or the fact that the individual recorded cells, for whatever reason, were less recruited by the induced activity on these occasions. I did not perform extracellular recordings in conjunction with the whole-cell recordings, which could have determined if this was due to a decreased amount of network activity, or simply the presence of certain cells that were less in tune to the rhythm generating kernel of the early cortical network (Silva et al., 1991).

4.6 Chapter summary

The findings of this chapter describe the maturation of the neocortical network. Specifically I have focused on pyramidal neurons located in layers 2/3 and 5 of both motor and adjacent somatosensory cortex. In terms of their intrinsic membrane properties, I find that pyramidal cells display somewhat similar developmental trajectories, however it seems that in somatosensory cortex, layer 5 pyramidal cells may mature slightly earlier than layer 2/3 cells. There were also some instances where specific electrophysiological properties were found amongst certain populations. For example, rebound spiking and a large voltage sag in response to hyperpolarizing current pulses were preferentially associated with cells recorded from deep layers, and in particular those cells recorded in layer 5 of the somatosensory cortex.

When looking at the maturation of excitatory inputs onto the same pyramidal cells, it appears there is a global pattern of network development that occurs independently of the location of the recorded cell. Initially, inputs recorded at -60mV are primarily located within the immediate layer. Over the forthcoming days there is a shift, away from these local domains, towards the mature, canonical pattern of input. By P17-21 the input onto recorded neurons has reached the organization that corresponds to the canonical circuit. This is brought about by an enhancement in layer 2/3 input onto layer 5 pyramidal neurons and a significant upregulation in the amount of layer 4 input received by layer 2/3 cells of the somatosensory cortex. The cells of the network also receive stronger inputs over the course of development and there seems to be a continued increase in total input up until P17-21.
By focusing on layer 5 neurons of the somatosensory cortex, I find that at P7-8 the emergence of the mature pattern of input is predicted by NMDAR mediated, silent inputs and NMDAR biased inputs from layer 2/3. This suggests that these NMDAR mediated inputs act as a substrate for the transition away from local input, towards that predicted by the canonical circuit. The gradual AMPAification of these synapses over development provides a mechanism by which this could occur. To support this idea I find that at mature ages, when the transition from local input to canonical input has occurred, there are few NMDA only or NMDA biased inputs observed, with the majority of inputs from layer 2/3 having a strong AMPAR contribution when mapped at -70mV.

Because activity is important in promoting the transition from silent to mature synapses (Durand et al., 1996; Isaac et al., 1997; Liao et al., 1999, 2001), I next chose to look at the influence of rhythmic network activity on the integration of cells during development. Exposure to brief periods of activity was found to preferentially potentiate the inputs onto those cells recorded at early developmental ages (<P12), when silent synapses were also more frequently observed. Thus it seems that there is a period of synaptic integration that occurs prior to P12. During this time cells are shifted away from local domains, towards the mature input organization of the canonical network. This is likely brought about by the ongoing activity of the early network, which functions to recruit NMDAR mediated inputs from layers that will form the future primary source of synaptic input.

4.7 Discussion

4.7.1 Comparative maturation of the intrinsic electrophysiology of cortical pyramidal neurons

The physiological development of cortical neurons has been studied across numerous regions of the cortex, from prefrontal to visual (McCormick & Prince 1987; Kasper et al., 1994; Zhang, 2004; Oswald & Reyes, 2008; Rheims et al., 2008). Across all these regions it has been found that neurons undergo a maturation of intrinsic membrane properties, such as faster membrane kinetics, faster maximum firing frequencies, taller spikes, lower input resistance and more hyperpolarized membrane potentials.
This maturation is brought about by changes in the expression of various ion channels within the plasma membrane of the neurons, in addition to growth of the membrane itself as the morphology of the neuron develops (Kang et al., 1996; Grosse et al., 2000; Picken-Bahrey & Moody, 2003). The findings of this chapter reveal that there is considerable maturation in the intrinsic membrane properties of cortical neurons recorded from both deep and superficial layers of the somatosensory and motor cortex, which agrees with the findings of others who have looked at similar aspects of pyramidal cell development (McCormick & Prince 1987; Kasper et al., 1994; Zhang, 2004; Oswald & Reyes, 2008; Rheims et al., 2008).

Because of the inside out development of the cortex, and the late specification of superficial cortical neurons relative to those in deeper layers (Angevine & Sidman, 1961; Rakic, 1974), it is possible that there may be some developmental delay in the intrinsic electrophysiology of superficial neurons. When comparing the electrophysiological properties of superficial pyramidal neurons relative to those recorded from layer 5, it does not appear that there is any initial developmental delay in the electrophysiological properties of layer 2/3 pyramidal neurons. At P5-8 many of the parameters that are known to undergo prominent maturation, and therefore good markers of developmental maturity (such as input resistance, spike half-width, tau and maximum firing frequency), are similar between cells recorded from both deep and superficial layers. However, at both P9-12 and P13-16 there are a number of significant differences between layer 2/3 and layer 5 pyramidal cells. This is particularly noticeable in the somatosensory cortex. In many cases where significant differences are observed, it seems that layer 5 cells possess properties that are indicative of greater maturity than cells in layer 2/3. This suggest that although at early ages there is no difference between the layers, layer 5 SSC pyramidal cells undergo a more rapid development across a number of electrophysiological properties between P9 and P16. It remains to be seen if this represents the start of a divergence in these properties, because by P17-21 many of the parameters are no longer significantly different. However this may in part be influenced by the somewhat smaller sample of cells recorded at P17-21 relative to earlier ages. A similar divergence may occur in the motor cortex, although it appears that the properties of layer 2/3 and 5 pyramidal cells in this region are more evenly matched across development than those in the SSC.
4.7.2 Development of distinct electrophysiological firing types

Pyramidal neurons of the cortex fall into one of a number of firing types that are known to possess distinct electrophysiological properties (Chagnac-Amitai et al., 1990; Connors & Gutnick, 1990; Schubert et al., 2001). It has been reported that the electrophysiological diversity amongst cortical pyramidal cells emerges towards later stages of this study (Kasper et al., 1994; Flint et al., 1997). To support this I observe an increase in the prevalence of neurons with an initial double at the initiation of the spike train at later ages, and others have reported the increased presence of the bursting pyramidal subtype (Kasper et al., 1994; Flint et al., 1997). However only one bursting pyramidal cell was recorded in this study, which was not included in the data reported in section 4.2, and the frequency of initial double cells was relatively low (peaking at 25%). It is therefore unlikely this significantly influences the instances of diversity amongst electrophysiological properties observed in this chapter, particularly as in the instances where variations in intrinsic electrophysiology are reported, many are observed prior to the emergence of electrophysiological subtypes (section 4.2.7, Kasper et al., 1994; Flint et al., 1997). However, because these distinct electrophysiological populations are known to preferentially segregate into individual layers (Silva et al., 1991; Agmon & Connors, 1992; Huggenberger et al., 2009), dividing mature pyramidal based simply upon the layer from which they were recorded, underestimates any physiological differences that occur amongst subpopulations of pyramidal cells contained within each layer. Therefore any observed variation likely represents a lower bound of electrophysiological diversity present between layers and regions within the adult cortex.

4.7.3 Laminar and regional diversity in intrinsic electrophysiology

In terms of the membrane properties of cells located in different layers or regions, I did observe some instances of laminar or regional variance. Other studies have looked at the electrophysiological variation in the properties of pyramidal cells recorded from layer 2/3 and 5 in other cortical regions (Atzori et al., 2004; Rheims et al., 2008; Huggenberger et al., 2009). Spike threshold appears to be one property that shows laminar variance between deep and superficial layers, with one study in auditory cortex finding that layer 2/3 neurons have more hyperpolarized thresholds than layer 5.
cells (Atzori et al., 2004), whilst another study recorded from unspecified cortical regions showed the reverse (Rheims et al., 2008). My findings from somatosensory cortex seem to agree with those of Rheims et al., (2008), and differ from those of Atzori et al., (2004). The data reported for the motor cortex in section 4.2.4 also provides a third scenario where neither layer has a significantly different spike threshold.

This suggests that there may be some degree of inter-areal difference in the electrophysiological properties of cells recorded from individual layers. Further support for inter-areal variation in intrinsic electrophysiology is found when looking at the response of cells to hyperpolarizing current. I report that in the somatosensory cortex there is a prominent voltage sag in response to hyperpolarizing currents in layer 5 pyramidal neurons (section 4.2.6), with this phenomenon most likely caused by a greater $I_h$ current (McCormick & Huguenard, 1992; Mitra & Miller, 2007). In motor cortex I find that the $I_h$ current is less prevalent and there does not appear to be such a prominent laminar bias to its expression (section 4.2.6). However, a recent study has highlighted that the $I_h$ current is only expressed in a subpopulation of layer 5 Mctx pyramidal cells, the corticospinal projecting neurons (Sheets et al., 2011). Because I recorded from an undefined population of layer 5 cells in the Mctx, it is possible that my sampling of corticospinal cells was relatively low- explaining the limited number of cells displaying a prominent $I_h$ current. However, whilst this provides greater clarity on the presence of $I_h$ in the motor cortex, this does not necessarily mean that no difference exists between the two cortical regions. Whilst $I_h$ expression in the motor cortex seems to be specifically enriched in corticospinal neurons (Sheets et al., 2011), it is possible that the greater number of layer 5 SSC cells showing a prominent $I_h$ current can be explained by it being more broadly expressed amongst cells in this region (section 4.2.6). A more focused approach to dissecting this issue may yield some important insight, as the $I_h$ current may underlie differences in the integrative capacity of translaminar inputs and the selective recruitment of distinct cortical microcircuits (Sheets et al., 2011). In addition it has been shown to influence the oscillatory activity of cortical neurons (Dickson et al., 2000; Erchova et al., 2004) and the preferential expression of this current in layer 5 may underlie a specific role in rhythm generation (Silva et al., 1991; Berger et al., 2009). Because a prominent $I_h$ current is observed from the earliest ages of this study
(fig. 4.2 C-D), it is also possible that it may play a similar role in the generation of the early activity patterns of the cortical network (Rocha et al., 2006).

Although the electrophysiology of cortical neurons has been studied in numerous layers and regions (McCormick & Prince 1987; Kasper et al., 1994; Dickson et al., 2000; Zhang, 2004; Erchova et al., 2004; Rheims et al., 2008; Oswald & Reyes, 2008; Sheets et al., 2011), it is difficult to reliably compare these individual studies due to differences in the recording conditions, species of animal used for the study and age recordings are made. To date there seem to have been very few studies that look at inter-areal differences in intrinsic firing properties, or even interlaminar variation (see however Atzori et al., 2004; Rheims et al., 2008; Huggenberger et al., 2009). The data reported here provides some initial, although tentative, evidence that whilst the properties of pyramidal neurons located across layers and regions may be broadly similar, there are subtle differences in how individual electrophysiological phenomena segregate. However much more needs to be done to understand this variation and the potential role it plays in cortical processing of individual sensory modalities.

4.7.4 The emergence of the canonical circuit

The canonical cortical circuit is defined by certain intracortical pathways that function as the major conduits for the transfer of activity between individual layers of the neocortex (chapter 1, section 1.2; Gilbert & Wiesel, 1983; Douglas & Martin, 1991). The organization of this circuit can be summarized as follows: sensory input, relayed via the thalamus, arrives at layer 4, it then passes to layer 2/3 then on to layer 5, before exiting to subcortical structures. In agreement with this, and the studies of others who have used LSPS to map the organization of inputs within this circuit (Dantzker & Callaway, 2000; Schubert et al., 2001, 2007; Hooks et al., 2011), I find that at mature ages the primary source of input is that predicted by the canonical circuit; i.e. layer 2/3 cells receive their main source of input from layer 4, whilst the primary input source onto layer 5 cells is from layer 2/3 (section 4.3). As reported by others, layer 5 pyramidal cells in the SSC also receive strong inputs from layer 4 (Feldmeyer et al., 2005; Schubert et al., 2006). I found that although layer 4 input does not form the primary input onto layer 5 pyramidal, it does show strong pA/pixel values which in many cases are comparable to the input strength from layer 2/3 (fig.
The situation in layer 2/3 of the motor cortex is slightly different to the somatosensory cortex, as the motor cortex lacks a layer 4 (Anderson et al., 2010; Hooks et al., 2011). However the pattern of input observed here, with input primarily emanating from within layer 2/3, is in agreement with other mapping studies from this region (Hooks et al., 2011). It must also be noted that the amount of layer 2/3 input received by layer 5 pyramidal cells decreases at greater depths within the cortex (Anderson et al., 2010). Layer 5 pyramidal cells were primarily targeted for recording amongst superficial regions of layer 5. To support this there were very few pyramidal cell morphologies recovered that showed large tufted apical dendrites and only a single cell was recorded that showed pronounced repeat bursts, with both of these properties more characteristic of IB pyramidal cells in layer 5b (Schubert et al., 2006). Therefore it is unlikely that the location in layer 5 that pyramidal cells were recorded from (primarily 5a/superficial 5b), greatly influences the findings reported here. Overall, the fact that there is strong agreement between the mature input maps reported in this study and those from other laboratories, suggests that the measures taken to maximize the accuracy of the LSPS mapping protocol, reported in chapter 3, were effective.

One of the questions posed in this thesis was how, and when does this pattern of input emerge during development? Initially inputs onto cortical pyramidal cells, mapped at -60mV, were weak and, although diffusely spread throughout the mapping grid, seemed to be slightly biased towards the immediate layer. Previous research has shown that during the first postnatal week cortical neurons are coupled via gap junctions, to produce local calcium domains (Yuste et al., 1995; Kandler & Katz, 1998) and it may be that this helps promote the formation of early local connectivity. However, this does not provide us with the complete picture of synaptic connectivity in the immature network. By only mapping inputs at -60mV, we neglect the role of NMDARs, which play a prominent role at developing synapses (Rumpel et al., 1998, 2004; Hall & Ghosh, 2008). By mapping P7-8 SSC pyramidal neurons at +40mV, where NMDAR mediated inputs could be observed, I uncovered translaminar NMDAR dependant input from layer 2/3 onto layer 5, which was not apparent when mapped at close to resting membrane potential. Although this showed that NMDAR mediated connectivity plays an important role in translaminar input at early ages, I was unable to reliably map NMDAR mediated intralaminar input, due to the influence
of local GABAergic connectivity (see section 4.4.1). However, it has been reported that silent synapses are an intermediary step in the formation of local, intralaminar connectivity in other cortical layers (Ashby & Isaac, 2011). So it is also possible that there may be a significant NMDAR contribution to local inputs recorded at P7-8.

Thus it would appear that the canonical pattern of input emerges through a gradual transition from local gap junction coupled domains (Yuste et al., 1995; Kandler & Katz, 1998) and weak local connectivity (section 4.3), through NMDAR mediated silent and NMDA biased translaminar inputs (section 4.4), before the eventual emergence of AMPAR dependant inputs at later ages (sections 4.3 & 4.4). Support for this conclusion is found in other studies where the transition from NMDAR mediated input to AMPAR input has been reported to occur at pathways that link distinct components of the canonical circuit. The thalamocortical synapse (Isaac et al., 1997) and intralaminar connectivity within layer 4 (Ashby & Isaac, 2011) have both been shown to undergo this transition. Furthermore silent synapses are found in layer 2/3 and 5 pyramidal cells (Rumpel et al., 2004) and on the basal dendrites of layer 2/3 pyramidal cells (Busetto et al., 2008), which overlap with ascending axons from layer 4 (although the presynaptic source of input was not confirmed in the aforementioned study). Taken with the data reported here, it would appear that silent synapses play an important intermediary stage in the translaminar coupling of the early network and the formation of the canonical circuit.

Interestingly, silent synapses may also play a similar role in the preferential coupling of sister cells and the formation of functional minicolumns in the neocortex (Yu et al., 2009, 2012; Li et al., 2012). Two recent papers from the lab of Song-Hai Shi have studied the connectivity present amongst clonally related sister cells in the neocortex (Yu et al., 2009, 2012). They find that during the period between P1-5, sister cells are preferentially coupled via gap junctions, but that this drops off at around P5-6 (Yu et al., 2009). In contrast AMPA receptor mediated synaptic coupling is not present between sister cells at P1-5, and emerges around P10, with limited connectivity between P6-9 (Yu et al., 2012). Although connectivity mediated by NMDARs was not assessed by the authors, the period during which there seems to be a drop off in connectivity between sister cells (P6-9), coincides with the period during which I find that there is prominent NMDAR mediated coupling in the neocortex (P7-8, section
Although it is entirely possible that the connectivity between sister cells is lost at P6, only to reemerge at P10, it seems more probable, particularly given the importance of functional connectivity during development in the formation of sister cells with similar receptive fields (Li et al., 2012), that there is a transition from gap junction coupling at P1-5 (Yu et al., 2009), to immature NMDAR mediated synaptic coupling between P6-9 (section 4.4.2), followed by the conversion to AMPAR mediated inputs at P10-13 (Yu et al., 2012).

Now that we have discussed the steps taken by developing synapses in the formation of canonical connectivity, we can focus on when it emerges. Although it seems that there is translaminar synaptic connectivity between cortical layers at early ages, this connectivity is primarily mediated via NMDARs and silent synapses, which are functionally distinct from the AMPARs that underlie synaptic transmission between the layers at mature ages (Mayer et al., 1984; Rumpel et al., 1998; Ye et al., 2005). Therefore the emergence of the mature, AMPAR mediated, pattern of canonical connectivity seems to occur from around P12. By this stage AMPAR mediated synaptic inputs prevail within the cortex, with silent synapses infrequently observed (section 4.4; Rumpel et al., 1998; 2004). As such, translaminar signaling is able to occur independently of coincident depolarization in the individual layers. The mapping studies reported here show that cells recorded after this point (P13-16 and P17-21), now receive prominent inputs from the layer that is the main source of canonical connectivity at mature ages (section 4.3), and the majority of recorded input maps are significantly organized around inputs from this layer (fig. 4.14). It also seems that by P12 the thalamocortical system and intralaminar connectivity in layer 4 has reached relative maturity (Isaac et al., 1997; Daw et al., 2007; Ashby & Isaac, 2011). Taken together these findings indicate a developmental AMPARification of immature and silent synapses during the period prior to P12, which is important in setting up the canonical network. However this is not to say that the individual components of the circuit mature at exactly the same time, as it appears that the development of the thalamocortical system is slightly advanced, with silent synapses absent by P8-9 (Isaac et al., 1997), and there are some temporal differences in the expression of silent synapses within layers 2/3 and 5 (Rumpel et al., 2004).
Although the canonical circuit seems to be in place by around P12, I still observe continued maturation in the synaptic inputs of cortical pyramidal neurons after this time. This seems to be in agreement with the emergence of sensory evoked responses amongst layer 2/3 pyramidal cells, which increase rapidly from P12 onwards (Stern et al., 2001), paralleled by a strengthening of sensory input relayed from layer 4 (Bureau et al., 2004; fig. 4.12 A). Although the situation in layer 4 itself may be somewhat different (Stern et al., 2001; Ashby & Isaac, 2011), a similar phenomenon is observed in layer 5 pyramidal cells; with considerable enhancement in layer 2/3 input post P12 (figs. 4.12 & 4.13 C). At first this may seem somewhat paradoxical, because the period during which there is a strong increase in total input onto cortical pyramidal cells (post P12), is not in agreement with the phase of development during which I show rhythmic activity, elicited by exposure to 0Mg ACSF, is able to augment synaptic input (pre P12, section 4.5). This can be explained by differences in the molecular composition of individual cortical synapses, in addition to changes in the activity patterns of the network as it matures. The differences in network activity and synaptic plasticity pre and post P12, and their influence on the development of the cortical circuit, will be discussed in the following sections.

4.7.5 Activity dependant synaptic integration during early postnatal development

Throughout development the cells of the cortex are exposed to a variety of in vivo activity patterns (see chapter 1, section 1.4.1; Golshani et al., 2009; Yang et al., 2009) that likely underlie the activity dependent integration of cells into the developing network (Katz & Shatz 1996; Liao et al., 2001; Wang & Kriegstein, 2008). To examine this in more detail I elicited activity in the slice with 0Mg ACSF, which has been used successfully to study synaptic integration in dissociated cultures (Liao et al., 2001) and is known to elicit activity in acute in vitro slices (Silva et al., 1991; Flint et al., 1997). Recording the changes to synaptic inputs in response to 0Mg ACSF induced activity revealed that there was an early integrative phase, prior to P12, that coincides with the period when synchronous activity is observed in the neocortex both in vivo (Golshani et al., 2009) and in vitro (Garaschuk et al., 2000; Allene et al., 2008).
Eliciting activity using 0Mg ACSF was able to produce novel responses at sites that had previously shown no synaptic inputs (see fig. 4.20 A-D). During development the *de novo* formation of spines can be induced by the focal uncaging of glutamate adjacent to a pyramidal cell dendrite (Kwon & Sabatini, 2011). This process occurs in an NMDAR and activity dependant fashion, with the probability of inducing spine growth proportional to the frequency and dose of glutamate uncaged and reliant upon the conductance of NMDARs (Kwon & Sabatini, 2011). It is also known to preferentially occur during the developmental window prior to P12 (Kwon & Sabatini, 2011). This would support the theory that the period leading up to P12 is a period of activity dependant integration and synapse formation.

Equally it provides a method by which the ongoing activity I elicited in the slice is able to induce synapse formation through spinogenesis and AMPA receptor recruitment. In agreement with the data outlined in this chapter, Kwon and Sabatini found that this process occurred rapidly (<1 minute) and in a number of cases produced stable spines that were still observed after 30 minutes without further stimulation.

Whilst the findings of Kwon & Sabatini show that it is possible to produce a functional synapse *de novo*, an alternative mechanism that may explain the findings of section 4.5 is to potentiate synapses that are already present but either AMPA silent or show few synaptic AMPARs (Liao *et al.*, 2001; Abrahamsson *et al.*, 2005, 2008; Hanse *et al.*, 2009). A number of papers have been published in the hippocampus that report a novel form of long-term potentiation (LTP) dubbed “developmental LTP” that functions in a different manner to LTP in the adult (Abrahamsson *et al.*, 2005, 2008; Hanse *et al.*, 2009). In its developmental form synaptic potentiation can only happen if a previous period of synaptic depression has occurred (Abrahamsson *et al.*, 2008). Thus at early ages these labile synapses most likely fluctuate between an integrated state and a depotentiated state, depending upon the activity they experience *in vivo* (Groc *et al.*, 2006). Synchronous burst activity, akin to that elicited in section 4.5, and known to occur *in vivo* (Golshani *et al.*, 2009; Yang *et al.*, 2009), would act to integrate cells into the network and could explain the observed integration of cells in response to activity (section 4.5).

Interestingly, the low frequency stimulation of the synapse that brings about synaptic depression in developmental LTP is akin to baseline non-specific synaptic release that
Chapter 4- Postnatal network maturation of neocortical pyramidal neurons

is frequently observed in cortical slices. This may suggest that the prevalence of NMDA only and NMDA biased synapses reported in section 4.4.2, is increased due to the in vitro slice preparation used to study them (Groc et al., 2006; Hanse et al., 2009). The removal of the cells from the activity patterns of the early cortical network (Golshani et al., 2009; Yang et al., 2009) would shift them from a situation that maintained them in an integrated state and transfer them to one where the majority of activity is spontaneous release that could potentially induce synaptic depression. The ability of low frequency stimulation to depotentiate synapses until they become AMPA silent has been reported in the hippocampus (Montgomery & Madison, 2002; Xiao et al., 2004). AMPA silencing has been shown to occur within 30 minutes and work in almost 50% of cases (Montgomery & Madison, 2002). Given that my slices were left for at least one hour prior to recording, and for those cells recorded towards the end of each session a number of hours, it is possible that an ongoing process of synaptic depression as described above could have contributed to the presence of silent or depressed synapses at early ages (section 4.4.2). The induction of activity within the slice, which is more akin to their native state in vivo, would then have potentiated these inputs, leading to the observed augmentation of synaptic input (section 4.5.3).

Both early labile synapses and the period of glutamate induced spine formation have been shown to occur over a similar developmental window to the period of activity dependant integration observed in this study (Groc et al., 2006; Hanse et al., 2009; Kwon & Sabatini, 2011). They have also both been shown to occur in a protein kinase A (PKA) dependant fashion (Abrahamsson et al., 2008; Kwon & Sabatini, 2011), and as such they may even be part of a similar plasticity process. Either way, it appears that these mechanisms may explain the activity dependant synaptic integration of pyramidal neurons prior to P12 (section 4.5)

4.7.6 Distinct molecular pathways that regulate plasticity pre and post P12

The data reported in this chapter suggests that there are two distinct phases of synapse maturation. Initially cells are integrated into the network by bursting activity prior to P12 (section 4.5), however the inputs onto individual neurons continue to develop post-P12; with a large increase in total input observed in pyramidal neurons of all
layers and regions up until P17-21 (sections 4.3.3 & 4.3.4). Over the course of postnatal development there are a number of known changes in the molecular pathways involved in synaptic plasticity, which may explain the distinct integration strategies of cells pre-and post-P12 (Monyer et al., 1994; Sheng et al., 1994; Yasuda et al., 2003).

Developmental LTP and the de novo formation of spines are known to rely upon protein kinase A (PKA) and can be stimulated by the addition of forskolin (Abrahamsson et al., 2008; Kwon & Sabatini, 2011). In contrast, mature LTP is known to be dependant on calcium calmodulin dependant protein kinase type II (CaMKII)(Silva et al., 1992; reviewed in Lisman et al., 2002; Merrill et al., 2005). The transition between these two phases of LTP has been shown to occur at around P12 (Yasuda et al., 2003; Abrahamsson et al., 2005, 2008; Hanse et al., 2009; Kwon & Sabatini, 2011). At this time there is also a transition in the subunit composition of the NMDA receptor. The NMDAR is comprised of numerous distinct subunits whose expression is developmentally regulated. In addition to the obligatory NR1 subunit, the NR2B subunit is the main subunit expressed in the immature cortex (Gray et al., 2011). However from P7 onwards there is a gradual increase in the expression of the NR2A subunit whose expression stabilizes at around P15 (Monyer et al., 1994; Sheng et al., 1994). Importantly, the function of CaMKII is dependent upon its interaction with these subunits (Foster et al., 2010), however the outcome of this interaction during development varies depending on the NMDAR subunit that is expressed (Wang et al., 2011).

The transition from PKA mediated plasticity to CaMKII mediated plasticity has been shown to influence the spike time dependence of LTP at the developing layer 4 to 2/3 synapse (Itami & Kamura, 2012). Early, PKA mediated LTP shows bidirectional potentiation, i.e. plasticity occurs when the postsynaptic cell fires either before, or after the presynaptic input (Itami & Kamura, 2012; however see Banerjee et al., 2009 for reports of spike time dependant LTD prior to P12). In contrast mature, CaMKII mediated plasticity was reported to produce LTD if postsynaptic firing occurred prior to the synaptic input, and LTP only when firing occurred post the synaptic input (Itami & Kamura, 2012; see also Markram et al., 1997; Feldman, 2000; Sjostrom et al., 2001). The later, CaMKII dependant, form of plasticity was found to increase
rapidly from around P13 onwards (Itami & Kamura, 2012). Because the function of CaMKII is dependant upon its interaction with the NMDAR, specifically the C-terminal tail (Foster et al., 2010; Wang et al., 2011), it is likely that the developmental transition from NR2B to NR2A (Monyer et al., 1994; Sheng et al., 1994) may play an important role in the shift from PKA to CaMKII mediated plasticity. Support for this comes from studies that have shown that the developmental expression of individual NMDAR subunits differentially influences homeostatic synaptic plasticity and AMPAR insertion during synaptogenesis (Gray et al., 2011; Wang et al., 2011). However it is also possible that other factors play a role in this transition as well, such as developmental alterations in the postsynaptic density (PSD) (Bourne & Harris, 2008), such that it becomes better able to retain CaMKII (Shen & Meyer, 1999), or changes in intracellular phosphorylation pathways that may influence the relative activity of PKA and CaMKII (see Blitzer et al., 1998). It is also likely that this is not the only factor that will influence synaptic plasticity, with changes in metabotropic glutamate receptor expression and endocannabinoid signaling also occurring during postnatal development (Blue et al., 1997; Bender et al., 2006; Deshmukh et al., 2007; Larsen et al., 2010). Finally, it is also possible that the precise mechanisms that regulate synaptic plasticity at individual synapses are dependant upon the specific pathway under examination (Banerjee et al., 2009).

