

illuminating the Mechanisms of Human Synaptic and Neural Network Dysfunction in Down Syndrome

by

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In Loving Memory of Arnie Brockman

Abstract

Human cortical circuit assembly is critical to neurodevelopment, but our understanding of this process in Down Syndrome (DS) remains incomplete. Previously, a model for studying DS neurodevelopment was created by xenotransplanting induced pluripotent stem cell (iPSC) derived neurons, made from DS individual fibroblasts, into the somatosensory cortex of an immunodeficient mouse. The transplanted graft cells were then imaged longitudinally using multiphoton microscopy. In this thesis, I develop an alternate graft type within the framework of the previous model, in order to study synaptic structural stability and spontaneous and oscillatory calcium activity in a model of the early DS brain. In addition to utilizing cells derived from an individual whose genetics have not been studied using this model-type, I have also demonstrated the ability to utilize this model for extended long-term imaging, with a pilot study of animals imaged at 8 months post-transplantation. In this study, 2-photon images were taken of axonal en passant boutons (EPB) and of somatic calcium signals over time. EPB turnover rate (TOR) and EPB size (strength) changes were quantified using EPBscore software. Calcium imaging was analyzed via MATLAB/Fiji scripts to determine event frequency, integral, and amplitude. I found no significant difference in EPB TOR, EPB strength, spontaneous somatic calcium activity, or oscillatory calcium activity between DS and isogenic control grafts. Therefore, differences in synaptic stability and somatic activity outcomes have been normalized across genotypes. Correlative analysis was used to determine whether normalization of SATB2+ and GFAP+ cell levels contributed to this rescue. GFAP+ cell numbers significantly correlate with synaptic stability outcomes, but no others. Overall, this study concludes that trisomy 21-related dysregulation in this graft is not sufficient to alter axonal bouton stability and neuronal activity compared to control neural networks of similar scale and cellular composition. I hypothesize that the lack of effect on synaptic structural stability and spontaneous and oscillatory activity may be correlated to an increased rate of astrogliogenesis in my grafts. As such, this graft model of DS neurodevelopment provides a foundation upon which to study the mechanisms behind early fetal changes in DS neurodevelopment, specifically related to the role of astrocytes in DS. Additionally, this model could be used as an excellent *in vivo* astrocyte research model, in WT or diseased cells, due to the high volume of developed astrocytes. Future research will include comparative transcriptomic analysis of this DS graft type to identify mechanisms of early DS neurodevelopmental dysfunction.

Statement of Originality

The work detailed in this thesis is the original work of Elizabeth Anne Brockman. All contributions of other individuals are acknowledged in the contributions section. Appropriate referencing is used when the work of other researchers is summarized, mentioned, or displayed in this thesis.

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Contributions

All two-photon imaging and analysis of two-photon imaging was performed by Elizabeth Anne Brockman (EB). All graphs were made by EB. All statistical analyses and were performed by EB. Perfusions and slicing of mouse brains were performed by EB and by the following lab members: Dr. Maria Tortora (MT), Dr. Shabana Khan (SK), Marcos Sintes (MS), and Victoria Menne (VM). Graft characterization was performed equally by EB, MT, and SK. Craniotomy surgeries were performed by Dr. Vincenzo De Paola (VDP) and EB. Craniotomy assisting was performed by EB, MS, MT, and SK. MATLAB scripts for analyzing calcium activity data were provided by our collaborator Dr. Samuel Barnes. *In vitro* cell growth and maintenance was performed by EB, MT, and SK.

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Chapter 1

Background

1.1 The Developing Human Neocortex and the Structure and Activity of its Neurons

1.1.1 The Neocortex: Anatomy & Development

The human neocortex is the outermost region of the brain and the largest portion of the cerebral cortex. It is composed of six distinct layers; layer I (the outermost layer), to layer VI (the deepest layer). They are grouped into three distinct functional strata; the supragranular layers (layers I, II, III), the granular layer (layer IV), and the infragranular layers (layers V, VI) [1]. These six layers are present in all four major cervical lobes (temporal, frontal, parietal, occipital) and within all functional subregions of the neocortex [2], with the exception of the mature adult motor cortex which has been shown to lack a distinguishable layer IV [3].

Each layer of the neocortex has a distinct function, therefore the composition and organization of each varies. Layer I, the molecular or plexiform layer, is a thin layer composed of axons and few somas. The nerves in this layer run parallel to the surface of the brain. Pyramidal neurons, cells of Martinotti, and other common neuronal subtypes form synapses in this layer [4], [5]. Layer II, otherwise known as the external granular layer, contains pyramidal and stellate cells

[4], [5]. Layer III, the external pyramidal layer, contains similar cell types to layer II, but the pyramidal neuron somas become progressively larger as they approach Layer IV [4], [5]. The neurons in Layers II and III send their axons into deeper regions of the brain and/or cortex, while receiving input from synaptic connections in Layer I. Layer IV, the internal granular layer, receives input from long, afferent neurons [4], and contains a dense, horizontal white matter tract [5]. This white matter tract is known as the external band of Baillarger [4], [5], and is the reason that Layer IV is known as the main “input receiver” layer, particularly when discussing the primary somatosensory cortex. The somatosensory cortex has a large internal granular layer due to the vast amounts of afferent signal intake and processing. Layer V, known as the ganglionic layer or the internal pyramidal layer, is referred to as the “output layer”, particularly in the motor cortex [6]. This layer contains large pyramidal cells and is responsible for many large efferent tracts that exit the cortex and proceed to the spinal cord, allowing for movement. The difference between Layer IV and V in the primary motor and primary somatosensory cortices illustrates a crucial detail; the size and number of cells present within each cortical lamina will differ based on the functional need of the larger cerebral region. Finally, Layer VI, also known as the multiform layer or fusiform layer, contains fusiform cells which largely project to the thalamus [4], [5]. Interneurons and glia are found throughout all cortical laminae.

Cortical lamination provides a road map of fetal neocortical development. At ~ 4 weeks post-fertilization (wpf), the nervous system has developed into three primary vesicles; the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain) [7]. By 6wpf, the prosencephalon has developed into two secondary vesicles; the telencephalon and the diencephalon [7]. The neocortex develops from the telencephalon, along with the corpus striatum. Within the telencephalon, neurogenesis occurs at the ventricular zone (VZ) and the newly formed immature neurons migrate from this zone near the lateral ventricle towards the developing neocortex. As more cells migrate, the neocortex expands and develops, using temporary scaffolding zones. The preplate, or the primordial plexiform layer (PPL), is created with the first cortical progenitors from the VZ (see Figure 1.1. As the cortical plate (CP) begins to develop, the PPL undergoes a splitting process [4], [6], [8], [7], [9]. The PPL splits into

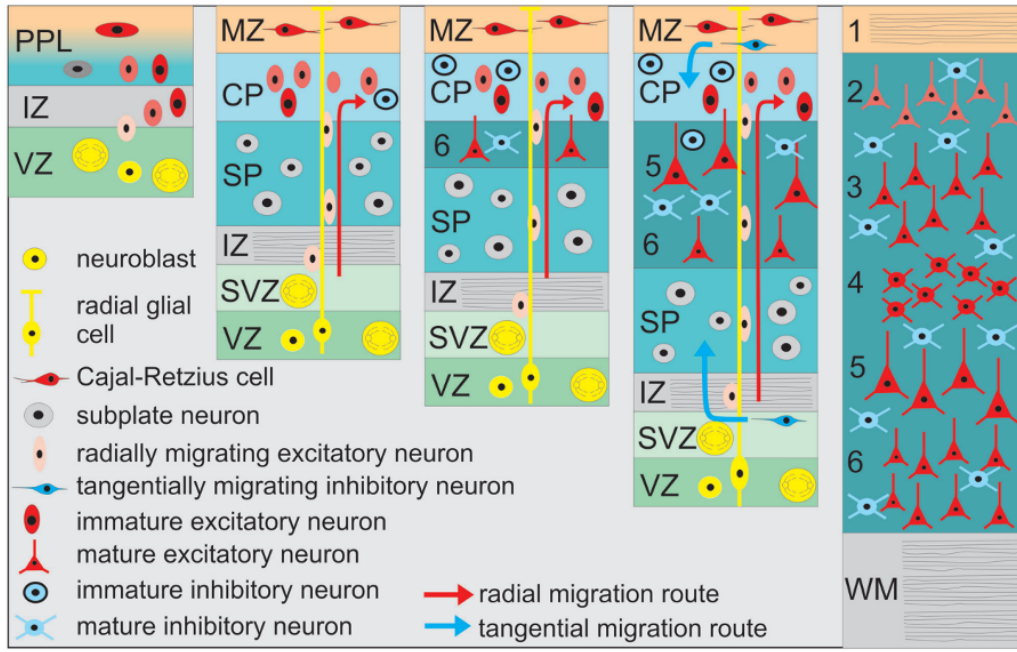


Figure 1.1: Cartoon-style diagram of the development of the cortex by reverse lamination. Figure taken from Luhmann *et al.* [9]. PPL = primordial plexiform layer, IZ = intermediate zone, VZ = ventricular zone, SVZ = subventricular zone, CP = cortical plate, MZ = superficial marginal zone, SP = subplate, WM = white matter. Numbered regions = cortical layers. For permissions, see Appendix Table 2.

two layers; a superficial layer called the marginal zone (MZ), and a layer called the subplate (SP) underneath the cortical plate (CP). At early stages of development, new migrating cells originate from the VZ, while at later stages, the sub-ventricular zone (SVZ) has developed and new migrating cells can originate from there. The level of migration from the VZ and SVZ will increase over time, using VZ radial glia as migratory aids. New cells formed in the VZ and SVZ migrate along the length of radial glia, which are connected to the VZ and superficial marginal zone (MZ) and therefore provide a direct avenue of migration to the CP following the radial migration pathway (see red arrow in Figure 1.1 [9]). Cells in the CP begin to mature and drop down, forming cortical layer VI. The intermediate zone (IZ) is a zone largely containing white matter tracts, which becomes the white matter (WM) regions of the cortex. Following development of Layer VI, cells migrating up to the CP then begin to form Layer V, and so on until all cortical layers have been formed [4], [6], [8], [7], [9].

Reverse cortical layer development has been confirmed experimentally in rodents using H-thymidine injection birth-dating studies in pregnant rats [6]. In humans, this process is ongoing

at 14wpf. By 28wpf, cortical folding has commenced, creating sulci and gyri. Cortical lamination as a generalized method of cortical development is conserved in many species, including humans, rodents, and non-human primates [10]. Figure 1.2B illustrates the reverse cortical lamination process in both humans and mice. Important differences include the presence of the outer and inner SVZ in humans, the persistent Cajal-Retzius cells that are maintained in Layer I, and an overall larger cortex with a higher number of neurons [10]. Furthermore, major changes in development occur at different intervals for humans, macaques, and rodents (see Figure 1.2C. For example, Layer V neurons are produced between ~ 10.5 -13 postconceptional weeks (pcw) in humans, $\sim$ embryonic day (E) 60-68 in macaque, and $\sim$ E13-14.5 in mice [10]. Additionally, the radial glia fibers used to form the scaffolding zone for reverse cortical development begin to degrade into a discontinuous scaffold at ~ 14 pcw in humans, $\sim$ E72 in macaque, and $\sim$ E15 in mice [10].

Inverted cortical layer development is highly genetically regulated. An important gene involved in the regulation of this process is *Reeler*, encoding the glycoprotein *reelin* in mice [11], [12], [13]. *Reeler* mutant homozygous recessive mice do not attain proper separation of the PPL into the MZ and SP, therefore reverse cortical lamination fails [11], [12]. The typical phenotype seen in homozygous recessive *Reeler* mice includes ataxia, tremor, and dystonia along with deficits in lamination of the cortex [11]. In humans, *Reeler* mutations are associated with the neurodevelopmental disorder lissencephaly [14].

The neurogenesis present during fetal neurodevelopment drops dramatically by birth. Neurogenesis remains only in two adult brain regions; the SVZ, which feeds new cells to the olfactory bulbs, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), which feeds new cells to the hippocampus for continuous memory circuit refinement [6], [15]. Significant reductions in neurogenesis during fetal development have meant the division of this process into two phases: the expansion phase and the neurogenic phase. During the expansion phase, neuronal progenitors derived from the VZ divide in a symmetric manner, meaning parent progenitor cell creates two equivalent daughter cells called radial glia. Radial glia are cells which maintain processes that stretch from the VZ to the pial surface [7]. Large numbers of radial glia are produced during this phase, providing the scaffold necessary for laminar development.

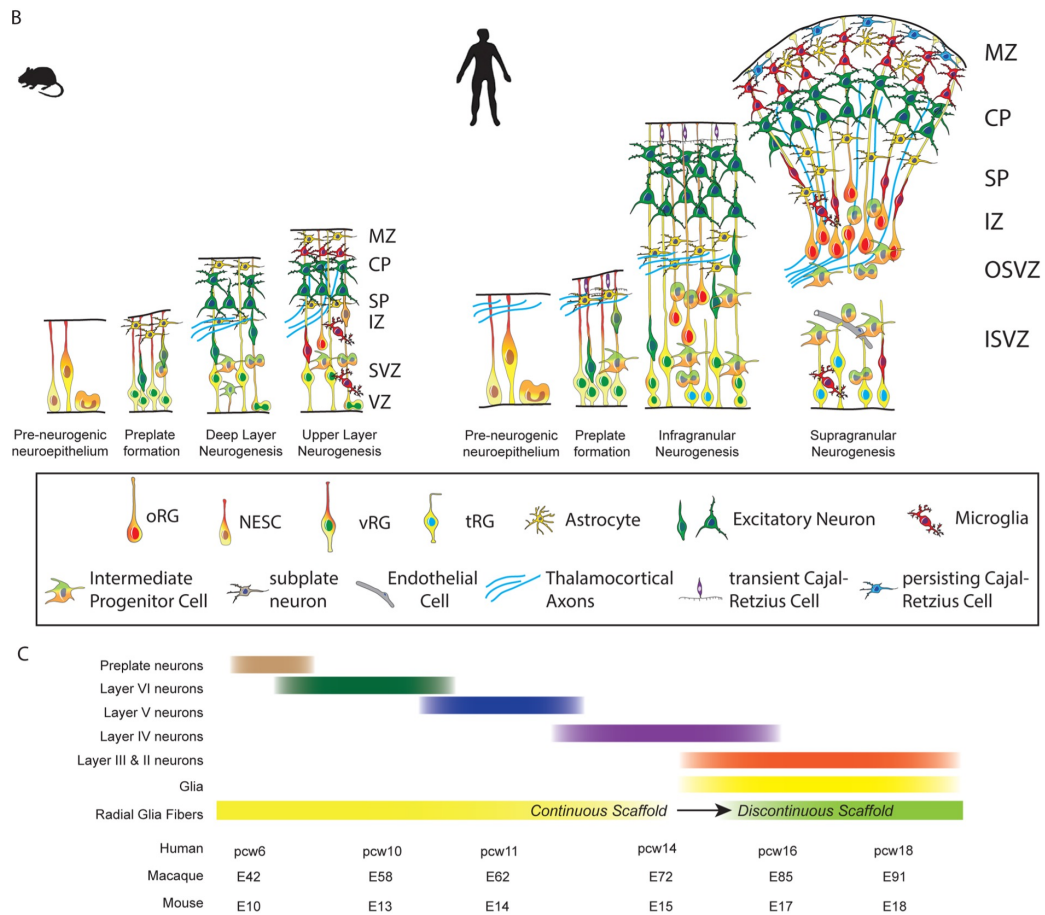


Figure 1.2: Illustration of cortical development via reverse cortical lamination in multiple species. The top section (B) of the illustration depicts the overall development of the cortex in mice (left) and in humans (right). The bottom section (C) of the illustration is a Gantt chart detailing when each cortical cell type develops in human, macaque, and mouse by embryonic (E) day and/or postconceptional week (pcw). Adapted from Cadwell *et al.* [10]. For permissions, see Appendix Table 2.

At later stages of embryogenesis, symmetric division becomes less common and is replaced by asymmetric division, thus beginning the neurogenic phase. In this phase, postmitotic neurons and/or intermediate progenitors bud off from the radial glia. Many of the cells produced from this point onwards are not mitotic, therefore the rate of mitosis quickly declines. This decline continues until the only neurogenic regions are the SVZ and the SGZ of the DG.

Developing cells differentiate into mature neurons and glia based on intrinsic and environmental factors. Common intrinsic factors that affect differentiation include Sonic hedgehog (Shh), Achaete-scute homolog 1 (Ascl1), and other morphogens and transcription factors [16], [17], [18]. Often these transcription factors are also used as the targets of antibodies for labelling of specific cortical cell subtypes, as variation in their expression can indicate developmental stage

or layer identity. Examples include the chromatin remodelling protein special AT-rich sequence binding protein 2 (SATB2), a target for immunohistochemical labelling of Layer II/III neurons [19], [20], and T-Box brain transcription factor 1 (TBR1), a target for labelling deep layer cortical neurons due to its role in specifying corticothalamic neuronal differentiation [21], [20]. Many groups have demonstrated experimentally that altering the environment of progenitor cells can have a significant effect in the differentiation of progenitor cells. For example, fibroblast growth factors (FGFs) added to media increase the likelihood of developing mature neurons from progenitors *in vitro* [6]. This has led to the development of many methodologies for culturing and differentiating cells to get specific mature cell culture profiles. This has been applied to *in vitro* primary cell cultures, as well as to induced pluripotent stem cell (iPSC) cultures and/or iPSC-derived cultures, both 2D and in 3D applications. Sanes, Reh, and Harris [6] describe how environment can alter final cell types from an early stage of vertebrate development, as researched by Richard Dorsky and colleagues [22]. When starting with a multipotent neural crest progenitor, one can end up with Schwann cells, sensory neurons, autonomic neurons, smooth muscle cells, or pigment cells depending on the presence of specific inductive factors like Wnt, Nrg-1, and TGF β 1-3, and the time at which they are introduced.

In summary, the human neocortex is a six-layered collection of pyramidal neurons, Martinotti cells, stellate cells, Purkinje cells, inhibitory interneurons, astrocytes, oligodendrocytes and other cell types that form synaptic connections and project to other parts of the brain and body. It is derived from the telencephalon and the subsequent early cortex formed by the PPL, IZ, and VZ. Novel progenitor cells follow the path of radial glia from the VZ to the CP in a process of reverse lamination. Deep layer neurons, such as TBR1+ cells, are developed first and upper layer neurons, such as SATB2+ cells, are developed last. The neurogenic process gradually declines throughout the course of embryogenesis. Development of the cortex can be studied *in vitro* in a 2D (flat dish culture) or 3D (eg: organoid) manner using primary culture cells or iPSCs. Since the type of mature cells produced depend on both intrinsic factors and environmental factors, small changes in expression of transcription factors and morphogens or changes to environmental factors have a significant effect on the cell types produced.

1.1.2 The Synapse: Function, Formation & Turnover

The synapse is the point of connection and information transfer between two neurons, which includes an approximately 20nm gap called the synaptic cleft. In most cases, information is passed from the pre-synaptic terminal bouton to the post-synaptic dendrite in the form of neurotransmitters. This form of synaptic communication is referred to as chemical neurotransmission or chemical signalling. Chemical signalling is the method used by most human neurons. However, some neurons such as retinal neurons use electrical signalling instead. This involves connected gap junctions, the pores of which are formed by hemichannels called connexons, used to pass information [7], [23], in which case no synaptic cleft is present. This thesis focuses on chemical signalling via the release of neurotransmitters.

Neurotransmitters are molecules secreted by fused vesicles at the pre-synaptic terminal that bind to receptors on the post-synapse. They act through fast or slow physiological actions and belong to one of three major categories: amino acids (including glutamate, γ -aminobutyrate (GABA), and glycine), amines/purines (including acetylcholine, dopamine, serotonin, and adrenaline), and peptides (including somatostatin and vasopressin) [7]. The most common excitatory neurotransmitter is glutamate, and the most common inhibitory neurotransmitters are GABA and glycine. All three of these type I amino acid neurotransmitters are generally fast-acting and together comprise 90% of neurotransmission in the CNS [7]. Neurotransmitters are collected into presynaptic vesicles prior to release. For large neurotransmitters like peptides, Golgi-derived large dense-core vesicles are used. These dense-core vesicles must be transported to the pre-synapse from the soma. For small neurotransmitters, small synaptic vesicles (SSVs; <50nm diameter) are used, which are recycled within the synaptic terminal bouton itself and are therefore more energy efficient. SSVs are maintained in the active zone of the presynaptic terminal bouton until they are triggered to fuse to the bouton membrane by calcium influx. This calcium influx is caused by the transient opening of calcium channels in the terminal bouton membrane, an event triggered by an AP [24]. As the vesicles fuse, the neurotransmitters are released into the synaptic cleft and travel to the post-synapse to bind to receptors on the post-synaptic membrane. The section of the post-synaptic dendrite nearest to

the membrane, which includes the neurotransmitter-binding receptors as well as structural proteins, is called the post-synaptic density (PSD) [24]. The neurotransmitter-binding receptors of the PSD can be metabotropic or ionotropic, and each neurotransmitter usually has multiple receptor types to which it can bind. For example, acetylcholine can bind to ionotropic nicotinic receptors and can also bind to metabotropic muscarinic receptors [7]. Briefly, ionotropic receptors are fast-acting receptors containing ion channels. When the receptor binds, the ion channel opens, creating an influx of ions that alter the membrane potential of the post-synaptic cell, resulting in a subsequent post-synaptic potential (PSP) which is either inhibitory (iPSP) or excitatory (ePSP) in nature. Examples of ionotropic receptors include NMDA receptors and AMPA receptors. In contrast, metabotropic receptors are slow-acting receptors which are coupled to a G-protein complex. When a ligand binds to a metabotropic receptor, it creates a conformational change in the associated G-protein complex on the intracellular side of the membrane. The activated G-protein complex will subsequently activate a second messenger. That second messenger alters other ion-channel receptors in the vicinity by phosphorylation, causing them to open and iPSPs or ePSPs to travel down the dendrite to the soma. Examples of metabotropic receptors include metabotropic glutamate receptors (mGluR), and metabotropic GABA (GABA_B) receptors [25]. The difference in speed of action between these two receptor types is based on how directly they interact with the ion channels that create PSPs in the post-synaptic cell. Speed of action has also been shown to affect the distribution of receptors in the PSD, as fast-acting ionotropic receptors tend to be positioned directly across the synaptic cleft from the central point of pre-synaptic vesicular fusion, while slower-acting metabotropic receptors are placed at the edges of the PSD [24].