Regardless of the cause of this transition, it appears that there is a shift in the plasticity mechanisms that regulate synaptic function, and that this occurs at around P12 (Groc et al., 2006; Kwon & Sabatini, 2011; Itami & Kamura, 2012). The early PKA dependant, timing independent, form of plasticity seems better suited to broad integration, such as a burst based plasticity rule (Butts et al., 2007), and would thus be able to explain the integration of cells after exposure to 0Mg ACSF induced activity prior to P12 (section 4.5). At later ages the plasticity of the network shifts towards pyramidal cell integration mediated by spike time dependant plasticity (Markram et al., 1997; Sjostrom et al., 2001; Itami & Kimura, 2012). This may be less well suited to the integration of cells via burst activity (section 4.5), but is still able to drive synaptic potentiation and explains the continued strengthening of LSPS evoked synaptic inputs that occurs post P12 (section 4.3). These two forms of plasticity seem to support the idea that there is an initial broad integration of cells into the network,
followed by the consolidation of inputs that are of functional importance to the network (Larsen et al., 2010; Itami & Kimura, 2012).

4.8 Conclusions

The results of this chapter broadly define the physiological properties and synaptic connectivity of pyramidal neurons spread throughout different layers and regions of the neocortex. The period of this study encompasses a phase of development where the neocortex undergoes considerable change. We begin just as synaptic connections start to form, and start to influence the activity patterns of the cortex (Dupont et al., 2006; Allene et al., 2008), and end at a time when the neocortical network has taken on its mature structure (Dantzker & Callaway, 2000; Hooks et al., 2011). The data reported in preceding sections provides a timeline for the gradual emergence of the canonical pattern of connectivity in pyramidal cells of the neocortex. I reveal that there is a global maturation of translaminar connectivity that occurs in both layer 2/3 and 5, such that the nascent neocortex undergoes a gradual emergence of the mature, canonical pattern of input between P5 and P21.

In layer 5 of the somatosensory cortex the emergence of canonical input from layer 2/3 is predicted by NMDAR mediated synaptic inputs from that layer. These NMDAR inputs likely act as a substrate for the observed shift away from local domains and help guide the formation of the mature canonical pattern of translaminar input. Both the period where NMDAR mediated inputs predominate and when the network is found to undergo this laminar transition in input focus, coincide with the period where I was able to integrate cells into the network with bursting activity and when activity of this sort abounds within the neocortical network (Golshani et al., 2009). This period of development therefore seems to represent an integrative phase, where synapses are formed and the network begins to take on its mature structure.

The activity dependant recruitment of AMPA receptors over this initial integration period could happen due to one of two mechanisms, either the de novo formation of spines, or through the potentiation or indeed un-silencing of immature synapses (Hanse et al., 2009; Kwon & Sabatini, 2011). Both mechanisms are known to occur during this period of integration and are capable of bringing about the observed
changes in AMPA receptor input within time frames that are relevant to the experimental setup utilized in this study. The high frequency bursts elicited using zero magnesium ACSF are akin to the activity that preferentially promotes developmental LTP and spinogenesis (Abrahamsson et al., 2005, 2008; Hanse et al., 2009; Kwon & Sabatini, 2011), supporting the idea that these mechanisms explain the preferential integration of cells prior to P12. Given the synapses at this age are functionally labile (Abrahamsson et al., 2005, 2008; Groc et al., 2006; Hanse et al., 2009), it also raises the possibility that the slicing procedure acted to promote the depotentiation of synaptic inputs. By removing cells from the ongoing activity of the network, that had previously acted to maintain their integration, and transferring them to a slice, where activity is primarily mediated by low frequency spontaneous release that produces synaptic depotentiation, it is possible that I may have produced an increase in depotentiated/NMDA biased synapses. As such it is possible that the activity elicited in the slice simply acted to mimic the activity experienced by the cells in vivo and restored the synapses to their native state.

Why activity no longer integrates cells post P12 is unclear; however it is known that the mechanisms by which plasticity is elicited pre and post P12 are distinct (Yusada et al., 2003; Groc et al., 2006; Markram et al., 1997; Sjostrom et al., 2001; Itami & Kimura, 2012). This may be due to molecular changes at the synapse, most notably the transition from NR2B to NR2A NMDAR subunits, which is known to influence the synaptic integration of cells into the network (Gray et al., 2011; Wang et al., 2011) and also occurs at around P12 (Monyer et al., 1994; Sheng et al., 1994). Given the sparsification of the network that occurs post P12 (Golshani et al., 2009; Rochefort et al., 2009) it seems reasonable that the neocortex no longer requires an integration strategy that is driven by large-scale synchronous bursts, once activity of this sort is less prevalent within the network.

By P12 the thalamocortical system, which relays sensory information to the cortex, has reached relative maturity; with both thalamocortical synapses and interlaminar connectivity within layer 4 having undergone a period of AMPAification and network organization (Isaac et al., 1997; Stern et al., 2001; Daw et al., 2007; Ashby & Isaac, 2011). This point also coincides with eye opening, the unblocking of the auditory canal and the start of active whisking, and as such seems to be a period of sensory
awakening in the cortex (Shnerson & Pujol, 1983; Mosconi et al., 2010; Rochefort et al., 2011). This is not to say that the early network does not received input from the periphery (Yang et al., 2009, 2012; Minlebaev et al., 2011), but it is possible that its influence is distinct from that at later ages, due to differences in the plasticity mechanisms of the cortex (Yasuda et al., 2003; Itami & Kimura, 2012). The transition towards sparse coding of sensory information (Golshani et al., 2009; Rochefort et al., 2009), and a more temporally defined plasticity mechanism (Markram et al., 1997; Sjostrom et al., 2001; Itami & Kimura, 2012), may be an important step towards preferentially potentiating synaptic inputs that are of behavioral relevance, and to promoting the formation of stable cell assemblies within the network (Song et al., 2000).

4.9 Further work

Upon submission of the data contained within this chapter for publication, the reviewers raised two points regarding the NMDAR mapping reported in section 4.4. These issues and the steps taken to clarify the role of NMDAR signaling by others in the lab are reported below. The data can be found in the published manuscript (Anastasiades & Butt, 2012).

4.9.1 Are the NMDAR mediated responses from layer 2/3 synaptic or extra-synaptic?

It is important to determine if glutamate spillover (Asztely et al., 1997) plays a part in the observed translaminar NMDAR mediated inputs reported at P7-8, as there is a prominent extracellular pool of NMDARs particularly at early stages of development (Harris & Pettit, 2007). To perform this study we pre-incubated the slice with Bicuculline plus the NMDAR open channel blocker MK-801 (Hardingham et al., 2002). This acts to block synaptic NMDA receptors that are opened during the network activity that is produced by blocking inhibition with Bicuculline. Pre-incubating the slice in this manner will block all synaptic NMDARs but leave those that are at non-synaptic sites open. Therefore, if the translaminar NMDAR mediated inputs from layer 2/3 to 5 are not observed after slice pre-incubation, it will confirm that the inputs elicited using LSPS are synaptic in nature. This was found to be the case, with the translaminar NMDAR inputs at P7-8 lost after slices were pre-
incubated in Bicuculline plus MK-801. Thus we confirmed that the inputs reported in section 4.4 are mediated by synaptic NMDARs.

4.9.2 Do changes in the rectification properties of AMPARs over development influence the prevalence of NMDA only inputs?

It has been reported that early in development synaptic AMPARs have a distinct subunit composition to that found at more mature ages, and that this causes increased rectification at positive holding potentials (Kumar et al., 2002). To account for this we measured the somatic responses to uncaging at P7-8, under normal conditions and in the presence of the NMDAR blocker AP-5. Using the values recorded at the soma, we were able to correct for any rectification in the synaptic responses. Despite doing so we still found that there were a number of NMDA only and NMDA biased responses at early ages.
Chapter 5- Maturation of Nkx2-1 derived interneurons in the nascent neocortex

5.1 Introduction

Cortical GABAergic interneurons are a heterogeneous population of cells that possess distinct morphologies, electrophysiological properties, axonal targeting strategies and expression of immunohistochemical markers (Markram et al., 2004; Ascoli et al., 2008; discussed in chapter 1 sections 1.3.5 & 1.3.6). The source of the heterogeneity amongst cortical interneurons can be linked back to their developmental origins (Nery et al., 2002; Butt et al., 2005), with this knowledge primarily having been brought about by the application of genetic fate mapping to study the neurogenic regions of the embryonic telencephalon that give rise to cortical interneurons of different subtypes (Miyoshi et al., 2007, 2010; Gelman et al., 2008, 2011).

Fate mapping is a technique that allows researchers to study the mature properties of cells within a biological system (in this case the brain) based upon their developmental origins. It involves selectively labeling a given population of neurons at a point in development, so that it is possible to discriminate them from the remaining cells at later ages. This not only allows researchers to track the final location of the labeled cells in the mature brain but, as they can be distinguished from the surrounding cells, it is also possible to study their physiological properties in detail. By applying different genetic strategies to conditionally fate map cortical interneurons, others have found that the time and place in the telencephalon from which each cell is specified during embryonic development correlates with the mature subtype of interneuron produced (Nery et al., 2002; Butt et al., 2005, 2008; Miyoshi et al., 2007, 2010; Gelman et al., 2008, 2011; see also Wonders & Anderson, 2006; Butt & Corbin, 2011 for reviews on the subject). These studies have also led to the development of numerous driver lines that allow researchers to study discrete subpopulations of cortical interneurons (Taniguchi et al., 2011).

In this study I made use of the Nkx2-1iCre mouse line (Fogarty et al., 2007) crossed with a Z/EG reporter (Novak et al., 2000) to conditionally fate map Nkx2-1 derived cortical interneurons derived from the medial ganglionic eminence (MGE). Nkx2-1 is
a transcription factor involved in the specification of a diverse range of cortical interneurons, which occupy both deep and superficial layers of the neocortex (Fogarty et al., 2007; Butt et al., 2008). This population therefore seemed an attractive one to study, as it allowed me to compare the organization of inputs onto both deep and superficial interneurons of the same subtype, in addition to comparing these inputs with pyramidal neurons recorded from the same layers (see chapter 4, section 4.3). It would also be possible to evaluate the inputs received by interneurons of different subtypes, with distinct network roles (Silberberg & Markram, 2007; Berger et al., 2009; Cardin et al., 2009; Moore et al., 2010; Wilson et al., 2012).

Given the developmental nature of this thesis, I wanted to see how both the inputs and the intrinsic electrophysiology of Nkx2-1 derived interneurons emerged during postnatal development. By recording from Nkx2-1 derived interneurons during early postnatal periods, I was also able to uncover their physiological properties during the phase of development when GABA mediated network activity is thought to facilitate pyramidal cell integration (Allene et al., 2008; Wang & Kriegstein, 2008), and Nkx2-1 interneuron subtypes in particular are thought to play an important role in the orchestration of this activity (at least in the hippocampus, Bonifazi et al., 2009; Picardo et al., 2011). Given that there is a strong overlap between the period of GABA driven network activity, and when I find similar activity can integrate cells into the network (chapter 4 section 4.5), I hoped it would be possible to provide some insight into the findings of chapter 4 with which I could build a hypothetical model of how GABAergic interneurons and cortical pyramidal neurons may interact to drive the formation of the mature cortical circuit.

5.2 Characterization of the mature electrophysiological subtypes labeled using the Nkx2-1iCre;Z/EG genetic strategy

Nkx2-1 is expressed in numerous subtypes of cortical interneurons that arise from the MGE as well as the POA and the region adjacent to the sulcus that abuts the LGE and CGE (Fogarty et al., 2007; Miyoshi et al., 2007; Butt et al., 2008; Gelman et al., 2008, 2011; Sousa et al., 2009). By looking at patterns of gene expression within the Nkx2-1 domain it has been suggested that there are a number of distinct sub-domains along the dorso-ventral axis (Flames et al., 2007). These sub-domains may well
influence the specification of individual subpopulations of mature interneuron, as
studies have shown that there is also a dorso-ventral bias in subtype specification;
with the ventral MGE preferentially giving rise to PV positive interneurons, whilst the
dorsal MGE, which includes the ventral aspect of the Nkx6.2 expressing region,
primarily gives rise to SST positive cells (Wonders et al., 2008).

The Nkx2-1iCre mouse line used in this study has been reported to possess a
recombination deficit, such that labeling should not be observed in the dorsal most
aspect of the MGE where Nkx6.2 is also expressed (Fogarty et al., 2007). Evidence
for the Nkx6.2 expressing region as a novel source of cortical interneurons is
considerable (Fogarty et al., 2007; Wonders et al., 2008; Sousa et al., 2009), however
a thorough electrophysiological characterization of interneuron subtypes that emerge
from the non-Nkx6.2 expressing domain of the MGE has not been performed. It was
therefore important that I defined the mature interneuron subtypes labeled using the
Nkx2-1iCre;Z/EG genetic strategy. This allowed me to compare the labeled subtypes
to those reported in the numerous studies mentioned above (and in chapter 1 sections
1.3.4 & 1.35) that have used fate mapping to uncover the physiological subtypes of
interneurons that arise from the various distinct neurogenic niches of the developing
telencephalon (Fogarty et al., 2007; Miyoshi et al., 2007; Butt et al., 2008; Gelman et
al., 2008, 2011; Sousa et al., 2009).

The Petilla terminology was set up in order to provide a universal framework for the
categorization and reporting of interneuron properties (Ascoli et al., 2008). In keeping
with the spirit of Petilla, I have tried to adhere to the electrophysiological
classifications outlined therein. However in some cases I felt that this nomenclature
was insufficiently detailed to differentiate between the subtypes reported in this
section (specifically between NFS type-1 and NFS type-2 populations). In such cases
I resort to the nomenclature provided by Miyoshi et al., 2007, which makes a
distinction between the electrophysiology of these individual interneuron subtypes.

5.2.1 Electrophysiological subtypes of Nkx2-1 derived cortical interneurons

Under 40x magnification EGFP labeled cells were chosen at random from the
somatosensory regions of the cortex. This section outlines the different interneuron
subtypes recorded from labeled cells in layers 2-5 of the somatosensory cortex between P14-21 (n=43). Upon entering whole-cell mode a number of current input protocols were performed on the cells, as outlined in the materials and methods (chapter 2, section 2.3). Firstly a ramp of slowly increasing current was inserted into the cell, which allowed the current input required to elicit a threshold spike to be approximately measured. This protocol also revealed the presence, or not, of a subthreshold oscillation and, once action potentials had been elicited, any stuttering in the firing patterns of the cell at close to spike threshold. Both of these factors were an initial indicator suggestive of fast spiking (FS) subtypes (Bracci et al., 2003). FS interneurons were the most numerous of the interneuron subtypes recorded (47% of total). Initial clues that I was recording an FS cell were the previously mentioned properties at close to spike threshold, in addition to the large amount of current that was typically required to reach threshold (indicative of a low input resistance), and the presence of high frequency spontaneous EPSCs in the baseline of the trace, producing a saw-tooth like effect. However the primary determinate used to distinguish them was their rapid (>100 Hz), minimally adapting spike trains in response to current injection and their narrow spike half-widths (<1ms) with large, quick action potential afterhyperpolarizations (AHPs) (typically >20mV, McCormick et al., 1985; fig. 5.1 A). The population of recorded FS cells could be divided into one of three previously reported FS subtypes. These were based upon the properties at the point of spike threshold and were, classical FS interneurons, (fig. 5.1 A) those with threshold spikes delayed from the point of initial current input (dFS, fig. 5.1 A & Goldberg et al., 2008), and stuttering responses (FSstut, fig. 5.1 A & Miyoshi et al., 2007).

The second largest population of recorded neurons was the non-fast spiking (NFS) interneurons (35% of total). As the name suggests, this population of neurons are distinguished from FS cells by their slower maximum firing frequencies. In addition they typically have higher input resistance, a greater degree of spike train adaptation and slower spike kinetics. NFS type-1 interneurons (fig. 5.1 C) were the most similar to FS cells as they possessed lower input resistance values and on occasions had spike half-widths of <1ms. However they do not reach the rapid maximum firing frequencies of FS cells and show quite pronounced adaptation. This population also has particularly shallow AHPs and tall spikes (fig. 5.1 C).
Chapter 5- Maturation of Nkx2-1 derived interneurons in the nascent neocortex

Figure 5.1

**Figure 5.1 Main intrinsic electrophysiological profiles of Nkx2-1 derived interneurons.** A-D show the responses of recorded neurons to 500ms square current pulses. A shows the responses of FS interneurons to, from top to bottom, hyperpolarizing current stepped to -100mV and a stuttering spike response at close to threshold, current protocols eliciting a threshold spike from a delayed FS, and classical FS subtype, and the response at close to maximum firing frequency. B shows the responses of a rIB interneuron, from top to bottom, to hyperpolarizing current stepped to -100mV, current protocols eliciting a threshold spike from -80mV, and from resting membrane potential, and the response at close to maximum firing frequency. C and D top panels show the responses to hyperpolarizing currents stepped to -100mV, the middle panels show responses to current protocols eliciting a threshold spike and the bottom panels show responses at close to maximum firing frequency for NFS type-1 and type-2 respectively. Boxed regions show close-ups of the afterhyperpolarization of the threshold spike for the cells in C and D.

NFS type-2 interneurons possess higher input resistance values and wider spike half-widths (>1ms), their action potentials are also shorter than those of NFS-type 1 and they possess deeper AHPs. However the time to peak of these AHPs is far slower than those of FS interneurons, as is their maximum firing frequency (fig. 5.1 D). As with NFS type-1 interneurons, NFS type-2 show pronounced adaptation over the course of a 500ms current input (fig. 5.1 D). Amongst the NFS population there were a number of delayed NFS subtypes (dNFS, fig. 5.2 A), which could be distinguished by a delayed threshold spike, brought about by a ramped depolarization in response to initial current input (fig. 5.2 A).
Rebound intrinsic bursting (rIB) cells (16% of total) possessed a pronounced burst of more than 2 action potentials in response to release from hyperpolarized holding potentials (<-80mV, fig. 5.1 B). Their spike threshold was also typically close to their resting membrane potential (fig. 5.1 B), such that they have also been reported as low threshold spiking cells (LTS, Kawaguchi, 1995). In addition, one initial intrinsic bursting (iIB, 2% of total) cell was recorded which showed a pronounced burst (2 or more action potentials) in response to depolarizing input from resting membrane potential (fig. 5.2 B), but not in response to release from hyperpolarizing potentials (<-80mV fig. 5.2 B). Overall the major subtypes of interneuron present in the labeled
population were FS, NFS (types 1 and 2) and rIB; with iIB and dNFS subtypes observed at lower frequency (only one iIB and three dNFS cells were recorded). The breakdown of mature subtypes recorded from labeled cells in the somatosensory cortex of the \textit{Nkx2-1iCre;Z/EG} mouse can be seen in figure 5.2 (D).

5.2.2 Spatial localization of interneuron subtypes

At the time of recording each cell notes where made on the predicted laminar location. In addition, a photomicrograph of the recording pipette relative to the pial surface was taken to confirm laminar location and assist in the allocation of mapping data to the correct layers (chapter 3, section 3.6.4). By recording the distances from the pial surface for each recorded cell, a depth profile for each of the three main subtypes reported in the previous section, fast spiking, non-fast spiking and intrinsic bursting (FS, NFS and IB), could be produced to determine their laminar occupancy. The sampling range was between 150-900\textmu m from the pial surface; outside this range EGFP positive cells were observed but were not recorded. Layer 5 cells were distinguished from those in more superficial layers based upon the photomicrographs taken of the recording pipette and the location of Ctip2 staining (fig 5.2 C).

I found that both FS and NFS subtypes were located across all layers of the cortex within the sampling range (layers 2-5, fig. 5.2 C). In contrast IB cells were only ever observed at depths corresponding to layer 5 of the cortex, (>550\textmu m, fig. 5.2 C). This was not caused by any sampling bias towards deep layers, as at mature ages 21 out of 43 cells were recorded from layer 5 the rest being recorded primarily from layer 2/3. The cells were all chosen at random from within the respective layers, therefore this effect is also unlikely to be caused by any sampling or laminar bias in the recordings. Identical electrophysiological protocols were applied to cells from both deep and superficial layers, with all cells tested for rebound spikes in response to current steps to -100mV, so it is also highly unlikely the laminar bias shown by IB cells is due to any difference in the current injection protocols used to assay subtype identity.
5.3 Distinguishing between interneuron subtypes through development

In section 5.2 I defined the mature electrophysiological subtypes labeled using the Nkx2-1iCre;Z/EG genetic strategy. Using this fate mapping approach it has been possible to correlate the embryonic domain in which these cells are specified, with the mature electrophysiological subtypes produced and their localization amongst individual layers of the neocortex. However it is also of interest to determine when the heterogeneity inherent within this population emerges. Although interneurons and GABAergic signaling are known to be important in the activity patterns of the early postnatal cortex (Ben-Ari, 2002; Allene et al., 2008; Bonifazi et al., 2009), less is known about the physiological diversity amongst interneurons at this time. Furthermore, greater understanding into changes in interneuron physiology during development will provide insight into the emergence and development of cells that have specific roles in the adult network (Long et al., 2005; Doischer et al., 2008).

The Nkx2-1iCre mouse line labels a diverse range of cortical interneurons, with three main classes (FS, NFS and IB), each with a number of distinct subtypes contained within them (section 5.2.1). These cells are known to play varied roles in the adult cortex (Silberberg & Markram, 2007; Berger et al., 2009; Cardin et al., 2009; Moore et al., 2010; Wilson et al., 2012) and early born interneurons, which primarily arise from the Nkx2-1 positive MGE (Miyoshi et al., 2007), are important in the GDP activity of the developing hippocampus (Picardo et al., 2011). By recording from this line through development I can perform a comparative study into the emergence of their network properties and compare these findings with other studies into the maturation of interneuron physiology; which have mostly focused later in development, and primarily on FS cells (Doischer et al., 2008; Okaty et al., 2009; Goldberg et al., 2011; Oswald & Reyes, 2011; see however Kinnischtzke et al., 2012). However, in order to track the properties of individual interneuron subtypes through development, I required a method that would allow me to discriminate between subtypes at early ages.

Populations of interneuron often show non-overlapping expression of one or more of a number of immunohistochemical markers (Gonchar & Burkhalter, 1997; Lee et al., 2010; Xu et al., 2010; Rudy et al., 2011). Immunohistochemistry may therefore have
offered a way to distinguish FS cells from the remaining populations; as NFS and IB
subtypes are both known to express SST (Cauli et al., 1997; Butt et al., 2005. N.B.
The cells were termed regular spiking non-pyramidal cells and burst spiking non-
pyramidal cells in these papers as they were produced prior to the introduction of the
Petilla classification system, however their electrophysiological properties largely
conform to those termed NFS and IB here and in Miyoshi et al., 2007). Work by an
MSc student in the lab had indicated that SST expression was approaching mature
levels by P7 (S. Raffiq, unpublished data), therefore SST immunohistochemistry
should have allowed me to segregate FS cells from the other Nkx2-1 interneuron
subtypes at early ages. Although FS cells can be defined by their expression of PV
(Chow et al., 1999; however also see Blatow et al., 2003), PV was not of use as its
expression is developmentally delayed, such that it is only weakly expressed from
P9/10 onwards and does not reach reliable expression levels until P14 (S. Raffiq,
unpublished data; del Rio et al., 1994).

Immunohistochemistry requires the recovery of the cell post recording, in order to fix
and stain the tissue with the appropriate antibodies. Unfortunately in the vast majority
of cases either the morphology of the recorded interneurons was not recovered or the
cells did not fill in the first place, making immunohistochemical analysis impossible.
A similar shortfall would have occurred had I made attempts to use morphology to
distinguish the subtype of immature interneurons (McDonald et al., 1982 a, b, c).
Therefore, whilst it may have been possible to use immunohistochemistry and
morphology to distinguish between cells at early ages, this was not feasible for the
data reported here due to technical difficulties encountered over the course of this
study.

Unlike both morphology and immunohistochemistry, the electrophysiological
properties of individual neurons were obtained for all recorded cells. Therefore an
approach to distinguish between subtypes using electrophysiology would be more
appropriate. It was not possible to simply look at the individual electrophysiological
parameters of the cells, as the properties of early interneurons are less well known
(its one of the reasons for this study). Phase space analysis of action potentials has
been used by other laboratories to distinguish between interneuron subtypes (Tricoire
et al., 2010), and has proven successful at distinguishing between FS and NFS
5.3.1 Use of phase space plots to separate interneuron subtypes

Interneurons were recorded between P5-21 and were divided into three age groups. From P14 individual interneuron subtypes have previously been distinguished based upon their electrophysiological properties (Butt et al., 2005; Miyoshi et al., 2007), cells recorded from P14 onwards were therefore classed as mature. P9-13 was deemed intermediate as during this period an upregulation in properties associated with the FS phenotype has been reported (Du et al., 1996; Goldberg et al., 2011) and all cells earlier than this were classed as immature (P5-8). As phase space analysis is capable of distinguishing between FS and NFS interneurons (Daw et al., 2007), which are two of the main classes of interneuron reported in this study (section 5.2.1), I applied phase space analysis to cells recorded at each of these ages to confirm it was suitable to distinguish between FS and NFS populations across development.

Phase space plots involve calculating the differential of the membrane voltage with respect to time across one or more action potentials and plotting the resultant values with respect to the membrane potential of the cell at which the value occurred (Naundorf et al., 2006). Firstly I applied phase space analysis to mature cells that had been previously classified in section 5.2.1 (fig. 5.3 A). This confirmed that FS and NFS cells each had distinct phase space profiles that were similar to those reported by Daw et al., (fig. 5.3 A). The phase space plots of NFS cells possess a pronounced bump in the trace, indicating altered kinetics at the point of action potential initiation,
and a greater degree of variation between individual spikes, due to adaptation in the individual spike properties (Daw et al., 2007). In contrast FS cells show tightly overlapping, consistent plots that lack a bump at the point of action potential initiation (fig. 5.3 A). Four cells had ambiguous plots that were slightly reminiscent of both subtypes, hindering their classification using this approach alone.

Figure 5.3

Figure 5.3 Phase space plots to distinguish between interneuron subtypes across development.
A shows phase space plots for each of the interneuron subtypes outlined in section 5.2.2. From top left, NFS type-1, NFS type-2, dNFS, iIB, rIB and FS. The values along the x-axis correspond to membrane potential (mV) and the y-axis is the rate of change of membrane voltage with respect to time (mV/ms$^{-1}$). Arrows indicate the pronounced bump at the point of action potential initiation, which is characteristic of NFS subtypes. B response of P8 NFS cell to 500ms current pulse of 60pA and at close to maximum firing frequency and phase space plot of the same cell. C same as B but for a P6 FS interneuron in response to a 490pA current input. The phase space plots in B and C are produced as in A. D close-up of the threshold spike of the cells in B and C. The light grey trace is the NFS cell and the dark grey trace the FS cell. Both spikes are plotted to the same scale for comparison.

Although there was a degree of variation between the individual NFS subtypes, IB cells showed phase space plots that were broadly similar to those of NFS interneurons, with a pronounced bump at the point of action potential initiation, and adaptation across multiple spikes (fig. 5.3 A). Thus it was not possible to distinguish between NFS and IB cells using phase space analysis, however IB cells at mature
ages could be distinguished from NFS cells thanks to their characteristic bursts (fig. 5.1 B & fig. 5.2 B).

Applying phase space analysis to cells recorded from the earliest age range of this study showed that cells with phase space plots indicative of FS subtypes possessed intrinsic electrophysiological properties that were more FS like (possessing rapid maximum firing frequency, narrow spike half-widths and large amplitude shorter latency AHPs, fig 5.3 C-D) and that those cells with NFS phase space profiles conform to what would be expected from an immature NFS interneuron (i.e. slower relative maximum firing frequency and spike half-widths. fig 5.3 B, D). The phase space plots for each subtype at immature ages are in strong agreement with those reported by Daw et al., (2007) for these two distinct interneuron subtypes recorded at immature ages, they are also comparable to those reported for mature interneurons of the same subtype at mature ages (fig 5.3 A-C).

I also wanted to ensure that the phase space plots I generated were the same across a range of current inputs. This would allow me to be confident that if assigning a cell using phase space analysis, the point on the current frequency curve from which the spikes were taken would have no bearing on the correct allocation of the cell. Phase space plots were made from a sample of 16 interneurons at both P5-8 (n=6) and P14-21 (n=10), with the number of spikes elicited, and therefore the current input, varied between 1 and 20 action potentials. I found that the shape of the plot was consistent at both age ranges, which corresponded to the two extremes of this study (fig 5.4 A-D). The plots were also consistent across a large current/frequency range (2-40Hz), with both threshold spikes and those elicited at points below spike failure able to produce plots with an overall shape that was characteristic of the presumed subtype (figs. 5.3 A & 5.4 A-D).

Phase space plots therefore appear to be a reliable method to distinguish between interneurons subtypes across the entire period of this study and are consistent over a large firing frequency range. The phase space plots of FS cells and NFS cells reported here agree with those reported for layer 4 FS and non-FS interneurons of the neocortex at similar ages to this study (Daw et al., 2007). They also strongly correlate
with the subtype of each recorded cell that was apportioned upon initial analysis of intrinsic electrophysiological properties at P14-21 (section 5.2.1).

Figure 5.4

**Figure 5.4 Phase space plots of interneurons at early and late developmental stages.** A-D show the responses of interneurons recorded at different ages and taken at varying current inputs. The panels are of increasing current input such that the cell responded with, from left to right, one, five, ten and twenty action potentials. A is a P17 NFS interneuron, B an immature (P5) NFS interneuron, C is a mature (P18) FS interneuron, D is an immature (P8) FS interneuron. The values along the x-axis correspond to membrane potential (mV) and the y-axis is the rate of change of membrane voltage with respect to time (mV/ms⁻¹).

This supports the use of phase space analysis as an accurate method to distinguish between FS and NFS subtypes across the entire developmental period of this study. For the remaining sections of this chapter cells recorded outside the mature age range described as being FS or NFS were assigned based upon their phase space plots. As rIB cells show phase space plots that are similar to NFS cells, rIB cells were distinguished from NFS cells based upon their rebound bursts in response to
hyperpolarizing current stepped to -100mV (fig. 5.1 B). At each stage of development there was also a population of cells that could not be easily classified into any either subtype using phase space analysis. I recorded 4 such cells at mature and intermediate ages and 11 at immature ages. With the exception of the cells at mature ages, which could be classified using alternative approaches (section 5.2.1), these cells were excluded from further analysis.

5.4 Electrophysiological maturation of Nkx2-1 derived interneurons

The preceding section of this chapter described the use of phase space analysis to separate recorded interneurons into either FS or NFS subtypes from early postnatal ages (P5) until adulthood (P21). Using this approach to separate the subtypes I can now look at the intrinsic membrane properties of the recorded cells to track the maturation of individual interneuron subtypes contained within the developing cortical network. Previous studies looking at the development of cortical FS interneurons have supported a late physiological emergence of the FS phenotype (Du et al., 1996; Goldberg et al., 2011). However these studies were comparing the properties of early cells against their mature counterparts, as opposed to the properties of other neurons at that age, and in particular other interneurons. Producing an age matched comparison of electrophysiological parameters between FS and NFS populations throughout postnatal development will help uncover the degree of overlap between the electrophysiological properties of these two populations at early ages.

5.4.1 Developmental maturation of interneuron intrinsic properties

Phase space analysis was able to separate between FS and NFS populations throughout development, with rIB cells found to possess similar phase space profiles to NFS subtypes (section 5.3.1). At mature ages it was possible to distinguish between NFS and rIB populations due to rIB cells distinctive rebound burst in response to hyperpolarizing currents (fig. 5.1 B). However at intermediate and immature ages the prevalence of cells that possessed this phenotype decreased relative to mature ages (discussed in more detail in section 5.4.6). Because of this the intrinsic membrane properties of rIB cells are only reported at mature ages, as this was the only age where
they were reliably observed. The properties of the one iIB cell recorded at mature ages is not reported.

To compare the maturation in the intrinsic electrophysiological properties of FS and NFS neurons I divided the recorded cells into the three age ranges outlined in section 5.3.1, immature P5-8, intermediate P9-13 and mature P14-21. The number of cells of each subtype recorded in each age group is shown in table 5.1. Between immature (P5-8) and mature ages (P14-21) I observed significant maturation in a number of intrinsic membrane properties of both FS and NFS interneurons (table 5.1). Both subclasses possess significantly faster spike half-widths, lower input resistance, faster firing frequencies and faster tau at P14-21 than at P5-8 ($p < 0.001$ for all parameters except NFS tau $p < 0.05$, table 5.1). Spike train adaptation matures in both FS and NFS cells but unlike other parameters, rather than both subtypes showing a trend in the same direction, FS cells show a significant decrease in percentage adaptation ($p < 0.01$), whilst NFS cell adaptation increases over development ($p < 0.05$, table 5.1). The delay to the trough of the spike afterhyperpolarization (AHP) occurs at a more rapid rate, especially in FS cells ($p < 0.001$), which are known to possess rapid, large amplitude AHPs (McCormick et al., 1985; Kawaguchi, 1993). AHP amplitude, along with spike delay, spike threshold, spike height and resting membrane potential did not show any significant change over the course of development ($p > 0.05$, table 5.1).

Given that some of these parameters are known to distinguish between FS and NFS cells at mature ages (section 5.2.1, McCormick et al., 1985; Miyoshi et al., 2007), this finding suggests that there may be some distinction between the electrophysiological properties of FS and NFS interneurons from the earliest ages of this study.

5.4.2 Comparison between FS and NFS subtypes throughout development

To determine if there were any differences between the properties of FS and NFS cells at each stage of development, I performed age matched statistical analysis between the two populations (i.e. immature FS with immature NFS) across all of the electrophysiological parameters shown in table 5.1. When comparing between FS and NFS interneurons at the immature age range, I found that they exhibited significant differences in many of the parameters traditionally used to distinguish FS cells later in development (McCormick et al., 1985; Kawaguchi, 1993, 1995).
Chapter 5- Maturation of Nkx2-1 derived interneurons in the nascent neocortex

Table 5.1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FS</th>
<th>NFS</th>
<th>rIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV) (n=18)</td>
<td>-63±1</td>
<td>-62±1</td>
<td>-64±1</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-36±1</td>
<td>-35±1</td>
<td>-34±1</td>
</tr>
<tr>
<td>Spike height (mV)</td>
<td>64±2</td>
<td>65±1</td>
<td>63±1</td>
</tr>
<tr>
<td>Spike half-width (ms) *</td>
<td>1.6±0.1</td>
<td>1.2±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Spike delay (ms)</td>
<td>209±25</td>
<td>224±30</td>
<td>300±39</td>
</tr>
<tr>
<td>Rm (MΩ) *</td>
<td>598±46</td>
<td>312±24</td>
<td>215±14</td>
</tr>
<tr>
<td>Max. Freq. (Hz) *</td>
<td>76±4</td>
<td>99±6</td>
<td>151±7</td>
</tr>
<tr>
<td>Initial Freq. (Hz) *</td>
<td>89±4</td>
<td>117±6</td>
<td>165±7</td>
</tr>
<tr>
<td>Final Freq. (Hz) *</td>
<td>71±3</td>
<td>91±6</td>
<td>142±7</td>
</tr>
<tr>
<td>Adaptation (%) *</td>
<td>21±1</td>
<td>23±2</td>
<td>15±2</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>54±6</td>
<td>29±4</td>
<td>13±2</td>
</tr>
<tr>
<td>AHP delay (ms)</td>
<td>18±2</td>
<td>14±1</td>
<td>6±1</td>
</tr>
<tr>
<td>AHP amp. (mV) *</td>
<td>23±1</td>
<td>21±1</td>
<td>21±1</td>
</tr>
</tbody>
</table>

* Values shown as ± are SEM. Greyscale shading indicates degree of significance relative to P5-8 for cells of the same subtype. Light grey p<0.05, darker grey p<0.01, darkest grey p<0.001. Boxes with no colour show no significant difference relative to P5-8 (p>0.05). Red stars (*) indicate parameters that are significantly different across all three age groups between FS and NFS cells (p<0.05). For FS and NFS cells red boxes indicate parameters that are significantly distinct when compared between FS and NFS interneurons only at the age the box is present (p<0.05). For rIB cells all parameters that are significantly different between FS and NFS cells are also significant for rIB cells. Blue boxes indicate parameters that are also significantly different between NFS and rIB cells (p<0.05).

Table 5.1 Intrinsic membrane properties of individual interneuron subtypes across development

Intrinsic membrane parameters of recorded interneurons. Rm input resistance, Max. Freq. maximum firing frequency, Initial Freq. maximum frequency in first 100ms of current pulse, Final Freq. maximum frequency in last 100ms of current pulse, AHP delay, delay to peak of action potential afterhyperpolarization, AHP amp, peak amplitude of action potential afterhyperpolarization. Values shown as ± are SEM. Greyscale shading indicates degree of significance relative to P5-8 for cells of the same subtype. Light grey p<0.05, darker grey p<0.01, darkest grey p<0.001. Boxes with no colour show no significant difference relative to P5-8 (p>0.05). Red stars (*) indicate parameters that are significantly different across all three age groups between FS and NFS cells (p<0.05). For FS and NFS cells red boxes indicate parameters that are significantly distinct when compared between FS and NFS interneurons only at the age the box is present (p<0.05). For rIB cells all parameters that are significantly different between FS and NFS cells are also significant for rIB cells. Blue boxes indicate parameters that are also significantly different between NFS and rIB cells (p<0.05).

For example, FS cells at mature ages have significantly lower input resistance, faster maximum firing frequencies and narrower spike half-widths than NFS neurons.
(p<0.001 fig. 5.5 A-C). Each of these properties was also found to be significantly different at the immature age range, with immature FS cells firing at faster frequencies, possessing narrower spike half-widths and lower input resistance than NFS cells recorded at the same age (fig. 5.5 A-C, p<0.001 for spike half-width and maximum firing frequency p<0.01 for input resistance).

Figure 5.5

Many other parameters were found to differ significantly between immature FS and NFS cells: these were spike threshold, spike height, initial firing frequency, final firing frequency, spike train adaptation and spike afterhyperpolarization amplitude (p<0.001 for all except spike height p<0.01 and adaptation p<0.05, table 5.1). Furthermore despite these properties showing considerable maturation in many cases, all of these factors displayed this difference over the course of development (table 5.1, p<0.001 between FS and NFS interneurons for all previously listed parameters at both...
intermediate and mature ages). Most of the time any maturation that occurred acted to maintain or strengthen any pre-existing differences between the two populations; however a few exceptions were observed. Tau was initially similar amongst both subtypes, but underwent a developmental divergence such that by later ages it was significantly distinct between FS and NFS/rIB populations (table 5.1. \( p<0.01 \)). The time to afterhypoprarpolarization peak showed this trend in reverse, being significantly distinct at immature ages \( (p<0.05, \text{table 5.1}) \), but not at intermediate or mature ages \( (p>0.05) \).

5.4.3 Delayed relative maturation of FS spike properties

The ability of FS cells to fire fast, brief action potentials is due to the expression of potassium channels of the Kv3 subfamily, particularly Kv3.1 (Chow et al., 1999; Erisir et al., 1999), whose developmental expression increases steadily from P9/10 (Du et al., 1996). This increased expression correlates with findings from other laboratories that suggest a delayed electrophysiological emergence of FS cells based upon the late maturation of properties such as maximum firing frequency and spike half-width, which are associated with rapid conductance (Goldberg et al., 2011). To see how this late developmental profile contrasts with other interneuron subtypes, I compared the normalized values of both spike half-width, maximum firing and input resistance for NFS and FS cells recorded between P5-21 (fig. 5.5 D-E). Input resistance was chosen because like both of the other properties it is known to be significantly different between FS and NFS interneurons from the earliest ages of this study. Furthermore input resistance undergoes significant maturation, allowing me to track the time-course of the developmental change and compare it to that of spike half-width and maximum firing frequency.

As reported previously (Goldberg et al., 2011), the maximum firing frequency and spike half-width of FS cells showed a delayed non-linear pattern of maturation (fig. 5.5 D-E). In contrast NFS interneurons showed a linear maturational profile for both properties (fig. 5.5 D-E). The rapid non-linear phase of FS maturation occurred from P9-10 onwards, with normalized spike half-width and maximum firing frequency values significantly less than those observed for NFS cells until P16 \( (p<0.05, \text{fig. 5.6 D-E}) \). This effect was not observed in input resistance, which does not depend upon
the rapid conductance of Kv3.1. The maturation of input resistance followed a fairly similar, linear pattern of maturation regardless of cell subtype (fig. 5.5 F).

5.4.4 Comparison between the electrophysiology of interneurons of the same subtype recorded in layer 5 and layer 2/3

As discussed in chapter 1 (section 1.3.3), the cortex develops in an inside out fashion, with earlier born cells populating deep layers and later born cells forming the superficial layers (Angevine & Sidman, 1961; Rakic, 1971). To determine if this may have influenced the developmental maturity of cells located in distinct cortical layers, I compared their electrophysiological properties within type (i.e. FS with FS and NFS with NFS), at each of the three developmental ages mentioned previously. With the exception of threshold spike delay, I found no statistical difference between cells located in layer 2/3 and 5 for each of the parameters shown in table 5.1 (p>0.05). Spike delay was found to be greater in layer 2/3 FS cells than those located in layer 5 at both immature, intermediate and mature time points (p<0.01 for immature and intermediate, p<0.05 for mature. Immature layer 2/3 n=7, delay 297ms±35, layer 5 n=8, delay 158ms±20, intermediate layer 2/3 n=12, delay 311ms±36, layer 5 n=8, delay 101ms±32, mature layer 2/3 n=5, delay 410ms±48, mature layer 5 n=7, delay 222ms±68). This finding is likely due to the increased proportion of dFS subtypes known to be present in layer 2/3 (Goldberg et al., 2008).

Amongst NFS cells the only significant difference was in spike delay at intermediate ages, with layer 5 cells found to possess greater threshold spike delay than those in layer 2/3 (p<0.01, layer 2/3 n=9, delay 159ms±33, layer 5 n=7, delay 324ms±30). However, there was no significant difference in spike delay at immature (p>0.05, layer 2/3 n= 4, delay 303ms±70, layer 5 n=12, delay 264ms±20), or mature ages (p>0.1, layer 2/3 n=6, delay 281ms±36, layer 5 n=7, delay 197ms±38). Unlike FS cells, the spike delay properties in NFS cells did not show a strong trend towards differences between the layers. This may in part be due to the distinct electrophysiological properties displayed by individual subclasses of NFS cells (see section 5.2.1) and different relative sampling of these subtypes in each age group.
5.4.5 Comparison between the electrophysiology of rIB and other interneuron subtypes

The properties of mature rIB interneurons were found to be similar to mature NFS cells across many of the parameters tested (table 5.1). The only significant differences that were observed was in their input resistance, which was significantly higher than both NFS cells and FS cells \((p<0.001, \text{fig. 5.6 C})\), their spike threshold, which was significantly more hyperpolarized (table 5.1, \(p<0.05\) relative to NFS and \(p<0.001\) for FS) and their action potential afterhyperpolarization amplitude, which was significantly smaller than both FS and NFS subtypes (table 5.1, \(p<0.001\) for both FS and NFS). Apart from these differences the properties of NFS and rIB cells appeared to be fairly matched, and rIB cells were significantly distinct across all parameters where FS and NFS cells were previously found to differ at mature ages (maximum firing frequency, initial firing frequency, final firing frequency, spike height, percentage adaptation, spike half-width and tau. \(p<0.001\) for all parameters between FS and rIB except spike height \(p<0.01\)). The properties that make rIBs distinct from NFS neurons are those that increase their relative excitability and produce cells with a low threshold firing (LTS) phenotype (Kawaguchi, 1995). This is emphasized in figure 5.1 (fig. 5.1 B) where their high input resistance and hyperpolarized spike threshold mean that the threshold spike occurs close to resting membrane potential and with minimal current input.

5.4.6 Late emergence of the rIB phenotype

At mature ages rIB cells formed an independent subtype of interneuron that could be distinguished from other cells by their distinctive burst when released from hyperpolarizing potentials (fig. 5.1 B). Their remaining properties were found to be largely similar to NFS interneurons, with the only differences being their higher input resistance, more hyperpolarized spike threshold and shallower AHP amplitude (table 5.1 and section 5.4.5). The use of conditional genetic fate mapping in these experiments ensures that the labeled population comprises the same cell types at each stage in development. Therefore as long as a sufficient number of cells are recorded at each age, any cells that are found in one sample should be found in other sample groups at a similar frequency. This was not observed to be the case for rIB
interneurons, which formed a significant proportion of cells recorded at mature ages (16%), but occurred at much lower frequencies at intermediate and immature ages (6% and 3% respectively). This suggests that either there may have been some sort of sampling error in the earlier two age groups, or that there is a delayed developmental expression of the rIB phenotype, causing them to be preferentially observed at later ages.

Firstly I calculated the total number of cells recorded in each age group, which revealed that the number of cells recorded at each age was similar (40 neurons at immature ages, 47 at intermediate and 42 at mature). Given that rIBs are only observed in layer 5 (fig. 5.2 C), I next looked at the number of cells specifically located in this layer. This was also found to be similar across ages (21 mature, 18 intermediate and 21 immature) and therefore unlikely to account for the observed drop in recorded rIB cells. A larger proportion of rIB neurons appeared to be located in deeper parts of layer 5 compared to NFS and FS cell types so it is possible that, even though there was no significant laminar bias in the sampling, there may have been some intralaminar sampling variance between the three ages that could explain the observed differences. Values for the average depth of all recorded cells located within layer 5 showed that there was a discrepancy in the average depths from which cells were recorded (mature 723±26, intermediate 624±22 and immature 630±27). The average depth of rIBs was 712±34; this meant there was a trend towards significance between the recording depths of rIBs and immature neurons (p=0.07) and a significant difference at intermediate ages (p<0.05). Whilst this may explain the reason why fewer rIB cells were recorded at these earlier ages, it seems further work is necessary to unequivocally determine if the decreased frequency of rIBs cells at later ages is due to a sampling error, or the late developmental emergence of this particular electrophysiological phenotype.

5.5 Cluster analysis of interneuron intrinsic membrane properties

During preceding sections of this chapter I have reported the postnatal maturation of interneuron electrophysiological properties. I have shown that at immature ages cortical interneurons possess distinct phase space plots (fig. 5.3 B-C) and that FS and NFS subtypes are significantly distinct across a number of electrophysiological
parameters (fig. 5.5. A-C; table 5.1). However the preferential observance of rIB cells at older ages (section 5.4.6) and the late developmental maturation of FS cells (section 5.4.3) also suggest that the full diversity of electrophysiological subtypes within the Nkx2-1 population may not be realized until later ages. To look at this in more detail I applied cluster analysis to the intrinsic data recorded at the three age ranges reported previously in this chapter. This allowed me to look at the data on a cell-by-cell basis, whilst simultaneously comparing across multiple electrophysiological parameters. To segregate the populations I used a number of physiological properties that I had shown were significantly different between FS and NFS interneurons across development (section 5.4.2). These were: spike threshold, spike height, spike half-width, input resistance, maximum firing frequency, AHP amplitude and AHP delay. This group of parameters also contains the three that have been shown to distinguish rIB cells from NFS populations at mature ages (table 5.1 and section 5.4.5).

5.5.1 Cluster analysis at mature ages

Initially I performed k means cluster analysis on the intrinsic data recorded from cells at P14-21. To perform this analysis I set the number of clusters to three, as I have shown that there are three main populations of interneuron labeled in the Nkx2-JiCre mouse FS, NFS and rIB (the one iIB cell was excluded from the sample). The division of cells into each cluster was in agreement with the allocation of cells in section 5.2.1, with one cluster dominated by FS cells, one cluster primarily containing NFS cells and one cluster rIBs. Although the segregation of the three main subtypes into separate cluster was strong, there were some cells that did not segregate based upon their subtype. There were 5 NFS type-1 cells that were found in the FS cluster (cluster 1). The presence of these cells in this cluster is likely due to their low input resistance and narrow spike half-widths; properties they share with FS cells. Within the NFS cluster (cluster 2) there were two FS cells and two rIB cells. The FS cells were recorded at P14 and therefore slightly less mature, with higher input resistance and slower maximum firing frequency than other FS cells contained within the mature data set (although both fired at >100Hz). The two rIB cells had shorter spike heights and lower input resistance than other rIBs recorded at this age. The remaining rIB cells formed a third distinct cluster (cluster 3) with one NFS cell also found within this group.
The silhouette value for each cell compares within cluster distances with between cluster distances (Rousseeuw, 1987) and is an indicator of how close a cell is to other clusters. Silhouette values range between 1 and -1, with cells possessing high positive silhouette values correctly allocated, whilst those data points whose silhouette values are close to -1 are in an incorrect cluster. Figure 5.6 shows the silhouette values for each of the cells after performing k means clustering at mature ages (number of groups = 3). The majority of cells have a silhouette value greater than 0.8, indicative of good separation from the remaining clusters. The cells that show lower silhouette values were those cells mentioned above that were allocated into a different group to their subtype; the NFS type-1 cells that fell into the FS group, the two FS and rIB cells in the NFS group and the NFS cell in the rIB group.

**Figure 5.6**

![Silhouette values of mature cells grouped with k means clustering](image)

**Figure 5.6 Silhouette values of mature cells grouped with k means clustering.** Silhouette values for individual cells after performing k means cluster analysis on the intrinsic membrane properties of cortical interneurons recorded at mature ages with the number of groups set at 3. Cluster 1 represents primarily FS like neurons, cluster 2 NFS like neurons and cluster 3 rIB like neurons. The distance metric used to perform this analysis was Euclidean distance squared.

The high silhouette values for the cells in each cluster indicate that k means clustering with 3 groups, using the parameters outlined above, produces clusters where the majority of cells are well separated from cells in other clusters. To further confirm the
reliability of the groups allocated using k means clustering, I performed hierarchical clustering on the same data set (fig. 5.7). Unlike k means clustering, hierarchical clustering groups the data over varying scales- from each data point forming an individual group, to all the data combined into a single cluster. It is then possible to see which degree of clustering best fits the data. Hierarchical clustering produced similar clustering to that observed using k means, with an FS group, NFS group and rIB group (fig. 5.7).

Figure 5.7

**Figure 5.7 Dendrogram of the result of hierarchical clustering of mature interneurons.** Dendrogram produced after performing hierarchical clustering on all cortical interneurons recorded at mature ages (P14-21). The shaded circles represent individual cells, with the subtypes shown in the key at bottom right. The distance metric used to perform this analysis was Euclidean distance squared. Cluster 1 represents primarily FS like neurons, cluster 2 NFS like neurons and cluster 3 rIB like neurons. Each cluster approximately corresponds to those clusters shown in figure 5.6.

When comparing between the allocation of cells using k means and hierarchical clustering I found that there was an agreement of 97.6%, the only difference being that one of the NFS type-1 cells in the NFS group was switched to the FS cluster when using hierarchical clustering (the cell with a negative silhouette value in fig. 5.6). The strong agreement between the clusters generated with these two distinct algorithms suggests that the allocation of cells is reliable. Thus FS, NFS and rIB cells can be said to fall into three discreet clusters at mature ages, with a population of NFS type-1 interneurons contained within the FS cluster.
5.5.2 Cluster analysis at intermediate ages

I next performed k means cluster analysis on the data recorded at intermediate ages, using the same intrinsic parameters and number of groups as at mature ages. Again there was one cluster that was dominated by FS cells (cluster 1), but also contained 3 NFS type-1 interneurons that had more rapid spike half-widths and lower input resistance than other NFS cells recorded at this age. The second cluster was a mixed group containing 6 NFS, 9 FS and 2 rIBs (cluster 2). Many of the FS cells that were contained within this group were recorded at P9 (n=5) and had slower kinetics than other FS cells in the data set. The third cluster primarily comprised NFS cells with the exception of 1 rIB (cluster 3). Plotting the silhouette values for the cells after k means clustering again showed that many of the cells have high silhouette values (fig. 5.8).

Figure 5.8

Figure 5.8 Silhouette values of intermediate cells grouped with k means clustering. Silhouette values for individual cells after performing k means cluster analysis on the intrinsic membrane properties of cortical interneurons recorded at intermediate ages with the number of groups set at 3. Cluster 1 represents primarily FS like neurons, cluster 2 a mixed population of neurons and cluster 3 NFS like neurons. The distance metric used to perform this analysis was Euclidean distance squared.
Amongst the FS group (cluster 1) those cells with lower silhouette values correspond to the three NFS type-1 and two FS cells recorded at P9. The lower values in the NFS group (cluster 2) correspond to one of the rIB cells, some of the older FS cells and one NFS type-1. The final cluster has a number of cells that show lower silhouette values (cluster 3, fig. 5.8). As the third cluster seems to be less well defined than the others at intermediate ages and given that rIB cells are less prevalent at this time (section 5.4.6), it was important I validated the legitimacy of imposing three clusters on the data. Performing hierarchical clustering, where the number of defined clusters is not pre-defined, produced a strong agreement with the groupings produced using k means clustering. Using hierarchical clustering there are still 3 main clusters (fig. 5.9), with 98% of the cells found in the same cluster as when using k means- the only exception being a FS cell that switched from the mixed group (cluster 2) to the FS group (cluster 1). The agreement in the allocation of cells using these two methods confirms the reliability of this analysis and that despite the drop in the number of rIBs at intermediate ages the data still segregates into 3 discrete clusters.

**Figure 5.9**

Dendrogram produced after performing hierarchical clustering on all cortical interneurons recorded at intermediate ages (P9-13). The shaded circles represent individual cells, with the subtypes shown in the key at bottom right. The distance metric used to perform this analysis was Euclidean distance squared. Cluster 1 represents primarily FS like neurons, cluster 2 a mixed population of neurons and cluster 3 NFS like neurons. Each cluster approximately corresponds to those clusters shown in figure 5.8.
5.5.3 Cluster analysis at immature ages

Performing cluster analysis at immature ages appeared to be less effective at segregating the populations than at intermediate or mature ages. Whilst one cluster was comprised of NFS cells and the one rIB recorded at this age (cluster 3) the other two clusters contained a mixture of FS and NFS cells. Cluster 1 contained cells recorded at later ages (64% of cells recorded at P8) and which typically possessed faster spike kinetics and lower input resistance. The other group (cluster 2) was made up of NFS and FS cells with varied properties and evenly distributed between earlier and late ages within the recording window (50% of cells recorded at P5/6, and 50% recorded at P7/8). The silhouette values for the clusters are still high, with the exception of some cells in cluster 1 (fig. 5.10).

**Figure 5.10**

*Figure 5.8 Silhouette values of immature cells grouped with k means clustering.* Silhouette values for individual cells after performing k means cluster analysis on the intrinsic membrane properties of cortical interneurons recorded at intermediate ages with the number of groups set at 3. Clusters 1 and 2 represent mixed interneuron subtypes, with cluster 2 cells possessing faster kinetics. Cluster 3 contained primarily NFS interneurons. The distance metric used to perform this analysis was Euclidean distance squared.
There is also a strong agreement between the groups allocated using k means clustering and hierarchical clustering (fig. 5.11), with 90% of cells falling into the same groups as determined by both methods. The fact that more mature cells were segregated into a discrete cluster (cluster 2), suggests that age may be an important factor in the discrimination of subtypes at this early stage of development. To test this hypothesis I performed k means cluster analysis on cells recorded solely at P7-8 (n=22), or at P5-6 (n=18). At P7-8, 3 NFS cells occupied one cluster, the second cluster contained a mixture of 4 FS and 5 NFS and the third 3 NFS and 7 FS cells. At P5-6 one cluster was occupied by 6 NFS cells, the next by 2 NFS, the single rIB recorded at this age and a single FS cell and the final cluster was occupied by 5 FS cells and 2 NFS. Although the separation into clusters occupied by a single subtype appeared slightly more accurate when reducing the age range of the sample, there still appears to be some overlap between the populations, particularly at P7-8. Thus it would appear that the individual subtypes become less distinct at earlier stages of development, as they are unable to be reliably separated by cluster analysis across multiple parameters at this time.

**Figure 5.11**

*Figure 5.11 Dendrogram of the result of hierarchical clustering of immature interneurons.* Dendrogram produced after performing hierarchical clustering on all cortical interneurons recorded at immature ages (P5-8). The shaded circles represent individual cells, with the subtypes shown in the key at bottom right. The distance metric used to perform this analysis was Euclidean distance squared. Cluster 1 represents older cells with faster firing properties, cluster 2 a mixed population of neurons and cluster 3 NFS like neurons. Each cluster approximately corresponds to those clusters shown in figure 5.10.
Support for this can be seen in table 5.2, which shows the centroid (center point) values for each of the clusters calculated using k means clustering at the three developmental ages. At mature ages the centroid values for the three populations (FS cluster 1, NFS cluster 2 and rIB cluster 3) are distinct, indicating that the individual clusters are separated along each of the dimensions corresponding to the 7 electrophysiological parameters used in this analysis. The centroid values also agree with the electrophysiological properties previously reported for the interneuron subtypes that each of the clusters represent (section 5.2.1; table 5.1; McCormick et al., 1985; Miyoshi et al., 2007).

Table 5.2

<table>
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<th>Spike Height (mV)</th>
<th>Spike Half-width (ms)</th>
<th>R_in (MΩ)</th>
<th>Max. Freq. (Hz)</th>
<th>AHP delay (ms)</th>
<th>AHP amp. (mV)</th>
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Table 5.2 Centroid values of intrinsic electrophysiological parameters produced via k means clustering. Centroid values produced after performing k means clustering on the intrinsic electrophysiological properties of Nkx2-1 derived cortical interneurons recorded at immature (P5-8), intermediate (P9-12) and mature ages (P14-21). The number of clusters in each case was set to 3. Cluster 1 represents FS like cells, cluster 2 NFS like cells and cluster 3 rIB like cells. The seven electrophysiological parameters used in the cluster analysis are shown in row 1. R_in, input resistance, Max. Freq, maximum firing frequency, AHP delay to peak of action potential afterhyperpolarization, AHP amp, peak amplitude of action potential afterhyperpolarization.
For example cells in the FS cluster (cluster 1) possess narrow spike half-widths, low input resistance, high maximum firing frequency and shallow, rapid spike AHPs, whilst rIBs in cluster 3 have a more hyperpolarized threshold and higher input resistance than cells in the other clusters (table 5.1 & table 5.2). As we move earlier in development the centroid values for many parameters become less distinct, or do not show the patterns observed at mature ages. By immature ages the spike threshold value for each of the three clusters is almost identical, cells in clusters 2 and 3 no longer have taller spikes than those in cluster 1 and cells in cluster 3 go from having rapid, shallow spike AHPs to possessing the slowest AHPs with the largest amplitude. The same is true, albeit to a slightly lesser extent, at intermediate ages. Thus it would appear that whilst at early ages there is some diversity in the intrinsic electrophysiology of Nkx2-1 derived cortical interneurons (section 5.4.2), cells that possess the full array of electrophysiological properties used to distinguish between individual subtypes are not frequently observed until later stages of development (P14 onwards).