Synapses are crucial to CNS function, which is why it is so important to understand their formation and function. Synaptogenesis is a complex process requiring the involvement of both the pre-synaptic cell and post-synaptic cell. Synapses can be classified by signalling method (eg: chemical, electrical), and by the parts of the cells forming the synapse (eg: axodendritic, axoaxonal, axosomatic). For this thesis, synaptogenesis is discussed in terms of the axodendritic synapse. As such, the presynapse is a terminal bouton (also known as axon terminal or synaptic bouton) and the post-synapse is a dendrite. For axons and dendrites to be formed, neurites

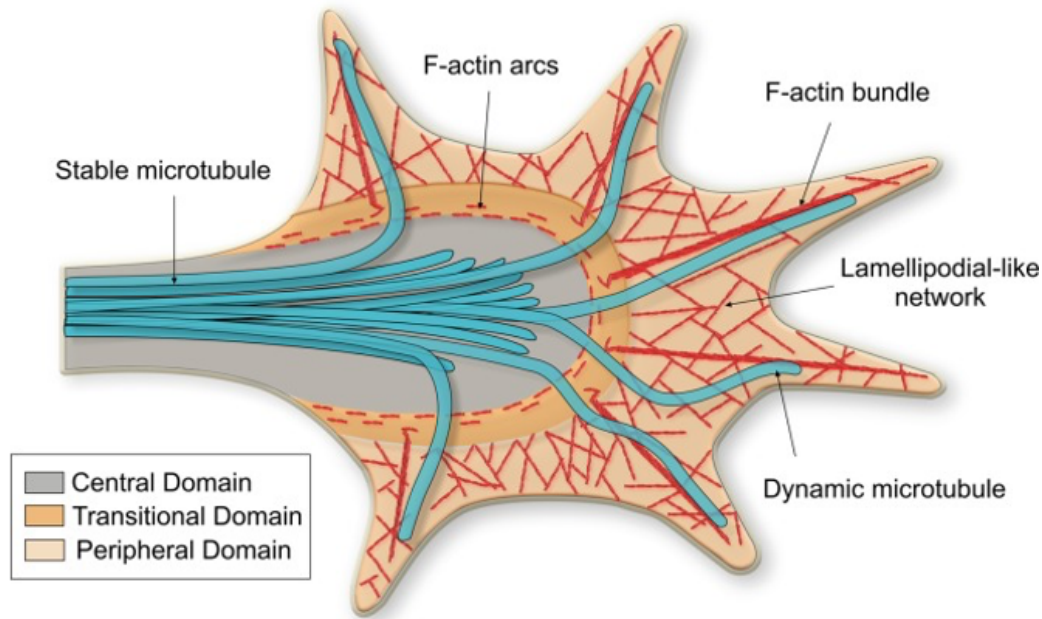


Figure 1.3: Illustration of the neuronal growth cone, including the three major domains (zones): the central zone, the transition zone, and the peripheral zone. The peripheral zone contains both filopodia and lamellipodia. The F-actin bundles making up the filopodia (long points extending outward) navigate the environment allowing for directional neuron growth. Image taken from Cammerata *et al.* [28]. For permissions, see Appendix Table 2.

must spread outwards from the soma, elongating from their growth cones. Growth cones are transient structures located on the terminal ends of developing neurites that allows them to grow, elongate, search their environment for potential connections, and develop synapses. They were first identified by Ramon y Cajal in 1890 [26] and their anatomy can be divided into three distinct regions: the central zone, the transition zone, and the peripheral zone (containing the lamellipodia and filopodia) [27], [28] (see Figure 1.3). At the transition zone, the microtubules of the neurite give way to F-actin bundles, which protrude outwards into the peripheral zone in a fan-like array to determine their best path. The F-actin bundles protruding outward at regular intervals make up the filopodia, while spaces between the filopodia in the peripheral zone are filled with F-actin networks that are shorter and criss-crossed. These actin network regions are the lamellipodia [27], [28].

As growth cones elongate and move, they react to chemotropic molecules in their environment to help them navigate [29]. These chemotropic molecules can be either attractant or repellant and can be introduced to the growth cone as molecules attached to the surface on which they are moving, or molecules that are diffuse within the environment [27], [29]. They bind to

growth cone receptors, creating subsequent downstream effects on the cytoskeletal proteins in the filopodia and lamellipodia [30]. Attractant chemotropic molecules will create growth of filopodia and lamellipodia around them, while repellants will cause the elimination of F-actin structures [30]. Growth cones continue to elongate in this directed manner until they find another cell with which to form a synapse. Examples of relevant chemotropic molecules include slits, netrins, and semaphorins [31], [32].

As neurites progress into their environment, stretching and pathfinding by means of chemotropic molecules, they find other neurons with which to form synaptic connections. The first step in synaptogenesis is making contact. Often, the first contact occurs between filopodia. The development of a functional synapse takes ~ 1 -2 hours after contact [8]. This process would take longer if not for the pre- and post-synaptic proteins that are localised in the neurites prior to contact [8].

Neuroligins and neuroligins are two classes of transmembrane proteins that are crucial for CNS synaptic development and organization. Neuroligins are found in the pre-synaptic membrane of developing neurites. They bind with partner neuroligins expressed on the developing post-synapse. Examples of neuroligins (and their neuroxin partners) in the CNS are: Netrin G-1 (Netrin GL-1), SynCam (SynCam), and Neuroligin (Neuroligin, Dystroglycan, LRRTM) [33] (referencing figure 48-18). Neuroligin/neuroligin binding provides structural stability for the developing synapse, while also promoting synapse development, differentiation, and organization [33]. The presence of a neuroligin on a neurite induces the formation of post-synaptic factors including ionotropic/metabotropic receptors and PSD properties on another neurite upon contact, and vice versa [21]. However, neuroligins mainly affect the pre-synapse, performing functions such as localization of docking proteins and pre-synaptic neurotransmitter-filled vesicles, while neuroligins mainly affect the post-synapse, performing functions including the recruitment of receptors to the post-synaptic membrane [23]. The physical connection created by neuroligin/neuroligin binding and their downstream effects are both crucial in synaptogenesis and synaptic maturation/maintenance.

Synaptogenesis is also affected by the presence of and interactions with glial cells. In the CNS,

synapses are encapsulated by astrocytes. Astrocytes contribute significantly to the development and maturation of synapses due to secretion of factors such as glypicans and thrombospondins [21]. Glypicans bind to receptor protein tyrosine phosphatases on the pre-synapse, creating the release of neuronal pentraxin 1, which in turn increases the expression of AMPA receptors on the post-synapse [21]. Thrombospondins bind to a subunit of calcium receptors on the post-synapse [21], promoting synaptic maturation by increasing vesicular glutamate transporter 2 (VGlut2) recruitment to the post-synaptic membrane [34]. Both glypicans and thrombospondins are secreted in high concentrations during early stages of development, but their secretion rate drops off after birth [34]. Their expression is replaced by factors such as Hevin, which increases post-birth and remains highly expressed during adulthood [34]. Astrocytic cholesterol secretion has also been shown to promote pre-synaptic development [33]. Additionally, microglia play a role in synaptogenesis [33]. They have been demonstrated to play a part in synapse elimination, an essential process in neural plasticity and brain development [33].

As the synapse forms, both the pre-synapse and the post-synapse thicken and enlarge. At the same time, important internal functions occur in each to form the regions necessary for synaptic function. These include the recruitment of receptors (eg: NMDARs, AMPARs) to the post-synapse, development of the PSD (both receptor presence and scaffolding proteins), development of the pre-synaptic active zone, and production/transport of pre-synaptic vesicles and neurotransmitters to the active zone. At later stages, the process of synaptic plasticity allows for strengthening and enlargement or weakening and elimination of synapses based on activity levels. As synaptogenesis is important in cortical development, so activity-dependent fluctuation is important in synapse turnover. To address this topic, two crucial neural circuit element terms must be introduced: bouton and spine. These terms describe structures of the excitatory synapse based on their location on the neuron. Boutons are axonal protrusions, therefore are usually indicative of the enlarged pre-synapse [8]. Spines are dendritic protrusions and are therefore indicative of the post-synapse [8]. Boutons and spines also have differing morphological characteristics. Boutons are classified into two sub-types: en-passant boutons and terminaux boutons [35], [36]. Terminaux boutons (TBs) are bulb-like boutons protruding from the axon, while en-passant boutons (EPBs) are varicosities along the axon. It is common

for an axon to have both TBs and EPBs, as such the TB/EPB ratio is indicative of variation in axon type [35], [36]. For example, L6 axons have a TB density of approximately 107 TBs/mm and an EPB density of approximately 143 EPBs/mm [36], [37],[38]. Along axons whose somas originate in layers 2, 3, and 5, TB density drops off dramatically (approximately 2 TBs/mm) while EPB density increases (approximately 160 EPBs/mm) [36].

In this thesis, I investigate the turnover of EPBs along iPSC-derived axons. EPBs were selected as they are more common than TBs across multiple cortical layers [36], which are represented in our graft. Note that the term graft is used regularly in this thesis and refers to a developed collection of injected human cells grown or growing inside the mouse brain (see [39]). Since the presence of an EPB implies the presence of a synapse, analyzing the number of EPBs and their rate of development/elimination provides information on neuronal synaptic plasticity and network morphology. The use of EPB survival and turnover analysis to study synaptic development and plasticity is not a new one. Not only was it used as a basis for the development of our current xenotransplantation *in vivo* model (see Real *et al.* [39] and/or the Methods section of this thesis for details [xenotransplantation refers to transplantation of matter from one species into another]), it was developed for general use in research of axon dynamics [40], and has been applied as an analytical method in research of aging [41], axonal injury [37], [38], and diseases, including Alzheimer's disease [35] and other tauopathies [42]. These findings suggest that EPB survival and turnover are critical factors in normal brain function which are affected by aging, injury, dementias, and other diseases. In their 2013 papers [37], [38], Canty *et al.* determined that TB-rich axons and EPB-rich axons act differently post-lesion, allowing for prediction of regenerative ability prior to injury based on the density/type of boutons. They found that TB-rich axons, which are largely cortical layer VI axons, have a higher rate of bouton loss on the proximal size of the injury and have a higher rate of post-lesion regeneration than EBP-rich axons [37], [38]. In addition to increase regenerative ability, TB-rich axons maintain axonal dynamic change (eg: elongation, turnover, & retraction) with age, a trait not shared by EPB-rich axons, which have significantly decreased bouton survival rates over time [41]. By demonstrating the relationship between fluorescent intensity of GFP-labelled EPBs and EPB size, Grillo *et al.* [41] were able to ascertain that persistent

(stable) EPBs demonstrate significant increases in the rate of bouton size change when aged, compared to young adult equivalents. Since bouton size is directly correlated to the strength of a synapse, Grillo *et al.* [41] provide rationale for studying the intensity of GFP-labelled bouton fluorescence; it is a correlative measurement of the strength of a synaptic connection. Fluctuations in fluorescent intensity over time, when properly normalized to the axon backbone intensity, provide information about the plasticity of presynaptic boutons and synapses.

In summary, synapses are the information passing centers in the nervous system. Each synapse is comprised of a pre-synapse, a post-synapse and a synaptic cleft. Signals can be chemical or electrical in nature, with chemical signals requiring neurotransmitter release from pre-synaptic vesicles and their binding at post-synaptic ionotropic or metabotropic receptors. Their binding creates iPSPs or ePSPs in the post-synaptic cell, allowing for signal transmission, which will be discussed in more detail in the next section. For this to occur, synaptogenesis must first take place. This process requires the growth cone of the neurite to follow the path laid out by chemotropic molecules in its environment until it reaches another neurite. Binding of neurexin/neuroligin pairs, along with signalling from the neurons and adjacent glial cells, allows the synapse to be formed by recruitment of receptors to the presynapse, forming the PSD, and vesicles/neurotransmitters to the pre-synapse, forming the active zone. The presence of synapses is indicated by TBs or EPBs in axons and by spines in dendrites (mainly in the case of excitatory transmission). Fluctuation in TB and EPB turnover rate, survival rate, and size (which can be measured indirectly using the fluorescent intensity of GFP normalized to the axon backbone) are all features which change based on age, cell type, disease status, and injury. Research has shown that deep layer neurons are often more TB-rich, with boutons more prone to loss after injury, and axons that are more regenerative, and that maintain dynamic fluctuations with age. EPB-rich axons, commonly found in upper layers (I, II, III) of the neocortex, tend to have more stable boutons, less regenerative capability after injury, and have a turnover rate more dramatically affected by the aging process. These EPB-rich axons are the focus of this PhD project and will be addressed again in the results section.

1.1.3 Spontaneous and Oscillatory Neuronal Network Activity

The chemical synapse contains a pre-synaptic terminal, a post-synapse, and the synaptic cleft. Upon pre-synaptic calcium influx, vesicles in the active zone fuse to the terminal membrane, releasing neurotransmitters into the synaptic cleft. Neurotransmitters bind to ionotropic or metabotropic receptors on the post-synaptic membrane. In the case of ionotropic receptors, ion channels are opened, most commonly creating a sodium influx into the post-synaptic dendrite. Metabotropic channels do the same, but with activation of the G-protein complex as an intermediary step. In both cases, an ion influx creates a change in the membrane potential of the post-synaptic dendrite. This change to the membrane potential can either be depolarizing, in that the membrane potential becomes more positively charged, or hyperpolarizing, in that the membrane potential becomes more negatively charged. These depolarizing and hyperpolarizing signals are referred to as iPSPs (hyperpolarizing) or ePSPs (depolarizing). iPSPs and ePSPs (collectively referred to as PSPs) are conducted to the soma via the post-synaptic dendrite for summation.

At the summation stage, all PSPs that reach the soma within a small window of time are summed, both temporally and spatially. The time frame for summation varies based on multiple factors, including conductance of the dendrite [43], [44]. In both temporal and spatial summation, all negatively and positively charged PSPs are added to determine net depolarization or hyperpolarization. If the summated final membrane potential is depolarized to threshold membrane potential levels, then an AP will be commenced at the axon hillock. Thresholds vary based on cell type and thresholds are lower at the axon initial segment (AIS) to assist in initial AP development. The threshold must be met, otherwise the signal will not be propagated. The majority of CNS neurons have a resting membrane potential of approximately -70mV and a threshold membrane potential of approximately -55mV. Importantly, the intensity of APs does not vary. As such, APs are referred to as “all-or-nothing” phenomena.

Threshold depolarization at the axon hillock triggers a cascade effect of opening and closing ion membrane channels. First, sodium channels open when the threshold potential is attained, causing a fast axonal sodium influx, accentuating the depolarization of the axon membrane. As

the membrane potential becomes more positively charged, the axon attempts to maintain homeostasis by opening potassium channels and beginning to close the sodium channels. At peak depolarization, the axon membrane will reach a charge of approximately +30-40mV, although this varies by cell type and firing rate [45]. The sodium channels close while the potassium channels open in larger numbers, creating an efflux of potassium from the axon. This efflux reverses the depolarization, bringing the membrane potential back towards resting potential. The potassium channels close as resting membrane potential approaches and is subsequently reached, at which point overcorrection occurs, resulting in a short period of hyperpolarization. The membrane regulates itself to reach resting membrane potential using leak channels. AP depolarization begins near the axon hillock and continues at the Nodes of Ranvier along the axon until the depolarization reaches the axon terminal and triggers the opening of voltage-gated calcium channels. Vesicles then fuse to the pre-synaptic terminal for neurotransmitter release and the process starts again in the next cell. Importantly, this depolarization is sped up by myelination of the axons, which is provided by oligodendrocytes in the CNS and Schwann cells in the PNS. The myelin sheaths of these glial cells provide insulation to sections of the axon, with Nodes of Ranvier between each sheath to propel the signal. This method of propagation, called saltatory conduction, avoids the loss of charge and increases speed by requiring depolarization and repolarization via channel opening only at the Nodes of Ranvier. The most highly myelinated neurons are the fastest, reaching speeds up to 150m/s [23].

In most cases, chemical signals originate from a stimulus. This creates a chain reaction of signals, allowing information to reach the brain and for a response (eg: movement) to be relayed to the appropriate regions of the body (eg: muscles). However, the human brain is also capable of spontaneous activity, a phenomenon which regulates the early development of neural networks. In the neocortex, this network development is particularly important, as network formation and communication are crucial to higher cognitive functions. Spontaneous activity can take many forms and is characterized by the number of neurons involved, the frequency and amplitude of the signal, and the type of synapses and receptors required. Spontaneous network-level activity, when synchronized, may be classified as oscillatory. Neural oscillations are synchronous, patterned activity events that can occur at frequencies between 0.05 and

500+Hz [46], [47]. Frequency is a defining factor of oscillation sub-type identification. The sub-types of neural oscillations, as defined by frequency range, are: ultra fast ($\sim 200\text{-}500\text{+Hz}$), fast ($\sim 70\text{-}200\text{Hz}$), gamma ($\sim 30\text{-}70\text{Hz}$), beta ($\sim 30\text{-}13\text{Hz}$), alpha ($\sim 8\text{-}13\text{Hz}$), theta ($\sim 3.5\text{-}8\text{Hz}$), delta ($\sim 1\text{-}3.5\text{Hz}$), and slow delta ($\sim 0.1\text{-}1\text{Hz}$) [48], [49], [50], [46]. The term “slow oscillations” is often used as a general descriptor for all oscillations between 0.1Hz and 4Hz, therefore encompassing both slow delta and delta frequencies [51]. Some publications describe up to four categories of slow oscillations [46]. Oscillations can be found across large brain regions or localized within a confined neural network. Oscillations have been found in various CNS regions in humans [46], [52], [53], [50], non-human primates [54], [55], rodents [46], [51], [56], [57], ferrets [58], [59], and zebrafish [60]. A well-studied example of this phenomenon is the process by which circadian rhythms are regulated [47].

Neural oscillations have been shown to be essential for integration of signals and networks, allowing a more comprehensive level of information sharing throughout the brain [53], [61]. A pertinent quote by Rodolfo Llinàs, cited by Olaf Sporns in his 2016 book [62], discusses how sensory inputs provide information by affecting the already present oscillatory activity in the brain, rather than being the lone active region. This requires spontaneous oscillatory activity to be occurring intrinsically and without stimulation in the brain, a process which also promotes the development of complex and precise functional neuronal networks [62]. Sporns [62] concludes, based on literature, that spontaneous oscillatory activity comes both from each neurons’ intrinsic ability for spontaneous activity, and from the perpetuation of other spontaneous signals throughout connected networks. This has since been corroborated by other researchers [63], [64]. Spontaneous oscillatory networks can be overlapping, therefore the spontaneous firing of a few neurons can create multi-network spontaneous firing patterns in response [64]. This makes isolating the specific functions of single-cell and network-level spontaneous firing difficult. Nonetheless, some have been identified. Elucidated functions of neural oscillations include circadian rhythms [47], motor control and timing of movements (oscillations in the inferior olive; [61]), and olfactory information encoding [61]. CNS wiring is another crucial function of neural oscillations which is particularly relevant to this thesis, as it ties neural oscillation to early neural development. Multiple researchers have determined that activity oscillations

and their patterns directly effect the development of synapses and neural circuits [65], [66], [67] (see Figure 1.4). CNS activity-dependent patterning and wiring has been studied extensively in the retina and ocular nervous system [65], [67]. These data have shown that calcium activity creates long-term potentiation and depression, changing the connections and altering the strength of synaptic boutons and dendritic spines [65], [68]. Calcium activity is also linked to the navigation of growth cones [68]. Sur and Rubenstein [65] depict the need for oscillatory patterned activity in the developing brain, while tying these functions directly to bouton and spine strength and turnover. Figure 1.4 summarizes how oscillations relate to cortical development in the rodent brain. Importantly, burst activity is present in the somatosensory cortex *in vivo* at the first postnatal week of development [69]. The mouse brain microenvironment used to grow our iPSCs has developed to adulthood, thus patterned somatosensory perception is present and mature, refined oscillation will be occurring in all regions, thus affecting our grafted cells.