5.6 Excitatory synaptic input maps

To date LSPS studies into the inputs of cortical interneurons have been limited, with one study examining a diverse array of interneuron subtypes in layer 2/3 (Xu & Callaway, 2009) and a further recent study focusing on FS and LTS interneurons in layer 5 of the motor cortex (Apicella et al., 2012). My aim was to study the organization of excitatory inputs onto layer 2/3 and 5 interneurons in the somatosensory cortex and look at how both the location and subtype of a cell influences its inputs. By recording at different ages I would also be able to track any changes in the organization of these inputs that occurred as the cells matured. The developmental aspect of this study is important, as in the previous chapter we find that the inputs onto pyramidal cells undergo a shift in input organization over the course of development (chapter 4, section 4.3). As the developmental organization of inputs onto interneurons had not been studied prior to this, it was not possible to determine if this is a global phenomenon within the network, or one restricted to pyramidal neurons. Mapping the inputs onto immature interneurons will also allow me to look at their network connectivity during a phase in development when they are
important in orchestrating the activity of the network (Allene et al., 2008; Picardo et al., 2011).

To track the organization and maturation of Nkx2-1 derived interneurons I recorded from randomly selected EGFP positive neurons located across layers 2-5 of the somatosensory cortex from P5-21. Thanks to my ability to distinguish between interneuron subclasses at early ages (section 5.3.1), it was possible to follow the network integration of both FS and NFS populations as they wire-up within the cortex.

5.6.1 Mapping of excitatory synaptic inputs onto cortical interneurons

The method for calibrating the laser outlined in chapter 3 (section 3.3) was based around firing the laser at the soma of the recorded neuron and tuning the laser power according to the cells intrinsic excitability. Upon initial firing of the laser at interneurons, with identical laser settings to those used for pyramidal neurons of the same age, it became apparent that many interneurons were more sensitive to the uncaged glutamate (fig. 5.12 A-C). Thus had the laser power been tuned to the recorded interneuron, it would not have had sufficient intensity to excite pyramidal neurons and therefore I would have been unable to elicit excitatory inputs onto the recorded interneurons. To counter this I used the same approach taken when mapping NMDAR mediated inputs onto cortical pyramidal cells (chapter 4, section 4.4.1), and used the average values for previously recorded pyramidal cells at the four developmental ages reported in chapter 4. A plot of the laser intensity values used for mapping the inputs onto interneurons can be seen in figure 5.12 (D), and shows that these values overlap strongly with the values previously used to excite pyramidal cells. Therefore all the technical considerations outlined in chapter 3 would be accounted for in these experiments, allowing for accurate mapping of excitatory inputs onto cortical interneurons through development.

5.6.2 FS inputs

FS cells were shown to occupy each layer from 2-5 (fig. 5.2 C), allowing a comparative study of the development of FS cell synaptic inputs across layers. To this
end 46 FS cells were recorded located in layers 2/3 (n=16), 4 (n=11) and 5 (n=19) and their input maps constructed as described in chapter 3. Recorded cells were divided into the same age windows reported in section 5.3.1, P5-8 being immature, P9-13 intermediate and P14-21 mature.

**Figure 5.12**

**Figure 5.12 Calibrating laser intensity for mapping excitatory inputs onto interneurons using LSPS.** A-C show an example response of a cortical interneuron when mapping was performed in current clamp at a laser intensity that had been used to map pyramidal neurons of the same age. A shows a current clamp map of a cortical interneuron with action potentials indicated by coloured pixels and subthreshold responses indicated by greyscale pixels. A white circle indicates the location of the soma of the recorded cells, white squares indicate individual uncaging sites shown in panel C. Dashed lines demarcate the laminar boundaries and each pixel represents a 50x50µm region of the cortex. B shows the expanded current clamp trace of one entire sweep of the map for the cell shown in panel A. C shows individual current clamp traces from the corresponding uncaging sites highlighted in A, the black line below trace 4 represents the 100ms laser pulse. D shows a graph of the laser intensity used to map layer 2/3 and 5 cortical pyramidal neurons at different ages and the laser intensities used to map interneurons over the same developmental period.

5.6.2.1 Layer 2/3 FS inputs

Inputs onto layer 2/3 FS interneurons were found to primarily arise from within the immediate layer. Throughout development layer 2/3 was the primary source of excitatory input, with the percentage of input from within each layer found to be similar at immature (fig. 5.13 A, C, D. n=4, layer 2/3 input 84±8%, layer 4 input
5±3% layer 5/6 input 11±5%), intermediate (fig. 5.14 A, C, D n= 8, layer 2/3 input 64±4%, layer 4 15±3%, layer 5/6 21±2%), and mature ages (fig. 5.15 A, C, D n= 4, layer 2/3 69±7%, layer 4 13±6%, layer 5 18±4%). The only significant shift in input organization over the course of development was a decrease in the total amount of layer 2/3 input between immature and intermediate layer 2/3 FS cells (p<0.05), which occurred without any significant compensatory enhancement in inputs from either of the other layers (p>0.05 for both layer 4 and 5/6).

**Figure 5.13**

**Figure 5.13 LSPS maps of excitatory input onto layer 2/3 FS interneurons at immature ages.** A and D show example input maps of layer 2/3 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A and D corresponds to 80pA per uncaging site. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.
Despite the majority of input arising from within their host layer, layer 2/3 FS cells appear widely integrated, often receiving inputs from all layers of the cortex (figs. 5.13, 5.14 & 5.15 A, B, D, E). Although the input maps were dominated by large amplitude, inputs from layer 2/3 (figs. 5.13, 5.14 & 5.15 A, B, D, E), inputs from layer 4 were often of similar magnitude and the pA/pixel values for layer 4 were in many cases larger than expected based upon their contribution to the percentage of total input (figs. 5.13, 5.14 & 5.15 C, F).

Figure 5.14 LSPS maps of excitatory input onto layer 2/3 FS interneurons at intermediate ages. A and D show example input maps of layer 2/3 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 80pA per uncaging site and in D corresponds to 120pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.
This can be explained by the smaller size of layer 4 relative to layers 2/3 and 5/6, such that even though the inputs from each pixel in layer 4 are on occasions quite large, the fewer pixels from this layer diminishes their overall contribution to the total input.

**Figure 5.15**

![Figure 5.15 LSPS maps of excitatory input onto layer 2/3 FS interneurons at mature ages. A and D show example input maps of layer 2/3 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 300pA per uncaging site and in D corresponds to 160pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

When looking at the inputs onto layer 2/3 FS cells, it seems that there is a strengthening of input over the course of development. When compared to inputs at immature ages (fig. 5.13 F, layer 2/3 12±4 pA/pixel, layer 4 2±1 pA/pixel, layer 5/6
The pA/pixel values showed a trend towards larger values at later ages (figs. 5.14 & 5.15 F). At intermediate ages the pA/pixel values were found to be larger than immature ages, with inputs from layers 4 and 5/6 significantly enhanced ($p<0.05$ for both, layer 4 $13\pm3$ pA/pixel, layer 5/6 $6\pm1$ pA/pixel). Although twice that observed at immature ages, input from layer 2/3 only showed a non-significant trend towards stronger inputs at intermediate ages ($p=0.07$, intermediate layer 2/3 $24\pm4$ pA/pixel). At mature ages the pA/pixel values for inputs from layer 4 were significantly enhanced relative to immature ages ($p<0.05$, layer 4 $12\pm3$ pA/pixel), but those from other layers were not ($p>0.1$, layer 2/3 $29\pm10$, layer 5/6 $8\pm4$), nor were the mature pA/pixel values for any of the layers significantly different when compared to intermediate ages ($p>0.1$).

5.6.2.2 Layer 5 FS inputs

Layer 5 FS cells showed a pattern of input that was distinct from their superficial counterparts. At immature ages (n=6) the input onto layer 5 FS cells was primarily divided between layers 2/3 and 5/6, with prominent EPSCs evoked from both layers (fig. 5.16 A-E, immature layer 2/3 input 40±10%, layer 4 14±6%, layer 5/6 46±12%). The organization of observed excitatory inputs did not change between immature and intermediate ages, with no statistical difference found in the percentage input derived from any layer (fig 5.17 A-E, $p>0.1$ for all layers. Intermediate cells n=6, layer 2/3 44±4%, layer 4 12±2%, layer 5/6 44±4%). For mature layer 5 FS cells (n=7), again there was no significant change in the proportion of inputs derived from each layer relative to both immature and intermediate ages (fig. 5.18 A-E, $p>0.1$ for all ages and layers. Mature layer 2/3 input 32±7%, layer 4 18±4%, layer 5/6 50±8%).

In the previous section I found that layer 2/3 FS interneurons showed a developmental enhancement in pA/pixel values. Looking at the pA/pixel values for layer 5 FS cells I also find an upward trend over the course of development. When compared to immature ages (fig. 5.16 F, layer 2/3 $9\pm3$ pA/pixel, layer 4 $9\pm4$ pA/pixel, layer 5/6 $5\pm1$ pA/pixel), intermediate pA/pixel values are not significantly enhanced despite tending towards larger values across all layers (fig. 5.17 F, $p>0.1$, layer 2/3 $16\pm4$ pA/pixel, layer 4 $12\pm2$ pA/pixel, layer 5/6 $10\pm2$ pA). By mature ages a significant increase in pA/pixel amplitude was observed for inputs from layer 5/6 (fig. 5.18 F,
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$p<0.01$ relative to both immature and intermediate, mature layer 5/6 21±3 pA/pixel), however the values for layer 2/3 and 4 were not significantly enhanced (fig. 5.18 F, layer 2/3 18±7 pA/pixel, layer 4 22±6 pA/pixel). Therefore, despite a global trend towards larger inputs, the only significant enhancement is in those inputs derived from within layer 5/6.

Figure 5.16

Figure 5.16 LSPS maps of excitatory input onto layer 5 FS interneurons at immature ages. A and D show example input maps of layer 5 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A and D corresponds to 60pA per uncaging site. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

The reason for the fact that inputs were only significantly enhanced in layer 5/6 may have been due to a greater degree of variation observed in the inputs from layers 2/3
and 4. Inputs from these layers occupied a much greater range of values than inputs from layer 5/6, particularly at immature and mature ages (figs. 5.16, 5.17 & 5.18 F). This suggests that strong local inputs are a consistent property of all layer 5 FS cells, with inputs from other layers showing greater variability.

**Figure 5.17**

*Figure 5.17 LSPS maps of excitatory input onto layer 5 FS interneurons at intermediate ages. A and D show example input maps of layer 5 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 60pA per uncaging site and in D corresponds to 80pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.*
Figure 5.18

Figure 5.18 LSPS maps of excitatory input onto layer 5 FS interneurons at mature ages. A and D show example input maps of layer 5 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 120pA per uncaging site and in D corresponds to 140pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 FS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

5.6.2.3 Layer 4 FS inputs

Fewer FS cells were recorded from layer 4 (n=11 figure 16), with 3 cells recorded at immature, 6 at intermediate and only 2 at mature ages. The primary focus of this thesis is to report the development of layers 2/3 and 5, however as a number of layer 4 cells were recorded their properties are shown in figure 5.19.
Figure 5.19

Figure 5.19 LSPS maps of excitatory input onto layer 4 FS interneurons. A, D and G show example input maps of layer 4 FS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A, D and G corresponds to 80pA per uncaging site The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are indicated by white squares, with the corresponding traces shown in panels B, E and H. B, E and H show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 4 FS cells. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 4 FS cells. I shows the total input received by individual layer 4 FS cells at different ages. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.
Most layer 4 cells received the majority of their inputs from within layer 4 (fig. 5.19 A, D), although they also showed inputs from layer 2/3 and 5/6, particularly from those regions adjacent to the layer 4 boundary (fig. 5.19 A, D). In addition, there was a population of layer 4 FS cells (n=3) that showed prominent layer 2/3 input (fig. 5.19 G-H). Overall the main source of input onto layer 4 FS cells is from layer 2/3 (layer 2/3 input 40±5%, layer 4 input 33±3%, layer 5/6 27±3%). However, the pA/pixel values are found to be strongest from within their host layer 4 (fig. 5.19 F, layer 2/3 10±2 pA/pixel, layer 4 16±2 pA/pixel, layer 5/6 5±1pA/pixel), indicative of a strong recurrent circuit within this layer (Ashby & Isaac, 2011).

5.6.2.4 Development of FS input strength

In the previous sections I have shown that layer 2/3 and 5 FS cells show an increase in pA/pixel values over the course of development. Although this was only found to be significant for the inputs from some layers, this is likely influenced by the variability in translaminar input onto these cells (discussed later in section 5.7). To better examine any potential increase in input over development, I calculated the total input received by individual FS cells recorded in each age range. In both layer 2/3 and layer 5 FS cells I find a trend towards increased input at later ages (fig. 5.20 A, D). In layer 2/3 there is a significant increase in total input between immature and intermediate ages (p<0.05, fig. 5.20 A, immature total input 771±167pA, intermediate 2180±281pA), but the input onto mature cells is not significantly enhanced (fig. 5.20 A, p>0.1 relative to both immature and intermediate, mature input 2676±951). This is perhaps due to increased variability in the inputs observed at this age and a slightly smaller sample. Looking at layer 5 FS cells I find that relative to immature ages (immature input 958pA±137), there is a significant increase in total input at both intermediate (fig. 5.20 B, p<0.05, intermediate input 1896pA±363) and mature ages (fig. 5.20 B, p<0.01, mature input 3277pA±598), with an increase significant at the 90% confidence level between intermediate and mature ages (p=0.056). Due to the limited number of layer 4 cells recorded it is difficult to make solid conclusions about any maturation in their input. However the recorded data does not suggest a strong developmental enhancement (fig. 5.19 I).
The observed increase in input amongst layer 2/3 and 5 FS cells could have been caused by an increase in the number of LSPS evoked EPSCs or an increase in their overall size. In both layers I observed a significant increase in the number of LSPS evoked EPSCs over development (fig. 5.20 B, F). In layer 2/3 there is a significant increase at both intermediate and mature ages when compared to the immature age group (fig. 5.20 B, \( p<0.05 \) for mature, \( <0.01 \) for intermediate, immature \( 84\pm11 \) EPSCs, intermediate \( 184\pm21 \) EPSCs, mature \( 195\pm47 \) EPSCs). This was also the case in layer 5, with a significant increase in the number of recorded EPSCs relative to immature ages at both intermediate and mature stages (fig. 5.20 F, \( p<0.01 \) mature, \( <0.05 \) intermediate, immature \( 125\pm17 \) EPSCs, intermediate \( 204\pm32 \) EPSCs, mature \( 247\pm30 \) EPSCs).

**Figure 5.20**

(A) Development of FS inputs. A-F show the maturation of excitatory inputs onto layer 2/3 and 5 FS interneurons. A shows the maturation of total input for layer 2/3 cells recorded at each of the three age ranges used in this chapter. B as A but for number of LSPS evoked EPSCs. C as A but for average amplitude of LSPS evoked EPSCs. D-F as A-C but for layer 5 FS cells. The red lines in each panel indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean. * = \( p<0.05 \), ** = \( p<0.01 \).
In layer 2/3 FS cells the average size of recorded EPSCs seemed to increase between the immature and intermediate age, before stabilizing between intermediate and mature (fig. 5.20 C, immature 9±1pA, intermediate 12±1pA, mature 13±2pA). However the increase in EPSC amplitude between immature and both intermediate and mature ages was only found to be significant at the 90% confidence level ($p=0.09$ for both), with no significant difference between intermediate and mature ages ($p>0.1$). In layer 5 the maturation of the size of EPSCs went counter to that observed in layer 2/3. Rather than increasing rapidly between immature and intermediate ages, the size of EPSCs showed a small non-significant increase (fig. 5.20 F, $p=0.08$, immature 8±0.5pA, intermediate 9±1pA). There was then a larger increase in the size of EPSCs between intermediate and mature ages, such that mature average EPSC size was significantly enhanced relative to both immature and intermediate ages ($p<0.01$ for immature, $p<0.05$ for intermediate, mature average EPSC size 13±1pA).

Overall it appears there is a developmental enhancement in total input onto layer 2/3 and 5 FS interneurons, even if the temporal profile of these shifts may vary slightly between deep and superficial layers. This enhancement of total input is caused by increases in both the number and size of LSPS evoked EPSCs. This developmental augmentation of synaptic input does not appear to influence the laminar organization of the inputs, which remain relatively constant throughout development (sections 5.6.2.1 & 5.6.2.2).

5.6.3 NFS inputs

To uncover the organization and maturation of excitatory inputs onto NFS interneurons, I recorded 32 NFS cells located in layers 2/3 (n=16) and 5 (n=16) of the somatosensory cortex between P5-21. As with FS interneurons the location from which NFS cells were recorded had a strong influence on their input organization.

5.6.3.1 Layer 2/3 NFS inputs

As was found for FS cells, layer 2/3 NFS interneurons receive the majority of their inputs from within their own layer (figs. 5.21, 5.22 & 5.23 A-E). Only two immature layer 2/3 NFS interneurons were recorded in this study, making strong conclusions
about their properties difficult. In both cases their inputs were primarily derived from within layer 2/3 (fig. 5.21 A-E, layer 2/3 60%, layer 4 11%, layer 5/6 32%). However there was some variation in their inputs, with one cell receiving minimal input from other layers (fig. 5.21 A-B), whilst the other appeared widely integrated with inputs from all layers of the cortex (fig. 5.21 D-E).

**Figure 5.21**

![LSPS maps of excitatory input onto layer 2/3 NFS interneurons at immature ages.](image)

Figure 5.21 LSPS maps of excitatory input onto layer 2/3 NFS interneurons at immature ages. A and D show example input maps of layer 2/3 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A and D corresponds to 80pA per uncaging site. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set.
At intermediate ages (n=6) the percentage laminar input appears similar to cells recorded at immature ages (p>0.1 for all layers, fig. 5.22 C, layer 2/3 67±9%, layer 4 13±4%, layer 5/6 21±7%). Layer 2/3 inputs are still the most prominent and, as was found at immature ages, some cells receive inputs almost entirely from this layer, whilst others appear to integrate more widely with the network (fig. 5.22 A-B, D-E).

**Figure 5.22**

![Figure 5.22 LSPS maps of excitatory input onto layer 2/3 NFS interneurons at intermediate ages. A and D show example input maps of layer 2/3 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 80pA per uncaging site and in D corresponds to 100pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.](image-url)
At mature ages (n=4) there was no significant difference in laminar input organization when compared to both immature and intermediate ages ($p>0.1$, fig. 5.23 C, layer 2/3 65±5%, layer 4 11±2%, layer 5/6 25±4%). Again I also found that there are some layer 2/3 NFS cells that are widely coupled to the network and others that receive primarily local inputs (fig. 5.23 A-B, D-E).

**Figure 5.23**

Figure 5.23 LSPS maps of excitatory input onto layer 2/3 NFS interneurons at mature ages. A and D show example input maps of layer 2/3 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 80pA per uncaging site and in D corresponds to 100pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 2/3 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.
So whilst it seems there is little alteration to the laminar organization of excitatory inputs onto layer 2/3 NFS cells over the course of development, there appears to be some variation in the organization of inputs within the population as a whole.

The pA/pixel values for layer 2/3 NFS cells indicate a bias towards strong input from layer 2/3 (fig. 5.21, 5.22 & 5.23 F). There is no significant alteration in the pA/pixel strength between immature (layer 2/3 16 pA/pixel, layer 4 1 pA/pixel, layer 5/6 1 pA/pixel) and intermediate ages ($p>0.1$ layer 2/3 16±4 pA/pixel, layer 4 8±3 pA/pixel, layer 5/6 4±1 pA/pixel). However, given the limited sample at immature ages it is difficult to make solid conclusions about this point. When comparing the pA/pixel values at mature ages I find no significant difference relative to intermediate ages (layer 2/3 10±2 pA/pixel, layer 4 5±2 pA/pixel, layer 5/6 3±1 pA/pixel $p>0.1$ for all layers), the only significant difference being an increase in the pA/pixel values from layer 5 when compared to immature cells ($p<0.05$). This suggests that unlike layer 2/3 FS cells, there is little increase in the inputs onto NFS cells over the period of this study.

5.6.3.2 Layer 5 NFS inputs

Layer 5 NFS cells showed a pattern of input that was similar to layer 5 FS cells, with inputs primarily derived from deep layers and an additional prominent superficial layer input (figs. 5.24, 5.25 & 5.26 A-E). Comparing the laminar organization of inputs between cells recorded at immature ages ($n=6$, fig. 5.24 C, layer 2/3 input 26±5%, layer 4 12±3%, layer 5/6 62±5%) and intermediate ages ($n=6$, fig. 5.25 C, layer 2/3 36±9%, layer 4 19±3%, layer 5/6 45±9%) I found there was no significant difference, although there was a trend towards an increase in the percentage of layer 4 input at intermediate ages (layer 4 $p=0.053$, layer 2/3 and 5/6 both $p>0.1$). When compared to both immature and intermediate ages, the inputs at mature ages ($n=4$) showed no significant difference in their laminar organization ($p>0.1$ for all layers relative to all ages, fig. 5.26 C, mature layer 2/3 27±11%, layer 4 19±8%, layer 5/6 55±14%). Therefore as was found for FS and layer 2/3 NFS interneurons, it does not appear that there is any significant maturational shift in the laminar organization of excitatory inputs onto layer 5 NFS cells.
Figure 5.24 LSPS maps of excitatory input onto layer 5 NFS interneurons at immature ages. A and D show example input maps of layer 5 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A and D corresponds to 60pA per uncaging site. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50μm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

Despite layer 5 NFS cells showing a bias in the percentage of total input towards layer 5/6, the pA/pixel values were fairly similar across layers- with layer 4 inputs found to be relatively strong despite contributing the least to the total input (figs. 5.24, 5.25 & 5.26 C, F). Comparing the relative strength of inputs between immature (layer 2/3 3±1pA/pixel, layer 4 4±2 pA/pixel, layer 5/6 5±0.5pA/pixel) and intermediate ages, I found a trend towards stronger layer 4 input at intermediate ages ($p=0.07$, layer 4
12±4 pA/pixel) but not in the values for inputs from other layers (p>0.1, fig. 22 F, layer 2/3 7±3 pA/pixel, layer 5 7±3 pA/pixel).

Figure 5.25

Figure 5.25 LSPS maps of excitatory input onto layer 5 NFS interneurons at intermediate ages. A and D show example input maps of layer 5 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 60pA per uncaging site and in D corresponds to 80pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.

This was likely due to a subgroup of intermediate cells that showed particularly strong layer 4 input compared to other cells recorded at intermediate ages and those observed at immature ages (fig. 5.24 & 5.25 F). When compared to both immature and intermediate ages, the inputs at mature ages showed no significant difference in their
pA/pixel values for any layer (\(p > 0.1\) for all layers and ages, fig. 5.26 F, layer 2/3 4±1 pA/pixel, layer 4 7±2 pA/pixel, layer 5/6 9±5 pA/pixel). Therefore as was found for layer 2/3 NFS interneurons it seems there is little enhancement in input strength amongst layer 5 NFS cells over the period of this study.

**Figure 5.26**

![Figure 5.26 LSPS maps of excitatory input onto layer 5 NFS interneurons at mature ages. A and D show example input maps of layer 5 NFS interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A corresponds to 60pA per uncaging site and in D corresponds to 80pA. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50\(\mu\)m region of cortex. Individual uncaging sites are highlighted by white squares, with the corresponding traces shown in panels B and E. B and E show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 NFS cells recorded in this age group. The red lines in C and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean. ](image-url)
5.6.3.3 Development of NFS input strength

Unlike FS interneurons there did not appear to be such a strong increase in the pA/pixel values recorded from NFS cells. When looking at the total input onto both deep and superficial NFS populations it is apparent that there is little developmental enhancement in input (fig. 5.27 A, D). I found that the values were fairly constant through development, with the only significant difference a decrease in input between intermediate and mature layer 2/3 cells ($p<0.05$, fig. 20 A, D, layer 2/3 immature 1151pA, intermediate 1475±196pA, mature 943±112pA. Layer 5 immature 640±74pA, intermediate 1159±296pA, mature 1035±235pA). This difference was brought about by a greater degree of variation in total input onto cells recorded at intermediate ages, whilst at mature ages this variation was not observed, with inputs more tightly grouped (fig. 5.27 A).

**Figure 5.27**

A-F show the maturation of excitatory inputs onto layer 2/3 and 5 NFS interneurons. A shows the maturation of total input for layer 2/3 cells recorded at each of the three age ranges used in this chapter. B as A but for number of LSPS evoked EPSCs. C as A but for average amplitude of LSPS evoked EPSCs. D-F as A-C but for layer 5 NFS cells. The red lines in each panel indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean. * = $p<0.05$, ** = $p<0.01$. 

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Examining the composition of the inputs at each of the three ages in more detail, I found that in layer 2/3 there was no significant alteration in the number of LSPS evoked EPSCs, or their average amplitude ($p>0.05$, layer 2/3 NFS immature 136 EPSCs, intermediate 143±11 EPSCs, mature 116±10 EPSCs. Size of EPSCs, immature 9pA, intermediate 10pA±1, mature 8pA±1). In layer 5 the values for the number (immature 98±13 EPSCs, intermediate 146±36 EPSCs, mature 113±16 EPSCs) and size of LSPS evoked EPSCs (immature 7pA±1, intermediate 8pA±1, mature 9pA±3) were also relatively consistent through development ($p>0.1$ for all except between immature and intermediate number of EPSCs where $p=0.06$). Overall it appears that the inputs onto NFS cells are relatively constant over the developmental period of this study, with little maturational shift in either the organization or magnitude of LSPS evoked inputs.

5.6.3.4 Comparison with FS cell inputs

When comparing the inputs between layer and age matched FS and NFS interneurons I found that there was no significant difference in the percentage of inputs derived from each layer between the two populations at any of the three developmental age ranges ($p>0.05$ for all layers and ages). However, there were differences in their degree of integration. Total input onto layer 2/3 FS cells was significantly enhanced relative to layer 2/3 NFS cells at mature ages ($p<0.05$) and significantly enhanced at the 90% confidence level at intermediate ages ($p=0.06$, fig. 5.28 A-C). The difference at mature ages was brought about by significantly enhanced pA/pixel values for inputs emanating from layers 2/3 and 4 ($p<0.05$), which are the layers that form the primary inputs onto layer 2/3 FS and NFS cells (sections 5.6.2.1 & 5.6.3.1). No significant difference in pA/pixel values was observed for any layer at intermediate or immature ages ($p>0.1$). In addition to pA/pixel values I also compared the number and size of LSPS evoked EPSCs between the two populations. I found that layer 2/3 NFS cells received significantly fewer and smaller EPSCs at mature ages than layer 2/3 FS cells ($p<0.05$), but not at earlier ages ($p>0.1$ at both intermediate and immature ages for both EPSC amplitude and number of LSPS evoked EPSCs, fig 5.28 A-C).

In layer 5 I also find that FS cells are more integrated than their NFS counterparts (fig. 5.28 D-F). FS cells received significant more total input than NFS cells at both
immature and mature ages ($p<0.05$ for both), although not at intermediate ages ($p>0.1$). This increase in input was brought about by a greater number of LSPS evoked EPSCs onto layer 5 FS interneurons at both immature and mature ages ($p<0.05$, fig. 5.28 D-F). The only significant difference in pA/pixel values between the two cell types was in the input from layer 5 at mature ages ($p<0.05$, all others $p>0.05$). The findings discussed here seem to reiterate those of the previous section, whereby NFS cells show similar laminar input structure to FS cells of the same layer but do not show a strong enhancement in input over the course of development (fig. 5.27 A-F), leaving them less integrated relative to FS cells at mature ages (fig. 5.28 D-F). However, in layer 5 it also seems that FS cells may be more integrated than NFS cells at earlier stages of development as well.