Neural oscillations are hypothesized to be linked to human consciousness [70], specifically low amplitude (12-70Hz) oscillations [53]. Spontaneous oscillatory activity has also been tied to the function of the default brain network [71] and to fMRI data on resting state networks [72]. When evaluating spontaneous activity at the network level, periods of time can be classified into states of activity, known as ‘UP’ or ‘DOWN’ states [63], [73]. ‘UP’ states are spontaneous firing active periods, the dynamics of which are comparable to stimulation-response activity, while ‘DOWN’ states are periods of network-level silence [57], [63]. Luczak and colleagues [57] studied these UP and DOWN states in the somatosensory cortex of anaesthetized and unanaesthetized rats using implanted electrodes. Their findings indicate that at the onset of UP states, the signal travels outward within the network in a pattern of sequential activation that is present regardless of the presence of anaesthetics. Network sequential spread of activity is also independent of wave direction. When one network is being stimulated by another spontaneous network, the stimulated network maintains a consistent direction of signalling, regardless of the wave direction of the stimulating network. Additionally, UP state activity decays over time from point of onset [57].

Importantly, Luczak *et al.* [57] did find significant differences between anaesthetized and

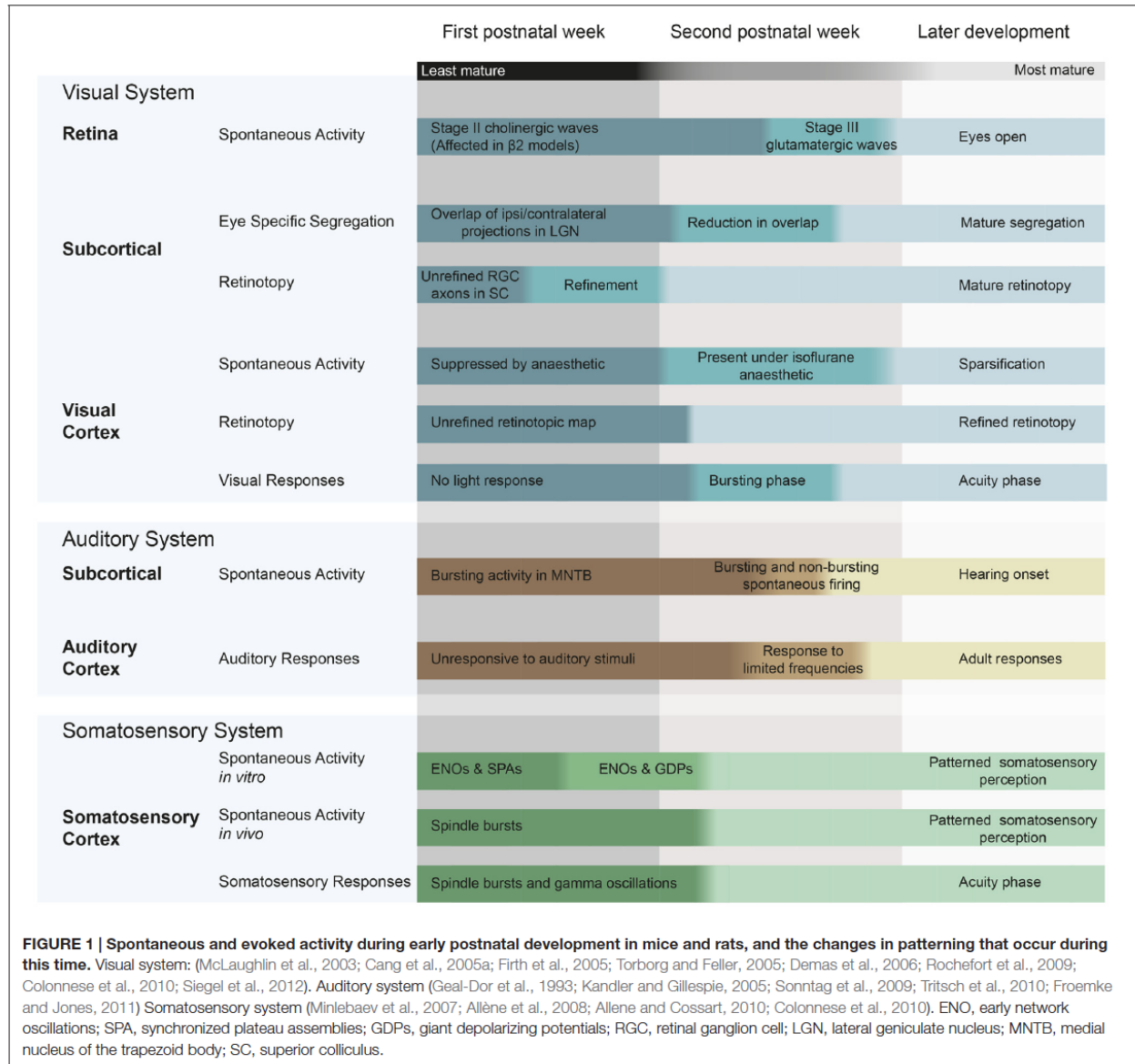


Figure 1.4: Neuronal oscillations in the developing auditory, somatosensory, and visual cortices of mice and rats. This figure is taken from Leighton and Lohmann's 2016 paper published in *Frontiers Neural Circuits* [69]. The original figure title has been kept to maintain the source lists for these data, as well as the abbreviation lists. For permissions, see Appendix Table 2.

unanaesthetized rat spontaneous activity. Namely that the sequential firing of the network was significantly faster in unanaesthetized rats, suggesting that the presence of anaesthetics increases the latency from UP state onset in further removed neurons. They were also not able to identify clear UP and DOWN states in unanaesthetized animals that were awake and alert, but were able to see them clearly in unanaesthetized animals that were asleep or groggy, as well as in anaesthetized animals. As my experiments perform activity measurements while the mice are under light (0.5 - 0.7%) anaesthesia, these findings are likely not an exact representation of activity in awake individuals. However, the similarities highlighted by Luczak *et al.* [57] in UP state onset and spontaneous sequential spread between anaesthetized and unanaesthetized rodents indicate that data from lightly anaesthetized mice would still prove useful, perhaps being indicative of sleeping or groggy states.

Spontaneous oscillatory activity in the brain is crucial for human brain function, both at baseline (eg: DBN function) [74] and when stimulated [61]. This is not only demonstrated by the aforementioned work (eg: circadian rhythms, functional integration, development) but also by the research that demonstrates the link between spontaneous oscillatory activity and neurological disease. Many neurological diseases are associated with oscillatory activity malfunctions, including epilepsy [75], schizophrenia [52], bipolar disorder [49], and Down Syndrome (DS) [39]. Since higher brain function relies on oscillations, it is logical that perturbations to oscillatory function create disordered symptoms and affect cognitive development. To understand the relationship between neuropsychiatric disorders and oscillatory activity, these disorders must be studied from early stages of development when the disordered activity first emerges. This thesis adds to the understanding of how activity and oscillations are affected in DS using an *in vivo* xenotransplantation model of early neuronal development.

1.2 The Use of Induced Pluripotent Stem Cells in Early Development of Neuropsychiatric Disorders

1.2.1 Induced Pluripotent Stem Cells: A Brief History

Induced pluripotent stem cells (iPSCs) are stem cells created by reprogramming a mature cell type, usually fibroblasts, into their pluripotent predecessors. This term was first published in 2006, when Takahashi and Yamanaka [76] identified a quartet of transcription factors able to create iPSCs from adult mouse fibroblasts: Sox2, Oct3/4, c-Myc, and Klf-4. They confirmed that the iPSCs were pluripotent, but germline competency was not achieved. Maherali *et al.* [77] studied these newly developed iPSCs using the Takahashi and Yamanaka [76] protocol and were able to determine that epigenetic factors are also reprogrammed using this method. That same year, Yamanaka and colleagues demonstrated switching the selection factor from Fbx15 to NANOG solved the issue of germline competency [78]. At the same time, Yu and colleagues [79] had utilized Takahashi and Yamanaka's work to attempt iPSC development in human lines. Their experiments proved that c-Myc is not an appropriate factor for human cell reprogramming, as it can lead to cell death, and therefore they developed a different set of transcription factors for reprogramming human fibroblasts into iPSCs: Oct4, Sox2, Nanog, and Lin28 [79], [80]. This finding allowed for the creation of human-derived iPSC lines, a revolutionary tool for researching disease. The following sections discuss how iPSCs have been used to produce cortical neurons and the use of these iPSC-derived neurons as models of neurodevelopmental disorders.

1.2.2 Developing Cortical Neurons from Human iPSCs

The development of patient-derived human iPSCs created a novel avenue for studying cortical development and neuronal function in disease without invasive biopsies. One of the first groups to develop cortical neurons from iPSCs was Marchetto *et al.* [81], who produced neurons from Rett Syndrome patient iPSCs. They demonstrated that embryoid bodies can be produced

from iPSCs via the introduction of both FGF2 and Noggin. Replating of these embryoid bodies permitted maturation into neural rosettes and subsequent treatment with additional FGF2 combined with rosette dissociation gave rise to neural progenitors [81]. They confirmed that control neural progenitors go through normal X-inactivation and could proliferate normally. Research on neurological and neuropsychiatric disorders using iPSC-derived neurons expanded [82],[83]. In 2012, Shi *et al.* [84] published work on the dependence on retinoid signals in differentiation of human iPSCs to cortical cells, also publishing their differentiation methodology in a detailed protocols paper [85]. Their methodology is precise and detailed, producing cortical cultures containing both deep layer (CTIP2+, TBR1+) and upper layer (SATB2+) cortical neurons that follow typical human developmental progression. Akin to Marchetto *et al.* [81], the protocol by Shi *et al.* [85] also uses FGF2 and Noggin, but requires rigid timing based on the morphological appearance of the developing cell cultures, a factor that introduces increased experimenter variability. For example, a critical step in this protocol is to ensure a uniform neuroepithelial cell layer before proceeding [85]. Instructions which are not based on a specific timing or a quantitative measurement create variability across different experimenters. This protocol has since been used as the basis for neural differentiation in many papers [86], [87], [88], [89], including the publication upon which this project was based, performed by past members of my research group [39]. Since the Shi *et al.* [85] protocol is effective at producing excitatory cortical neurons, Real *et al.* [39] used a variation of this protocol to develop their xenotransplantation model. The cultures developed were mature and formed connections but did not have inhibitory interneurons. This benefited Real *et al.* [39] as it decreased the number of variables in the grafts, providing a simpler cell culture with which to test their new model. Following up on the 2018 paper and the establishment of the xenotransplantation graft model, my project was developed with the goal of expanding on their findings, specifically that trisomy 21 (Ts21) grafts demonstrated increased synaptic structural stability and decreased oscillatory activity compared to controls. One way in which I hoped to expand on their work was by increasing the number of cell types within the graft, providing a culture more akin to the developing brain. Specifically, I hoped to create grafts that contained inhibitory interneurons which were previously lacking, thereby creating a model where decreased oscillatory activity

could be evaluated more accurately. To do this, an alternative protocol was required. We collaborated with the group of Professor Dean Nizetic (Queen Mary University London), who are experts in neural stem cell differentiation and DS [90], [91], [92]. They had developed multiple DS iPSC lines derived from DS fibroblasts and were using a variation on a method published by Gibco (MAN008031, “Induction of Neural Stem Cells from Human Pluripotent Stem Cells Using Gibco PSC Neural Induction Medium”). The MAN008031 protocol has been used to develop neuronal lines from iPSCs for the study of various diseases including Huntington’s [93], autism [94], and other neurodevelopmental disorders [95]. After the development of neural progenitors, the Nizetic group use brain physiology media containing brain-derived neurotrophic factor (BDNF), glial-cell derived neurotrophic factor (GDNF), and other supplements to drive neuronal maturation. This contrasts the neuronal differentiation step of the Real *et al.* [39] protocol, which utilizes an N2B27 medium.

While these two protocols are most relevant for the work of this thesis, they are not the only protocols being used for the development of neurons/glia from iPSCs. Yu *et al.* [80] developed neuronal progenitors, maturing them using a media that includes 20ng/mL of Wnt3a to be added for 3 weeks *in vitro*. This protocol was also used the following year by Mertens *et al.* [96] to develop neuronal cultures from iPSCs for studying bipolar disorder. The mature cells produced using this protocol are mainly excitatory neurons, but unlike the Nizetic and Shi *et al.* [85] protocols, GABAergic neurons are also present (<10% of cells) [96]. A variation of this protocol has also been used to successfully develop astrocytic cultures from iPSCs [97]. Some available protocols create cultures with a broader spectrum of included cell types. One such protocol produced cultures containing glutamatergic excitatory neurons, dopaminergic neurons, GABAergic neurons, and astrocytes [98], [99]. Unfortunately, licensing constraints on commercially available products restrict our ability to comparatively analyze available differentiation protocols.

In summary, iPSCs can be differentiated into neuronal progenitors, neurons, and glia using a wide range of protocols. It can be difficult to compare these protocols due to the nature of the media/supplements involved and their licensing. As such, many similar protocols have been developed. Small variations in the cell types produced have an immense effect on the

cell phenotype and the disease effects that are visible. When studying neurodevelopmental disorders, any change to the cell types present leads to altered development and can change the expected disease phenotypes, as we will see when comparing the Nizetic protocol and Shi *et al.* [85] protocol. This thesis utilizes a protocol created by the Nizetic lab, based on the MAN008031 protocol by Gibco (referred to as the “Nizetic protocol” for this thesis). Comparisons will be regularly made between the Nizetic protocol and the Shi *et al.* [85] protocol which was used in the Real *et al.* [39] paper in developing the *in vivo* xenotransplantation model.

1.2.3 Patient-derived iPSCs as Models of Neurological and Neurodevelopmental Disorders

Fibroblast-derived iPSCs provide donor-specificity and a human genetic background that is ideal for neurodevelopmental disease research. They can be used to create lines of various genetically matched cell types, thus replacing invasive nervous system biopsies with simple skin-punch biopsy procedures. These cultures can be expanded, stained, and frozen for many years. For these reasons, iPSC-derived neurons have become increasingly common as models for neurodevelopmental disorders, particularly for complex multifactorial disorders which are difficult to model and may be complicated by comorbidities (eg: DS, autism) [100].

As stated in Section 1.1.2a, Okita, Ichisaka, and Yamanaka [80] developed the first iPSCs from human fibroblasts. With the successful development of human iPSCs from fibroblasts and the confirmation of healthy karyotype maintenance [80], researchers began to investigate whether this method could be used to study disease. The first paper confirming that iPSC disease lines can be created from human fibroblasts was published by Park *et al.* [101]. They focused on neurological diseases, producing cell lines for 10 different diseases including Down Syndrome (see Table 1.1). In all cases, the iPSC lines had the same genetics as their parent cells and maintained karyotypes that were otherwise healthy (aside from mutations due to the disease in question) [101].

Other publications quickly followed that of Park *et al.* [101], producing human iPSC lines for

Table 1.1: Table listing the diseases made into iPS cell lines from fibroblasts or bone marrow cells by Park *et al.* in their 2008 publication [101]. Molecular defects for each disease, along with the age and sex of the donor individual are listed in the table. For permissions, see Appendix Table 2.

Name	Disease	Molecular Defect	Donor Cell	Age	Sex
ADA	ADA-SCID	GGG > AGG, exon 7 and Del(GAAGA) exon 10, <i>ADA</i> gene	Fibroblast	3 M	Male
GD	Gaucher disease type III	AAC > AGC, exon 9, G-insertion, nucleotide 84 of cDNA, <i>GBA</i> gene	Fibroblast	20 Y	Male
DMD	Duchenne muscular dystrophy	Deletion of exon 45–52, <i>dystrophin</i> gene	Fibroblast	6 Y	Male
BMD	Becker muscular dystrophy	Unidentified mutation in <i>dystrophin</i>	Fibroblast	38 Y	Male
DS1, DS2	Down syndrome	Trisomy 21	Fibroblast	1 Y, 1 M	Male
PD	Parkinson disease	Multifactorial	Fibroblast	57 Y	Male
JDM	Juvenile diabetes mellitus	Multifactorial	Fibroblast	42 Y	Female
SBDS	Swachman-Bodian-Diamond syndrome	IV2 + 2T > C and IV3 – 1G > A, <i>SBDS</i> gene	Bone marrow mesenchymal cells	4 M	Male
HD	Huntington disease	72 CAG repeats, <i>huntingtin</i> gene	Fibroblast	20 Y	Female
LNSc	Lesch-Nyhan syndrome (carrier)	Heterozygosity of <i>HPRT1</i>	Fibroblast	34 Y	Female

neurological diseases including familial dysautonomia [102], Fragile X Syndrome [103], Timothy Syndrome [104], [82], microcephaly [105], Phelan-McDermid Syndrome [106], Alzheimer’s Disease [107], [108], [109], and Rett Syndrome [110], [111], [81]. In these experiments, patient-derived iPSCs were differentiated into neurons and/or glia with the goal of improved understanding of each disease in question. Excitatory cortical neurons [81], [82], [107], neuronal progenitors [82], inhibitory neurons [82], and dopaminergic neurons [82] have all been produced in this manner using a range of differentiation protocols. In most cases, differentiation led to mixed cultures containing two or more of these nervous system cell types [82].

DS was one of the first neurological conditions with a developed patient-derived iPSC line [101]. DS iPSCs have been used to study many aspects of DS, including the role of astrocytes in the DS brain [112], [113], the effects of Ts21 on synaptic activity [114], and the role of APP and BACE2 in DS Alzheimer’s-like phenotypes [115], [90], [85]. Patient-derived iPSC research on related disorders such as autism spectrum disorder or Alzheimer’s disease have also aided in understanding of DS [116], [117], [118], [119]. Many donor-specific DS iPSC lines have been created [120], and stored as stock lines for research use. An additional benefit to the use of iPSC-derived neurons is the use of paired isogenic controls. These controls are genetically matched, the only difference in DS control lines being the absence of trisomy [121]. The production of isogenic control lines originated with revertant disomic lines, the products of mutations from

passaging/expanding the cell line [122], [39]. In their revertant disomic cultures, Li *et al.* [122] found unwanted mutations in ACTA2, PTRF, and RPL39L. Thus, an alternative was found, namely the use of disomic paired cells from mosaic Ts21 individuals. In other neurological conditions, CRISPR-Cas9 would be used to generate the isogenic control lines, but this process is not feasible for adding or deleting an entire chromosome. While CRISPR-Cas9 is not used in this instance, our mosaic Ts21 disomic lines provide an equivalently valid control.

Despite the innumerable benefits of patient-derived iPSCs for DS research, there are some drawbacks to using this model type. The *in vitro* environment exploited in many iPSC studies lacks vascularization, immune cells, aspects of cell-to-cell developmental interaction, and other microenvironmental supports. Exploration of cortical circuit dynamics and long-term cortical development is enhanced by the presence of the aforementioned *in vivo* microenvironments. This plays a part in why mouse and *Drosophila* models are used to study DS, providing crucial information on synapse formation, including the role of cell adhesion molecules in synapse development [123], [124]. Models such as 3D organoids and lab-on-a-chip structures further emphasize certain limitations of *in vitro* iPSCs. For example, organoids suffer from internal necrosis due to lack of vascularization [125]. Other limitations in both 2D and 3D iPSC models include the lack of proper modelling of metabolism and metabolic stress [126]. The xenotransplantation model used in this thesis addresses many of these concerns by utilizing the mouse microenvironment to improve iPSC growth and development.

In the next section, I will discuss DS as a neurodevelopmental condition. I will break down the genotype, phenotype, and development of DS, with the goal of outlining current areas of research need, current techniques and tools used in DS research, and how models like patient-derived iPSCs can be utilized when studying early DS synaptic development.

1.3 Down Syndrome: A Neurodevelopmental Condition

1.3.1 Down Syndrome: History & Epidemiology

DS is a genetic neurodevelopmental condition caused by a trisomy of chromosome 21 (Ts21), affecting ~ 1 in 700 individuals worldwide [127], [128], [129]. The DS phenotype varies widely. All individuals with DS present with a degree of cognitive and learning disability [130]. IQ levels in DS individuals range from 30-70 with the average being below 50 [131]. Other common characteristics of Ts21 include abnormalities in craniofacial features, memory deficits, hypotonia, and cardiac anomalies [132], [133]. Congenital heart defects are present in approximately 40% of DS individuals [134]. The presence and severity of each symptom can be affected by whether the trisomy is complete, partial, or mosaic [135], [136]. Additionally, individuals with DS develop an early-onset Alzheimer's-like dementia in adulthood, usually presenting in their 30's or 40's [137], [138], [139].

DS has been present in human society long before it was named, characterized, or understood. Evidence of DS has been found as far back at the ninth century, as skulls with similar features and dimensional characteristics have been discovered from that period [140]. However, past doctors and researchers lumped DS into a large category of mental illnesses, all of which were poorly understood. The first description of DS as an independent syndrome was devised in the mid-1860's by John Langdon Down [141], [142]. It is after Down that the syndrome takes its name. Despite Down's work, the genetic basis of DS was not understood. This stemmed from the lack of technological and medical advancement necessary to elucidate the genetic properties of DS. The necessary discovery that allowed DS research to be propelled forward was the 1956 discovery that the human genome contained 46 chromosomes, separated into pairs [143]. This provided Lejeune *et al.* [127] with the information necessary to publish the first paper confirming that DS is caused by an extra copy of chromosome 21. This led to the coining of the term "Trisomy 21". Lejeune's work has since been built and improved upon to characterize DS in further detail, including much about how DS affects the brain. This research led to an increase in medical developments, and improved treatment options. As such,

the average life expectancy of DS individuals has risen dramatically. In 1929, the average life expectancy for someone with DS in Western Europe (data largely from the UK) was only 9 years [140], [144]. Thanks to continued advancements in medicine, the median lifespan of individuals with DS has increased. In 2011, the median UK lifespan expectancy for individuals with DS was calculated to be 58 years [145].