Figure 5.28

**Figure 5.28.** Comparison between the LSPS evoked inputs onto FS and NFS interneurons across development. **A-C** the average total input, average number of LSPS evoked EPSCs and average amplitude of LSPS evoked EPSCs respectively for FS and NFS cells recorded in layer 2/3 at P5-8, P9-12 and P14-21. **D-F** same as **A-C** but for cells recorded in layer 5. Error bars show SEM ± from the mean. * = $p<0.05$.
5.6.4 rIB inputs

rIB neurons (n=10), were located solely within layer 5. The values reported here are for all recorded rIB interneurons, as I found little difference between the properties of the seven mature rIB cells and those recorded at earlier ages. Including/excluding the three cells recorded prior to P14 has little overall effect on the percentage laminar input (moving the values by approx 1% for each layer), or pA/pixel (less than 0.5pA/pixel variation). It also does not influence the results of any of the statistical tests performed in this section. As with other layer 5 interneurons, rIB cells received the majority of their input from layer 5/6 with additional prominent input from layer 2/3 (fig. 5.29 A-H, layer 2/3 input 27±4%, layer 4 input 11±2%, layer 5/6 input 63±5%). The relative amount of their input derived from each layer was found to be similar to both FS and NFS interneurons, with no significant difference observed when comparing between the three populations (p>0.05 for all layers and subtypes). rIB cells received inputs from all layers of the cortex (fig. 5.29 A-H), but layer 5/6 provided the strongest pA/pixel values (fig. 5.29 F, layer 2/3 4±1 pA/pixel, layer 4 5±1pA/pixel, layer 5/6 8±2 pA/pixel). Unlike FS and NFS cells from layer 5, there appeared to be less variation in the organization of inputs onto rIBs, with a lower range for the percentage of total input, or the pA/pixel values for inputs derived from layer 4 than found in other interneuron subtypes (fig. 5.29 C, F, layer 4 input range 5-17% of total). rIB cells showed comparable levels of integration to mature NFS cells recorded from layer 5 (n=4), with similar values for total synaptic input (p>0.1, NFS 1035±235pA, rIB 946±130pA), as well as the average number and amplitude of LSPS evoked EPSCs (p>0.1 for both, NFS 118±18 EPSCs, amplitude 10pA±3, rIB 127±18 EPSCs, amplitude 8pA±1). Comparing the average pA per pixel data showed that NFS cells also had similar values to rIB cells across all layers (p>0.1). When comparing rIB cells to mature layer 5 FS cells (n=7) the amount of total input onto FS cells was significantly elevated (p<0.001, FS input 3277pA±598), as were the values for average number of EPSCs and average size of EPSCs (p<0.01). The pA/pixel values for FS cells were also significantly larger than rIB cells across all layers (p<0.05 for layer 2/3 and 4 and <0.01 for layer 5/6). This supports the findings of previous sections, which showed that FS interneurons received enhanced excitatory input compared to other Nkx2-1 derived interneuron subtypes (fig 5.28 A-F & section 5.6.3.4).
Figure 5.29

Figure 5.29 LSPS maps of excitatory input onto layer 5 rIB interneurons. A, D and G show example input maps of layer 5 rIB interneurons with the age the cell was recorded indicated at the bottom left of the panel. White circles indicate the location of the soma of the recorded cells and dashed lines demarcate the individual layers. Filled white circles show sites where direct responses masked the observance of synaptic inputs. Colours represent increasing levels of input according to the scale bar on the bottom left, maximum input in A, D and G corresponds to 90pA per uncaging site. The histograms adjacent to the individual maps show the percentage of total input emanating from each row of the LSPS mapping grid. Each pixel represents a 50x50µm region of cortex. Individual uncaging sites are indicated by white squares, with the corresponding traces shown in panels B, E and H. B, E and H show two traces from each uncaging site. The black lines at the top of each panel indicate the firing of the laser pulse, which lasted 100ms. C shows the percentage of total input from layers 2/3, 4 and 5/6 received by all layer 5 rIB cells. F shows the pA/pixel values from layers 2/3, 4 and 5/6 received by all layer 5 rIB cells. I shows the total input received by individual layer 5 rIB cells at different ages. The red lines in C, and F indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean.
Given that rIB neurons were recorded predominantly at later ages (see section 5.4.6) it is difficult to make solid conclusions regarding the maturation of their inputs. However, all cells recorded, regardless of age, showed little variance in their overall degree of integration (fig. 5.29 I). The degree of rIB integration at mature ages was also significantly less than that of layer 5 FS cells, suggesting there is limited developmental enhancement over the period of this study, and if it does take place, is considerably less than that observed in layer 5 FS cells.

5.6.5 ANOVA analysis of synaptic inputs onto Nkx2-1 derived interneurons

Unlike pyramidal cells in the preceding chapter, both FS and NFS interneurons do not show a prominent developmental alteration in the percentage of total input received from each layer and seem to receive prominent, focused inputs from the earliest ages of this study. To quantify the organization of synaptic inputs onto FS and NFS interneurons over development, I applied one-way ANOVA to interneuron maps recorded in layers 2/3 and 5 as reported in the preceding chapter for layer 2/3 and 5 pyramidal neurons (section 4.3.5). This revealed that the synaptic input maps for the majority of FS and NFS interneurons produced p values that fell within the 95% confidence level regardless of the age recordings were made (fig. 5.30).

Figure 5.30

**Figure 5.30 ANOVA analysis of interneuron input maps.** ANOVA p values for FS and NFS interneurons recorded at each of the 3 developmental ages in this study. The individual circles indicate the p value for each cell, with the subtype distinguished in the key at bottom right. The significance threshold of p=0.05 is demarcated by a dashed line.
Chapter 5- Maturation of Nkx2-1 derived interneurons in the nascent neocortex

The percentage of cells that show significantly organized inputs, as determined by one-way ANOVA analysis, is relatively constant for both subtypes across development. At P5-8 only 14% of NFS and 11% of FS cells have a $p>0.05$, at P9-13 these values are NFS 15% and FS 8% and at P14-21 NFS 0% and FS 9%. Taken with the findings of the preceding sections, it seems inputs onto FS and NFS interneurons are significantly organized around their mature laminar sources of input from the earliest ages of this study.

5.7 Variation in the laminar source of input onto Nkx2-1 interneurons

The input maps recorded from Nkx2-1 derived interneurons showed some degree of variation in their organization. In both layer 2/3 and 5 I observe that there is consistent local connectivity derived from the immediate layer onto all cells (sections 5.6.1-4), however translaminar inputs from other layers appear more variable (for example compare the inputs of the cells in fig. 5.26 A, D with fig. 5.25 A, D, or those in fig. 5.23 A with fig. 5.22 D). In layer 2/3 this phenomenon is observed in both FS and NFS subtypes and centers around variation in the amount of layer 4 input. There is considerable range in the amount of total input received from layer 4, with FS and NFS cells receiving between 1-32% and 2-26% of total input from layer 4 respectively. In layer 5 a similar distinction can also be made between cells that receive inputs of varying degree from both layer 2/3 and 5. As with layer 2/3 this seems to occur across all populations mapped in this layer. Previous studies of both pyramidal cells and interneurons have shown that the location of cells within each layer may be an important determinate of the source of a cell's inputs (Feldmeyer et al., 2005; Anderson et al., 2010; Hooks et al., 2011; Apicella et al., 2012). The following sections will examine what role the sublaminar location of the cell may play in the observed variation in translaminar input. Because it was found in preceding sections that the laminar organization of excitatory inputs onto Nkx2-1 derived interneurons does not change over development, the data reported in this section is primarily based upon normalized inputs pooled across development.
5.7.1 Variation in inputs emanating from layer 4

Although layers 2 and 3 are frequently grouped together as a single entity, it has been shown there is some degree of variation in their synaptic connectivity (Hooks et al., 2011). Furthermore layer 5 is also often subdivided into layers 5a and 5b, which are known to possess distinct subtypes of neuron with different input organizations (Molyneux et al., 2007; Anderson et al., 2010; Sheets et al., 2011). In layer 2/3 it has been reported that pyramidal cells recorded in close proximity to layer 4 receive a greater proportion of their inputs from that layer than do those pyramidal cells located more superficially (Hooks et al., 2011), and in layer 5 there is a pathway from stellate cells in layer 4 to pyramidal cells recorded close to the layer 4/5 boundary (Feldmeyer et al., 2005).

To determine if the proximity of the recorded cell to layer 4 may explain the variation in layer 4 input, I used the soma position from the synaptic input map to calculate the distance from the layer 4 boundary for each cell. Cells whose soma pixel was adjacent to layer 4 were recorded as 50µm from the boundary, cells that were a further pixel away as 100µm and so on. This separated the cells into 50µm bins. I then correlated this distance from the layer 4 boundary with the percentage of total input received from that layer (fig. 5.31 A-B). In layer 2/3 I find that there is a significant trend towards greater layer 4 input for cells whose soma is located closer to the boundary with layer 4 (fig. 5.31 A). Cells that were recorded with their soma pixel adjacent to layer 4 (within 50µm n=14) and those recorded within 100µm (n=6) had significantly more layer 4 input than those recorded at 150µm (n=7, fig. 5.31 A, p<0.001 for 50µm and <0.01 for 100µm. Average layer 4 input 50µm 20±2%, 100µm 20±4%, 150µm 7±2%). Only one cell was recorded at 200µm from the layer 4 boundary, but this cell also showed less layer 4 input than those located within 100µm of layer 4 (fig. 5.31 A). This trend was observed across both FS and NFS subtypes, as when comparing the inputs received by layer 2/3 FS and NFS cells, within subtype, I also found that cells recorded within <100µm from the layer 4 boundary receive increased layer 4 input than those that were recorded more superficially (p<0.01 for FS and <0.05 for NFS).
Layer 5 cells were recorded over a much larger range than in layer 2/3 (100-400μm from the layer 4 boundary), with none observed directly next to layer 4 (fig. 5.31 B). Cells recorded within 100μm (n=6) and 150μm (n=5) did tend to receive more layer 4 input than many cells located at greater distance from layer 4 (fig. 5.31 B, layer 4 input 100μm 20%±5, 150μm 16%±3, 200μm 12%±2, 250μm 21%, 300μm 20% 350μm 8%, 400μm 12%±8). However this was not significant and there were also a number of cells that received strong layer 4 input recorded at greater depths in layer 5 (for example the cells in fig. 5.26 A, D), which produce a second peak in layer 4 input at 250μm and 300μm, (although only one cell was recorded at 250μm and two at 300μm). Because fewer cells were recorded at greater depths (one at 250μm, two at 300μm and 350μm and four at 400μm) and the lack of cells recorded directly adjacent to the layer 4 boundary, it is not possible to make any solid conclusions regarding the role of the distance from layer 4 in the amount of input received from that layer. However the presence of a population of cells with prominent layer 4 input at greater depths within layer 5 (figs. 5.26 A, D & 5.31 B) suggests that in layer 5 the situation may be less clear-cut than in layer 2/3.
5.7.2 Sublaminar variation in input onto layer 5 interneurons

When observing the input maps onto interneurons recorded in layer 5, it appeared that whilst the majority of cells received prominent input from layer 2/3 (fig. 5.32 A, E), some cells had little or no superficial input (fig. 5.32 B, F). This was reminiscent of the situation reported in layer 5 pyramidal cells and interneurons of the motor cortex, where the amount of layer 2/3 input correlates with the distance from the pial surface (Anderson et al., 2010; Apicella et al., 2012). To uncover if this was also the case for Nkx2-1 interneurons in the somatosensory cortex, I calculated the relative layer 2/3 input onto cells recorded within layer 5 at various depths from the pial surface. This revealed that cells recorded at a depth greater from the pial surface showed less layer 2/3 input compared to those located more superficially within layer 5 (fig. 5.32 C, D, G.).

To look in more detail at the influence of pial depth on the amount of input from layer 2/3, I divided layer 5 cells into 100µm bins based on the depth of the recording pipette tip (and therefore the cell soma) from the pial surface. The data for all recorded interneurons shows that there is a significant drop in layer 2/3 input for cells recorded at depths greater than 800µm from the pial surface (fig. 5.32 G, p<0.05 for 8-900µm compared to both 5-600µm and 6-700µm. Layer 2/3 input 4-500µm 36%±13, 5-600µm 38%±4, 6-700µm 37%±5, 7-800µm 30%±6, 8-900µm 18%±6). This was also found to be the case when looking at individual subtypes, with FS and rIB cells mapped at depths greater than 800µm found to receive significantly less layer 2/3 input than cells of the same subtype mapped more superficially in layer 5 (p<0.05 for both FS and rIB). Data for NFS cells is not reported as only one NFS cell was mapped at depths greater than 800µm, however it too seemed to receive little layer 2/3 input. The influence of the recording depth on layer 2/3 input can be seen in the example cells shown in figure 5.32 (A, B, E, F) and the population data for FS and rIB cells (fig. 5.32 C, D). These findings show that, as observed in pyramidal and interneuron populations in the motor cortex (Anderson et al., 2010; Apicella et al., 2012), there is a distinction in the amount of input received from layer 2/3 by Nkx2-1 interneurons based upon the depth they are recorded from in layer 5 of the somatosensory cortex.
In addition to the depth from the pial surface, the sublayer within layer 5 that cells are recorded from influences their laminar input properties (Anderson et al., 2010; Apicella et al., 2012). For the majority of the cells reported in previous sections the sublaminar boundary between layer 5a and 5b could be discerned on the photomicrographs taken of the cortex prior to mapping. This allowed me to separate cells into those recorded from either layer 5a or layer 5b and compare the laminar
breakdown of excitatory inputs onto Nkx2-1 derived interneurons recorded in these two distinct sublayers. The population of cells used for this analysis consisted of 15 FS cells, 6 from layer 5a and 9 from layer 5b, 7 rIB cells all from layer 5b and 9 NFS cells, 5 from layer 5a and 4 from layer 5b. Analysis of their normalized inputs revealed that layer 5a cells received a greater amount of input from layer 2/3 and layer 5a than cells recorded in layer 5b, whose inputs were preferentially biased towards layer 5b (fig. 5.33 A-C). There were also a population of cells that were recorded in layer 5b, 50µm from the border with layer 5a, that showed input patterns more alike layer 5a cells- with a high percentage of input from layers 2/3 and 5a (fig. 5.33 A-C). However those cells that showed higher 5a input were not always the same cells that showed high layer 2/3 input.

Figure 5.33

Comparing the inputs onto layer 5a and 5b cells within subtype I find that FS cells in layer 5b (n=7) receive significantly less layer 2/3 input than layer 5a cells (n=8, fig. 5.33 A, p<0.05, layer 5a FS cells 42%±2, layer 5b FS cells 18%±9) and significantly more layer 5b input (fig. 5.34A, p<0.01, layer 5a FS cells 18%±3, layer 5b FS cells 53%±12). The difference in input from layer 5a is only significant at the 90% confidence level (p=0.051) with layer 5a FS cells receiving more layer 5a input than layer 5b cells (fig. 5.34 A, layer 5a FS cells 22%±4, layer 5b FS cells 14%±2). There

Figure 5.33 Variation in input based upon distance from layer 5a/b boundary. A-C show the percentage of total input received by layer 5 interneurons based upon their distance from the layer 5a/b boundary. Cells are recorded between P5-21 and pooled irrespective of age. A-C show the percentage layer 2/3, 5a and 5b input respectively. Coloured circles indicate individual cells with the corresponding subtypes shown in the key at bottom right. The dashed line in A-C distinguished the layer 5a/b boundary.

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was no significant difference in layer 4 input between the two populations ($p>0.1$, layer 5a FS cells 15%±2, layer 5b FS cells 15%±5).

**Figure 5.34**

![Figure 5.34](image_url)

*Figure 5.34 Sublaminar variation in excitatory input onto layer 5 FS and NFS cells. A and B show the percentage of total input received by layer 5a/b FS and NFS cells respectively. A and B show the percentage of total input received from layers 2/3, 5a and 5b by FS and NFS cells located in layer 5a and 5b. Coloured circles represent individual cells, with the sublayer cells were recorded from indicated in the key to bottom right. The red lines indicate the mean average for that data set and the fainter red blocks indicate the SEM ± from the mean. * = $p<0.05$, ** = $p<0.01$. For NFS cells the only significant difference between layer 5a (n=5) and 5b cells (n=4) is in the amount of layer 2/3 input (fig. 5.34 B, $p<0.05$, layer 5a NFS cells 33%±7, layer 5b NSF cells 12%±6). However as was found for FS cells, NFS cells located in layer 5a tended towards a greater percentage of total input from their host sublayer over the opposing one (fig. 5.34 B, layer 5a input 29%±5, layer 5b input 22%±6). The same was also found for layer 5b NFS cells (fig. 5.34 B, layer 5a input 22%±6, layer 5b input 48%±17). As with layer 5 FS cells there was no difference in layer 4 input based on sublayer ($p>0.1$, layer 5a layer 4 input 15%±5, layer 5b layer 4 input 21%±8). Comparing the inputs between the subtypes I find no significant difference between the percentage of total input received from each layer for layer 5a FS and NFS cells, or between layer 5b FS, NFS or rIB cells ($p>0.05$ for all layers between all cell types).
5.8 Cluster analysis of inputs onto \textit{Nkx2-1} derived interneurons

The findings of the previous section (section 5.7) provide some insight into the variation that is observed in the excitatory input onto \textit{Nkx2-1} interneurons. In a number of cases this can be correlated to the location the cells were recorded from, with the amount of layer 4 input in layer 2/3 cells related to the distance of the soma from the layer 3/4 boundary (fig. 5.31 A), and the percentage of layer 2/3 input onto layer 5 cells dependant upon the depth of the cell in the cortex relative to the pial surface (fig. 5.32 A-G). In addition, there seem to be other sources of variation that occur within the \textit{Nkx2-1} interneuron population- for example the amount of layer 4 input received by layer 5 cells (compare figs. 5.7 & 5.18 D), or layer 5 input received by layer 2/3 cells (figs. 5.13 & 5.14 D). To look in more detail at the variation inherent amongst the input maps of \textit{Nkx2-1} interneurons, I performed hierarchical cluster analysis to determine how they segregated into defined groups based around their inputs from discrete layers. This allowed me to look for similarities and differences in the input patterns within each subtype, and to discover how many individual input motifs are present within each population, and equally the population as a whole. It also provided a way to look in more detail at any changes in input organization that may occur over the course of development.

Hierarchical cluster analysis was chosen because, unlike k means clustering used for the intrinsic membrane properties (section 5.5), hierarchical clustering examines that data across a number of degrees of clustering. This was important because in the intrinsic analysis I was aware that there were three main electrophysiological subtypes of interneuron within the labeled population, whilst in this analysis I wanted to look at the variation inherent within the population without imposing any \textit{a priori} constraints upon the number of clusters. The data used for this analysis was the normalized inputs from individual layers, as this removed the influence of any developmental maturation in input strength. The sample comprised cells where it was possible to distinguish the sublaminar border between layer 5a and 5b- it contained: 16 layer 2/3 FS, 10 layer 4 FS cells, 15 layer 5 FS (6 layer 5a and 9 layer 5b), 15 layer 5 NFS cells, 9 layer 5 NFS cells (6 layer 5a and 3 layer 5b) and 7 layer 5 rIB cells (all layer 5b).
5.8.1 FS input clusters

FS cells were the most numerous of the recorded interneurons amongst the \textit{Nkx2-1} population (section 5.2.1) and where recorded across layers 2-5 (section 5.2.2), including at depths greater than 800µm in layer 5b (section 5.7.2). I find 8 distinct clusters amongst the FS cell population (fig. 5.35 A-H), 3 of them are primary observed amongst layer 2/3 cells (clusters FS 1, FS 2 & FS 3), 2 amongst layer 4 cells (clusters FS 4 & FS 5) and 3 in layer 5 cells (clusters FS 6, FS 7 & FS 8). In addition there was one cell that did not fall within a given cluster, this was a layer 5b cell that received input from layer 2/3 and 5b. Regardless of which cluster they are in, all FS cells receive input from their immediate layer, with varying amounts of input from other layers. Variation in the amount of translaminar input gives rise to two broad categories of clusters, those whose inputs are primarily local (fig 5.35 A, D, H) and those that receive translaminar input in addition to local input (fig 5.35 B, C, E, F, G).

With the exception of the local clusters (FS 1, FS 4, FS 8), the remaining input motifs can be defined by the source and amount of translaminar input they receive. As we have seen previously, the location of a cell within the cortex can influence the presence, or not, of translaminar connectivity (section 5.7). Because of this, many of the input motifs are dominated by cells recorded from a given layer or sublayer. However, there are also cells present within some clusters that are not in agreement with the majority of other cells; with layer 4 and layer 5 cells in FS 2 and FS 3 and layer 2/3 cells in FS 5 (fig. 5.35 B, C, E). These cells appear to be those that receive a greater ratio of translaminar to local input. For example, the layer 4 and 5 cells in FS 2 and FS 3 are those cells whose inputs are preferentially biased towards layer 2/3, so that rather than falling within those clusters associated with their own layer, that receive layer 2/3 input (FS 5 and FS 6), they localize with layer 2/3 cells that receive layer 4 or layer 5 input. This can be seen in more detail when looking at the normalized input received by cells in each cluster (table 5.3).
Figure 5.35 Patterns of excitatory input amongst FS interneurons. A-H show the average input maps produced for individual clusters as determined by hierarchical clustering of the inputs onto FS interneurons. Each map was calculated by aligning the maps of cells contained within that cluster by their laminar boundaries and then averaging across individual pixels. The inputs per pixel were normalized to the total input received by each cell, such that each map is a spatial representation of the average normalized input derived from individual pixels in each layer for all cells contained within that cluster. Dashed lines indicate the laminar boundaries. The white circles represent the soma positions of cells contained within each cluster. For each circle the strength of the line shading indicates the frequency that cells in that location were present within each cluster, with the most prevalent soma locations indicated by strong white circles and those that were less frequent by more transparent circles. The colour scale on each map represents increasing amounts of input with the scale indicated at bottom left. The scale varies in each map and corresponds to 6% of total input in A & D, 4.5% of total input in E & H and 3% of total input in B, C, F & G. The input motifs shown here correspond to those recorded in table 5.3. A is motif FS 1, B FS 2, C FS 3, D FS 4, E FS 5, F FS 6, G FS 7 & H FS 8.

The cluster analysis confirms many of the findings of previous chapters regarding the influence of the location of the cell on the source of its inputs (section 5.7), in addition to providing greater insight into other types of variation within the data. In layer 2/3 we find that there are those cells that receive primarily local input (FS 1) and those that receive input from other layers (FS 2 & FS 3)(fig 5.35 A-C). Those cells in FS 1
are found to be located on average further from the border with layer 4 than those that receive a greater amount of layer 4 input (FS 3)(table 5.3).

### Table 5.3

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<th>5a/5b dist (L5)</th>
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**Table 5.3 Summary of input motifs amongst Nkx2-1 derived interneurons.** Table 5.3 shows the properties of each of the clusters produced by performing hierarchical cluster analysis on the inputs of FS, NFS and rIβ interneurons. The table shows from left to right, the name of each cluster and the number of cells contained within that cluster, the amount of total normalized input received from layer 2/3, layer 4, layer 5a and layer 5b/6, the age that cells were recorded at within each cluster (percentage of cells recorded at immature/intermediate/mature), the percentage of cells contained within the cluster recorded from superficial layers (percentage of cells recorded in layer 2/3/layer 4), the percentage of cells recorded in layer 5 (percentage of cells recorded in layer 5a/layer 5b), the average distance from layer 4 of all those cells recorded in layer 2/3, the average distance from the layer 5a/5b boundary of all those cells recorded in layer 5. Values ± are SEM except in FS 5 and NFS 1 where n=2.

However even amongst those cells that do receive translaminar input (FS 2 & FS 3), there seems to be a degree of variation, with FS 3 found to receive a greater amount of
translaminar input than FS 2 (table 5.3). Within layer 4 cluster analysis confirms that for layer 4 cells there is a distinction in the amount of layer 2/3 input they receive (see fig. 5.19 A-H), with those cells whose inputs are biased towards layer 4 found in FS 4 whilst the remaining cells are observed in FS 2, 3 & 5 depending on the amount of layer 2/3 and 4 input they possess (fig. 5.35 B-E; table 5.3). Within layer 5, layer 5a cells mostly receive prominent layer 2/3 input (fig. 5.35 C, F), with those that receive stronger input from superficial layers found to cluster with layer 2/3 cells in FS 3 (table 5.3). Layer 5b cells receive inputs from layer 2/3 (fig. 5.35 B, C), layer 4 (fig. 5.35 G), or solely layer 5 (fig. 5.35 H). As expected cells located deep in layer 5 lack layer 2/3 input (fig. 5.35 G, H; table 5.3). However this population is not homogeneous, as there is also a subpopulation of layer 5b cells that receive strong layer 4 input (fig 5.35 G; table 5.3).

Many of the inputs motifs appear to be present across development (table 5.3). In instances where some motifs are not present at all ages, or appear biased towards a particular age group, many of these differences can be explained by variations in sampling frequency. FS 4 and FS 5 are primarily comprised of layer 4 cells, which were not recorded at such a high frequency in this study (section 5.6.2.3). The increased prevalence of FS 7 and 8 at mature ages and the lack of mature cells in FS 6 can be explained by the fact that 92% of all cells recorded at mature ages were located in layer 5b. Given that FS 7 and 8 are solely observed in layer 5b and FS 6 primarily in layer 5a (table 5.3), this would suggest a sampling bias as opposed to a developmental drop in cells possessing layer 2/3 inputs. The lack of cells in FS 7 and 8 at earlier ages can be explained by the fact that these motifs are typically observed at greater depths within layer 5b (table 5.3), however only one cell was recorded at a depth of >800µm outside of mature ages (see fig 5.32 G) and it was not included in the data set used for this cluster analysis, as the layer 5a/5b boundary could not be clearly discerned. Similarly the decrease in FS 1 over development can be explained by an increased bias towards layer 2/3 cells recorded in close proximity to layer 4 at later ages, with 66% of FS cells located >100µm from layer 4 at immature ages, 38% at intermediate and 0% at mature. Because cells that lack layer 4 input are more likely to be recorded at a distance of >100µm from layer 4 (fig. 5.31 A) this will likely influence the observation of this motif at later ages.
5.8.2 NFS input clusters

Performing cluster analysis on the inputs onto NFS cells produced 5 clusters (fig. 5.36 A-E). Within layer 2/3 there are 3 clusters (NFS 1, 2 & 3), one where the inputs are confined to layer 2/3 (NFS 1) and the remaining 2 showing translaminar input (NFS 2 & 3), but distinguished by differences in the amount of layer 4 and 5 input they receive (fig. 5.36. A-C; table 5.3). Amongst layer 5 cells I find 2 clusters and an orphan cell that was not part of any cluster (fig. 5.36 D-F). One cluster (NFS 4) is primarily comprised of layer 5a cells and receives strong layer 5a and layer 2/3 input (fig. 5.36 D) whilst the other contains a greater percentage of layer 5b cells and receives strong input from layer 5 and 4, but not from layer 2/3 (fig. 5.36 E).

The patterns of input observed amongst NFS cells are reminiscent of those previously described for FS cells (section 5.8.1). The motifs present amongst layer 2/3 NFS cells (NFS 1, 2 & 3) can also be found amongst their FS counterparts (FS 1, 2 & 3 respectively) and the layer 5 NFS motifs, NFS 4 and 5, are similar to FS 6 and 7 (figs. 5.35 A-G & 5.36 A-E). The orphan cell amongst the NFS population also appeared to have a pattern of input that was similar to that observed in FS cells of the FS 8 cluster (figs. 5.35 H & 5.36 F). This orphan cell was the only NFS cell that was recorded at a depth of greater than 800µm from the pial surface, with this pattern of input previously having been shown to occur in cells recorded at greater than this depth (fig. 5.32 A-G). When comparing the percentage of input derived from each layer amongst the above mentioned input motifs it also appears that the NFS input patterns are remarkably similar to those observed amongst FS cells (table 5.3).