The effects of Ts21 vary across brain regions and change throughout development [146]. Studying the molecular mechanisms of brain development in DS is a complex undertaking, as the syndrome is affected not only by protein-coding gene overexpression and downregulation, but also by alterations to non-coding factor expression [147]. For example, the five non-protein coding micro-RNAs from the long arm of chromosome 21 have been found to be more highly expressed in DS than gene dosage effects would predict [148]. They have also been shown to cumulatively affect the expression of 3600+ protein coding genes [148]. To understand how DS research has expanded, it is crucial to understand the genetic basis of the syndrome.

1.3.2 The Genetic Basis of Down Syndrome

Trisomies are generally not inherited [149], although some rare cases do exist [150]. Instead, they are caused by malfunctions in the mitotic or meiotic cell division processes. Trisomies can occur with any chromosome [151], [152], [153], [154], [155], but only three autosomal trisomies are viable [156]. These are DS (Trisomy 21), Edward Syndrome (Trisomy 18), and Patau Syndrome (Trisomy 13). Only DS is viable into adulthood. The few patients that are born with Edward or Patau syndrome [157] will rarely survive longer than one year [158].

The mutation mechanism responsible for Ts21 in 90-95% of DS individuals is called non-disjunction [159]. Non-disjunction is a malfunction of cell division, usually occurring in meiotic anaphase when the paired homologues (Meiosis I) or the sister chromatids (Meiosis II) are aligned and subsequently pulled apart towards the centrosomes. In this situation, non-disjunction presents as uneven division of the homologues or chromatids by the spindle fibres, producing one daughter cell with an extra chromosomal copy of the affected homologue or chromatid (see Figure 1.5). The other daughter cell will be missing chromosomal material,

resulting in an unviable monosomy. Ts21 mutations occur primarily during parental gamete meiosis, with only approximately 5% of cases resulting from mitotic mutations [160]. In approximately 90% of cases, malfunctions to maternal gametes (oocytes) are the cause of trisomic genotypes in offspring [161], [162]. As maternal age increases, so does the likelihood of oocytic mutations and the production of oocytes with an extra chromatid [162]. Fertilization of an oocyte with an extra chromatid from a meiotic non-disjunction or translocation mutation results in trisomy. The outcome of meiotic nondisjunction is demonstrated in Figure 1.5. This nondisjunction can occur either in meiosis I or meiosis II, which will result in trisomy in 50% or 25% of the daughter cells respectively upon fertilization. When the trisomy is derived from maternal oogenesis, 75% of cases are meiosis I mutations, while meiosis II mutations account for the other 25% [160].

The main alternative mutation method which creates trisomy is Robertsonian Translocation, occurring in less than 10% of cases [164], [165]. Robertsonian Translocations are characterized by the fusion of the large ends of two separate acrocentric chromosomes (see Figure X). When a chromosome is acrocentric, its centromeres are far from the centre, thus presenting unevenly with a short arm and a long arm [166]. In humans, chromosomes 13, 14, 15, 21, 22, and Y are acrocentric, with the remainder being metacentric or submetacentric [167]. The hallmark of translocation is the fusion of the chromosomes as a consequence of the chromosomes exchanging arms. The long arm from chromosome A is exchanged with the short arm of chromosome B and the new pairs fuse together. This fragment of chromosome created by the fusion of the two small ends is usually lost. These lost ends contain nucleolus organizing regions and their loss does not affect translocation carriers [168]. The loss of the short arms does not appear to change the function of the translocated chromosomes in carriers. The fusion of the two long arms creates a larger metacentric chromosome [166] (see Figure 1.6) Despite carrying the genetic material of two chromosomes, translocated metacentric chromosomes are treated as one chromosome by the cell during mitosis/meiosis, thus creating an avenue for the development of trisomy.

Robertsonian Translocations can be carried by the individual in which they develop without any physiological effects [170]. However, carriers of Robertsonian Translocation may pass on a

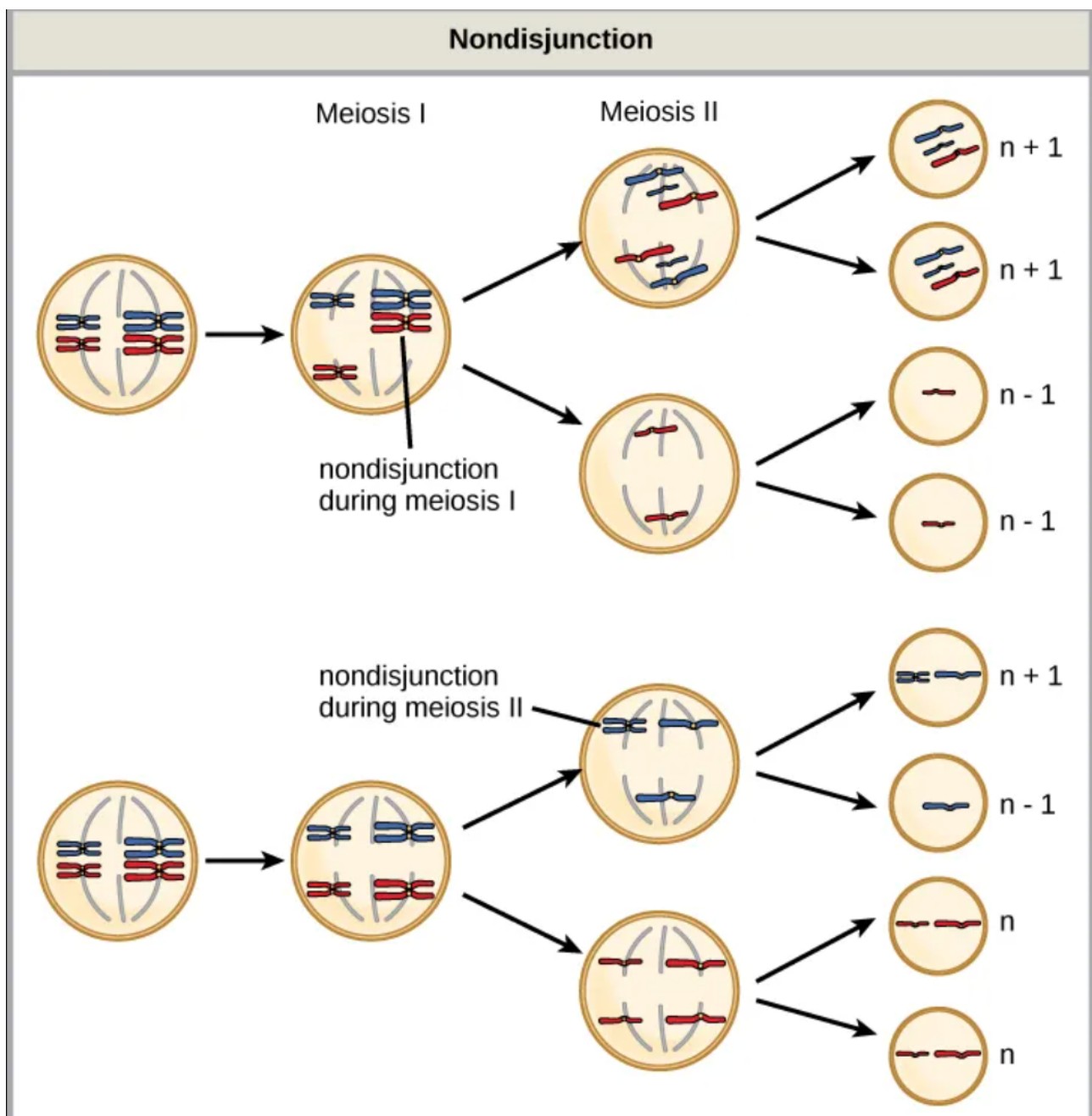


Figure 1.5: Depiction of non-disjunction mutations occurring in both Meiosis I (top) and Meiosis II (bottom). This figure illustrates chromosomes being pulled apart during Metaphase/Anaphase, and how nondisjunction occurs at this point in cell division. Each case, the number of chromosomes in the daughter cells are depicted both visually and numerically. The value “ n ” refers to daughter cells that will be disomic once fertilization, while “ $n-1$ ” indicates a daughter cell that would be monosomic upon fertilization and “ $n+1$ ” indicates a daughter cell that would be trisomic upon fertilization. Source: Zedalis and Eggebrecht, 2018 [163]. For permissions, see Appendix Table 2.

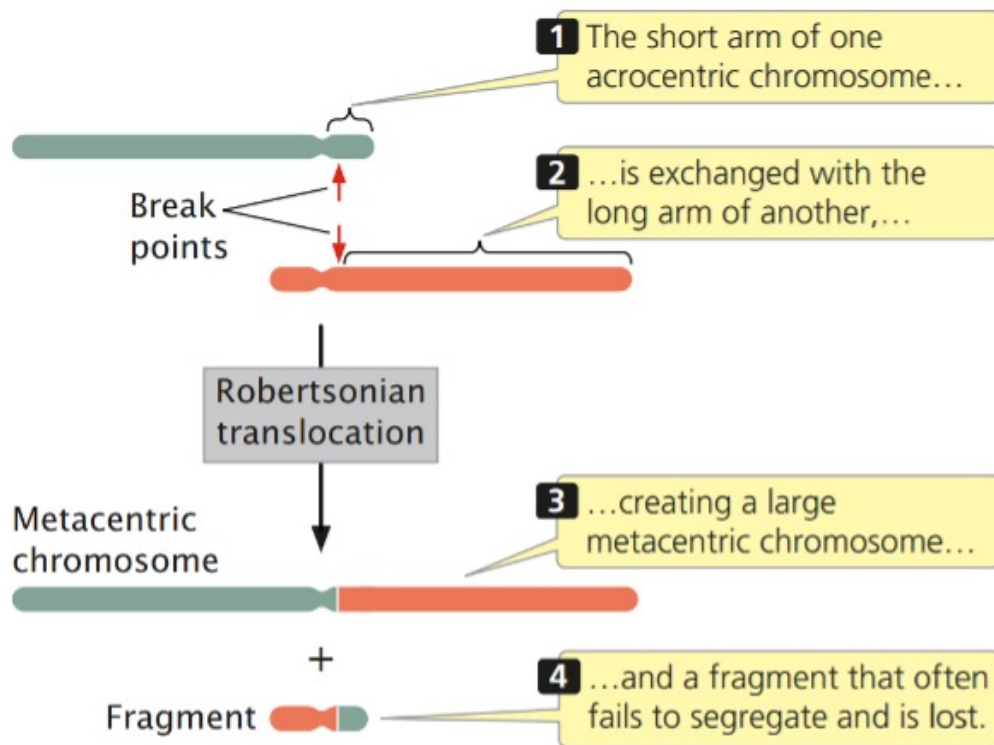


Figure 1.6: Fusion of two chromosome long ends creating a Robertsonian Translocation. From Pierce, 2019 [Genetics: A Conceptual Approach, 7th Ed., page 233] [169]. For permissions, see Appendix Table 2.

translocated chromosome along with a copy of the original chromosome, leading to a trisomic gamete if fertilized. For example, a 14/21 translocation carrier could pass on the translocated 14/21 chromosome and a full copy of chromosome 21. If fertilized, this fetus would have DS [171], [172].

Approximately 1 in 1000 individuals worldwide are Robertsonian Translocation carriers [173]. The genetics of a carrier's offspring will depend on the meiotic division of the gamete. If the translocation is passed on, the child may have a balanced translocation, an unbalanced translocation, or a mosaic translocation [174]. Alternate segregation leads to a balanced translocation, producing an asymptomatic carrier with 46 chromosomes, like their carrier parent (see Figure 1.7). Unbalanced translocations, caused by adjacent segregation, produce gametes that would be monosomic or trisomic when fertilized, therefore containing either 45 or 47 chromosomes respectively (see Figure 1.7). Mosaic trisomy can occur via adjacent segregation, the difference being that not all cells in this individual would be trisomic. The last type of segregation is 3:0 segregation, which is rare and is not viable [175], as the offspring would have either double

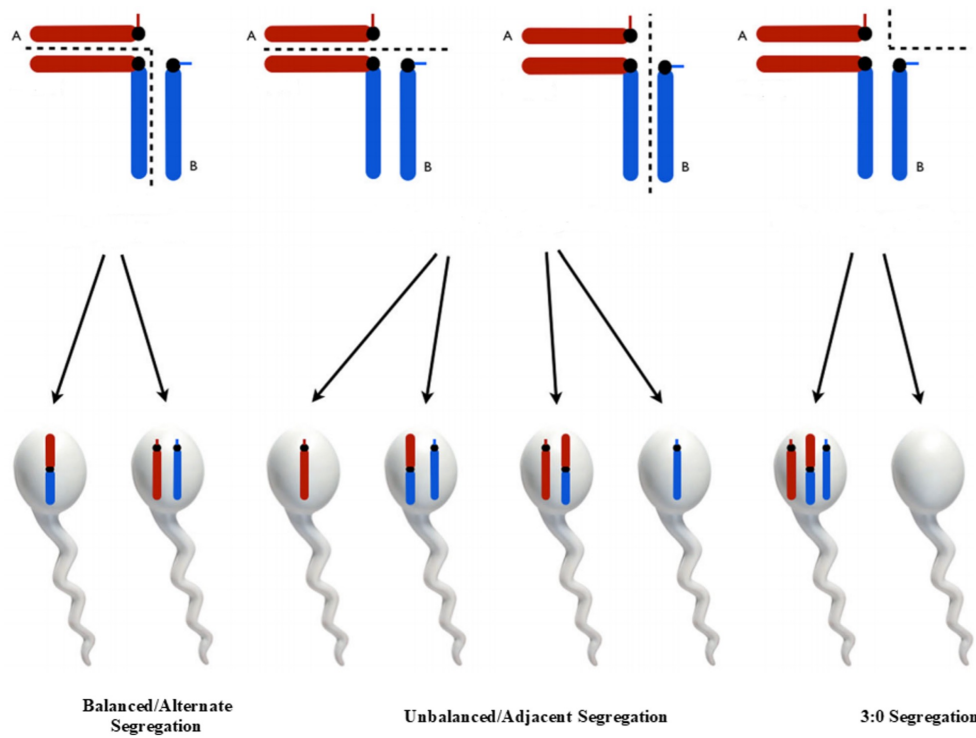


Figure 1.7: Meiotic segregation of chromosomes in a Robertsonian Translocation carrier parent before fertilization. This diagram depicts this process in sperm. Alternate segregation (left) leads to a balanced Robertsonian Translocation carrier and chromosomally normal cell. Adjacent segregation (centre, also called 2:1 segregation) can be performed two different ways. In both cases one chromosome would be trisomic and the other monosomic when fertilized. The final group (right) depicts 3:0 segregation which not viable. Adapted from Lamotte *et al.*, 2018 [175]. For permissions, see Appendix Table 2.

trisomy or double monosomy (see Figure 1.7).

Non-disjunction and translocation mutations are the main methods of developing trisomy. Most DS individuals have a full trisomy, but there are some with partial or mosaic trisomies. As the DS samples in this thesis are from a mosaic individual, I will briefly highlight some of the differences presented in these cases. Mosaic trisomies represent 2-3% of DS cases [159], [176]. In these individuals, some cells are trisomic while others are disomic. The causes of mosaic trisomy are the same as full or partial trisomy in that either non-disjunction mutations [177] or translocations [178] create the trisomy in affected cells. However, the presence of both trisomic and disomic cells in the same individual indicates an increased likelihood of trisomy via mitotic mutations [160]. DS individuals with a higher percentage of disomic cells have decreased phenotypic severity and increased life expectancy [179].

1.3.3 Early Neuronal Development in Down Syndrome

Alterations to brain development in fetal stages lay the foundation for neurological DS phenotypes in childhood, adulthood, and old age. The fetal DS brain has a significantly reduced volume, deficits in cell migration/proliferation, lamination defects, and deficiencies in overall cell number and in neuron number in major brain regions such as the cortex, hippocampus, thalamus, and cerebellum [180], [181], [131], [182], [183], [184]. Whole brain volume deficits are significantly detectable in DS fetuses from the second trimester onwards when compared to age-matched controls [180]. Evidence has been found demonstrating increased neuronal apoptosis and reduced serotonin levels in the fetal DS brain [131], [182]. DS fetal brains also have shorter neurites and abnormal morphologies when cultured *in vitro* [185]. Furthermore, a tendency towards gliogenesis over neurogenesis has been identified in DS fetal brains [131].

Based on mouse studies, Ts21 significantly affects neuronal activity in the brain, altering both excitatory and inhibitory neuronal activity and creating an excitation/inhibition (E/I) imbalance. GABAergic inhibitory neuron dysfunction is a prominent mechanistic theory for cognitive, intellectual, and learning deficits in DS [186], [183]. Due to the significant role of GABA_A and GABA_B receptors in neurodevelopment and synaptic formation [183], they have been researched heavily in this context. Studies have demonstrated that GABAergic interneurons are produced in higher numbers in Ts65Dn mouse models [183]. Overproduction of GABAergic interneurons contributes to increased inhibitory GABAergic activity in the DS mouse model (Ts65Dn and Ts1Cje) brain and related long-term potentiation (LTP) deficits [187] and increased long-term depression (LTD) [188], [186]. A study by Best *et al.* [189] revealed that inhibitory dysfunction in the Ts65Dn hippocampus is driven specifically by GABA_B receptors, creating an altered GABA_A/GABA_B ratio.

The E/I imbalance in Ts21 cannot be solely attributed to alterations of inhibitory GABAergic signals. Deficits in excitatory glutamatergic neuron number and function also contribute to DS phenotypes [183]. Oscillatory calcium activity has been found to be reduced in cortical excitatory neurons of both mouse and xenotransplanted iPSC models of DS [56], [190], [39]. Deficits in excitatory neuron activity in DS have been linked specifically to *DYRK1A* triplication in

mouse studies [56]. When *DYRK1A* overexpression is normalized, abnormal LTP and increased spine number are rescued [191].

More generally, functional MRI (fMRI) studies have shown alterations to neural activity in the frontal cortex and language centers [159], [192], however fMRI has proved to be a difficult tool for DS research in the past due to motor-related phenotypes present in these individuals [193]. Despite this difficulty, significant abnormal synchrony has been confirmed in the human DS cortex [194].

Ts21 also affects glial cells, which in turn create changes in the function of excitatory and inhibitory neurons. Ts21 astrocytes are shown to have significantly increased calcium transients, which inhibit neuronal excitation [190]. This increase in astrocytic activity is attributed to overexpression of *S100 β* , an Hsa21 gene [190]. The presence of Ts21 astrocytes in culture also increases synaptic formation and strength [190]. These effects may be related to the hyperactivation of the mTOR pathway found in Ts21 astrocytes, which also affects neuronal mTOR pathways via paracrine signalling [195].

Fetal developmental delays and deficits provide the foundation for the postnatal DS phenotype. Newborns with DS have brains of roughly average weight, despite reduced volume of cells in multiple brain regions including the amygdala, cerebellum, and prefrontal cortex [147]. Whole brain weight is average at birth, likely due to deficiencies in neural pruning in these individuals at early post-natal stages [159]. As these individuals age, whole brain weight decreases as volume reduction presents in other brain regions such as the parietal cortex and the hippocampus [147]. The table below details the fluctuation in volume within major regions of the DS brain in newborns, adults, and the elderly (see Table 1.2).

Compounding of the phenotypic abnormalities seen in the fetal DS brain results in many cognitive and intellectual deficits later in life. The table below summarizes common cognitive delays present in DS children and adults (see Table 1.3). These phenotypes include decreased average IQ, memory deficits (particularly non-verbal short-term memory), linguistic deficits, visuospatial perception deficits, and executive function deficits [181].

Table 1.2: Table from Dierssen *et al.*, 2012 [147] describing the effects of Ts21 on the size, volume, and weight of each brain region. Each column indicates how the size of various brain regions are affected by Ts21 at a different stage of human development: in newborns, in 20-50 year old adults, and in the elderly (individuals over 50 years old). Over 50 year olds are deemed as elderly in this paper due to reductions in expected lifespan and earlier onset of cognitive decline in DS. For permissions, see Appendix Table 2.

Brain region	Newborns	Adults (20–50 years of age)*	Elderly individuals (>50 years of age)*
Whole brain	Almost normal weight	Reduction in weight, brachycephalic	Smaller overall cerebral volumes
Prefrontal cortex	Reduction in volume	Reduction in volume	Reduction in volume
Parietal cortex	Normal or reduction in volume	Reduction in volume	Unknown
Temporal cortex	Narrow superior temporal gyrus	Reduction in volume of right middle or superior temporal gyrus	Decreased grey matter volume in posterior cingulate and entorhinal cortex
Hippocampus	Unknown	Reduction in volume	Unknown
Parahippocampal region	Unknown	Increase in size of the parahippocampal gyrus	Reduction in volume
Amygdala	Reduction in volume	Reduction in volume	Reduction in volume
Cerebellum	Reduction in volume	Reduction in volume	Reduction in volume
Brain stem	Reduction in volume	Increase in grey matter volume	Degeneration of locus coeruleus
Basal prosencephalon	Almost normal size	Normal	Degeneration of basal prosencephalon cholinergic nuclei (nucleus of Meynert)
*Adulthood begins later in people with trisomy 21 than in individuals without intellectual disability, although people with Down syndrome are considered to be elderly once they have reached 50 years of age.			