All of the motifs present amongst NFS cells therefore show a counterpart amongst the FS population, with the organization of their inputs from each layer found to be in strong agreement (figs. 5.35 A-H & 5.36 A-F; table 5.3). The lack of NFS cells with input patterns corresponding to FS 4 and 5 can be explained by the fact that no layer 4 NFS cells were mapped in this study. The frequency of each motif also appears to be consistent across development, with many of the motifs occupied by cells recorded at immature, intermediate and mature ages (table 5.3). The lack of immature cells in NFS 2 can be explained by the fact that only 2 cells were recorded at this time, with these cells found in NFS 1 and NFS 3.
Figure 5.36

Figure 5.36 Patterns of excitatory input amongst NFS interneurons. A-E show the average input maps produced for individual clusters as determined by hierarchical clustering of the inputs onto NFS interneurons. Each map was calculated by aligning the maps of cells contained within that cluster by their laminar boundaries and then averaging across individual pixels. The inputs per pixel were normalized to the total input received by each cell, such that each map is a spatial representation of the average normalized input derived from individual pixels in each layer for all cells contained within that cluster. Dashed lines indicate the laminar boundaries. The white circles represent the soma positions of cells contained within each cluster. For each circle the strength of the line shading indicates the frequency that cells in that location were present within each cluster, with the most prevalent soma locations indicated by strong white circles and those that were less frequent by more transparent circles. The colour scale on each map represents increasing amounts of input with the scale indicated at bottom left. The scale varies in each map and corresponds to 6% of total input in A, 4.5% of total input in B & D and 3% of total input in C & E. The input motifs shown here correspond to those recorded in table 5.3. A is motif NFS 1, B NFS 2, C NFS 3, D NFS 4, E NFS 5, F shows the input map of the one cell that did not fit into any cluster. Colours represent increasing levels of input according to the scale bar at bottom right, maximum input corresponds to 120pA per uncaging site. Each pixel represents a 50x50µm region of cortex. The open white circle indicates the soma with laminar boundaries shown by dashed white lines. Filled white circles show sites where direct responses masked the observance of synaptic inputs.
5.8.3 rIB input clusters

rIB cells were only recorded from layer 5 and their inputs could be divided into two discrete clusters (fig. 5.37 A-B). rIB 1 (fig. 5.37 A) is similar to FS 6 and NFS 4 (figs. 5.35 F & 5.36 D), receiving prominent layer 2/3 input and present amongst cells recorded more superficially in layer 5 (table 5.3). rIB 2 (fig. 5.37 B) is more alike FS 8 and the single NFS cell recorded greater than 800µm from the pial surface (figs. 5.35 H & 5.36 F). This input motif was biased towards cells recorded at greater depths within layer 5 (table 5.3). Unlike FS and NFS cells, amongst rIBs there was no layer 5 cluster associated with input from layer 4. This seems to confirm the observation made in section 5.6.4, where rIB cells showed little variation in the amount of layer 4 input they received (fig. 5.29 A-H). The lack of the remaining input motifs amongst rIB cells can be explained by the fact that they were not recorded from layers 4 and 2/3, such that input patterns associated with these layers (FS 1-5 and NFS 1-3) are unlikely to be observed.

Many of the input motifs in FS and NFS cells were found to be present across development, given that rIB cells were preferentially observed at later ages, it is difficult to draw strong conclusions on the prevalence of rIB input motifs at different ages. However, rIB 1 at least seems to be present at all three age ranges (table 5.3). As mentioned for FS cells (section 5.8.1), the lack of the rIB 2 motif at earlier ages can be apportioned to the lack of immature and intermediate cells recorded at depths greater than 800µm from the pial surface and the few rIB cells recorded at these ages. Because of the small sample amongst rIB cells it remains to be seen if the lack of a motif associated with layer 4 input onto layer 5 rIB cells is due to the limited number of recordings from this population, or if it represents a true divergence in the organization of inputs onto individual electrophysiological subtypes of Nkx2-1 derived interneurons. Apart from this it appears that whenever recordings were made in a given layer or sublayer, the input motifs observed are remarkably similar across subtypes (table 5.3). This suggests a global integration strategy for all Nkx2-1 derived interneuron subtypes.
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Figure 5.37

Figure 5.37 Patterns of excitatory input amongst rIB interneurons. A-B show the average input maps produced for individual clusters as determined by hierarchical clustering of the inputs onto rIB interneurons. Each map was calculated by aligning the maps of cells contained within that cluster by their laminar boundaries and then averaging across individual pixels. The inputs per pixel were normalized to the total input received by each cell, such that each map is a spatial representation of the average normalized input derived from individual pixels in each layer for all cells contained within that cluster. Dashed lines indicate the laminar boundaries. The white circles represent the soma positions of cells contained within each cluster. For each circle the strength of the line shading indicates the frequency that cells in that location were present within each cluster, with the most prevalent soma locations indicated by strong white circles and those that were less frequent by more transparent circles. The colour scale on each map represents increasing amounts of input with the scale indicated at bottom left. The scale varies in each map and corresponds to 3% of total input in A, 6% of total input in B. The input motifs shown here correspond to those recorded in table 5.3. A is motif rIB 1, B rIB 2.

5.9 Chapter summary

Whilst the focus of this study was primarily the postnatal maturation of the cortical network, and the subtypes of interneuron contained within it, thanks to the use of genetic fate mapping tools I was also able to uncover some novel information about the specification of interneuron subtypes during embryonic development. Previous studies into the Nkx2-1iCre mouse line have shown that Nkx2-1 derived interneuron subtypes, specified from the non-Nkx6.2 expressing region of the MGE, are in some ways distinct from those of the Nkx6.2 expressing domain (Fogarty et al., 2007). However the aforementioned study did not include electrophysiological data. In the above sections of this chapter I have provided an electrophysiological characterization
of EGFP labeled cells produced when using the *Nkx2-1iCre;Z/EG* genetic strategy to label cortical interneurons. By looking in more detail at the neuronal progeny of a subdivision of the embryonic telencephalon, it has become possible to compare these results with numerous other studies that have used conditional fate mapping to probe the diversity of electrophysiological subtypes produced during embryonic development (Miyoshi et al., 2007, 2010; Gelman et al., 2008, 2011; Sousa et al., 2009), and thus enhance our understanding of the genetic programs that underlie the specification of mature cortical interneurons.

Now aware of the mature subtypes labeled using this genetic strategy, I proceeded to look at the network properties of these cells throughout postnatal development. To aid in the distinction of interneuron subtypes at immature ages, I turned to phase space analysis of the spiking properties of interneurons, which has been shown to produce unique plots based upon the electrophysiological subtype of interneuron recorded (Daw et al., 2007; Tricoire et al., 2010). By applying this method I was able to distinguish between FS and NFS *Nkx2-1* derived interneuron subtypes from P5 onwards and study their intrinsic membrane properties and excitatory synaptic inputs from around the time they begin their synaptic integration into the network (Pengratz-Fuehrer & Hestrin, 2011). This revealed that there were early differences in the intrinsic properties of *Nkx2-1* derived interneuron populations that became more distinct as development progressed. Previous reports have found that FS interneurons show a delayed developmental maturation (Goldberg et al., 2011), and I report evidence to support this. Although there was some initial diversity amongst the electrophysiological properties of the immature cortex, cells recorded at P5-8 could not be reliably segregated into individual clusters based upon subtype at this time. This supports the idea that the diversity of electrophysiological firing patterns observed amongst this population of cortical interneurons does not emerge until later in development.

The inputs onto *Nkx2-1* derived interneurons also underwent a developmental divergence over a similar time frame to their intrinsic membrane properties- with a prominent upregulation in the inputs onto FS cells but not NFS cells. This developmental upregulation of FS inputs meant that by the time intrinsic diversity had developed amongst the *Nkx2-1* population, the two main interneuron subtypes
contained within this population also had distinct synaptic inputs. Despite this
difference in input strength, the laminar structure of inputs onto Nkx2-1 derived
interneurons was organized around a number of distinct input motifs, many of which
occurred across multiple electrophysiological subtypes. Unlike pyramidal cells in the
preceding chapter it also appears that these patterns of input are in place from the
point of initial integration into the network.

5.10 Discussion

5.10.1 Genetic specification of interneuron subtypes

One of the dogmas central to the study of neuronal diversity in the cortex is that the
mature subtype of a neuron is dictated by its time and place of emergence from the
ventricular zone of the embryonic telencephalon (see chapter 1, sections 1.3.3-4). This
study aimed to supplement the findings of a number of other papers on the
specification of interneuron subtypes (Fogarty et al., 2007; Miyoshi et al., 2007;
Sousa et al., 2009), by looking at the electrophysiological properties of the Nkx2-1
positive interneuron population of the MGE, labeled using the Nkx2-1iCre;Z/EG
mouse. The Nkx2-1iCre mouse used in this study has been shown to possess a
restricted region of recombination that does not include the Nkx6.2 expressing region
(Fogarty et al., 2007). Although I did not directly confirm the region of recombination
produced with the Z/EG reporter used in this study (which is distinct from the ROSA
reporter used by Fogarty et al., 2007), the Nkx2-1iCre mouse shows limited
expression of Cre within the Nkx6.2 expressing domain (Fogarty et al., 2007) and the
Z/EG reporter used here has been shown to produce decreased labeling compared to
reporter lines based around the ROSA locus (Miyoshi et al., 2007). It is therefore
unlikely the genetic strategy used in this study produced an expanded region of EGFP
labeling in the Nkx6.2 region relative to the study by Fogarty et al., (2007).

From amongst the labeled population I recorded four main subtypes of interneuron in
the mature somatosensory cortex FS, NFS type-1, NFS type-2 and rIB with a lower
number of dNFS and iIB cells. These populations are also reported in the
Olig2CreER\textsuperscript{tm} mouse (Miyoshi et al., 2007), which labels cells that emerge from the
MGE. However it appears there are some differences between the two populations. In
terms of the electrophysiological subtypes observed in this study, I did not record any
initial adapting or late spiking interneuron subtypes, which were reported in the
Olig2CreER\textsuperscript{tm} mouse (Miyoshi \textit{et al.}, 2007). Instead it would appear that the
electrophysiological populations reported here and by Sousa \textit{et al.}, (2009), combine to
produce the sum total of those cells labeled using the Olig2CreER\textsuperscript{tm} mouse. However,
the presence of dNFS, NFS type-1, rIB and iIB cells in the labeled population
reported in both this study and that of Sousa \textit{et al.}, (2009) suggests that either the
boundary between the two regions is not absolute, or alternatively that there is some
overlap between the regions of recombination and therefore EGFP labeling in the two
mouse lines used in these studies. In addition I also observe laminar differences in the
localization of some subtypes relative to Miyoshi \textit{et al.}, (2007). Whilst rIB
interneurons were reported to occupy both deep and superficial layers when fate
mapped using the Olig2CreER\textsuperscript{tm} mouse (Miyoshi \textit{et al.}, 2007), when labeled using the
Nkx2-1iCre;Z/EG mouse they were selectively localized to deep cortical layers (fig.
5.2 C). This observation was not due to any laminar bias in the number of cells
recorded and, as all cells tested for their response to hyperpolarizing current, nor was
it due to any difference in the electrophysiological protocols performed on deep and
superficial cells.

The likely explanation for the differences reported between this study and Miyoshi \textit{et al.}, is the use of a different genetic strategy to label cortical interneurons. The Nkx2-
1iCre;Z/EG mouse used here seems to label few neurons reportedly derived from the
Nkx6.2 positive sulcal region (Fogarty \textit{et al.}, 2007; Sousa \textit{et al.}, 2009), whereas the
Olig2CreER\textsuperscript{tm} used by Miyoshi \textit{et al.}, produces recombination that extends into more
dorsal aspects of the ganglionic eminences, including the Nkx6.2 expressing domain
(Miyoshi \textit{et al.}, 2007). A recent study by Gelman \textit{et al.}, (2011) revealed that the POA
produces more interneuron populations than previously expected, some of which have
also been reported to arise from the Nkx6.2 expressing region adjacent to the sulcus
(Sousa \textit{et al.}, 2009). Whilst sharing similar physiological properties, the spatial
distribution of labeled cells in the two samples was distinct. POA derived cells were
primarily located in deep layers (layers 5 and 6), whereas the populations arising from
the Nkx6.2 expressing region adjacent to the sulcus showed a second cluster of cells
populating layer 2/3 (Sousa \textit{et al.}, 2009; Gelman \textit{et al.}, 2011). This suggests that
Nkx6.2 may act to initiate an altered genetic program that allows cells normally
destined for deep layers to settle in layer 2/3. Given that rIB cells were fate mapped
from the *Nkx6.2* expressing region (Sousa *et al*., 2009), this hypothesis would explain
both the findings reported here regarding the lack of superficial rIBs, as well as those

In support of this hypothesis is an observation from spinal cord development that
shows that some Nkx6 proteins act to diminish, but not prevent, the expression of
other Nkx transcription factors (Vallstedt *et al*., 2001). Given the apparent mutual
exclusivity of MGE and CGE genetic programs (Butt *et al*., 2008) it is possible that
*Nkx6.2*, which is located at the boundary between these domains, acts to soften the
border between the two regions- allowing the production of MGE interneuron
subtypes that posses CGE laminar profiles (see Miyoshi & Fishell, 2011). Support for
this idea comes from observations that *CoupTFII*, a transcription factor that is
expressed in CGE interneuron subtypes and important for their specification
(Kanatani *et al*., 2008; Fuentealba *et al*., 2010), is also found to be expressed amongst
some MGE subtypes that display characteristics that likely place their origin in the
dorsal MGE and sulcus (Kanatani *et al*., 2008; Triciore *et al*., 2011), where *Nkx6.2* is
known to be expressed (Fogarty *et al*., 2007; Sousa *et al*., 2009).

5.10.2 Similarities and differences between NFS and FS interneurons

Attempts to uncover the roles played by individual interneuron subtypes in the
neocortex have primarily focused upon NFS/SST and FS/PV interneurons (Reyes *et
al*., 1998; Kapfer *et al*., 2007; Berger *et al*., 2009; Cardin *et al*., 2009; Fino & Yuste,
2010; Wilson *et al*., 2012), which comprise the two main populations of cells reported
in this chapter. These cells make attractive subjects for study because they are
relatively abundant within the cortex and we posses genetic lines that allow
researchers to selectively target them for electrophysiological recording or imaging,
or to manipulate them to uncover their role within the network (Taniguchi *et al*.,
2011). Studies into the molecular mechanisms that govern how these two cell-types
connect with other cells around them seem to highlight differences rather than
similarities in their individual approaches (Bartley *et al*., 2008; Gibson *et al*., 2009;
Chang *et al*., 2010; Fazzari *et al*., 2010). However SST and PV cells are derived from
the same progenitor region, express similar downstream genes, such as *Lhx6* and *Sox6*
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(Butt et al., 2005, 2008; Liodis et al., 2007; Zhao et al., 2008; Azim et al., 2009; Batista-Brito et al., 2009), are born over similar time scales and integrate into the same layers of the neocortex (Butt et al., 2005; Miyoshi et al., 2007; and fig 5.2 C). It is thus unlikely that their environment at the time of integration is largely different, suggesting cell intrinsic mechanisms regulate the processes that drive their integration and maturation. In support of this notion the consensus of opinion appears to be on the side of genetically defined programs to specify the subtype specific axonal targeting and integration of interneurons (Stepanyants et al., 2004; Huang, 2006; Fazarri et al., 2010), followed by activity driven maturation of intrinsic and synaptic properties (Bartley et al., 2008; Doischer et al., 2008; Chang et al., 2010; Goldberg et al., 2011; Miller et al., 2011); although not necessarily in CGE interneurons (Karayannis et al., 2012). If this is the case, what do the results described in this chapter tell us about the timescale over which these processes may occur, and can we begin to explain the distinct developmental pathways taken by PV/FS and SST/NFS interneurons as they integrate into the cortex?

5.10.3 Emergence of physiologically distinct subtypes amongst Nkx2-1 derived interneurons

Although it is known that the mature subtype an interneuron will become is specified during embryonic development (Nery et al., 2002; Butt et al., 2005; Miyoshi et al., 2007), less is known about when in development the heterogeneity inherent amongst mature interneurons manifests itself. The findings of this chapter show that although there is some initial diversity amongst the electrophysiological properties of the immature neocortex, this diversity is accentuated over the course of postnatal development. Using phase space analysis to segregate the subtypes, I found that the population data shows a number of electrophysiological parameters that are distinct between FS and NFS cells even at P5-8 (table 5.1). However, when using these parameters to perform cluster analysis at immature ages, I was unable to fully segregate these populations. This suggests that the complete set of characteristics that define a mature electrophysiological subtype have yet to be expressed and that there is a greater degree of overlap between the electrophysiological properties of FS and NFS cells at this time (P5-8). By P9-13 cluster analysis was mostly able to separate FS and NFS cells based upon subtype, the exceptions being some FS cells recorded at P9 that
segregated in the NFS cluster and a population of NFS type-1 cells in the FS cluster. Given the delayed developmental maturation of certain FS spike properties, which begins at around P9/10 (section 5.4.3; Du et al., 1996; Goldberg et al., 2011), it is possible that the P9 FS cells had yet to begin up regulating the Kv channels required for faster spike kinetics- an important determinate of the FS phenotype. NFS type-1 cells were also found in the FS cluster at mature ages suggesting their rapid spike dynamics, more akin to FS than NFS cells, place them in this population when analyzed in this way. At mature ages (P14-21) it was possible to reliably separate all three main subtypes (FS, NFS and rIB) across multiple electrophysiological parameters. From this point onwards it is also possible to reliably assign cells into one of the individual electrophysiological subtypes shown in figure 5.1 (see also Miyoshi et al., 2007) and thus it would appear that the diversity inherent to the Nkx2-1 derived interneuron population is fully realized at this time.

The late emergence of the electrophysiological properties of interneurons also agrees with studies by others who have looked at individual subtypes in isolation (Goldberg et al., 2011; Oswald & Reyes, 2011). This time frame also correlates with the emergence of other physiological properties that influence the network role played by individual subtypes and are known to emerge, or show prominent maturation, during the second and third postnatal week (Lahtinen et al., 2002; Long et al., 2005; Doischer et al., 2008; Oswald & Reyes, 2011). Interestingly, pyramidal cells also begin to show increased electrophysiological diversity at around this time (Kasper et al., 1994; Flint et al., 1997). Taken together these findings suggest that the electrophysiological diversity of the neocortex as a whole seems to emerge over a protracted period during the first two to three postnatal weeks.

5.10.4 Differences between the subtype segregation reported in this study and in Daw et al., (2007)

Earlier in this chapter I report that a study by Daw et al., (2007) was able to separate cells with FS and NFS phase space plots into two discrete clusters based upon their intrinsic membrane properties (section 5.3.1). Unlike Daw et al., (2007), when performing cluster analysis on cells distinguished by phase space analysis as being either FS or NFS subtypes, I was unable to fully segregate cells when using multiple
electrophysiological parameters. There are a number of reasons that may explain this. Firstly the GIN line used by Daw et al., to distinguish SST positive, NFS interneurons only labels a small proportion of SST positive cells (around 30%, Oliva et al., 2000), whereas the Nkx2-1iCre line used in this study labels between 70-80% of cortical SST positive interneurons (Fogarty et al., 2007; S. Raffiq, unpublished data). Because of this it is likely that the electrophysiological diversity of NFS populations reported here is greater than in the GIN line used by Daw et al., (2007). As all NFS subtypes seem to display similar phase space plots (fig. 5.3 A), it is possible that this increased diversity may explain the increased overlap between FS and NFS populations reported at early ages (section 5.5.3). The study by Daw et al., is also restricted to layer 4, whereas the focus of this study was on layers 2/3 and 5 and it is possible that there may be some variation in the subtypes of cells located in these layers (see Markram et al., 2004), or their development, which makes them distinct.

5.10.5 Diversity in early interneuron populations

Although it seems that the full electrophysiological diversity of the cortex does not emerge until later in postnatal development, the early inhibitory component of the cortex is not a homogeneous pool of neurons. We know that there is already a degree of morphological (Mcdonald et al., 1982 a, b, c) and immunohistochemical (S. Raffiq, unpublished data) distinction amongst interneurons at this age and, whilst they did not segregate into distinct clusters, FS and NFS cells show distinct phase space plots (fig. 5.3 B-C) and are beginning to take on the electrophysiological properties that will define them at later stages of development (table 5.1). A recent study from the Cossart lab, performed in the hippocampus, has shown that interneurons undergo a morphophysiological transformation during the first postnatal week (Allene et al., 2012). This transition brings about a change in their electrophysiological properties, which go from being universally immature and strongly adapting to become more alike mature interneuron populations (Allene et al., 2012). Taken with the data reported here, this seems to suggest that the period towards the end of the first postnatal week underlies the start of the electrophysiological diversification of cortical interneurons (at least in terms of their observable phenotype).
Overall it seems that, as reported for other interneuron populations (Batista-Brito et al., 2008; Di Marco Garcia et al., 2011), early Nkx2-1 derived neurons have already undergone a degree of physiological divergence over the course of their migration and initial integration into the network, and are on their way to forming the discrete subtypes that populate the adult cortex. This initial period in their development likely provides the opportunity for interneurons to upregulate factors that bestow them with the competency to respond to signals that will drive their subtype dependant maturation at later ages. These signals may be endogenous to the cortex (Huang et al., 1999; Fazzari et al., 2010; Wen et al., 2010), or derived from the periphery (Sugiyama et al., 2008) and not only influence the electrophysiological maturation of interneurons but their synaptic integration as well.

5.10.6 Development of excitatory inputs onto Nkx2-1 derived interneurons

After an initial phase of network integration the inputs onto the two main Nkx2-1 derived interneuron subtypes (FS and NFS) undertook distinct developmental trajectories. I observed a considerable enhancement in the inputs of layer 2/3 and 5 FS cells post P8 (fig. 5.20 A-F), but not those of other recorded interneuron types (figs. 5.28 A-F & fig. 5.29 I), or those FS cells located in layer 4 (fig. 5.19 I). Further work is needed to confirm the maturation of inputs onto both layer 4 FS cells and rIB interneurons, as only a limited sample were recorded in this study. However, it seems that rIB interneurons are in many ways similar to NFS subtypes, both in terms of their immunohistochemical marker expression (Butt et al., 2005) and many of their intrinsic electrophysiological properties (section 5.4.5). Given mature rIB cells show a similar degree of integration to that of mature NFS cells (section 5.6.4), it seems that this also extends to their synaptic inputs- discounting a considerable developmental rise. The increase in FS cell input in layers 2/3 and 5 seems to approximately match the developmental enhancement in pyramidal cell connectivity reported for cells recorded in the same layers in the previous chapter (chapter 4 section 4.3). If it is the case that the rise in pyramidal cell input drives the maturation of FS cell input, or visa versa, the observation that layer 4 FS cells display more constant input over development can be explained by reports that the network in this layer becomes fairly crystallized by P9-10 (Ashby & Isaac, 2011), such that no increase in layer 4 FS input is required to maintain network parity.
The distinct maturation of layer 4 may explain the lack of input onto FS cells recorded in that layer, however outside of layer 4 FS and NFS cells located in the same layer show subtype dependant differences in the maturation of their excitatory inputs. We know that the mechanisms of FS and NFS integration are distinct (Bartley et al., 2008; Gibson et al., 2009; Fazzari et al., 2010) and by looking in more detail at the pathways involved in their formation it is possible to find a number molecules that may explain the differences observed in their developmental integration. Interestingly, a number of these signaling pathways involve presynaptic pyramidal cell partners, providing a link between the rise in pyramidal cell inputs and those of FS cells.

5.10.7 Molecular pathways that govern interneuron integration

The difference in the developmental integration of FS and NFS interneurons adds further support to the fact that the mechanisms underlying the formation of excitatory synaptic inputs onto these two populations are distinct. Surveys of the literature suggest a number of candidates with the potential to drive the subtype specific integration of these two classes of cortical interneurons, the most well studied being ErbB4 a receptor for a number of factors, including neuregulin-1 (Wen et al., 2010). The ErbB4 neuregulin-1 signaling pathway underlies key processes in the proper formation and maturation of inhibitory circuits (Fazzari et al., 2010). Initially ErbB4 is expressed downstream of Lhx6 (Zhao et al., 2008), where it functions to promote correct migration towards the cortex (Yau, 2003; Anton et al., 2004; Flames et al., 2004). Once there its role switches towards integrating interneurons into the cortical network. It does so by promoting excitatory input onto PV positive FS cells, in addition to the formation of inhibitory outputs from PV cells onto pyramidal neurons, and GABA release from these synapses (Woo et al., 2007; Fazzari et al., 2010; Ting et al., 2011). Its role in the formation of excitatory inputs is likely mediated through its interaction with PSD-95 (Garcia et al., 2000; Huang et al., 2000; Longart et al., 2007), a molecule that is known to associate with postsynaptic densities in excitatory synapses (Cho et al., 1992) and acts to stabilize the postsynaptic structure (Keith & El-Husseini, 2008).

The role of ErbB4 in excitatory synapse formation makes it a suitable candidate to explain the different integration strategies observed between individual interneuron
subclasses in this study. The fact that it is initially expressed in migrating neurons under the control of \textit{Lhx6} (Zhao et al., 2008), which is found in both PV/FS and SST/NFS interneurons (Fogarty, \textit{et al.}, 2007; Du \textit{et al.}, 2008), may lead to some residual expression during the early phases of interneuron integration that could explain the similar levels of connectivity observed in FS and NFS interneurons at P5-8. In FS cells ErbB4 upregulation is known to occur over an equivalent period to the maturation of excitatory input reported in this study (Longart \textit{et al.}, 2007); explaining both this finding and the preferential expression of ErbB4 in PV positive FS interneurons at more mature ages (P30, Fazzari \textit{et al.}, 2010). Interestingly the point at which the upregulation of ErbB4 begins (P9/10), coincides with the increased expression of other factors that contribute to the FS phenotype, such as PV and Kv3.1 (del Rio \textit{et al.}, 1994; Du \textit{et al.}, 1996; Longart \textit{et al.}, 2007). This suggests a shift in the expression of a number of key genes in FS cells of the somatosensory cortex at around this time, and the beginning of large-scale transcriptional changes in FS cells (Okaty \textit{et al.}, 2009).

A further structure that shows developmental upregulation in FS cells is their perineuronal net (Okaty \textit{et al.}, 2009). In the neocortex this structure is primarily found in the extracellular matrix of FS cells (Hartig \textit{et al.}, 1999, 2001) and has been shown to be important in regulating the formation of inputs onto these cells, as well as having a more global influence on synaptic plasticity (Chang \textit{et al.}, 2010). Narp (neuronal activity–regulated pentraxin) is a molecule that acts to promote excitatory inputs onto FS cells (Chang \textit{et al.}, 2010). Narp function is dependent upon the presence of a perineuronal net, with loss of the net preventing the role of Narp in synaptic scaling of excitatory inputs to FS cells (Chang \textit{et al.}, 2010). Experiments where neuregulin and Narp signaling have been perturbed suggest that these two pathways both contribute to the maturation of FS inputs, but in slightly different ways.

Loss of the perineuronal net, and therefore Narp signaling, seems to perturb the activity dependent scaling of synaptic inputs (Chang \textit{et al.}, 2010), whilst loss of ErbB4/neuregulin signaling diminishes the number of excitatory inputs but appears to have no effect on their overall amplitude (Fazzari \textit{et al.}, 2010). Given that over the course of FS cell integration I report both an increase in the average number of LSPS
evoked EPSCs as well as their average size (fig. 5.20 A-F), this suggests that both of these mechanisms may play a role in the maturation of synaptic inputs amongst layer 2/3 and 5 FS cells reported in this study. Both ErbB4 (at least at mature ages, Fazzari et al., 2010) and the perineuronal net are particularly enriched in PV interneurons (Hartig et al., 1999, 2001; Chang et al., 2010) and their expression is known to be upregulated over a similar timescale to this study (Longart et al., 2007; Okaty et al., 2009). The lack of these molecules in NFS interneurons therefore provides an explanation as to why they do not show an increase in excitatory input over the same developmental period. Both Narp and neuregulin-1 molecules are released in an activity dependant fashion by cortical pyramidal neurons, causing them to enhance excitatory input onto FS cells in response to increased cortical activity (Ozaki et al., 2004; Chang et al., 2010; Fazzari et al., 2010). Therefore these molecular pathways provide two distinct mechanisms that may link the upregulation of pyramidal cell connectivity described in chapter 4 (section 4.3.3) with the maturation of FS inputs reported in this chapter (section 5.6.2.4).

5.10.8 Variation in the organization of inputs onto Nkx2-I derived interneurons

In addition to looking at developmental changes in the amount of input received by Nkx2-I derived interneurons, one of the other main reasons for this study was to map the laminar distribution of these inputs onto individual interneuron subtypes recorded in different layers of the neocortex. In terms of the organization of inputs onto Nkx2-I derived interneurons, I found that they broadly follow the pattern expected by the canonical circuit (Douglas & Martin, 1991), and in many ways mirrored the inputs shown by mature pyramidal cells in chapter 4 (section 4.3). However, due to the fact that labeled interneurons were more sparsely distributed within the cortex, when looking for cells to patch I recorded cells over a greater range of distances from the pial surface than pyramidal cells, and because of this encountered a number of instances of variation in the synaptic input maps produced (sections 5.7 & 5.8). This variation in input organization broadly conforms to pathways of excitatory connectivity previously reported for pyramidal cells in layers 2/3 and 5 (Schubert et al., 2007; Anderson et al., 2010; Hooks et al., 2011). However in many cases this represents the first observation of such phenomena in interneurons of the
somatosensory cortex. Interestingly it seems that the majority of these input motifs occur irrespective of the subtype of interneuron recorded from.