Table 1.3: A table modified from Bartesaghi *et al.*, 2022 [181] depicting how cognitive functions are impacted and/or impaired in DS. Cognitive functions listed are IQ, memory (verbal and non-verbal, short-term and long-term, and implicit), language, visuospatial perception, and executive functions. Subtypes of each are listed and average DS functionality is compared to typically developing (TD) individuals as well as individuals with other intellectual disability (ID) or neurodevelopmental disorders such as Williams Syndrome (WS) and Fragile X Syndrome (FXS). Other abbreviations: IQ = intellectual quotient; MA = mental age. For permissions, see Appendix Table 2.

Cognitive functions		Profile
IQ		IQs average 50 and range from 30 to 70
Memory	Verbal short-term memory	Impairment relative to MA-matched TD groups and other ID groups
	Nonverbal short-term memory	In comparison to MA-matched TD groups, comparable or poorer performance In comparison to other ID groups, comparable or stronger performance
	Verbal long-term memory	Impairment relative to MA-matched TD groups and other ID groups
	Nonverbal long-term memory	Impairment in visual associative memory, visual recognition, and spatial memory compared with MA-matched children Similar or stronger visual memory than groups with mixed ID or WS
	Implicit memory	Comparable to MA-matched TD groups
Language	Expressive and receptive language	Expressive language more affected than receptive language
	Lexicon	Lower performance on both comprehension and production tasks compared to MA-matched TD children
	Morphosyntactic skills	Particular impairment in expression and comprehension
Visuospatial perception	Visuospatial construction	Performance on block design task consistent with cognitive level; similar performance to other ID groups; stronger performance than WS; mixed findings on figure-copying tasks
	Mental rotation	Less accurate than MA-matched TD groups
	Closure	Impairments relative to MA-matched TD groups Poorer than other ID groups (e.g., FXS)
	Wayfinding	Particularly poor in comparison to TD individuals and other ID groups
Executive functions	Attention	Impairment in auditory sustained attention and visual selective attention
	Planning	Comparable accuracy but longer execution times than MA-matched groups
	Shifting	Lower performance than TD individuals and other ID groups on verbal shifting
	Inhibition	Impairment in the verbal modality Less severe visual inhibition deficit
	Working memory	Significant impairment relative to TD individuals; mixed findings in comparison to other ID populations

Abbreviations: DS, Down syndrome; FXS, fragile X syndrome; ID, intellectual disability; IQ, intelligence quotient; MA, mental age; TD, typically developing; WS, Williams syndrome.

Overall, Hsa21 trisomy affects all aspects of neurodevelopment; from development of the early fetal brain, to adult neurogenesis and aging. Understanding DS neurodevelopment will assist in the development of novel treatments and will inform the scientific community of specific mechanisms important to DS phenotypes present in childhood, adulthood, and old age.

These cognitive and intellectual delays begin from fertilization. Developing a complete understanding of the DS fetal brain will provide the building blocks necessary to understand the mechanisms behind DS phenotypes later in life. Our understanding of DS fetal neurodevelopment, including decreased cell number/brain volume, decreased spine formation, and inhibition of excitatory neuronal activity, is rooted in the use of DS models including iPSCs and rodent models. The following section will describe major rodent models of DS and their uses.

1.3.4 Rodent Models for Down Syndrome Research

DS is an incredibly complex syndrome to model. Differences between the human genome and that of other model organisms like rodents contributes to that complexity. Human chromosome 21 (Hsa21) is comprised of over 550 genes and putative genes, both coding and non-coding [196]. Protein coding genes account for ~220-240 of these [197]. Many of these genes are conserved in the mouse genome, but not all [198]. In their study of the DS genome, Herault *et al.* [197] found that 158 Hsa21 protein coding genes had homologous genes in the mouse genome. This would mean ~62-82 Hsa21 protein coding genes are not represented. Additionally, Hsa21 homologues in mouse are spread across three mouse chromosomes (Mmu): Mmu16, Mmu17, and Mmu10 [196]. Of the 158 homologous genes identified by Herault *et al.* [197], 102 are found on Mmu16, 37 are found on Mmu10, and 19 are found on Mmu17.

DS mouse model development began in earnest in the 1970's with the development of the Ts16 mouse model, a model trisomic for the entirety of Mmu16 [199], [200], [201]. The Ts16 model proved problematic, as Mmu16 contains many genes not homologous to Hsa21, including genes homologous to Hsa3, 8, 16, 18, and 22 [202], [203], [204]. Ts16 failed to incorporate the homologous genes on Mmu10 and Mmu17, while including trisomies of unrelated genes. These mice often died *in utero* and were prone to problematic phenotypes including heart defects

[205], growth deficiencies [133], severe edema [133], [202], open eyelids [133], brain hypoplasia and abnormal electrophysiological properties (eg: shortened AP) [202].

The Ts16 model was followed by the Ts65Dn model, which is widely used to this day. The Ts65Dn model is a distal Mmu16 trisomy, thus limiting the number of trisomic non-Hsa21 homologous genes. Ts65Dn was produced by irradiation of the sperm of a male mouse, which created a translocation mutation of the distal arm of Mmu16 ($\approx 16\text{Mb}$) onto Mmu17 [206], [196], [207]. Offspring from sperm with this translocation were born with a trisomy for the distal arm of Mmu16, which consists of over 100 Hsa21 homologues. The translocation breaking point on Mmu16 is D16Mit58, thus Ts65Dn includes the trisomy of homologues for many Hsa21 genes important in DS including *APP*, *DYRK1A*, and *SOD1* [206], [208]. Ts65Dn has provided insight into DS behavioural alterations and physiological abnormalities [209], [210], the genetics of the syndrome [187], and the future of DS treatment [211], [182], [212]. Ts65Dn mice display a range of similar phenotypes to DS including tremors/seizures, hydrocephalus, smaller size, craniofacial abnormalities, spatial/visual learning and memory deficits, behavioural anomalies and increased gliogenesis [203], [204]. However, significant differences exist between Ts65Dn and DS in humans, including the lack of Alzheimer's pathology (no amyloid plaques or tau tangles) and the absence of leukemias, which are often present in humans with DS [203], [206]. Furthermore, Ts65Dn mouse models contain triplication of additional genes not associated with Hsa21. The contribution of these additional trisomic genes likely effects phenotypic outcomes.

Researchers continue to develop new mouse models to tackle specific questions for which Ts65Dn was less well suited. These alternative mouse lines include models that triplicate targeted sections of the Hsa21 mouse homologues to study specific genes and their contributions to DS [197]. Examples include Dp/f_Yey, Dp/f_Yah, Ts1Cje, Ms1Cje, and Ts1Rhr [213], [204], [197].

Another popular mouse model type is the transchromosomal mouse model Tc1. Tc1 is a mouse line first developed in 2005 which incorporates Hsa21 directly into its genome [214], [197]. Mosaicism is common in this model, but Kazuki *et al.* [215] have recently managed to develop a Tc1 line that is non-mosaic. They inserted the entire long arm of Hsa21 into the mouse genome, however, many gaps were present. Prior to the Tc1 line from Kazuki *et al.*

[215], this model was full of gaps as deletions and duplications were common side-effects of the gene insertion method [196]. These mice demonstrate a number of traits similar to human DS including cardiac anomalies and short-term memory deficits [214], [216], [217]. However, these mice have no craniofacial anomalies [214] and hippocampal defects are debated [216], [218].

The vast number of available DS rodent models means that model selection is based on experimenter question and goal. Many researchers, like those utilizing the Dp/f_Yey and Dp/f_Yah models [197], focus on specific genetic regions. Others are more general, wishing to study behavioural changes, musculoskeletal alterations, learning and other broad phenotypic characteristics of DS. Often these phenotypes are studied using mouse models like Ts65Dn and Tc1 [217], [208], which provide a close overarching DS phenotype. They are also used to provide genetic information, helping to narrow down treatment options and solve the yet uncovered mysteries of DS [219], [220], [221]. Novel tools like CRISMERE have further increased the ability to use mouse models for DS study [197], but there is much to learn that cannot be learned from mouse models. As DS is very complex, it can affect not only Hsa21 but also all other regions of the human genome. Furthermore, prolonged mouse model use over the years has created unwanted genetic variability between past and present mouse lines [222]. As with any model, there are pros and cons to the use of mouse models in the study of DS. It is for this reason that researchers carefully select the best model to test their hypotheses. They may choose to use other model types entirely (eg: iPSCs, rodent *in vitro* cultures, organoids, fresh-frozen tissue, *Drosophila*) or in combination with rodent models. The next section will discuss one such combinatory method in the context of DS research: iPSC xenotransplantation.

1.3.5 The iPSC Xenotransplantation Model in DS Neurodevelopmental Research

Xenotransplantation, or the transplantation of cells, tissues, or organs of one species into another species, has been around since the 17th century [223]. Early attempts, from blood transfusions to organ donations, failed. Xenotransplantation was largely abandoned in favor of allotransplantation in the 1960's [223]. Over time the need for donated organs grew exponen-

tially, while the number of donations remained steady [224]. This is a continuing problem in medicine, and one that caused researchers to return to xenotransplantation. One of the most successful uses of xenotransplantation is the replacement of heart valves with porcine valves, a procedure still performed successfully today [225].

Since the 1960's, multiple types of xenotransplantation have been attempted in research. These include pig liver to human, pig liver to non-human primate (NHP), pig lung to human, pig cornea to NHP, and pig/sheep/NHP kidney to human. While most full organ transplants were unsuccessful, they provided information on transplant rejection, which researchers used to determine that cell xenotransplantation is an option with reduced rejection risk. Xenotransplantation has been tested in many cell types, including islet cells [226], hepatocytes [227], and neurons [228], [229]. These studies created an opening in the field for iPSC xenotransplantation research following the discovery of the transcription factors for human cell reprogramming to pluripotency [230], [231]. In the 1980's and 1990's, xenotransplantation studies using porcine fetal tissues were published, indicating that both fetal pancreatic tissues could be xenotransplanted into diabetic patients [232] and that these tissues could be used to extract islet cells for effective xenotransplantation [233]. However, xenotransplantation work was not truly propelled forward until the method for fibroblast reprogramming into pluripotent stem cells was developed. Studies of patient-derived iPSCs being generated into neurons and used for xenotransplantation experiments continued from 2008 onwards, with techniques being refined along the way. These experiments demonstrated that in immunodeficient rodents, pigs or NHPs, neurons and glia could be transplanted and would develop good connections, proper structure, and healthy electrical activity over time [230], [231], [234], [235], [235], [236], [237], [238], [239], [240], [241]. This technique has been refined and used to study spinal cord injury repair [240], [241], [242], and neurological disease [243], [39].

In 2018, my research group developed a novel *in vivo* model using iPSC-derived neuron xenotransplantation [39]. This publication was the first to use DS iPSCs to create the transplanted cell grafts inside a mouse brain. During craniotomy surgery they injected neuronal progenitors derived from iPSCs into the primary somatosensory cortex of a type of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model called NSGTM. A cranial window was

placed over the region to allow for longitudinal multiphoton imaging of the transplanted human graft cells over time. Initially, wild type (WT) cells were used to test the model and monitor graft development. They confirmed that graft cells become vascularized, that graft cells develop and mature over time, and that the neurons demonstrate pruning and refinement. The graft cells were active as tested by electrophysiology, and 92.5% (+/- 1.5%) of the connections formed by the graft cells were human-to-human, with only 7.5% (+/- 1.5%) of connections being mouse to human, as determined by rabies virus tracing. Once the model was proven to be effective, they demonstrated how this model could be used in the study of neurological disorders by creating grafts derived from DS iPSCs. The DS neuronal progenitors grew normally, with no significant differences found in axon elongation and retraction when compared to WT and revertant disomic controls. However, significant differences were found in en-passant bouton (EPB) and dendritic spine turnover rates (TOR) and survival rate (Fr) and in measures of neuronal oscillatory activity at 3-4 months post-transplantation (mpt). They found that DS graft dendritic spines had significantly increased Fr and significantly decreased TOR and that EPBs trend towards an increased Fr and had a significantly decreased TOR. This indicates an overall increase in synaptic structural stability in DS grafts compared to controls. To measure neuronal oscillatory activity, they measured calcium activity and identified that DS grafts had a significantly decreased number of ROIs containing burst activity, a significantly decreased burst frequency, and a significantly lower global activity ($\Delta F/F_0$) compared to WT and revertant disomic controls. This indicates an overall decrease in neuronal oscillatory activity in DS grafts compared to controls. Post-mortem immunohistochemistry was performed on perfused brain slices to characterize the cell types within the graft. Real *et al.* [39] demonstrated that their grafts contained neuronal precursor cells (Pax6+), dividing precursor cells (Ki67+), deep layer cortical excitatory neurons (TBR1+, CTIP2+), upper layer cortical excitatory neurons (SATB2+), astrocytes (GFAP+), but no inhibitory interneurons. Cell type distributions were found to be not significantly different between DS and controls in all cell types except for GFAP+ astrocytes, which were present in significantly larger quantities in DS grafts [39] (see Figure 1.8), and SATB2+ upper layer cortical neurons, which were present in significantly decreased quantities in DS grafts [244].

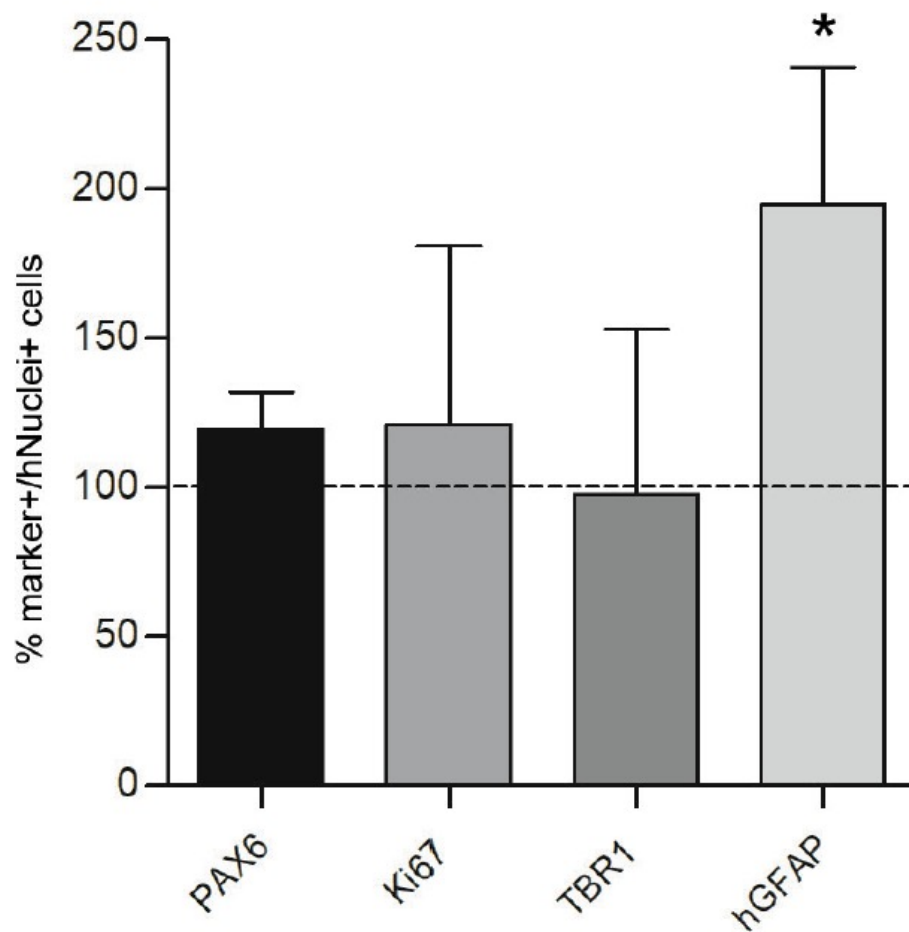


Figure 1.8: Figure showing the relative proportion of each cell type in Ts21-1 graft (n=3 for each group) at 5mpt when normalized against controls. From Real *et al.*, 2018 [39]. Reprinted with permission from AAAS. For permissions, see Appendix Table 2.

The findings highlighted in the 2018 paper were the basis for the development of this project and subsequent thesis. In the following sections, I will outline the aims and hypotheses of this project, which builds upon the work of Real *et al.* [39] to improve our understanding of early DS neurodevelopment.

Chapter 2

Aims & Hypotheses

2.1 Aims

This project was designed to investigate early neuronal cortical development in an *in vivo* xenotransplantation model of Down syndrome (DS) over an extended period of time (up to 8 months) using grafts derived from a protocol which simplifies neuronal differentiation and removes excess experimenter bias (Nizetic Protocol). The aims of this project are:

1. To develop cortical neuronal progenitor cells for *in vivo* xenotransplantation using a differentiation protocol which minimizes experimenter bias (Nizetic Protocol).
2. To confirm that Ts21 and isogenic control lines, produced from Down Syndrome fibroblast-derived iPSCs, can be differentiated using the Nizetic Protocol from Aim 1 and can effectively be xenotransplanted into the NSG mouse somatosensory cortex and longitudinally imaged with 2-photon microscopy.
3. To evaluate the stability of axonal en-passant boutons (EPBs) in Ts21 xenotransplanted grafts and compare their stability to isogenic control grafts at both 3 and 5 months post-transplantation (mpt).

4. To evaluate the spontaneous and oscillatory network calcium activity of Ts21 xenotransplanted grafts and compare their activity to isogenic control grafts at 1, 3, and 5 mpt.
5. To test the limits of the xenotransplantation graft model by maintaining a subset of animals to 7-8 mpt as a proof of concept that grafts remain healthy and can be longitudinally imaged at this timepoint to determine spontaneous and oscillatory calcium activity levels.

2.2 Hypotheses

Hypotheses for this project were developed based on the aims described above. My hypotheses were as follows:

1. That differentiation of neuronal progenitor cells using the Nizetic Protocol will produce grafts containing excitatory cortical pyramidal neurons and progenitor cells.
2. That Nizetic Protocol-derived neuronal progenitors will successfully develop inside the somatosensory cortex of an immunodeficient adult mouse. The development of en-passant boutons and calcium activity fluctuations is expected to be visible via longitudinal multiphoton imaging.
3. That repeatedly finding and imaging en-passant boutons from graft cell axons will be achieved from 3mpt onwards with 2-photon microscopy in both Ts21 and isogenic controls grafts. Based on the cell types produced with the Nizetic Protocol and after initial image screening, I hypothesized that I had developed a model where trisomy of chromosome 21 does not impact synaptic stability in early development, thus no significant difference would be found across genotypes.
4. That there is no significant difference between Ts21 and isogenic control grafts in spontaneous or oscillatory activity, matching the lack of effect of the additional chromosome 21 found when examining the axonal en-passant bouton stability. Furthermore, I hypothesized that somatic calcium activity would be measurable over time using 2-photon microscopy.

5. That spontaneous and oscillatory activity would be present and measurable at 7-8mpt in a proof of concept pilot subset of mice. This would demonstrate the extent to which this model can be utilized in the study of early neuronal development.

The end result of this thesis was the development of Ts21 and isogenic control neuronal grafts in which the presence of an extra copy of chromosome 21 does not affect synaptic stability and spontaneous or oscillatory activity at early stages of development. Furthermore, this thesis proves that longitudinal multiphoton imaging of calcium activity in these grafts can successfully be performed as far as 8 months after neuronal progenitor transplantation.

Chapter 3

Methods

3.1 Animal Care, Use, and Licensing

The animal work for this project was approved by Imperial College London's Animal Welfare and Ethical Review Body (AWERB) and by the UK Home Office prior to commencing experimentation. All research follows the UK and EU requirements for animal research, as laid out in the Animals (Scientific Procedures) Act 1986 (ASPA) [as amended in 2012] and in the European Directive 63/2010/EU. All animal work for this project was performed using adult NOD scid gamma (branded name: NSG) immunodeficient mice (JAX, Charles River UK). The full nomenclature for this mouse strain is NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (strain code 614). This strain has a NOD/ShiLtJ background and the severe combined immune deficiency mutation (scid) along with an IL2 receptor gamma chain deficiency. These mice have tested absent of mature T cells, B cells, Natural Killer cells, and Complement cells, and have defective dendritic cells and macrophages. Furthermore, they have a low incidence of lymphoma according to Charles River UK from which they are purchased (see full details on the Charles River UK website at <<https://www.criver.com/jax-nsg-mouse-variant-portfolio>>). The mice used in my experiments ranged in age from 2-5 months at the time of surgery, therefore are all adults. Both sexes were used equally for experiments. The mice were ordered from Charles River UK as needed and were stored in the MRC LMS animal facility (Central Biomedical

Services) for at least 1 week prior to surgery to acclimatize. Animals remained in the MRC LMS facility until experimental endpoint was reached. The mice were kept for 5-8 months post-surgery, depending on the experiment type. General care and maintenance of the animals (food, water, cleaning bedding, etc.) was performed by the full-time animal care staff in the facility, in coordination with the Named Veterinary Surgeon (NVS), the Named Animal Care and Welfare Officers (NACWO), the personal licence holder (Elizabeth Brockman) and the project licence holder (Dr. Vincenzo De Paola). Animals were given sterilized bedding and water due to their immunocompromised nature. All work performed on these animals was done under the following UK animal licences: the Imperial College London Establishment Licence (X32FDCFC1), Vincenzo De Paola's Neuroplasticity and Disease Group Project Licence (P07334938), and Elizabeth Brockman's Personal Licence (I21592226).