5.10.8.1 Sources of input onto layer 2/3 Nkx2-1 derived interneurons

In layer 2/3 I find that there is a degree of variation in the amount of layer 4 input received by layer 2/3 interneurons (section 5.7.1). As has been reported for layer 2/3 pyramidal neurons (Hooks et al., 2011), this correlated with the proximity of the cell soma to the granular layer (fig. 5.31 A). Other studies that have used LSPS to map excitatory inputs onto layer 2/3 interneurons have shown that an alternative source of variation in layer 4 input is the expression (or not) of calretinin (Xu & Callaway, 2009). In the aforementioned study the authors report that layer 2/3 SST positive cells that also express CR receive less layer 4 input than those that are CR-ve. Although this may contribute to a small amount of the variation in layer 4 input received by layer 2/3 SST positive, NFS interneurons in this study, it is unlikely to play a major role as there is limited co-localization with CR amongst EGFP labeled cells in the Nkx2-1iCre mouse (Fogarty et al., 2007; S. Raffiq, unpublished data).

The distinction between those cells that receive little or no input from outside layer 2/3, and those that are more widely integrated with other layers, was confirmed by cluster analysis of the laminar organization of excitatory inputs (section 5.8). However rather than forming a single cluster, those cells that were more widely connected could be separated further based upon the degree to which they integrate with other layers. Cells in the FS/NFS 2 cluster received a lower amount of input from layers 4 and 5, whilst those in FS/NFS 3 received more translaminar input, particularly from layer 5a (table 5.3). A previous study that looked into the variation in excitatory input onto distinct inhibitory subtypes in layer 2/3 of the somatosensory cortex revealed that there were three discrete input motifs (Xu & Callaway, 2009). As found here, these motifs centered on inputs from three layers- layer 2/3, layer 4 and layer 5a. Cells were found to receive inputs from either layer 2/3, layer 2/3 and 4 or layer 2/3 and 5a, with the subtype that was recorded seemingly the determining factor as to which pattern of input a cell received (Xu & Callaway, 2009). The variation in input observed onto Nkx2-1 derived interneurons also shows patterns that are similar to these, with cells receiving no layer 4 input (FS/NFS 1) and strong layer 4 input...
I also find that there is some variation in input from layer 5a, however the percentage of layer 5a input reported in Xu & Callaway (2009) is in the region of 40% of total input, which is far in excess of anything observed in this study (table 5.3). Furthermore cells that receive this strong layer 5a input also typically lacked layer 4 inputs (Xu & Callaway, 2009), whereas in this study I find cells that receive input from layer 5a also show input from layer 4 (FS/NFS 3, table 5.3). The input motif that centered around layer 5a input was common amongst chandelier, neurogliaform, irregular spiking and regular spiking populations (Xu & Callaway, 2009). Most of these populations were not recorded in this study, explaining the lack of this motif amongst the data reported here. Despite this the remaining two input motifs reported by Xu & Callaway (2009) appear to be present amongst Nkx2-1 interneurons. However is seems that amongst those layer 2/3 cells that do receive translaminar input, a further, perhaps more subtle, distinction can be made based on the amount of layer 5 input they receive. Unlike the findings of Xu & Callaway (2009), I find that instead of being restricted to particular subtypes, a number of input motifs are observed in both FS and NFS populations.

5.10.8.2 Sources of input onto layer 4 Nkx2-1 derived interneurons

Only a limited sample of layer 4 interneurons was recorded in this study, however even amongst this small population I still observe a degree of variation in their inputs. As with interneurons in other layers, layer 4 FS cells could be separated into those cells that received primarily local input, and those that received a large proportion of their input from outside their host layer. Although layer 4 cells received input from layer 5 (fig. 5.19 A-H) the main source of this variation centered on input from layer 2/3 (fig. 5.35 B-E). The presence of a similar divergence in input patterns has also been reported amongst layer 4 excitatory neurons; with spiny stellate cells receiving focused input from within their own barrel, whilst pyramidal neurons in layer 4 appear to be more broadly integrated with other layers of the home column (Schubert et al., 2007).
5.10.8.3 Sources of input onto layer 5 \(Nkx2-1\) derived interneurons

As reported for layer 5 pyramidal neurons and interneurons in the motor cortex (Anderson et al., 2010; Apicella et al., 2012), I find that the amount of input from layer 2/3 onto layer 5 interneurons varies based upon with depth of the cell soma from the pial surface (fig. 5.32 A-G). As expected, this meant that layer 5b interneurons received less layer 2/3 input than those recorded in layer 5a. However, as found for FS cells in the motor cortex (Apicella et al., 2012), the decrease in layer 2/3 input does not strictly follow the layer 5a/b boundary, with a population of superficial 5b interneurons still receiving prominent layer 2/3 input (fig. 5.33 A). Amongst the three input motifs observed in layer 5, the difference in the amount of layer 2/3 input received by layer 5 cells separates the input motif corresponding to the FS 6/NFS 4/rIB 1 clusters, from the remaining patterns of input. Amongst these further two distinct input motifs we find cells whose inputs are solely localized within layer 5 (FS 8 & rIB 2) and those that also receive prominent input from layer 4 (FS 7 & NFS 5).

The pathway from layer 4 to 5 is not particularly well studied (Thomson & Lamy, 2007; see however Feldmeyer et al., 2005; Schubert et al., 2007) and the only previous uncaging study that mapped the inputs onto layer 5 interneurons was performed in motor cortex (Apicella et al., 2012), which lacks a layer 4 (Shepherd, 2009; Anderson et al., 2010; Hooks et al., 2011). As such, this study represents one of the first mapping studies of layer 4 connectivity onto layer 5 cortical interneurons. Both layer 5a and 5b interneurons seem to receive some input from layer 4 (fig. 5.31 B), and amongst the input motifs present in layer 5 cells, one of them is defined by a prominent pathway from layer 4 to 5b (FS 7 & NFS 5, table 5.3). Therefore, as reported for pyramidal neurons (Douglas & Martin, 1991; Feldmeyer et al., 2005; Schubert et al., 2007), it seems that there is excitatory connectivity from layer 4 onto layer 5 interneurons. Unlike the other input motifs present in both layers 2/3 and 5 (where multiple subtypes have been recorded), the layer 4 to 5b motif (FS 7 & NFS 5) is the only one that does not appear to be present amongst all interneuron subtypes (table 5.3). Whilst a subpopulation of both NFS and FS layer 5b cells receive prominent layer 4 input, rIB cells seemed to show limited integration with layer 4, with no rIB correlate of the FS 7 & NFS 5 input motif observed. This could simply be due to a difference in sampling frequency, as fewer rIB cells were recorded in this
study. However an alternative explanation is that there is a difference in the role of rIB cells that separates them from FS/NFS interneurons. To support this hypothesis, it has been shown a similar distinction is observed in layer 5b pyramidal cells—whereby regular spiking pyramidal cells recorded in layer 5b receive stronger input from layer 4 than burst spiking pyramidal cells (Schubert et al., 2007). As the only layer 5b interneuron subtype that seems to lack strong layer 4 input also possesses a bursting phenotype (fig 5.1 D), this suggests that perhaps there is less integration between layer 4 and deep layer bursting cells—be they excitatory or inhibitory.

5.10.9 Accounting for variation in input maps

The results of the cluster analysis discussed in the preceding sections highlights a considerable degree of diversity in the laminar input structure of Nkx2-1 derived interneurons. This variation in input also appears to be present amongst individual subtypes, rather than simply being due to the heterogeneity of subtypes recorded (see Xu & Callaway, 2009). Many uncaging studies seem to focus on average data to report the inputs onto given populations (Bureau et al., 2004; Xu & Callaway, 2009; Apicella et al., 2012). Whilst this provides us with information about the primary sources of input onto a given cell type, located in a specific layer or region, it means that some of the variety present within the population is likely lost through averaging. It also assumes that the organization of inputs respects laminar or sublaminar boundaries, which, as we can see from the results of this chapter, does not always seem to be the case. By firstly looking at the diversity on a cell-by-cell basis and then performing cluster analysis to separate the different input motifs, I have been able to circumvent some of these issues. This has allowed me to provide a more accurate picture of the heterogeneity inherent within the excitatory synaptic inputs onto Nkx2-1 derived cortical interneurons and has also highlighted a number of input pathways that may have been lost if the data had simply been averaged without first taking this diversity into account. However, in order to perform this analysis I have had to group data recorded at all stages of development. The necessity and legitimacy of this approach shall be discussed in more detail in the following section.
5.10.10 Validity of normalized approach

Much of the analysis into the variation in laminar organization of inputs onto \textit{Nkx2-1} derived interneurons was performed on normalized data. This allowed my to group the data across development, as it discounts the enhancement in input strength observed amongst FS cells (section 5.6.2.4). I felt this was necessary as there was considerable input variability in the samples recorded at each age range, such that there was insufficient data to perform this analysis on any one of the three age groups in isolation. The reason that I felt confident in performing this analysis across ages, particularly as I have shown in the previous chapter that the laminar organization of inputs onto pyramidal neurons undergoes a considerable developmental shift (chapter 4, section 4.3), is that the data for interneurons seems to suggest that the laminar organization of inputs is relatively constant over development. To support this I find that the ANOVA analysis of the input maps shows that both NFS and FS interneurons possess significantly organized inputs from P5-8 (fig. 5.30). Also when performing the cluster analysis on the inputs I find that the majority of input motifs are present at both immature and mature ages (table 5.3).

There are instances where some motifs are not observed at a particular age. Many of these can be explained by differences in laminar, or sublaminar, sampling at each of the different ages (as discussed in section 5.8). However, there is no way to confirm the presence of these motifs at earlier ages without performing a more thorough and focused study of the input variation inherent within the \textit{Nkx2-1} population at each of the three ages outlined here, or at least at the two extremes of this study. Therefore, although it seems that many of the input motifs observed are present, at least to some degree, across the entire developmental period of this study, the findings presented in this chapter do not preclude the possibility that there is still some developmental alteration in the laminar organization of inputs onto \textit{Nkx2-1} derived interneurons, and therefore a shift in the relative contribution of cells with a particular input motif amongst the total population.
5.10.11 Differences in input between subtypes

Although it seems that there are a number of similarities between the laminar organization of inputs onto *Nkx2-1* derived interneurons, this is not to say that the inputs onto these cells, or indeed their roles within the network, are the same. There are a number of other factors that contribute to the functional properties of excitatory connectivity onto individual interneuron subtypes, and these will affect the influence of the synaptic inputs that are organized around these individual motifs. Firstly, as shown in sections 5.6.2.4 & 5.6.4, the inputs onto FS cells at mature ages are significantly stronger than those onto NFS and rIB cells. In terms of how this relates to input from a given layer, it seems that FS cells of the somatosensory cortex receive particularly strong inputs from both their host layer and the preceding layer of the canonical circuit (layer 4 input to layer 2/3 and layer 2/3 input to layer 5a). This seems to be in agreement with previous experiments that looked at the input onto layer 2/3 interneurons (Xu & Callaway, 2009), where it was reported that FS cells receive the strongest layer 4 input of any layer 2/3 interneuron population recorded in the somatosensory cortex (Xu & Callaway, 2009). This is also found to be the case in this study, with mature layer 2/3 FS cells showing stronger pA/pixel values from layer 4 than layer 2/3 NFS cells (section 5.6.3.4). All NFS cells recorded at this age clustered in either NFS 2 or 3, both of which receives input from layer 4 (table 5.3). This rules out the possibility that this finding is due to an increased prevalence of cells recorded from the NFS 1 cluster, which lack layer 4 input. However, because of the variation in laminar input amongst cells that conform to different input motifs (table 5.3), and the small number of cells of each motif recorded at mature ages, this analysis does not take into account the variation in translaminar input inherent within each inhibitory subtype- and ideally would be performed amongst cells of different subtypes that conform to the same input motif.

In layer 5 cells it appears that local connectivity is significantly enhanced onto FS cells relative to other interneuron subtypes (sections 5.6.3.3 & 5.6.4). However, again due to the variation in input encountered in this study and the fact that the majority of mature layer 5 cells were recorded from layer 5b (section 5.8), which receives less layer 2/3 input than 5a (section 5.7.2), it is not possible to confirm if FS cells also receive stronger layer 2/3 input than other *Nkx2-1* interneuron subtypes. It is
important that this issue is resolved, as a recent study by Apicella et al., (2012) has reported that LTS cells in layer 5 of the motor cortex receive greater layer 2/3 input than layer 5 FS cells (Apicella et al., 2012). This is perhaps in contrast to what would be expected given the findings reported for feed-forward excitatory input from layer 4 to 2/3 (Xu & Callaway, 2009 & section 5.6.3.4) and the increased input strength of layer 5 FS cells compared to NFS/rIB populations (section 5.6.3.3 & 5.6.4). As yet it is not possible to tell if the difference between the results expected for the somatosensory cortex and those reported by Apicella et al., is due to the inhibitory subpopulations labeled (they used the GIN line to label LTS cells), the cortical region under examination (SSC versus Mctx) or the age the studies were performed at. Further studies (discussed in section 5.11.6) will help clarify this issue.

In addition to differences in input strength it has been shown that the short-term plasticity of excitatory inputs varies based upon the interneuron subtype that is being targeted (Reyes et al., 1998; Kapfer et al., 2007; Levy et al., 2008). Whilst excitatory inputs from local pyramidal cells onto bitufted, SST positive interneurons show paired pulse facilitation, inputs onto multipolar, often PV positive, interneurons showed paired pulse depression (Reyes et al., 1998). Although the properties of this short-term plasticity are known to vary based upon the presence of various neuromodulators (Levy et al., 2008) and the frequency at which the presynaptic response occurs (Angulo et al., 1999), this mechanism provides a further difference in the excitatory inputs onto distinct subpopulations of cortical interneurons. As such, even in situations where the laminar organization of inputs onto two distinct inhibitory populations are similar, differences in the properties of these synapses will produce subtype specific responses. This is in keeping with the idea that individual interneuron populations are recruited differently depending upon the degree of excitatory drive within the network (Kapfer et al., 2007; also see review by Moore et al., 2010).

Whilst preceding sections focused on potential similarities in the excitatory inputs onto individual interneuron subtypes (section 5.9.9), here I have outlined some of the differences. In addition to excitatory inputs, interneurons also receive inhibitory connections from the surrounding network. These are known to show subtype dependant differences- even amongst interneuron subtypes whose patterns of excitatory input are remarkably similar (Xu & Callaway, 2009). As inhibitory inputs
were not assayed in this study, this may underlie a further distinction between the synaptic connectivity of the individual interneuron subtypes reported here. The interneuron subtypes that make up the labeled population in this study also show a diverse range of intrinsic electrophysiological properties (section 5.2.1) and are known to possess unique morphologies and regions of axonal targeting along the somatodendritic axis (Markram et al., 2004; Ascoli et al., 2008). All of these factors will combine to produce interneuron populations that play different roles in network computation in the mature neocortex (Silberberg & Markram, 2007; Berger et al., 2009; Cardin et al., 2009; Moore et al., 2010; Wilson et al., 2012).

5.11 Further work

5.11.1 Confirm the region of recombination and therefore EGFP labeling produced when crossing the Nkx2-1iCre line with the Z/EG reporter

In this study I show that the electrophysiological subtypes labeled using the Nkx2-1iCre;Z/EG mouse seem to dovetail with those reported to arise from the Nkx6.2 expressing region (Sousa et al., 2009) to produce the sum populations labeled using the Olig2CreER \( ^{TM} \) mouse (Miyoshi et al., 2007). Although this would suggest that the genetic strategy employed to label cells in this study produces a recombination deficit in the Nkx6.2 domain (as reported in Fogarty et al., 2007), because I make use of a Z/EG reporter line to label cells with EGFP (Novak et al., 2000) whilst Fogarty et al., used a Rosa26-GFP reporter, it would be ideal to confirm the region of EGFP expression during embryonic development. To do this I would produce sagital slices of embryonic brains at E11.5, when Nkx6.2 expression is prominent in the developing telencephalon (Sousa et al., 2009). It would then be possible to look at the overlap between EGFP labeling and Nkx6.2 expression to confirm that the recombination deficit reported by Fogarty et al., (2007) is also present when labeling cells using the Z/EG reporter. This will also provide some insight into the overlap between the interneuron populations reported here and those fate mapped from the Nkx6.2 domain (Sousa et al., 2009).
5.11.2 Confirm NFS phase space plots correlate with SST expression

Throughout this chapter I have used phase space plots to distinguish between interneuron subtypes. Whilst there is strong evidence that this approach was able to accurately assign interneurons into either FS or NFS subtypes, it would have been preferable to provide extra validation using immunohistochemistry. This is important, as it is likely that the subtypes of NFS interneurons reported here are more diverse than those reported by Daw et al., (2007). At mature ages all NFS/IB subtypes showed similar phase space profiles, suggesting that the use of phase space analysis is still valid across all these additional subtypes, however it would be ideal to perform somatostatin immunohistochemistry on recorded cells at P5-8 to see if its’ expression correlated with cells possessing NFS phase space plots. SST expression has been shown to display limited variation over the period of this study (S. Raffiq, unpublished data), therefore it would be possible to discriminate between NFS and FS cells using SST expression even at early ages (see Cauli et al., 1997; Butt et al., 2005). To confirm the correlation between SST immunohistochemistry and NFS phase space plots it would be necessary to record and fill individual EGFP labeled interneurons at P5-8 and then perform post-hoc immunolabeling with SST.

5.11.3 Confirm if the preferential observance of rIBs at later ages is due to the late maturation of the rebound burst phenotype

Earlier in the chapter I discussed the possibility that the observed late developmental maturation of rIB interneurons could have been caused by either a sampling error in the depth from which cells were recorded, or the late maturation of the rIB phenotype (section 5.4.6). To confirm which of the two is actually occurring it would be necessary to record a greater number of labeled interneurons at early ages, ensuring that recordings were made from greater depths within layer 5. If recording from a large number of cells in this region still failed to reveal cells with a rIB phenotype, it would support the late development of the rebound bursting response in these cells.
5.11.4 Uncover the maturation of layer 4 FS interneuron inputs

Although it was not a primary objective of this study, I have managed to record a small number of FS interneurons from layer 4. It appears that their inputs may show less developmental enhancement than that reported for layer 2/3 and 5 FS cells, however due to the number of cells recorded it is impossible to make a meaningful conclusion on this point. By recording more cells from layer 4, over a similar age range to that reported for layer 2/3 and 5 interneurons, it would be possible to confirm this. If it were found that layer 4 cells do not undergo further maturation in inputs, it would represent a laminar divergence in the integration strategies employed by cells of the same inhibitory subtype.

5.11.5 Provide further insight into developmental changes in the prevalence of individual input motifs

The cluster analysis that examined the variation in input onto individual interneuron populations was performed on data grouped across the entire developmental period of this study. This showed that many of the input motifs within the \textit{Nkx2-1} population were present from the earliest ages recordings were made. However there were some instances where cells located in a given layer or sublayer where not sampled at specific ages. Also as many of the clusters representing the input motifs are composed of a small number of cells (table 5.3) it makes precise reporting of their frequency at different stages of development impossible. To provide greater insight into these questions it would be necessary to produce a more complete study of this variation by undertaking targeted recordings of cells located in specific layers of the cortex. This does not have to be performed at all three of the ages used here, as if input motifs were found at similar frequency at both immature and mature ages it could be assumed that they are present across development. This would provide unequivocal confirmation that the organization of inputs onto \textit{Nkx2-1} derived interneurons is hardwired from very early stages in the formation of the cortical network.
5.11.6 Confirm the lack of layer 2/3 input onto layer 5b interneurons

The data reported in this chapter suggests that in somatosensory cortex there is a distinction between the superficial input onto interneurons located in layers 5a and deeper in layer 5b. One issue with this finding is that there is a possibility that by slicing the tissue I may have severed some connectivity, which may have caused the lack of superficial input onto those layer 5 interneurons recorded at greater depths. Although every attempt was made to preserve connectivity, at present this cannot be discounted. However, the sharp drop in layer 2/3 input at greater depths, the fact this is observed in a number of recorded interneurons, and that a similar phenomenon is known to occur in both pyramidal cells and interneurons of the motor cortex (Anderson et al., 2010; Apicella et al., 2012), means that if this were the case it would be a striking coincidence. Confirming the lack of superficial input onto deep layer cortical interneurons would be performed in a similar manner to Apicella et al. (2012) and other studies looking at layer 2/3 to 5 connectivity (Petreanu et al., 2009; Harwell et al., 2012), by expressing channel rhodopsin (ChR2) in layer 2/3 pyramidal neurons and optically stimulating them to record inputs onto layer 5a and 5b interneurons.

5.11.7 Look at differences in input strength between individual subtypes whose inputs conform to each motif

The recent study by Apicella et al., (2012) showed subtype dependent differences in both translaminar and intralaminar input onto layer 5 interneurons of the motor cortex. In contrast to the findings of Apicella et al., (2012) the data reported here seems to suggest that FS cells receive stronger translaminar coupling than NFS/rIB cells. This could be due to a regional divergence in the coupling of the layers, an issue brought about by the non-equivalence of the genetic lines used to label interneurons in the two studies, or perhaps a difference that occurs due to the ages the two studies were performed. As discussed in section 5.10.11, due to the variation in input received by Nkx2-1 interneurons, and different relative sampling of the individual input motifs at mature ages, it has not been possible to carry out a thorough investigation into differences in input strength onto individual interneuron subtypes. By recording a greater number of interneurons at mature ages and ensuring that there were a similar number of cells recorded amongst each input motif, it would be
possible to look in more detail at subtype dependant variation in the strength of inputs from specific layers.
Chapter 6 – The emergence of canonical connectivity in the neocortex

6.1 Introduction

‘Devotion to the cerebral hemispheres, enigma of enigmas, was old in me...the supreme cunning of the structure of the grey matter is so intricate that it defies and will continue to defy for many centuries the obstinate curiosity of investigators. That apparent disorder of the cerebral jungle, so different from the regularity and symmetry of the spinal cord and of the cerebellum, conceals a profound organization of the utmost subtlety which is at present inaccessible.’ Cajal autobiography

Since the time of Cajal we have made significant inroads towards understanding the complexity of the cortical network that was of such frustration to him. One aspect of these advances has been the study of the physiological properties of synaptic connections that occur between individual neurons of the neocortex. An important part of this work has been the increasingly detailed maps of synaptic connectivity, between neurons of distinct subtypes, located in different regions of the cortex (Dantker & Callaway, 2000; Xu & Callaway, 2009; Fino & Yuste, 2011; Hooks et al., 2011; Katzel et al., 2011). This has been facilitated by the advent of novel methodologies that allow for identification of the components of the network (Luo et al., 2008), and study of their network connectivity (Callaway & Katz, 1993; Fino et al., 2009; Jerome & Heck 2011; Nikolenko et al., 2011).

By using two such techniques, genetic fate mapping and LSPS, this thesis has aimed to provide further insight into the formation of the cortical circuit. I have focused on the emergence of synaptic connectivity, and the maturation, and diversification, of the intrinsic membrane properties of both pyramidal cells and interneurons. Through this I have been able to provide insight into the organization of the cortical circuit at early stages of its formation, and go on to track its postnatal maturation. This also affords me the opportunity to look at similarities and differences in the maturation of pyramidal cells and interneurons and, through this, look at how they might co-operate during network development. This later point is what I shall discuss in this chapter.
6.2 P12 as an important point in the postnatal maturation of the neocortex

Before we look at the organization of synaptic inputs onto individual network components, we must understand, broadly speaking, the maturation of the network as a whole. It is important to note that the immature cortex is a distinct environment from the mature. Prior to P12 the cells of the network have slower firing properties, in most cases are less integrated, and posses a number of synaptic signaling mechanisms, such as silent synapses and depolarizing GABA, that are to a large degree absent in the adult (Rumpel et al., 1998, 2004; Ben-Ari, 2002; Rheims et al., 2008). The activity of the early network is dictated by slower, synchronous activity patterns that are commonly observed within developing neuronal systems (Allene & Cossart, 2010; Blankenship & Feller, 2010; Kilb et al., 2011). However around P12 this global synchrony starts to disappear, as the network switches to sparse coding of sensory activity (Golshani et al., 2009; Rochefort et al., 2009). This time point also coincides with a number of changes in the properties of the cortex, with the periods pre-and post-P12 possessing distinct plasticity and homeostatic mechanisms, which will dictate how cells connect to the surrounding network (discussed in chapter 4 section 4.7.6; Burrone et al., 2002; Yasuda et al., 2003; Hanse et al., 2009; Gray et al., 2011; Kwon & Sabatini, 2011; Wang et al., 2011; Itami & Kimura, 2012). These differences in plasticity likely contribute to the observations of chapter 4 (section 4.5), where pyramidal cells are preferentially integrated by rhythmic network activity prior to P12. This itself further supports the notion that this period represents a distinct phase in cortical development. Although I primarily focus on these two distinct phases of development, pre-P12 and post-P12, it is important to note that the development of the cortex most likely occurs via a gradual transition between these states. Many of the processes outlined above, such as the prevalence of silent synapses, the reversal potential of GABA, the shift in NMDAR subunit expression and the sparsification of network activity, occur over the course of days rather than within a period of hours. Although for convenience they shall simply be referred to as pre-P12 and post-P12, it should be noted that this is not an absolute boundary and that the development of the cortex is a continuous process whereby the period towards the end of the second postnatal week seems to be a pivotal point in the transition from an immature self-organizing network, to a more mature functional one.
6.3 Summary of the postnatal maturation of excitatory inputs onto neocortical pyramidal cells

The two phases of cortical development were particularly evident in the maturation of excitatory input onto neocortical pyramidal cells. When recorded at close to resting membrane potential, pyramidal cells of the neocortex show a rapid emergence of the canonical pattern of input post-P12 (section 4.3). To examine this in more detail I focused on the development of the layer 2/3 to 5 pathway, which is a remarkably consistent feature of the neocortical network (Hooks et al., 2011) and was found to emerge over a similar developmental period in both somatosensory and motor cortex (section 4.3). I mapped layer 5 pyramidal cells at more depolarized membrane potentials (+40mV), where it was possible to elicit NMDAR mediated responses (Rumpel et al., 1998, 2004). At early ages (P7-8) layer 2/3 input was primarily mediated by NMDARs at +40mV. By mature ages (P14-16) I observed prominent inputs from layer 2/3 at both -70mV and +40mV, suggestive of an AMPAR component to the LSPS evoked inputs. Therefore the developmental transition towards AMPAR mediated input from layer 2/3 can be thought of as occurring through a gradual AMPAfication of previously silent or NMDAR biased synapses (section 4.4). As this happens the inputs onto cortical pyramidal neurons become increasingly more structured and by later ages they are organized, along the laminar axis of the cortex into focused hotspots of excitatory connectivity (as determined by one-way ANOVA, section 4.3.5).

6.4 Summary of the postnatal maturation of excitatory inputs onto Nkx2-1 derived neocortical interneurons

In chapter 5 I have shown that Nkx2-1 derived interneurons possess synaptic inputs from at least P5 onwards, which coincides with the transition from non-synaptic glutamate based cENOs to GABA driven cGDPs (Allene et al., 2008) and is in agreement with other studies of FS cell integration in the neocortex (Pengratz-Fuehrer & Hestrin, 2011). At early ages both FS and NFS populations possessed a similar degree of network connectivity, but over the course of development there was considerable enhancement in FS inputs, but not those onto NFS cells (chapter 5, sections 5.6.2.4 & 5.6.3.4). Nkx2-1 derived interneurons showed a degree of diversity
in the organization of their inputs. However, unlike pyramidal neurons, the inputs onto Nkx2-1 derived interneurons are significantly focused from the earliest ages they are recorded (as determined by one-way ANOVA, chapter 5, section 5.6.5). Importantly, these inputs are also organized around the mature patterns of network connectivity from this early stage of development (chapter 5, section 5.6). As such, a subpopulation of Nkx2-1 derived interneurons (those cells whose input motifs cluster them in FS/NFS 2 & 3 in layer 2/3 and FS 6/NFS 4/rIB 1 in layer 5) receive prominent translaminar inputs from the preceding layer of the canonical circuit from P5 onwards (chapter 5, section 5.8).

6.5 A role for interneurons in the maturation of the canonical circuit?

During the remainder of this thesis, I will examine how interneurons and pyramidal neurons may interact during the early postnatal period (<P12) to facilitate the correct maturation of the cortex. I have specifically chosen to focus on this phase of development because there are a number of studies that have highlighted the importance of GABAergic cells at this time (Allene et al., 2008; Wang & Kriegstein, 2008; Bonifazi et al., 2009; Picardo et al., 2011). However, we are yet to fully understand the role GABAergic interneurons play in assisting in the organization of the developing cortex. The data summarized above indicates that translaminar coupling between pyramidal cells prior to P12 requires coincident depolarization of both preynaptic and postsynaptic neurons. This is not the case for Nkx2-1 derived interneurons, raising the possibility that interneurons may play an important role in the translaminar coupling of the early neocortex and the formation of the canonical pattern of network connectivity, which appears to be hardwired onto them from an early age. However in order to do so they must be capable of transmitting this information on to other neurons, which means we must examine their outputs.