3.2 iPSC Lines: General Information & Collaborations

The induced pluripotent stem cell (iPSC) lines used in this project were provided by our collaborators in the Nizetic Lab at Queen Mary University of London (QMUL). These iPSCs were derived from fibroblasts collected by skin-punch biopsy from individuals with mosaic Down Syndrome. The fibroblasts were used to make iPSC lines of two types: chromosome 21 trisomic experimental lines (Ts21), and chromosome 21 disomic isogenic control lines (Ds21) (see Murray *et al.*, 2015 [245] for reprogramming protocol performed by the Nizetic Lab). This system allowed for each Ts21 line to have a genetically matched Ds21 control line. Specifically, this project utilized the paired lines NIZEDSM1 iD21-C3 and NIZEDSM1 iT21-C5 [245], which will henceforth be referred to as Ds21 and Ts21 respectively. These lines were expanded and frozen in liquid nitrogen by the Nizetic Lab members. They were differentiated as needed from iPSCs for all experiments. Importantly, genotypic analysis was performed on these lines by the Nizetic lab using semiquantitative microsatellite PCR to confirm the presence of Ts21 in the C5 line, that C3 is disomic for chromosome 21, and that two randomly selected euploid chromosomes (Chromosomes 18 and 21) were disomic in both lines [245].

3.3 iPSC Differentiation

The iPSC lines were thawed and differentiated into early neuronal progenitor cells using the “Induction of Neural Stem Cells from Human Pluripotent Stem Cells Using PSC Neural Induction Medium” protocol from Gibco (Publication No. MAN0008031, Life Technologies). This procedure was performed up to DIV24 by our collaborators at the Nizetic Lab at QMUL or by members of our laboratory (depending on the cell batch). Briefly, iPSCs were grown in culture until they reached 70-80% confluency, at which time they were lifted in clumps using Accutase (Sigma, A6964). The cells were counted and replated on Geltrex [®] (Gibco, A1413201) coated plates in iPSC culture media (StemPro [®] hESC SFM) and were incubated overnight. The next day, the cells were split and placed in iPSC Neural Induction Media (made by combining 490mL Neurobasal [®] Medium with 10mL Neural Induction Supplement [Gibco]) for incubation. The media was changed approximately 3 times over the next 5 days of incubation. After approximately one week (when the cells had reached confluency), the cells were again lifted with Accutase and were replated on Geltrex [®] coated plates in Neural Expansion Medium (made from 49mL Neurobasal [®] Medium, 49mL Advanced[™] DMEM-F12, and 2mL Neural Induction Supplement [Gibco]) with 5mM ROCK Inhibitor Y27632 (StemCell #72302). The following day, the media was changed to Neural Expansion Medium without ROCK Inhibitor, and media changes were continued every second day. From here, the cells were passaged to between passage 4 (P4) and P10.

For terminal differentiation into mature neurons and glia, the cells were plated onto Poly-L-ornithine (Sigma, P4957) and Laminin (Sigma, L2020) treated plates. The cells were incubated on these plates in Complete Brain Physiology Medium (made from 48mL Brain Physiology Basal Media [StemCell #05793], 1mL SM-1 Supplement [StemCell #05793], 0.5mL N2A Supplement [StemCell #05793], 0.5mL PenStrep, 0.5mL 1mM cAMP, 0.05mL 20 μ g/mL BDNF [Peprotech #450-02] 0.05mL 20 μ g/mL GDNF [Peprotech #450-10], and 0.035mL 200nM ascorbic acid [Sigma #A4403]) until use for immunohistochemistry or surgery. Media was changed every 3-4 days from this point onwards. After one week in Complete Brain Physiology Medium (DIV 24), the cells were transported to the LMS MRC facility from QMUL (for batches made

by Nizetic Lab). Media changes were performed for an additional week until the cells reached DIV 31-34. At this stage, the cells were transduced with a lentivirus and used for surgery or were fixed with paraformaldehyde (PFA, Fisher Scientific, 4% in PBS) and used for immunohistochemistry.

3.4 Lentiviral Transduction of Cells

All cells used for injection during surgery were transduced with a lentivirus (LV) one day prior to injection. Two LVs were used, one for imaging en-passant boutons (used for synaptic structural stability measurements) and one for calcium imaging (as a proxy for neural activity). For synaptic structural imaging, we employed LV-Synapsin-GFP (Signagen Laboratories, SL100271) which utilizes a human synapsin-1 promoter to tag neurons with GFP. It has a titer of 1×10^9 TU/mL and was used at a multiplicity of infection (MOI) of 10. For a single well from a 6-well plate containing 400,000 cells, we added 4.0 μ L of LV into 2.0 mL of media to get an MOI of 10. For calcium imaging, we used “LV[Exp]-SYN1>GCaMP6s(ns):P2A:{tdTomato}-SV40 promoter>Zeocin” [39]. This LV contains two different labels, joined within the vector by the self-cleaving peptide P2A, a member of the 2A peptide family derived from porcine teschovirus-1. The two labels that P2A joins together in the LV are: GFP-Calmodulin fusion protein 6 (GCaMP6), which acts as a neuronal activity proxy as conformational changes caused by calcium binding create increased chromophore brightness [246], and tdTomato which provides a cell filler marker within the neurons, illuminating their structure. The expression of both GCaMP6 and TdTomato is driven by the promoter for human synapsin-1. This LV was produced by Amsbio, has ampicillin resistance and a titer of 9.67×10^8 TU/mL. An MOI of 10 is also used for this LV.

3.5 Craniotomy Surgery and Cell Injection

The NSG immunodeficient mice (full nomenclature: NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ) described in Chapter 3.1 (adult, aged 2-5 months, both mixed sex) were initially anaesthetized using 3% isoflurane (IsoFlo ® 100% w/w inhalation vapour, liquid, Zoetis) to sedate them for surgery. This was decreased to 1-2% isoflurane dependent on the animal's size and breathing rate (average ~1.5%). In the surgical preparation suite, the anaesthetized mice received pre-operative injections of dexamethasone (intramuscular, 15µL), bupivacaine (subcutaneous, at surgical site, 30µL), carprofen (subcutaneous, 1mg/mL, 5mg/kg), and buprenorphine (subcutaneous, 0.03mg/mL, 0.1mg/kg). Hair was removed from the surgical site using a trimmer (Wahl ® BravMini Trimmer) and hair removal cream (Veet). The shaved region was sterilized with iodine (Videne, diluted in water). Eye gel (Lacrilube) was applied to the eyes to prevent dehydration.

The mouse was transferred from the preparation suite to the sterile surgical suite and placed in a stereotaxic frame. It was sedated with a continuous flow of isoflurane at approximately 1.5% throughout the remainder of the surgery. A non-sterile assistant monitored and recorded the respiration rate of the mouse and adjusted the isoflurane levels in real time to maintain a respiratory rate of 60-80 breaths/minute.

All surgical tools were autoclaved prior to use. Tools were organized on the surgical bench to maintain sterility as much as possible. The iodine-sterilized region of skin (top of head) was then removed above the somatosensory cortex by a sterile surgeon using sterile scissors. The edges of the skin were glued (Loctite ® Super Glue Gel) back to expose the skull region beneath. The exposed skull was cleaned and dried with sterile cotton swabs before being covered with a thin layer of glue and cement (DePuy CMW 1, Gentamicin Bone Cement). The only region left uncovered with glue was the region designated for cell injection (somatosensory cortex). Lidocaine was applied to this area and a drill (Stoelting Ideal Micro-Drill) was used to remove a circular piece of skull 4mm in diameter from above the right somatosensory cortex. The exposed brain was kept moist with filtered cortex buffer (125mM NaCl 7.21g, 5mM KCl 0.372g, 10 mM Glucose 1.802g, 10 mM Hepes 2.38g, 2 mM CaCl₂ 2 ml of 1M CaCl₂, 2 mM

MgSO₄ 2 ml of 1 M MgSO₄ in 1L distilled H₂O, pH equilibrated to 7.4 and filtered). A sterile glass needle (World Precision Instruments Inc., Borosilicate Glass Capillaries, Lot 1902328) pulled with no weight (step 1) using a needle puller (Narishige PC-10) at 72.8°C was used to aspirate ~4µL of cells. This needle was placed in the autoinjector micro pump unit (UMP-3, World Precision Instruments, USA) and was positioned just above the surface of the brain, in the center of the exposed cortical region. The needle was used to gently puncture the dura and the tip of the needle was then inserted approximately 500µm below the surface of the brain. Once in place, the needle was left there for 2 minutes before the cells were injected. The autoinjector injected 1 µL of cells into the somatosensory cortex at a speed of 300 nL/min. After cell injection, the needle was again left in place, this time for 5 minutes, to ensure that the cells were not pulled out upon removal of the needle. The needle was slowly removed and a glass coverslip (5 mm diameter, sterile) was placed over the exposed region to create a cranial window. The edges of the coverslip were sealed with glue. Once dried, the entire surgical region (where the skin was removed) was covered in a thicker layer of dental cement. A metal bar was placed on the cement cap as a method of stabilizing the animal during later imaging sessions.

Following surgery, the animals were monitored regularly for major changes in behaviour and fluctuations in weight. Post-surgical analgesics were provided both in the water (carprofen, 0.19mL undiluted carprofen per 250mL sterile water) and as jelly pellets (raspberry jelly with buprenorphine 50µL of 1:50 diluted buprenorphine per portion) for 3 days post-surgery. Post-surgical care was monitored by the personal licence holder with the assistance and advice of the Named Animal Care and Welfare Officer (NACWO), the Named Veterinary Surgeon (NVS), and animal welfare staff. After 3 days, post-surgical analgesics were stopped unless the mouse was unwell, at which time treatment was carried out in consultation with the NVS. The weight of the mice was monitored regularly; initially once a day for the first 7 days, then 1-2 times a week for the following weeks as needed. Detailed experimental endpoints were laid out in the project licence (P07334938, Protocol 2). When an endpoint from the project licence was met, such as the mouse had lost over 20% of its pre-surgery measured mass, it was culled in accordance with humane Schedule 1 procedures. The mice were perfused to preserve the brain tissue for experimentation when possible.

3.6 Two Photon (2P) Microscopy

3.6.1 General 2P Set-Up

All 2P (Prairie Technologies, Bruker) imaging was performed on injected graft cells under the cranial window of NOD scid gamma (NSG) mice that had undergone craniotomy surgery. Comparisons were made between mice with Ds21 grafts and mice with Ts21 grafts, as well as across multiple imaging timepoints. For GFP bouton imaging, the grafts were imaged at 3 months post-transplantation (mpt) and 5mpt. For GCaMP calcium imaging, the grafts were imaged at 1mpt, 3mpt, 5mpt, and 7-8mpt. Any animal for which a graft was not initially found was kept until at least 3mpt to check for graft development. At this point, any animals that still had no visible graft would be perfused and sliced to find deeper graft for immunohistochemistry. For all mice where graft was found successfully, imaging was performed at all listed timepoints. The mice were perfused after the final imaging timepoint (5mpt for GFP graft animals, 7-8mpt for GCaMP graft animals).

At the start of each imaging session, the mouse was anaesthetized in a box with 3% isoflurane. Once under sedation, they were transferred to the preparatory biological hood (ChemcapTM Filtration) where the glass window was cleaned with 70% ethanol and a sterile cotton tip, eye gel (Lacrilube) was placed on the eyes, and a mounting piece was screwed onto the metal bar attached to the cap. This was all done while under 2% isoflurane anaesthesia. With the mounting piece in place, the animal would be transferred to the microscope stage and would be placed on a warming pad (regulated using a Homeothermic Blanket Control Unit, Harvard). The isoflurane levels would be brought down to 1.5% while the mouse was being imaged and would remain there for the duration of the session (except during calcium imaging which required isoflurane levels of ~0.5-0.7%). A 4X (dry, Olympus, 0.13NA) lens was used to focus on the window through the microscope eyepiece and select a blood vessel bifurcation to be used as the 0,0 coordinates. The lens was switched for a 20X gel (Olympus, 0.95NA) lens and an image of the selected 0,0 location was taken with the 2P. All subsequent imaging sessions began by locating the 0,0 coordinates.

The microscope and laser were switched on and left to warm for ~ 10 minutes prior to use. All imaging with the 2P was run through Prairie View software (Prairie Technologies, Bruker). Imaging of GFP and GCaMP was performed at a wavelength of 920nm using channel 3, while tdTomato was imaged at a wavelength of 1040nm using channel 2. The 2P setup included the microscope itself as well as a power supply (Conoptics, Model 302RM), an amplifier and high voltage control box (Prairie Technologies, Dual Preamplifier & HV Control), a laser box with control panel (Coherent, MRU X1), and a broadband power/energy meter (Melles Griot, 13PEM001). The microscope and laser were located on a motion dampening table (Newport).

Upon completion of each imaging session, mice were returned to the biological hood warming mat to recover from anaesthesia. In accordance with animal welfare regulations, each animal was imaged for no longer than 90 minutes per session. Once the mouse had recovered successfully from the anaesthetic, it was returned to its cage in the animal facility.

3.6.2 En-Passant Bouton Imaging (GFP LV): Protocol & Settings

As stated above, all GFP 2P imaging was performed using 920 nm at channel 3 using a two-photon microscope (Prairie, Bruker). The laser was set to 650Hz and the pockels were set to provide a power at the back of the focal plane of ~ 40 -65mW. Images were taken at 3mpt and 5mpt, with each timepoint consisting of 3 days of imaging. Images were taken on Days 0, 2, and 4 at each timepoint. On Day 0, axons that contained visible en-passant boutons were selected and imaged with a 60X lens (Olympus, 1NA), using a zoom of 3.0, a field of view of 512x512 pixels, and a step size of 1.0 μ m. These settings provided images with size of 0.131 μ m/pixel. After taking the first image, the axon was followed along its length as much as possible. Multiple axons were selected and imaged at each Day 0. The images from Day 0 were duplicated at Days 2 and 4 using noted coordinates.

This imaging protocol allowed for comparative analysis of EPB presence over time. Calculations were performed to determine the frequency of boutons per μ m of axon, the turnover rate (TOR) of the boutons, the survival rate (Fr) of the boutons, and the fluorescent intensity of the boutons compared to the backbone axon. Analysis of boutons was performed in MATLAB (MathWorks

®), Version R2014a) using the EPBscore package (Version 8.6.2015; Song, Grillo *et al.*). Details on the specifics of this package can be found in literature [41], [40]. For a point along the axon to be considered a bouton, the intensity threshold required was at least 1.7X the fluorescent intensity of the backbone. Each bouton was compared to the backbone fluorescence of its own axon. Therefore, we controlled for background intensity and variations in the laser gain.

3.6.3 Calcium Activity Imaging (GCaMP LV): Protocol & Settings

The timepoints utilized for calcium activity were 1mpt, 3mpt, 5mpt, and 7-8mpt. This allowed for analysis of calcium activity changes over the course of many months of development.

Somas were located and imaged with the 2P microscope by tdTomato signal. These images (z-stacks) were taken using a 20X gel lens (Olympus, 0.95NA) at a zoom of 2.0, a field of view of 512x512, and a step size of 2-3. They were taken with channel 2 at 1040nm wavelength using a frame rate of 8 to achieve high quality images. These z-stack images were used to identify planes of interest for calcium imaging at that ROI. The planes containing somas of interest were noted, along with their z-coordinates, so that GCaMP6 activity could be measured at that location. The settings were then changed to channel 3 at a 920nm wavelength, with a field of view of 256x256 and a frame rate of 1, which provided a frequency of 3.03 frames/second for t-series imaging. At each selected plane, a t-series was taken containing 800-1200 frames (~4 minutes). Just prior to the beginning of the t-series, the isoflurane levels were decreased to ~0.5-0.7%. The decrease in anaesthetic level allowed for increased calcium activity. The level of anaesthetic was monitored closely, as variation in the size of the mouse affected the amount of isoflurane necessary to achieve light anaesthesia without waking. Additionally, required levels of anaesthetic decreased throughout the course of a 90-minute imaging session, due to acclimatization to the isoflurane over time. As such, the isoflurane level was altered slightly from mouse to mouse and over time.

T-series images (and their corresponding tdTomato z-stack) were analyzed using Fiji (ImageJ) macros and MATLAB (MathWorks ®, R2020a). The analysis of this dataset is described in the Data Analysis section.

3.7 Perfusion, Fixation, Slicing & Storage

3.7.1 Perfusion & Fixation of Whole Mouse Brains

Perfusions were performed on all imaged mice once they reached their experimental endpoint. Animals that reached a terminal endpoint prior to the experimental endpoint were perfused if the animal welfare staff/NVS deemed it permissible without causing further distress to the mouse. Otherwise, they were culled using approved Schedule 1 methods.

For perfusions, a lethal intraperitoneal injection (1.25mL ketamine, 0.5mL xylazine and 8.25mL sterile water mixture, ~0.5mL of this mixture injected into each animal) was first administered as the primary culling method. Once there was no longer a reflex response, the mouse was then pinned on its back to a board in a designated perfusion room. The chest was sterilized using 70% ethanol. The skin was then cut in a “V”-shape from the mid-chest up at an angle towards both shoulders using sterile tools. Subcutaneous layers of fat and tissue were cut similarly to reveal the ribs beneath. The tissue underneath the xyphoid process of the sternum was cut to allow visualization of the heart when looking upwards from the sternum, underneath the ribcage. The ribcage was also cut in a “V”-shape on either side. This allowed for the central section of the ribcage to be pulled up and away, exposing the heart. This central section of ribcage was pinned down to the board after being pulled over one shoulder of the mouse. At this point, the heart was exposed and still beating. The needle (27 gauge) of the perfusion machine (Masterflex C/L, Cole Parmer, Model 77120-62 [60RPM]) was carefully inserted into the left ventricle of the heart and clipped there. The flow sterile PBS ‘A’ was commenced using the perfusion machine. Sterile scissors were then used to cut the right atrium, allowing for exsanguination. 5mL of PBS ‘A’ was pushed through the system to exsanguinate the mouse. This was then swapped for 4% paraformaldehyde (4% in PBS, Fisher Scientific), of which 30mL was pushed through the circulatory system of the exsanguinated mouse to allow for fixation of the brain tissue. Once completed, the mouse was decapitated and the brain carefully extracted. The brain tissue was fixed overnight in 4% PFA at 4°C and then left in PBS ‘A’ (also at 4°C) until needed for experiments. All other tissue was disposed of as biohazardous waste in accordance

with Imperial College London, MRC LMS, and the UK Home Office regulations for animal use and waste disposal.

3.7.2 Vibratome Slicing of Whole Mouse Brains & Slice Storage

Fixed brains were sliced coronally into 40µm slices using a vibratome (Leica VT1000 S) to isolate slices of brain with injected graft cells. To perform this slicing, the brains were solidified into blocks of agarose gel. They were glued to the slicing platform, which was placed inside a box filled with cold PBS ‘A’ inside the vibratome. The brain was kept cool throughout the slicing process by ice which surrounded the PBS-filled vibratome box.

The slices were immediately collected and placed in wells filled with 0.01% NaN₃ (in PBS ‘A’). Slices were stored at 4°C in this solution until used for immunohistochemistry.

3.7.3 Fixation of *In Vitro* Cell Cultures & Storage of Fixed *In Vitro* Cells

When fixation was required for in vitro cell cultures, the media was removed and replaced with 4% PFA for 15 minutes at room temperature (RT). The cells were washed three times with PBS ‘A’ (10 minutes per wash, on a shaker, at RT). The cells could then be used or stored (4°C) as needed.

3.8 Immunohistochemistry

In this section, one “sample” refers to either one well of *in vitro* cells on a glass coverslip, or one coronal brain slice. Both were stored in one well of a 24-well plate throughout the staining procedure.

The sample was first blocked for 1 hour at RT on a shaker (GyroMini, Labnet International) using Blocking Solution (1% Triton X [Sigma] and 5% Donkey Serum [Sigma] in PBS ‘A’).

The Blocking Solution was replaced with 300 μ L of Antibody Solution (0.1% Triton X [Sigma] and 10% Donkey Serum [Sigma] in PBS 'A') per sample, containing the primary antibodies (see Appendix, Table 1) necessary for the desired staining. The concentrations used for each primary antibody are listed in Table 1 in the Appendix. The primary antibodies (in Antibody Solution) were left in overnight at 4°C on a shaker. The next morning, the sample was washed at RT with PBS 'A' (3 x 10 minutes per wash, on shaker) and the secondary antibodies were added. The secondary antibodies were added to 300 μ L of Antibody Solution per sample well. All secondary antibodies were used at a 1:1000 dilution in the Antibody Solution and were left in the sample well at RT on the shaker for 1 hour (covered in foil to prevent light exposure). They were then washed (PBS 'A', 3 x 10 minutes/wash, on shaker), and then 300 μ L of DAPI solution (1:10,000, diluted in PBS 'A') was added (RT, on shaker, 15 minutes), before another set of three PBS 'A' washes was performed. The samples were mounted using ProlongTM Gold Antifade Mountant (InvitrogenTM, #P36930). Mounted samples were stored at 4°C in a covered box.