6.5.1 Synaptic outputs of Nkx2-1 derived interneurons

The outputs from interneurons were not recorded in this study, meaning we shall have to rely upon evidence from research performed in other labs. This has shown that layer 5 FS neurons couple with both other local FS cells and pyramidal neurons from P5/6 onwards (Pengratz-Fuehrer & Hestrin, 2011), and that hippocampal FS cells also
form functional outputs from at least P6 onwards (Doischer et al., 2008). This is around the same time that we see synaptic inputs onto FS cells (chapter 5, section 5.6.2; Pengratz-Fuehrer & Hestrin, 2011) and when the early cortical network shifts towards synaptically driven GABA dependant cGDPs (Allene et al., 2008). FS outputs increase in amplitude and connection probability over a similar developmental period to FS inputs reported in chapter 5 (section 5.6.2.4; Pengratz-Fuehrer & Hestrin, 2011). Whilst NFS outputs at early ages are currently unknown, assuming their inputs and outputs mature at roughly similar times (as seems to be the case for FS interneurons), NFS cells are also likely to connect to pyramidal neurons from around P5/6.

In terms of the global, laminar organization of synaptic outputs from Nkx2-1 interneurons, little is currently known. At mature ages layer 2/3 somatostatin (and therefore most likely NFS) neurons form dense inhibitory connections to pyramidal cells in their immediate layer (Fino & Yuste, 2011). A recent study from the Miesenbock lab has also mapped the outputs of all cortical interneurons at mature ages and revealed that in most cases glutamatergic neurons receive the majority of inhibitory input from interneurons located in their immediate layer (Katzel et al., 2011). It is therefore likely that cortical pyramidal cells receive inhibitory input from local interneurons prior to P12.

At mature ages FS and NFS cells target different parts along the somatodendritic axis of cortical pyramidal cells (Markram et al., 2004). FS outputs typically target perisomatic regions (Martin et al., 1983), meaning these synapses should produce a stronger effect on the postsynaptic cell compared to those located more distally. This is because distal inputs may be subject to dendritic filtering, which would diminish their impact at the pyramidal cell soma (Rall, 1964; Magee, 2000). However, whilst it has been shown that some perisomatic synapses from basket cells of the hippocampus are present from P5 onwards (Seress et al., 1989; Seress & Ribak, 1990), there is also considerable maturation in axonal baskets at later ages (Chattopadhyaya et al., 2007), such that the increased efficacy of FS outputs may not be as pronounced at early ages. It is also unclear to what extent dendritic filtering would occur in the less developed morphology of immature pyramidal neurons (Zhu et al., 2000), or indeed the target location of NFS axonal projections at early postnatal stages. The targeting of
interneuron axonal projections along the somatodendritic axis of pyramidal cells, and their influence on postsynaptic cells, is of interest, and discussed in section 6.7.2. However, what is important is that, even if it transpires to only be layer 5 FS cells, a subpopulation of Nkx2-1 derived interneurons have functional outputs that target local pyramidal cells from P5 onwards (Pengratz-Fuehrer & Hestrin, 2011).

6.5.2 Hypothesis for the activity dependent maturation of early postnatal neocortical networks

Many developing neuronal systems exhibit spontaneous activity patterns, which are thought to help promote circuit organization and the functional integration of cells into the network (Katz & Shatz, 1996; Sanes & Lichtman, 1999). In the cortex the early network activity patterns are thought to be dependant upon GABAergic signaling (Ben-Ari, 2002; Allene et al., 2008), primarily internally generated (Golshani et al., 2009, Rochefort et al., 2009; however also see Yang et al., 2009; Minlebaev et al., 2011) and involved in the synaptic integration of cortical neurons (Wang & Kriegstein, 2008). Although the mechanisms that give rise to this activity have not been completely elucidated, early born interneurons comprise the GABAergic hub cells that are important for the propagation of GDPs in the hippocampal network (Bonifazi et al., 2009; Picardo et al., 2011). Importantly, many of the Nkx2-1 derived interneuron subtypes reported in chapter 5 are amongst the earliest cortical interneurons specified in the subpallium (Miyoshi et al., 2007), therefore it is possible they may play a similar role in the activity of the neocortex (Allene et al., 2008).

Given that early synchronous activity patterns are known to be important for the self-organization of a number of other neuronal networks (Katz & Shatz, 1996; Sanes & Lichtman, 1999; Petersson et al., 2003; Ackman et al., 2012), it is plausible that the activity of Nkx2-1 derived interneurons may function to promote the formation of the canonical circuit in the neocortex. Specifically I shall propose that Nkx2-1 derived interneurons play a role in promoting the synchronous interlaminar coupling of individual cortical layers and, through this, drive the synaptic integration of said layers. I shall primarily focus on the maturation of the layer 2/3 to 5 pathway, as this has been the focus of my previous uncaging studies looking at the role of NMDA
mediated inputs in development (chapter 4, section 4.4). However it is also possible that a process similar to that described below may play a role in the developmental integration of other cortical layers as well (discussed in section 6.6).

At early ages (<P12) translaminar glutamatergic connectivity onto layer 5 pyramidal cells is to a large extent mediated by NMDAR dependent, silent inputs from layer 2/3 (fig. 6.1 A-B). Because NMDA receptors have a voltage dependant Mg^{2+} block (Mayer et al., 1984), This means that translaminar coupling cannot occur without synchronous depolarization of the target cells in layer 5 (fig. 6.1 A, Malenka & Nicoll, 1997; Rumpel et al., 1998, 2004). Given that GABAergic signaling during early development is thought to be depolarizing (Owens et al., 1999; Ben-Ari, 2002), and able to remove the voltage dependant Mg^{2+} block from NMDA receptors (Leinekugel et al., 1995; Ben- Ari et al., 1997; Cserep et al., 2012), it is possible that local interneurons may provide the synchronous depolarization that is required for translaminar signaling between pyramidal cells to occur.

Figure 6.1

Figure 6.1 Model to explain the developmental AMPAification of layer 2/3 to 5 connectivity. A-C show schematic representations of a reduced microcircuit linking pyramidal cells in layer 2/3 and 5 of the neocortex at different stages of development. A during early stages of development (<P12), if NMDAR mediated input from layer 2/3 occurs without synchronous depolarization of the target layer 5 pyramidal cell (red triangle), a postsynaptic response is not evoked (X). B layer 2/3 activity recruits GABAergic interneurons (green circle) producing synchronous depolarization of layer 5 pyramidal cell, such that now synaptic transmission does occur. C the continued recruitment of NMDAR mediated inputs during early development in B produces AMPAification of the layer 2/3 to 5 pathway so that in the adult cortex direct AMPAR mediated synaptic transmission occurs between layer 2/3 and 5 pyramidal cells.
In such a scenario synchronous inputs from layer 2/3 pyramidal cells could drive interneurons in layer 5 to fire, thanks to the strong AMPA mediated connectivity between layer 2/3 pyramidal and layer 5 interneurons (fig. 6.1 B). GABAergic signaling has been shown to drive action potentials in developing layer 5 pyramidal cells (Rheims et al., 2008). Therefore GABAergic outputs from layer 5 interneurons would produce a short latency, disynaptic depolarization in layer 5 pyramidal neurons that would facilitate synaptic coupling between pyramidal cells in the two layers (fig. 6.1.B). Because NMDA receptor recruitment is an essential part of the transition from silent to mature AMPA containing synapses (Malenka &Nicoll, 1997), an ongoing process of this sort throughout early development would drive the recruitment of AMPA receptors to previously silent synapses, and would explain the emergence of AMPA mediated input from layer 2/3 onto layer 5 pyramidal cells (fig. 6.1 C; chapter 4, section 4.4). We can also see why perturbing GABAergic signaling during early development prevents the formation of AMPA mediated synaptic inputs (Wang & Kriegstein, 2008) and can hypothesize that if such experiments were repeated, the maturation of translaminar connectivity should be affected.

6.6 Conclusions

The method for coupling the layers during development discussed in this chapter focuses on the maturation of the layer 2/3 to 5 pathway in the somatosensory cortex. The layer 2/3 to 5 pathway has been reported to be a particularly conserved intracortical connection (Hooks et al., 2011), therefore it is quite possible that a process of this sort may underlie the coupling of these layers in other cortical regions. It is also possible that it may play a role in the coupling of other cortical layers as well. Certainly a population of layer 2/3 interneurons receive strong layer 4 input at early ages (motifs FS/NFS 2 & 3, chapter 5, section 5.8) and the basal dendrites of layer 2/3 pyramidal, which overlap with ascending layer 4 axons, have been shown to possess silent inputs (Busetto et al., 2008). Furthermore, layer 4 inputs go through a stage of NMDAR mediated connectivity, which is converted to AMPAR mediated input over the period prior to P12 (Isaac et al., 1997; Ashby & Isaac, 2011). However, the situation in layer 4 is likely more complex; due to the presence of the subplate, which itself is thought to promote associative LTP at thalamocortical terminals (Kanold & Shatz, 2006; Kanold, 2009). In fact, the hypothesis for the coupling of...
layers reported here is remarkably similar to the role of the subplate in thalamocortical synapse formation (Kanold et al., 2003; Kanold & Shatz, 2006). In both instances an intermediary connection acts to strengthen synaptic integration between two weakly coupled networks via associative LTP (Kanold, 2009). However, in this case GABAergic interneurons facilitate the AMPAification of connectivity between spatially separated layers of the cortex, as opposed to the primarily glutamatergic connections from the subplate that promote the integration of thalamus and layer 4 (Kanold, 2009).

The hypothesis outlined in this chapter for the coupling of individual layers of the neocortex (section 6.5.2) is based upon the fact that the inputs onto Nkx2-1 derived cortical interneurons are remarkably similar to the input observed amongst pyramidal neurons of the same layer (chapter 5 section 5.10.8; Feldmeyer et al., 2005; Schubert et al., 2007; Anderson et al., 2010; Hooks et al., 2011). There are a number of ways that this could be brought about, which when taken to their two extremes, can be summarized as follows. Firstly it is possible that the canonical network is hardwired into interneurons, who act as a scaffold to instruct a broadly integrated pyramidal cell population and, through a physiological mechanism such as the one outlined above, bring about the mature canonical pattern of connectivity within the network. A second alternative is that the canonical pattern of input is hardwired into pyramidal cells who, by their vastly superior numbers, are able to better direct incoming axons and therefore organize the cortical network via molecular cues. In terms of layer formation it seems that the latter, pyramidal-centric, model is best supported by experiment (Hevner et al., 2004; Pla et al., 2006; Lodato et al., 2011a). It has also been found, across numerous studies, that developing pyramidal cell axons are hardwired towards the correct laminar target from their initial formation, with limited arborization in non-target layers (Katz & Shatz, 1996; Callaway, 1998; Larsen & Callaway, 2006). Finally, a recent study has shown that diffusible ligands, secreted by pyramidal cells, are involved in the formation of the layer 2/3 to 5 pathway- at least in a subpopulation of pyramidal cells (Harwell et al., 2012). Thus it seems unlikely that the first, interneuron dependant, mechanism functions in isolation to organize cortical connectivity. What is more probable is that there is an initial period during which molecular cues regulate the formation of immature synapses (Harwell et al., 2012), before a period of physiological integration, driven through a mechanism such as that
described herein (see also Wang & Kriegstein, 2008). To advance our understanding of neocortical development it is important that we uncover the specific roles played by pyramidal cells and interneurons in these individual processes. This will allow us to discover how the interplay between these populations promotes the formation of the mature canonical cortical circuit.

6.7 Remaining questions

This chapter has discussed the organization of synaptic inputs onto neocortical interneurons and pyramidal cells during early postnatal development (<P12). Using these data I have outlined how these two neuronal populations might cooperate to drive cortical network formation. However there are currently a number of unknowns surrounding this hypothesis. The key questions, and how we might go about answering them, are outlined below-

6.7.1 Is the NMDA dependant translaminar coupling reported from layer 2/3 to 5 also present in other layers?

It remains to be seen if the NMDAR mediated translaminar coupling observed in layer 5 extends to other cortical layers. Repeating the NMDA synaptic input mapping onto layer 2/3 pyramidal cells would confirm if such a system is employed more globally across cortical layers, or simply between 2/3 and 5. Also within layer 5 it is known that pyramidal cells located at greater depths in layer 5b show significantly less layer 2/3 input than those cells located in layer 5a (Anderson et al., 2010). Given the findings of chapter 5 (section 5.7.2) it would be of interest to see if this phenomenon occurs because layer 5b pyramidal cells do not receive NMDA receptor mediated inputs from layer 2/3 (indicative of a molecular mechanism driving wiring specificity, see Harwell et al., 2012), or, if they do receive NMDA receptor mediated inputs, that they are not converted to AMPA containing synapses due to the lack of layer 2/3 input onto local interneurons (indicating a physiological mechanism).
6.7.2 Mapping the synaptic outputs from \textit{Nkx2-1} derived interneurons

As discussed in section 6.3.3 there are still a number of unknowns regarding the synaptic outputs from \textit{Nkx2-1} derived interneurons, particularly at early stages of development (<P12). By studying their outputs during early postnatal periods it will allow us to ensure their ability to couple the layers of the cortex. Such a study would follow the approach applied by Katzel \textit{et al.}, (2011), using the \textit{Nkx2-1iCre} line to drive channel rhodopsin (ChR2) expression. Whilst it would not be possible to separate the inputs that arise from FS and NFS neurons using this approach alone, we would be able to use other mouse lines to individually confirm the specificity of outputs from PV and SST interneurons during early development using paired recordings (such as SST-Cre and G42-GFP lines, Chattopadhyaya \textit{et al.}, 2004; Taniguchi \textit{et al.}, 2011; Apicella \textit{et al.}, 2012). It would also be possible to use subcellular ChR2-assisted circuit mapping, to map the location of inputs derived from \textit{Nkx2-1} interneurons along the somatodendritic axis of neocortical pyramidal cells (Petreanu \textit{et al.}, 2007, 2009). FS and NFS axons are known to target different locations on cortical pyramidal neurons (Markram \textit{et al.}, 2004), however it is less certain to what extent this occurs during early development (see however Seress \textit{et al.}, 1989; Seress & Ribak, 1990). The location of the axonal targets may be of importance, given GABAergic inputs from distal locations are perhaps less likely to produce strong somatic depolarizations than those that occur at, or in close proximity to, the soma. However, it is also possible that dendritic GABAergic inputs are responsible for the integration of inputs that occur at distal locations on pyramidal cell dendrites (see Petreanu \textit{et al.}, 2009).

In relation to the point above, it has also been shown that GABAergic signaling can drive action potentials in layer 5 pyramidal cells (Rheims \textit{et al.}, 2008). However little is known about which subtypes contribute to the GABA mediated currents evoked in such experiments. The studies mentioned above, in conjunction with extracellular or perforated patch recordings of pyramidal neurons (so as to maintain a physiological chloride reversal potential, Rheims \textit{et al.}, 2008), will be able to begin answering these questions and provide a more detailed understanding of how GABAergic outputs influence the early activity of cortical networks.
6.7.3 Determining the role of \textit{NKx2-1} derived interneurons in the translaminar coupling of cortical layers and AMPAification of silent synapses

The properties of synaptic inputs onto early interneurons and pyramidal cells have led us to believe that interneurons may play an important role in the translaminar coupling of the early network and the integration of layer 2/3 and layer 5 pyramidal cells (section 6.4). However this hypothesis still requires experimental validation. The studies outlined in the previous questions will provide more information on this subject but will not unequivocally prove a direct relationship between interneurons and the uncoupling of silent synapses, or the AMPAification of translaminar inputs. A simple way to test this would be to perturb GABAergic signaling during development. Blocking GABAergic signaling completely may lead to epilepsy, which could itself produce perturbations in the maturation of the network (Manent \textit{et al.}, 2007). A more prudent approach would be to minimize the depolarizing influence of GABAergic signaling. This could be performed either pharmacologically with Bumetanide, by hyperpolarizing \textit{Nkx2-1} interneurons using inward rectifying potassium channels (Di Marco Garcia \textit{et al.}, 2011) or by perturbing the reversal potential for GABAergic signaling during early development (Wang & Kriegstein, 2008). It would then be possible to use glutamate uncaging to see how this influenced the maturation of translaminar connectivity (as performed in sections 4.3 & 4.4).

The above described experiments would show that GABA driven signaling may be important for the recruitment of AMPA receptors to silent synapses, however it does not directly confirm the role in coupling of activity between layers 2/3 and 5. This could be ascertained \textit{in vivo}, by recording how activation of layer 2/3 neurons propagated to layer 5 in the presence and absence of intact GABAergic signaling. Individual whole-cell recordings from layer 5 neurons, in combination with electrical or optical stimulation of superficial layers, would confirm the propagation of activity between the layers (see Adesnik & Scanziani, 2010). This could be studied in control conditions and those where GABA signaling had been perturbed, to see the net effect on translaminar coupling. The advantage of voltage clamp is that it would allow us to hold the cells at different membrane potentials and isolate excitatory and inhibitory contributions to the evoked currents. An alternative approach is to apply a recent technical advance that allows for the simultaneous imaging of deep and superficial
layers *in vivo* (Cheng *et al.*, 2011). This would essentially involve recapitulating the work of Golshani *et al.*, (2009), but including deeper cortical layers and altered GABAergic signaling.
Chapter 7- Final summary and conclusions

7.1 Thesis summary

The dense web of connectivity in the neocortex is an intimidating structure to try and comprehend. One approach is to focus on development, by studying the genetic rules and physiological cues that give rise to the complex architecture of the cortical circuit. One of the aims of this thesis was to provide an overview of neocortical circuit formation. The idea being that by understanding the physiological properties of cortical neurons at different ages, and by studying the connectivity between these individual circuit components, it would be possible to produce a timeline of the physiological maturation of the neocortex. To achieve this I have produced a general assessment of the cortical network over the period of development between postnatal days (P) 5 and P21, providing insight into the changing inputs, intrinsic electrophysiology and outputs of cortical neurons during this phase of development.

In terms of the intrinsic electrophysiology of the neocortex it seems that in both interneurons and pyramidal cells there is a divergence of firing types as the network matures (chapters 4, section 4.2.7; chapter 5, sections 5.4 & 5.5). I find that there is a relative increase in the number of pyramidal cells that posses an initial double spiking pattern, which agrees with the work of others who have shown that there is a late developmental emergence in the diversity of pyramidal cell intrinsic firing properties (Kasper et al., 1994; Flint et al., 1997). A similar phenomenon is observed amongst Nkx-1 derived interneurons, whereby some initial diversity in the electrophysiological properties of FS and NFS cells is further accentuated over the course of development-such that the full complement of physiological firing types is present from around P14 onwards (chapter 5, section 5.5). This fits in with work from other labs that has shown a late emergence of the FS phenotype (Du et al., 1996; Doischer et al., 2008; Okaty et al., 2009; Goldberg et al., 2011; Oswald & Reyes, 2011). Taken together these findings indicate that there is considerable refinement in the intrinsic electrophysiological properties of cortical neurons over the period of this study. It also suggests that the full diversity of electrophysiological firing patterns observed in the adult develops in a coordinated fashion amongst both excitatory and inhibitory
neurons- emerging around the same time, towards the end of the second postnatal week.

In terms of the source of this diversity, the use of the Nkx2-1Cre;Z/EG mouse to label cortical interneurons has allowed me to correlate the mature interneuron subtypes recorded in this study with their point of origin in the developing telencephalon. This conditional fate mapping approach has provided an electrophysiological characterization of the interneuron subtypes derived from the non-sulcal, Nkx2-1 positive MGE (chapter 5, section 5.2). This study contributes to an ever growing body of work from other labs that is providing increasingly detailed spatiotemporal maps of interneuron specification, and as such a greater understanding of the distinct neurogenic domains that run along the dorso-ventral axis of the developing telencephalon (Flames et al., 2007; Fogarty et al., 2007; Miyoshi et al., 2007, 2010; Wonders et al., 2008; Sousa et al., 2009; Gelman et al., 2012; Inan et al., 2012). This knowledge moves us ever closer to the possibility of being able to target individual interneuron subtypes (for example see Inan et al., 2012), either to study their role within the network in greater detail (Woodruff et al., 2011), or to better harness the therapeutic potential of interneuron transplants (Sebe & Baraban, 2011).

It is not just the intrinsic electrophysiology of Nkx2-1 interneurons that was found to be diverse, with considerable heterogeneity observed amongst their synaptic inputs. The data reported in this thesis represents one of the most detailed mapping studies of interneurons produced so far, looking at input variation amongst cells recorded in layers 2-5 and onto 3 broad electrophysiological subtypes (however see also Xu & Callaway, 2009; Apicella et al., 2012). By sampling a number of distinct interneuron subtypes, across multiple layers, I have managed to uncover some of the similarities and differences in their synaptic inputs (chapter 5, section 5.7). It seems that all 3 of the main interneuron subtypes labeled in this study show inputs that conform to a number of distinct motifs, based around the degree of translaminar input they receive (chapter 5, section 5.8). The vast majority of these input motifs are found to occur across subtypes, and be hardwired from their initial integration into the network- with prominent, translaminar AMPAR mediated inputs observed from P5 onwards. This is not to say that the inputs onto the different subtypes cells are identical, with FS cells found to show a prominent developmental upregulation in their inputs, leaving them
more integrated than NFS/rIB cells by the time we reach the third postnatal week (chapter 5, section 5.6).

The development of pyramidal cell inputs was found to occur in a different fashion to that of interneurons. Pyramidal neurons showed a gradual emergence of the mature, canonical pattern of connectivity (chapter 4, section 4.3). This seems to be a fairly universal phenomenon, because it occurs in cells recorded from both deep and superficial layers of motor and somatosensory cortex. To examine the mechanism behind this in greater detail, I focused on the emergence of layer 2/3 input onto layer 5 pyramidal cells of the somatosensory cortex (chapter 4, section 4.4.2). This revealed that the mature pattern of input, from layer 2/3, was predicted by the presence of NMDAR mediated inputs. This adds further credence to the idea that NMDAR mediated, silent synapses function as immature precursors to fully formed AMPAR containing synapses (Liao et al., 1995, 1999, 2001; Durand et al., 1996; Isaac et al., 1997; Malenka & Nicoll, 1997) and suggests that in pyramidal cells, unlike Nkx2-1 interneurons, the mature pattern of excitatory input occurs through a process of gradual synapse AMPAification.

The AMPAification of silent synapses seems largely to have occurred by around P14-16 and was followed by a steady increase in the total input received by pyramidal cells up until P21 (chapter 4, sections 4.3.3-4). Because silent inputs are for the most part absent at this later stage of development (chapter 4, section 4.4.2; see also Rumpel et al., 1998, 2004), and the canonical pattern of input is now in place, this led to the idea that cortical pyramidal cells undergo two distinct phases of input maturation - a period of integration prior to P12, followed by a phase of synapse consolidation until at least P21 (the latest age of this study). To support this I find that prior to P12 rhythmic network activity, akin to that observed in vivo (Golshani et al., 2009; Yang et al., 2009), is able to augment the inputs onto individual cells and produce de novo AMPAR mediated synaptic inputs (chapter 4, section 4.5.3). Although there have been a number of studies that have suggested distinct plasticity paradigms during early development (Burrone et al., 2002; Yasuda et al., 2003; Hanse et al., 2009; Gray et al., 2011; Kwon & Sabatini, 2011; Wang et al., 2011; Itami & Kimura, 2012), many have been based on tightly controlled pairing of presynaptic and postsynaptic activity, or differences in homeostatic synaptic scaling. The data reported
Chapter 7- Final summary and conclusions

here provides further insight into how these distinct plasticity mechanisms influence the way in which cells are integrated. It seems that plasticity in the immature cortex is more akin to the burst-dependant plasticity reported by others (Butts et al., 2007), with the lack of this form of integration post P12 likely due to the transition towards a more mature form of cortical plasticity, which functions through a STDP based mechanism (Itami & Kimura, 2012).

Because the inputs onto Nkx2-1 derived interneurons were in place from the earliest ages of this study, whilst those of pyramidal cells were initially dependant upon synchronous coupling of cortical layers and emerged gradually over the course of development, this led to the idea that interneurons may be instructive in the organization of the canonical circuit. This was further supported by the observation that the input patterns amongst Nkx2-1 derived interneurons were remarkably similar to a number of input motifs observed amongst mature pyramidal cells recorded from the same layers of the neocortex (Feldmeyer et al., 2005; Schubert et al., 2007; Anderson et al., 2010; Hooks et al., 2011), such that they may act as a template for the emergence of cortical circuit in pyramidal cells. The fact that GABAergic signaling is depolarizing during the early integrative phase of cortical development (Ben-Ari et al., 1989; Rheims et al., 2008) provided a mechanism through which translaminar coupling could occur. This has led to a hypothesis as to how the ongoing activity of the early network (Allene et al., 2008; Golshani et al., 2009) may drive the translaminar integration of cortical pyramidal cells and the emergence of the canonical network (chapter 6, section 6.5.2).

7.2 A final overview of cortical development

Combining these findings we can start to build a model, although far from complete, of cortical development. Around P5 there is a gradual integration of cells into the network, which coincides with the transition from cENOs to synaptically driven cGDPs (Allene et al., 2008; Pengratz-Fuehrer & Hestrin, 2011). These early synchronous activity patterns (Allene et al., 2008; Golshani et al., 2009; Yang et al., 2009), act to increase synaptic inputs onto pyramidal neurons prior to P12, and drive the gradual emergence of the canonical pattern of input amongst these cells (chapter 4, sections 4.3 & 4.4). This integrative stage is driven by depolarizing GABA (chapter
6, section 6.5.2; Allene et al., 2008; Wang & Kriegstein, 2008) and the distinct plasticity mechanisms that are found at developing synapses (Monyer et al., 1994; Montgomery & Madison, 2002; Yasuda et al., 2003; Groc et al., 2006; Hanse et al., 2009; Wang et al., 2011). Occurring in tandem to this, we have a gradual maturation in the intrinsic membrane properties of cortical neurons (chapter 4, section 4.2; chapter 5, sections 5.4 & 5.5) and the development of the thalamocortical system, which will relay sensory derived input from the exterior (Kanold et al., 2003; Daw et al., 2007; Ashby & Isaac, 2011; Minlebaev et al., 2011).

By the end of the second postnatal week the canonical pattern of network connectivity has emerged, with both intralaminar connectivity and the thalamocortical system showing prominent integration (chapter 4, section 4.3; Daw et al., 2007; Ashby & Isaacs, 2011). By this point there have been a number of changes in both synaptic composition and GABAergic transmission, that signal an end to the integrative phase of network development (Rumpel et al., 1998; Yasuda et al., 2003; Rheims et al., 2008). It is likely that this also causes, or at least contributes to, the sparsification of network activity that also occurs around this time (Golshani et al., 2009; Rochefort et al., 2009). The end of the second postnatal week coincides with eye opening, the clearing of the auditory canal and the start of active whisking (Shnerson & Pujol, 1983; Mosconi et al., 2010; Rochefort et al., 2011), suggesting a sensory awakening of the cortical network. The plasticity of the cortex now favours integration mediated via STDP (Markram et al., 1997; Sjostrom et al., 2001; Itami & Kimura, 2012), which is likely important in promoting distinct cell assemblies within the network (Song et al., 2000). As GABAergic signaling is now inhibitory, it no longer functions to broadly integrate cells into the network (as proposed in chapter 6, section 6.5.2), but is now able to tightly control the coincidence of presynaptic and postsynaptic firing (Gabernet et al., 2005; Cruikshank et al., 2007; Kimura et al., 2010) and through STDP, alter the weights of these connections to promote those of behavioral relevance and eliminate those that are not (Markram et al., 1997; Sjostrom et al., 2001; Itami & Kimura, 2012). In addition, the emergence of distinct electrophysiological subtypes by this stage (chapter 4 section 4.2.7; chapter 5, section 5.5; Kasper et al., 1994; Flint et al., 1997; Goldberg et al., 2011) will increase the prevalence, and influence, of a number of network activity patterns (Flint et al., 1997; Long et al., 2005; Doischer et
al., 2008), which will contribute to the effective processing and transmission of sensory information within the cortex (see for example Sohal et al., 2009).

Across a broad spectrum of neuronal subtypes, we find there is a coordinated development of both synaptic and intrinsic membrane properties that all converge at the end of the second postnatal week. At this point the neocortical network undergoes a profound transition in its functional capabilities and is now well placed to integrate and process sensory information. By examining some of the mechanisms that underlie the formation of the cortex we find that from its initial layer formation (Lodato et al., 2011a), through the integration (chapter 6, section 6.5.2; Wang & Kriegstein, 2008) and consolidation of its synaptic inputs (Markram et al., 1997; Sjostrom et al., 2001; Cruikshank et al., 2007; Gabernet et al., 2010; Itami & Kimura, 2012), there is an intricate balance between interneurons and pyramidal cells. Because perturbations in this balance are thought to underlie a number of neurological conditions (Rico & Marin, 2011; Yizhar et al., 2011; Gandal et al., 2012) it is essential we continue to study the processes that define this interaction such that we are better able to understand the functional, and consequently dysfunctional, maturation of the cortical network.
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Appendix

Figure 1.1 A

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