The only variation from this protocol consisted of coronal slices that required the use of antigen retrieval prior to staining. This was necessary only when staining with the anti-hNuMa primary antibody (rabbit), as previous experimentation in our lab had shown that antigen retrieval greatly improved the efficiency of this antibody. To perform antigen retrieval, the slice was placed in a bath of 10mM Sodium Citrate Buffer (tri-sodium-citrate [AnalaR [®]]) diluted in distilled water, pH 6.0). It was microwaved at medium heat for 10 minutes in this buffer, with pauses during that time to ensure it did not bubble over. The slice was allowed to cool at RT in 0.01% NaN₃ solution before being placed in Blocking Solution for 1 hour as stated above. The staining procedure would then continue as described above.

3.9 Confocal Imaging of Immunohistochemistry Samples

Images of mounted coronal brain slices were taken as z-stacks using an SP8 confocal microscope (Leica Microsystems). All images were taken with a 20X lens at zoom 3 or with a 40X lens

at zoom 1, matching previous confocal imaging settings for graft characterization [39]. The z-stacks were taken as 16-bit images with a format of 1024x1024 and a speed of 400Hz. All images contained multiple wavelengths being detected, therefore all z-stacks were taken using the sequential scan function. All staining combinations contained DAPI for staining of all somas (mouse and human), as well as either human nuclei (hNu) or human NuMa (hNuMa) which were used to identify the human graft cells. At least one other stain would be used in each combination to quantify cell types within the graft. We conducted stainings with the following antibodies: the marker of proliferation antigen Ki67 (Ki67), the marker of early neuronal differentiation paired box 6 (Pax6), the inhibitory interneuron marker glutamic acid decarboxylase 67 (GAD67), the excitatory deep layer (Layer VI) cortical neurons marker T-box brain transcription factor 1 (TBR1), the excitatory deep layer (Layers IV and V) cortical neuron marker chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (CTIP2), the excitatory upper layer cortical neuron marker special AT-rich sequence-binding protein homeobox 2 (SATB2), the marker of neuronal migration doublecortin (DCX), the neural stem cell/progenitor marker nestin, and the astrocyte marker glial fibrillary acidic protein (GFAP) (see Appendix Table 1).

3.10 Data Analysis

3.10.1 Immunohistochemistry: Quantification for Graft Characterization

The graft characterization work was performed by myself (Elizabeth Brockman) and two other members of the De Paola Lab as a joint effort. Dr. Maria Tortora and Dr. Shabana Khan both produced large quantities of the graft characterization samples and data. These data are included in this thesis, along with the data that I collected and analyzed myself, so that all cell types within the grafts are described accurately. This data is crucial to understanding the effects of experiments on these grafts and was necessary for multiple projects. The analysis protocols used for graft characterization and analysis were standardized.

Stained slices were imaged with an SP8 confocal microscope (Leica Microsystems) as described above (see Chapter 2-8), and images were saved as .tiff files. The .tiff files were randomized to make the analysis blinded. A Fiji (ImageJ) macro called “BatchCellPosNegIdentification” (originally created by Richard Butler, modified by Chad Wilding [MRC LMS]) was used to count cells after median filtering the images. DAPI staining was counted using the “Huang” threshold setting. hNu and hNuMa stainings were counted using the “Huang” or “Manual” threshold settings, with variation dependent on the level of background signal present in the image. These images were then reviewed manually and adjusted to account for missing or overcounted regions. All other cell stainings were counted in relation to the DAPI count, meaning that a positive hNu (or other stain) was only counted if that cell was also DAPI+. All other nuclear stainings were counted in the same manner as hNu and hNuMa, either using “Manual”, “Huang” or “Isodata” threshold settings. Non-nuclear stainings were processed by utilising the macro to count DAPI+ and hNu+ or hNuMa+ cells, then counting the non-nuclear stain (eg: DCX, hGFAP, Nestin) manually based on colocalisation with DAPI and hNu/hNuMa and signal intensity compared to negative control slices or contralateral slice regions. All samples were counted in relation to DAPI+ cells and were checked manually before the blinding was removed. In each case, the settings were adjusted to remove background, based on matched control slices.

Once the blinding was removed, total values were tallied in Excel (Microsoft). Stainings were counted as the percentage of positive cells over the total hNu+ or hNuMa+ graft cells. At least 3 slices were used for each stain with each genotype. A minimum of 35 human graft cells (hNu+ or hNuMa+) were required per animal.

3.10.2 2P Image Analysis: Synaptic Stability (EPB) Dataset

Images taken with the 2P for synaptic stability imaging were saved as .tiff files and randomized. Randomization was performed by placing the three images of the same ROI (Day 0, Day 2 and Day 4) into a folder and labelling them “1”, “2”, and “3” so that the order of imaging is still available. All ROIs were put into folders in this manner and the names of the folders were then randomized using a Python script (produced by Calum Henderson with my input).

The script was run using Anaconda (Spyder) software. The .tiff files were processed in their groups of three using MATLAB (MathWorks ®, R2014a) and a program entitled EPBscore [40], [41]. As stated previously, a threshold of 1.7X the axonal backbone intensity was used to select for boutons along the axons. EPBscore was able to select boutons from each axon as I manually added each axon into the software after the images had been median filtered. The length of each axon was also measured by the software. To decrease program error and false positives from debris, I manually removed debris with Fiji (ImageJ) prior to inserting the images into EPBscore. Outputs from EPBscore were tallied in Excel (Microsoft). Each image set was then checked manually for errors in the software (e.g.: counting the overlap of two axons as a bouton). Additionally, axon length measurements were adjusted to eliminate overlap. Once manual checks were completed, the folders were unscrambled. The Excel files containing the EPBscore outputs were used to calculate turnover rate (TOR), survival rate (Fr), bouton density (boutons/ μm of axon), and average bouton fluorescent intensities. These were compared across genotypes by animal, by axon, and by bouton. Fluorescent intensity was directly extrapolated from the EPBscore output.

TOR (see 3.1) was calculated as the total gains (nG) plus the total losses (nL) over the total number of EPBs in all sessions (N) multiplied by two. The N value included repeats across multiple sessions.

$$\text{TOR} = (nG + nL)/(2xN) \quad (3.1)$$

Fr (see 3.2) was calculated as the sum of EPBs that survive at each time point (N_x) over the total number of EPBs present in session A (N_A). N_x did not include new EPBs gained in sessions B or C.

$$\text{Fr} = N_x/N_A \quad (3.2)$$

The density of EPBs (see 3.3) was calculated as the number of EPBs in each session for one

animal/cell (N_x) over the total length of the respective axons for that animal/cell (L_x) in micrometers.

$$\text{Density} = N_x / L_x \quad (3.3)$$

3.10.3 2P Image Analysis: Spontaneous GCaMP6 Calcium Activity

Analysis of spontaneous activity was performed using Fiji (ImageJ) macros for motion correction (Moco) [247] and 3D filtering. The t-series files were converted into 8-bit .tiff files for this processing to take place. Moco motion correction was performed by overlaying the 8-bit t-series .tiff files with average projections of the tdTomato z-stack of that region. Once corrected and filtered, final analysis was performed with Fiji by selecting all somas in the tdTomato images and measuring the mean grey value of those soma ROIs in the t-series images. Each soma was measured as an ROI and compared to a background region of the same dimensions. This allowed for the data to be normalized on the background of the same image. This data was added to MATLAB (MathWorks ®, R2020a) and the change in fluorescent intensity ($\Delta F/F_0$) was calculated using the equation below (see 3.4). In this equation, “x” refers to the soma (and matching background) being analyzed at that time.

$$\Delta F/F_0_x = (Soma_x - Background_x) / (Background_x) \quad (3.4)$$

$\Delta F/F_0$ values were established for each soma at each frame of the t-series. These data were saved as MATLAB variable files, with each soma having its own file. The soma files were amalgamated, with one column representing one soma and each row representing a single t-series frame. The amalgamated $\Delta F/F_0$ files were run through the following MATLAB script developed by Dr. Samuel Barnes:

```
X=INSERTFILENAMEHERE;
thr = 30;
```

```

time = 264;

Xthr = ((X>thr)+0).*X;

Xthr(isinf(Xthr)|isnan(Xthr)) = 0;

StoreInt = trapz(Xthr);

StoreInt = StoreInt.';

S2 = size(Xthr,2);

for i = 1:S2

    [pks,locs,widths,proms] =findpeaks(Xthr(:,i));

    Freq = size(pks,1);

    Amp = mean(pks);

    StoreFreq(i,1) = Freq/time;

    StoreAmp(i,1) = Amp;

end

OUTPUT=horzcat(StoreInt,StoreFreq,StoreAmp);

clearvars -except OUTPUT

```

This script produces the following outputs: integral ($\Delta F/F_0$), frequency (Hz), and amplitude ($\Delta F/F_0$). The script applied a threshold value as a cut-off for what was deemed to be activity. Optimization analyses were run and a threshold of $30\Delta F/F_0$ was deemed optimal, as it eliminates outlying activity while keeping low-level activity.

3.10.4 2P Image Analysis: Oscillatory GCaMP6 Calcium Activity

Analysis for oscillatory activity was commenced in the same manner as spontaneous activity analysis. However, once $\Delta F/F_0$ values were calculated for each soma, the process changed. First, the “corrcoef” function in MATLAB (MathWorks [®], R2020a) was used to find correlation coefficient for the activity of each individual cell with all other individual cells in a single t-series. This analysis method looked at all frames of the t-series for each soma. It provided information on activity over time, and how much the cells were firing in unison. Second, the

activity was evaluated by frame. This was performed by using the MATLAB code below (written by Dr. Samuel Barnes) to produce an outcome of “Perc”, which detailed the percentage of somas in a t-series that were active during each individual frame. Using this second analytical method, an 800-frame t-series produced 800 outputs, each one a percentage of cells active at that specific time.

The MATLAB code for the “Perc” output, as written by Dr. Samuel Barnes, is as follows:

```
out = (traces>1.2)+0;
S1 = size(out,1);
S2 = size(out,2);
outsum = sum(out,2);
Perc = outsum./S2;
```

Furthermore, the “Perc” outputs were evaluated at varying thresholds. This was both to ensure that our selected threshold of 30 was suitable for reporting outcomes, but also to evaluate how coactivity is affected by the presence or absence of low-level activity.

I also quantified oscillatory neural network activity by extracting the Strength of correlated somas using another script from Dr. Samuel Barnes. Once the calcium activity outputs were filtered by the selected threshold ($30\Delta F/F_0$) and a moving mean of 5 was applied using the MATLAB “movmean” function, a correlation coefficient analysis was applied and only significant correlations were extracted to subsequently determine the strength by use of the MATLAB “nanmean” function and dividing by 2. This provides an average strength of significant correlations for each soma.

```
[R,P] = corrcoef(INSERTDATAFILENAMEHERE);
R = ((R>0)+0).*R;
SIGr = ((P<0.05)+0).*R;
Strength = nanmean(SIGr,2);
```

3.10.5 Correlative Analysis

TOR values from the EPBscore dataset and Integral ($\Delta F/F_0$) values from the spontaneous somatic activity dataset were paired with immunohistochemistry cell counting values from the same brain for correlative analysis. These values were calculated as described in Sections 2.10.2 and 2.10.3.

3.11 Statistics

3.11.1 Power Calculations

Power calculations were performed to determine the necessary number of animals to reach a power of 0.8 or above, given a statistical significance of 0.05. These calculations were made based on the Real *et al.* [39] dataset, as this was used as a pilot study for the work done for this thesis. These calculations were performed as *a priori* calculations using G*Power (Version 3.1.9.6) software.

For synaptic stability experiments, the outcome variable used for power calculations was TOR. Initially, power calculations were performed using preliminary data from Real *et al.* [39] data. I used a two-tailed *a priori* power calculation and determined that based on TOR data an n-value of 39 animals would be required (Mean WT = 0.30; Mean Ts21 = 0.22; SD WT = 0.057; SD Ts21 = 0.15). As this number is too high to be feasible, we decided to calculate the power using a preliminary subset of animals from the current experiment. We hypothesized that graft composition differences may have an effect, which we determined to be correct. An *a priori* test was run using the first three animals from both genotypes to reach 5mpt. The mean of Ds21 was 0.1201 (SD = 0.043) and the mean for Ts21 was 0.18 (SD = 0.014). Based on these data, an n-value of 6 animals per genotype per timepoint was required. A few additional animals are present as some animals were available from the experiments of other lab members, thus adding to the total without the need for further surgeries. The lack of significance found altered the need for power calculations in further experiments, as detailed in the next paragraph.

For calcium activity experiments, the outcome variable used for power calculations was frequency (Hz), as measured at 3-5mpt by animal. The difference in our dataset was hypothesized to be large, therefore we used the matched variables from the Real *et al.* [39] dataset with the largest degree of difference; WT-1 and Ts-1. WT-1 had a mean of 0.033 and a standard deviation (SD) of 0.026. Ts-1 had a mean of 0.011 and an SD of 0.0018. Using a two-tailed *a priori* test (t-test family) with a Laplace parental distribution (see Appendix for work describing why Laplace parental distribution was used), the needed n-value for a power of at least 0.8 was found to be 9 animals per genotype per timepoint. However, upon collection of initial activity and EPB data the hypothesis changed. As no significant difference was the new hypothesis, power calculations were not performed.

For correlative analyses, a post-hoc power calculation was performed on the significant TOR and hGFAP+ number correlation from Figure 4.32. Using the obtained r-value of 0.9968 and an n-value of 3, I determined that the power is 0.89. This indicates that the correlation is sufficiently powered.

3.11.2 Other Statistical Analyses

Statistical analyses for all other experiments (excluding power calculations) were performed in Prism (GraphPad ©). In all cases, descriptive statistics and Shapiro-Wilk normality tests were run using GraphPad to determine optimal test type (e.g.: parametric, non-parametric). An appropriate test was selected in Prism based on normality and number of experimental variables (test types stated in Results sections for each experiment). For parametric data, unpaired parametric t-tests with Welch's correction or 1-way parametric ANOVAs with Tukey's multiple comparisons are used. For non-parametric datasets, either unpaired non-parametric t-tests (Kolmogorov-Smirnov or Mann-Whitney), or 1 or 2-way ANOVA with multiple comparisons (Dunn's for 1-way, Dunnett for 2-way) were used. Curve comparison for percentage of coactivity data across various thresholds was evaluated using simple linear regression. Correlative analyses were performed using Pearson's correlation. All reported values within the results sections are based on calculations performed in Excel and Prism. Statistical significance was set at 0.05 in

all cases.

Chapter 4

Results

4.1 Graft Characterization & Graft Size

The first two aims of this project were to develop cortical neuronal progenitor grafts using a differentiation protocol which minimizes experimenter bias and to confirm that this protocol for cell differentiation creates grafts that are functional *in vivo* and develop into cortical excitatory pyramidal neurons and neural progenitors. As stated in the Methods section, I used a method developed by Dean Nizetic's Lab at Queen Mary University of London to produce the novel graft type. This protocol reduces experimenter bias as it contains precise timing instructions and requires only the changing of media. Other protocols, such as the Shi *et al.* [85] protocol, require many additional steps that are based on the appearance of developing cells, therefore introducing variability from one experimenter to the next. I hypothesized that the use of the Nizetic Protocol, over other protocols previously used in my group, would decrease experimenter bias and be more easily reproducible long-term.

Characterization of the graft cells after 5 months of *in vitro* development was required to check if my initial aims had been met and the grafts had developed as expected, demonstrating good proliferation with a range of cortical cell types. Mouse brains containing graft were perfused following the final imaging session and were vibratome sliced into 40 μ m thick slices. Immunohistochemistry was performed on slices containing graft to characterize the abundance of cell

types within. The figure below (Figure 4.1) outlines the cell types present in ~ 5 mpt grafts formed from the injection of 20,000 cells into the primary somatosensory cortex. The average graft age at time of perfusion for the grafts used in these analyses is 5.44mpt for Ds21 and 5.0mpt for Ts21. The following cell types were positively identified in the grafts of both genotypes (n=3-5 animals/genotype/staining): paired box protein Pax6 (Pax6), antigen Ki67 (Ki67), special AT-rich sequence-binding protein 2 (SATB2), COUP-TF interacting protein 2 (CTIP2), T-box brain transcription factor 1 (TBR1), human glial fibrillary acidic protein (hGFAP), neuroepithelial stem cell protein (Nestin), and doublecortin (DCX). This indicates the presence of neuronal stem cells (Nestin+), neuronal progenitor cells (Pax6+), migrating neuronal progenitors (DCX+) and dividing neuronal progenitors (Ki67+). These are not mutually exclusive. Additionally, the grafts include mature excitatory neurons from cortical layer VI (TBR1+), cortical layer V (CTIP2+), and cortical layers II/III (SATB2+). Human astrocytes are also present as indicated by hGFAP+ cells. The mean percentage of positive graft cells for each genotype with their standard error of difference (SEd) are as follows: Ki67+: Ds21 mean = 4.99, Ts21 mean = 1.88, SEd = 4.06; Pax6+: Ds21 mean = 25.42, Ts21 mean = 38.92, SEd = 19.59; SATB2+: Ds21 mean = 25.42, Ts21 mean = 38.92, SEd = 10.93; CTIP2+: Ds21 mean = 6.57, Ts21 mean = 1.68, SEd = 3.33; TBR1+: Ds21 mean = 9.80, Ts21 mean = 25.79, SEd = 14.12; hGFAP+: Ds21 mean = 24.88, Ts21 mean = 34.14, SEd = 17.42; DCX+: Ds21 mean = 4.61, Ts21 mean = 6.82, SEd = 4.17; Nestin+: Ds21 mean = 6.95, Ts21 mean = 1.50, SEd = 3.07. No glutamic acid decarboxylase 67 (GAD67) positive inhibitory interneurons were found in either genotype.

Here, I have included multiple example images taken with a confocal microscope to highlight the graft characterization outcomes. Figure 4.2 depicts Ki67+ and Pax6+ staining in mouse brain slices containing human iPSC-derived graft cells. The other two progenitor cell stainings (DCX and Nestin) are represented by the example image in Figure 4.3. Excitatory layer-specific neuronal subtypes are all represented by example confocal images in Figure 4.4. Specifically, the figure depicts positive staining for upper layer excitatory neurons (SATB2+), and deep layer excitatory neurons (CTIP2+, TBR1+), as labelled. Finally, Figure 4.5 provides a representative image for human astrocytes (hGFAP+) within the graft.

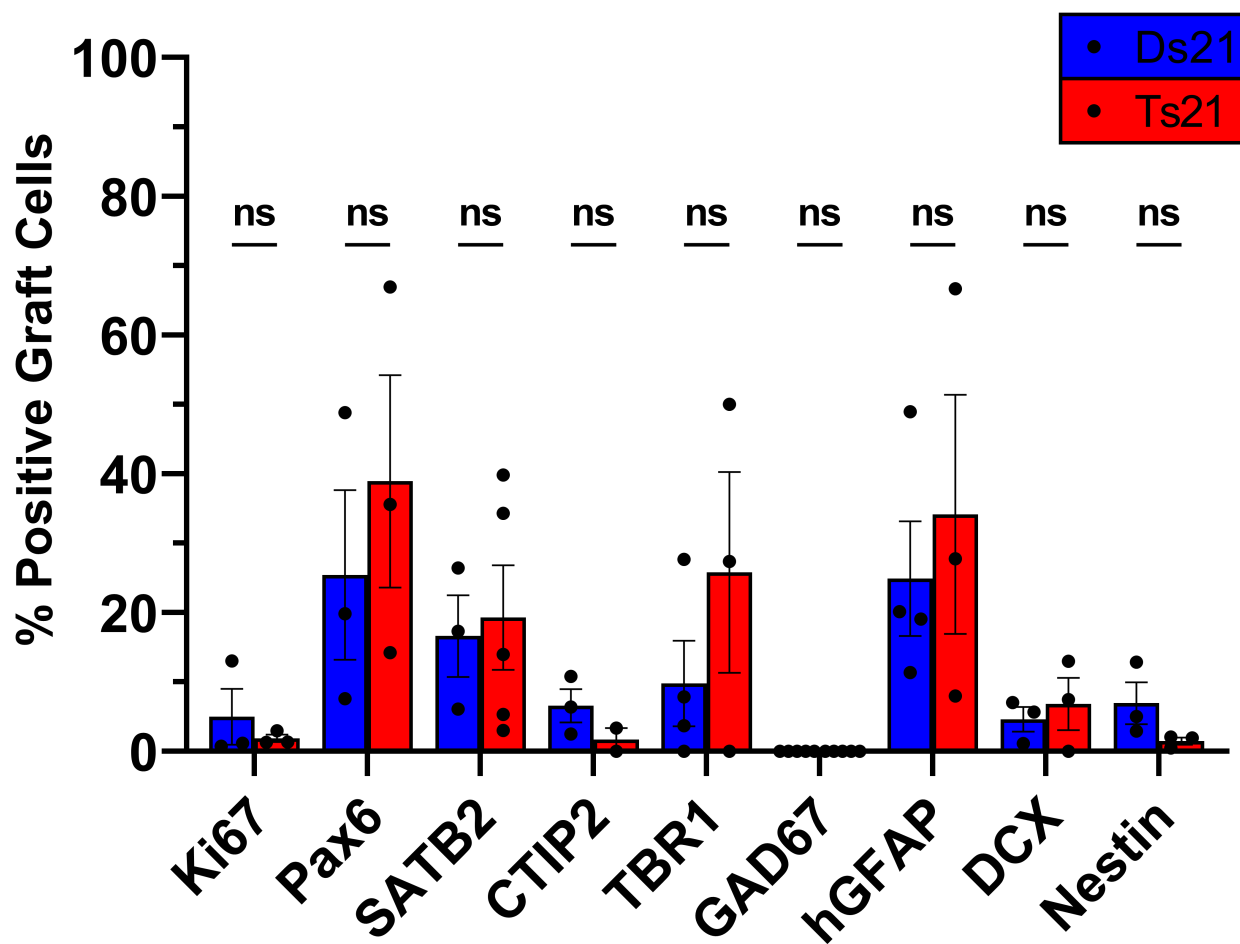


Figure 4.1: Characterization of cell type distributions within Ds21 and Ts21 grafts with 20,000 cell injections. Groups are mixed sex. Shapiro-Wilk normality test indicated that these data are normally distributed. Statistical analysis was performed using multiple unpaired (parametric) t-tests, one for each of the stainings, to check for differences between genotypes. No significant difference was found between Ds21 and Ts21 for any staining group (ns). P-values and mean values are as follows: Ki67: $p=0.49$, meanDs21=4.99, meanTs21=1.88; Pax6: $p=0.53$, meanDs21=25.42, meanTs21=38.92; SATB2: $p=0.81$, meanDs21=16.61, meanTs21=19.30; CTIP2: $p=0.24$, meanDs21=6.57, meanTs21=1.68; TBR1: $p=0.31$, meanDs21=9.80, meanTs21=25.79; hGFAP: $p=0.62$, meanDs21=24.88, meanTs21=34.14; DCX: $p=0.62$, meanDs21=4.61, meanTs21=6.82; Nestin: $p=0.15$, meanDs21=6.95, meanTs21=1.50.

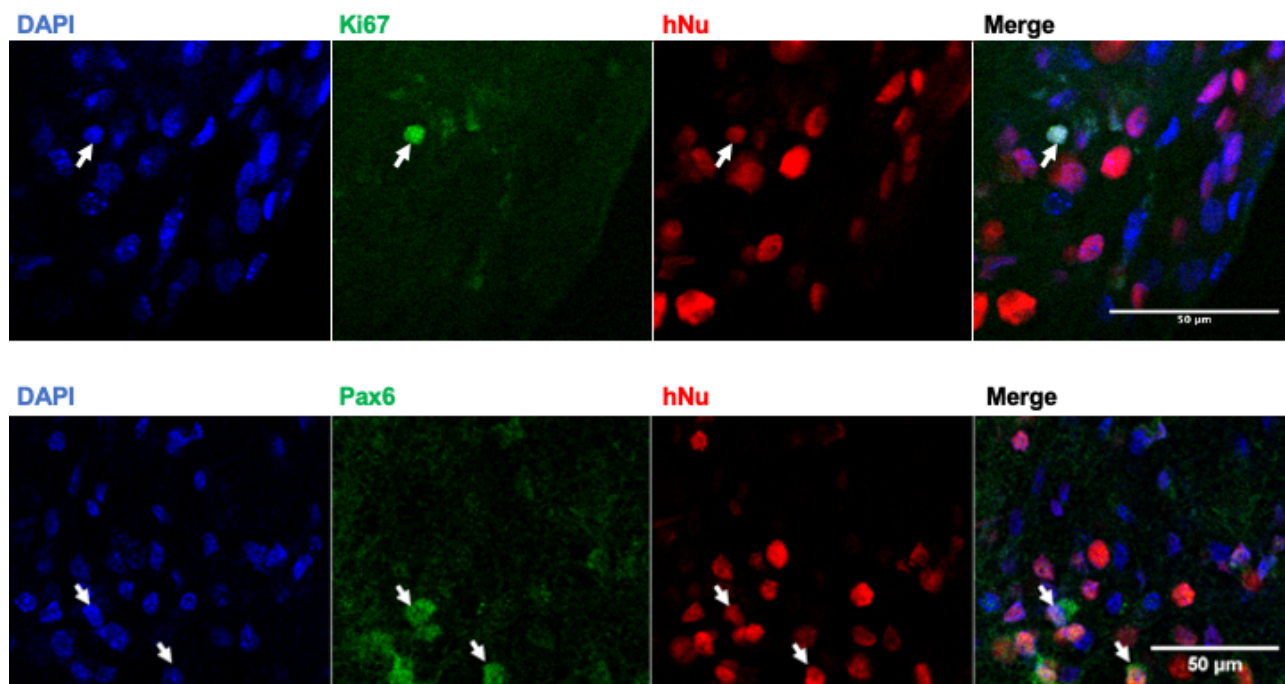


Figure 4.2: Representative confocal images of human grafts stained *ex vivo* for Ki67+ cells (top; green; Ds21 graft) and Pax6+ cells (bottom; green; Ts21 graft). In both cases, hNu (red) and DAPI (blue) stainings are present in the same slice. The right-hand side of each staining depicts the merged image. A scalebar depicting a length of 50 μm is present at the bottom right of both images. White arrows indicate the locations of representative cells that are positive for both hNu and the other stain in question. Note that the Pax6+ image was taken at a higher zoom than the Ki67+ image.

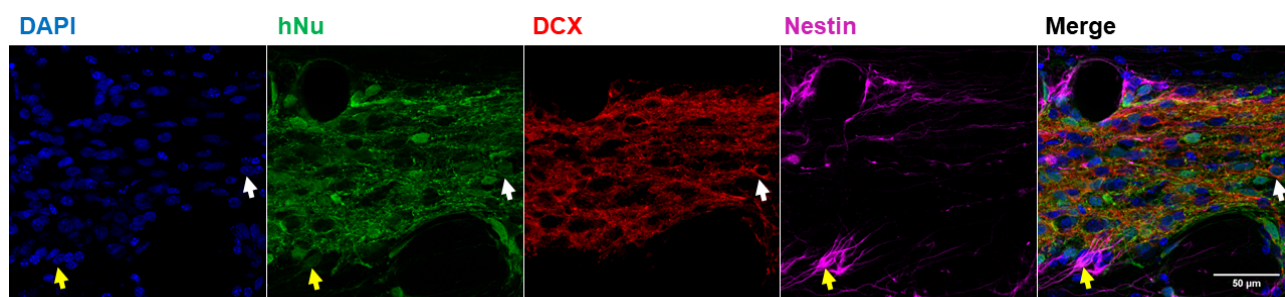


Figure 4.3: Representative confocal images of a Ds21 human graft stained *ex vivo* for DCX+ cells (red) and Nestin+ cells (pink). Both stainings were performed in the same slice, along with DAPI (blue) and hNu (green). The right-hand side depicts the merged image. White arrows indicate the location of a representative DCX+ and hNu+ cell. Yellow arrows indicate the location of a representative Nestin+ and hNu+ cell. Importantly, hNu staining (green) appears to be present in the cell processes as well as the nuclei. This is due to GFP+ stain from the LV, which is at the same wavelength.

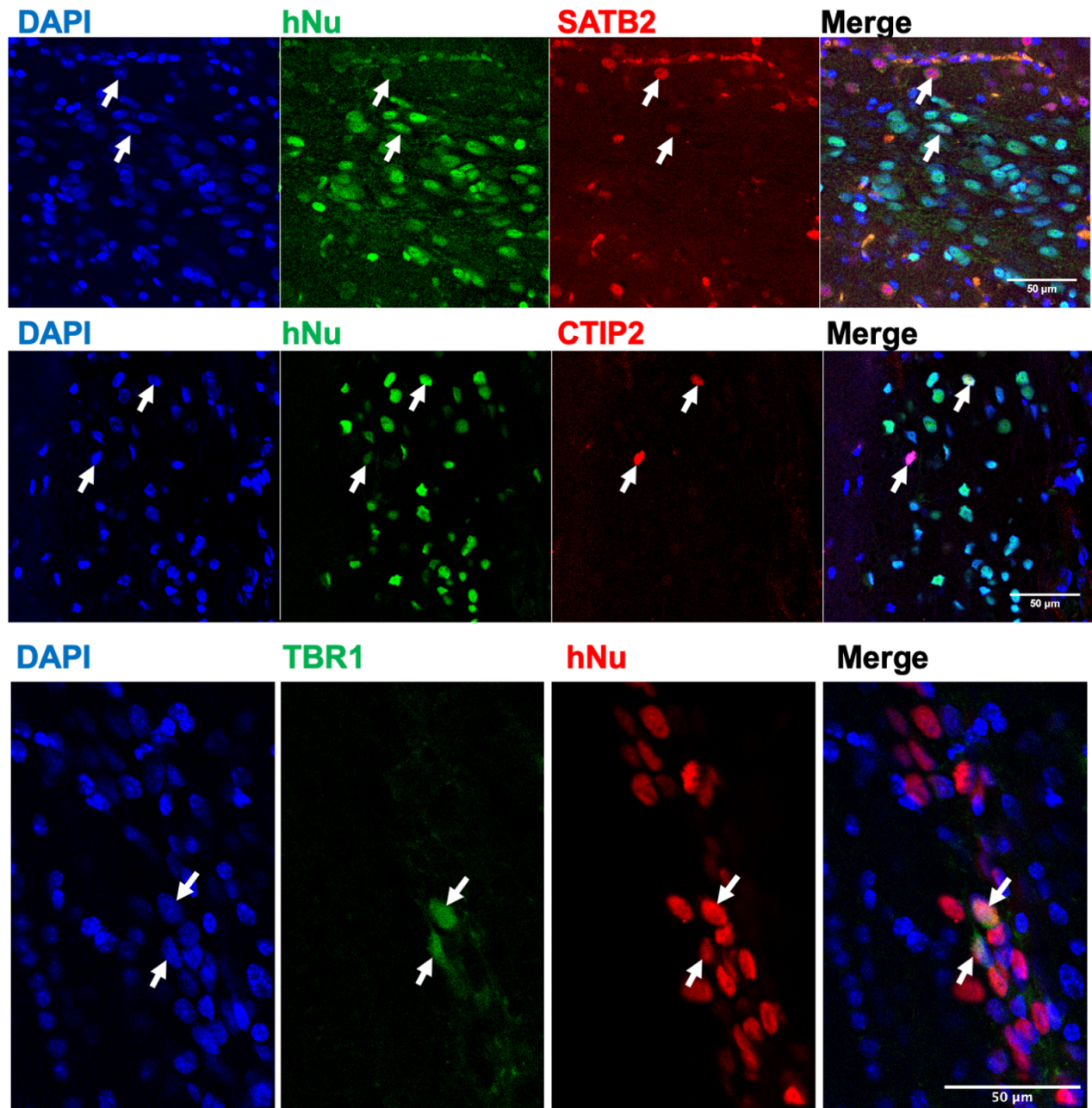


Figure 4.4: Representative confocal images of human graft slices stained *ex vivo* for excitatory neuronal layer-specific subtypes. The right-hand side depicts the merged image. White arrows indicate the location of representative hNu+ cells that are also positive for the cortical excitatory neuron stain in question. Top: SATB2+ staining (red) with DAPI (blue) and hNu (green) in a Ts21 slice. Middle: CTIP2+ staining (red) with DAPI (blue) and hNu (green) in a Ds21 slice. Bottom: TBR1+ staining (green) with DAPI (blue) and hNu (red) in a Ds21 slice.

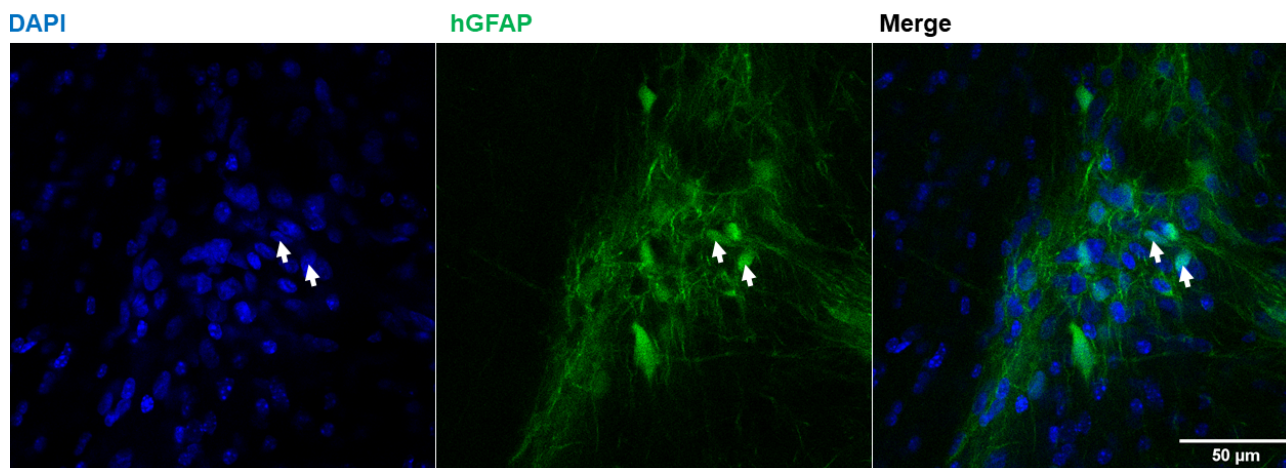


Figure 4.5: Representative confocal images of a Ds21 human graft stained *ex vivo* for human GFAP+ astrocytes (hGFAP+; green) and DAPI (blue). The right-hand side depicts the merged image. White arrows indicate the location of representative hGFAP+ cells that colocalize with DAPI.

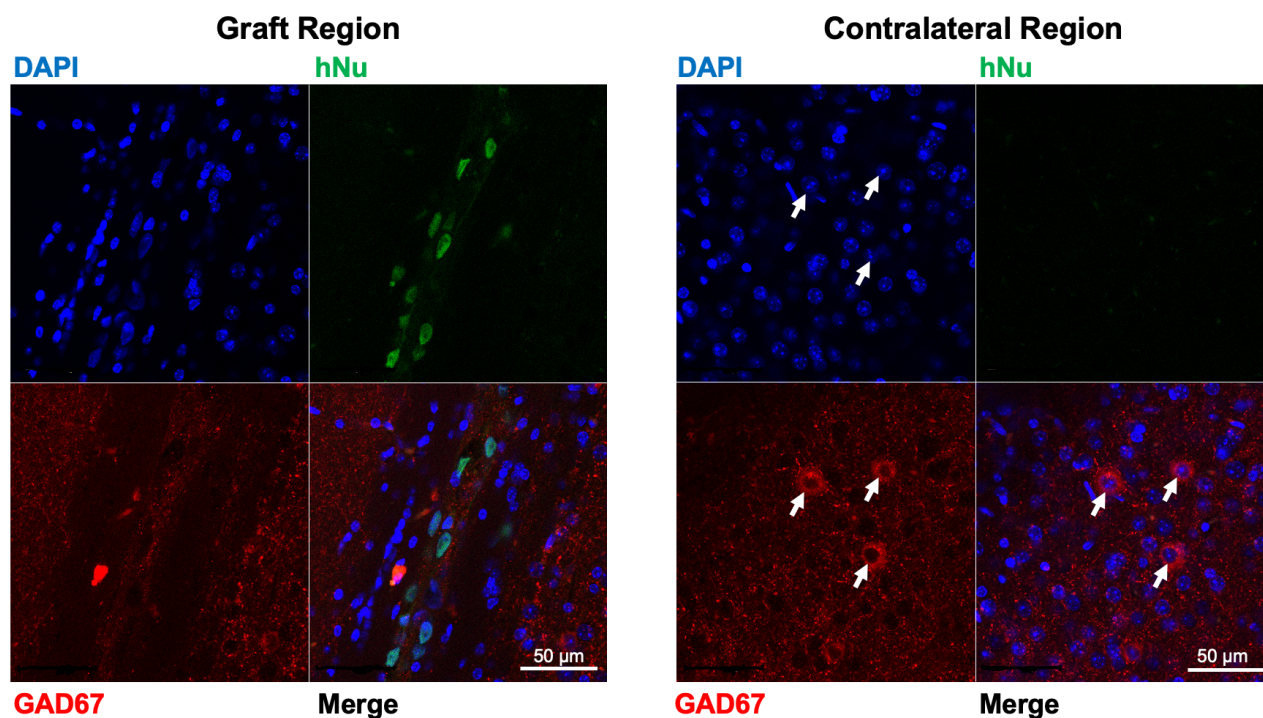


Figure 4.6: Representative confocal images of a Ds21 human graft stained *ex vivo* for GAD67+ inhibitory neurons (red), hNu (green) and DAPI (blue). Scalebars are present at the bottom right. Left: Image of the graft region of the slice. hNu+ cells are visible, but no GAD67+ cells colocalise with them. Note that the bright red mark is debris, as it does not follow the correct staining configuration as GAD67+ (hole in the center, somatic but not nuclear stain). Right: Image of the contralateral region of the slice containing no hNu+ graft cells but GAD67+ inhibitory mouse cells. White arrows indicate the location of GAD67+ mouse cells (no hNu+ cells present).

These results indicated that there is no significant difference in any cell type amount between Ds21 and Ts21 grafts at 5mpt. These grafts did not develop inhibitory interneurons, as can be seen in Figure 4.6. This absence of inhibitory interneurons is likely a result of altered development after injection of the cells into the mouse brain, as the Nizetic group have demonstrated the presence of inhibitory interneurons when growth is continued *in vitro*. Nonetheless, the normalization of cell types and the presence of a wide range of cell types in both genotypes (neuronal progenitors, dividing cells, upper layer excitatory neurons, and deep layer excitatory neurons) made this graft type both novel and relevant to the study of DS neurodevelopment. Given that this graft meets the aims of being functional, being able to be imaged longitudinally, and producing cortical excitatory pyramidal neurons with a protocol that decreases experimenter bias, I chose to proceed with this graft type and investigate synaptic structure stability and spontaneous and oscillatory activity.

In summary, a novel graft type was developed using an altered differentiation protocol (called the Nizetic Protocol). Five months after injection into the mouse brain, these grafts were characterized by immunohistochemistry of fixed slices. Characterization indicates that grafts are composed of neuronal progenitors, dividing progenitors, proliferating progenitors, astrocytes, upper layer excitatory cortical neurons, and deep layer excitatory cortical neurons. No inhibitory interneurons were found. No significant differences in the amount of each cell type across the genotypes was found, indicating normalization of amounts of each cell type across genotypes. Overall, the xenotransplantation of grafts produced using the Nizetic differentiation protocol was successful, creating grafts that could be imaged and represent a range of cortical excitatory neuronal subtypes. The next step in the project was to evaluate how the new differentiation protocol changed proliferation. One way in which this can be done is by examining the size of current grafts compared to previous grafts.

Graft size was evaluated as a method of investigating differences between current and past (Real *et al.* [39]) grafts and their differentiation protocols. This method of comparison was selected as it is indicative of the level of cell proliferation. Furthermore, a substantial change in graft size compared to previous work was visible by eye upon inspection of perfused graft slices. Change to graft size was not expected at a significant level, but after initial inspection

it became clear that this was the case. As such, I wanted to quantify the level of size change and attempt to narrow down the underlying causes of this change.

The size of grafts was compared by genotype and by total number of cells injected. Two different amounts of cells were injected. The reason for this was to investigate whether graft size affects measured outcomes (eg: EPB TOR, calcium activity) and to see if increasing the number of injected cells could make the graft size more comparable to those from the Real *et al.* paper. Importantly, the injection volume was constant at $1\mu\text{L}$ per animal, only the number of cells changed. These were also compared to a representative control graft from the Real *et al.* [39] work (Graft ID: AT60), in order to evaluate how much altering the differentiation protocol or iPSC line affected graft size. Figure 4.7 depicts representative 20X slide scanner images of fixed and stained brain slices containing graft. The slice from the Ds21 control brain AT60 from Real *et al.* [39] is included at the top. The middle images depict a Ds21 (left) and a Ts21 (right) slice containing grafts made with the Nizetic (current) protocol with an injection of 20,000 cells (in $1\mu\text{L}$). The bottom images depict a Ds21 (left) and a Ts21 (right) slice containing grafts made with the Nizetic (current) protocol with an injection of 100,000 cells (in $1\mu\text{L}$).

Figure 4.8 depicts the measured size of AT60 in relation to current Ds21 and Ts21 grafts at both 20,000 cell and 100,000 cell injections. A 2-way ANOVA with Dunnett's multiple comparisons was used to compare all factors to AT60. The ANOVA output was significant ($p < 0.0001$) and multiple comparisons indicate significant differences ($p < 0.0001$) between AT60 and all other groups. This means that the size differences visible by eye upon inspection are statistically significant as expected.

Figure 4.9 shows the same data as Figure 4.8 but without AT60 so that current graft sizes can be visualized and statistically analyzed. It is important to note that all points in a single column come from the same brain and that all graft slices from these reference brains are included. Separate unpaired non-parametric t-tests (Kolmogorov-Smirnov) were used to evaluate any differences between cell injection volumes within each genotype. A significant difference ($p = 0.005$) was found between 20,000 cell injection and 100,000 cell injection graft sizes in Ds21

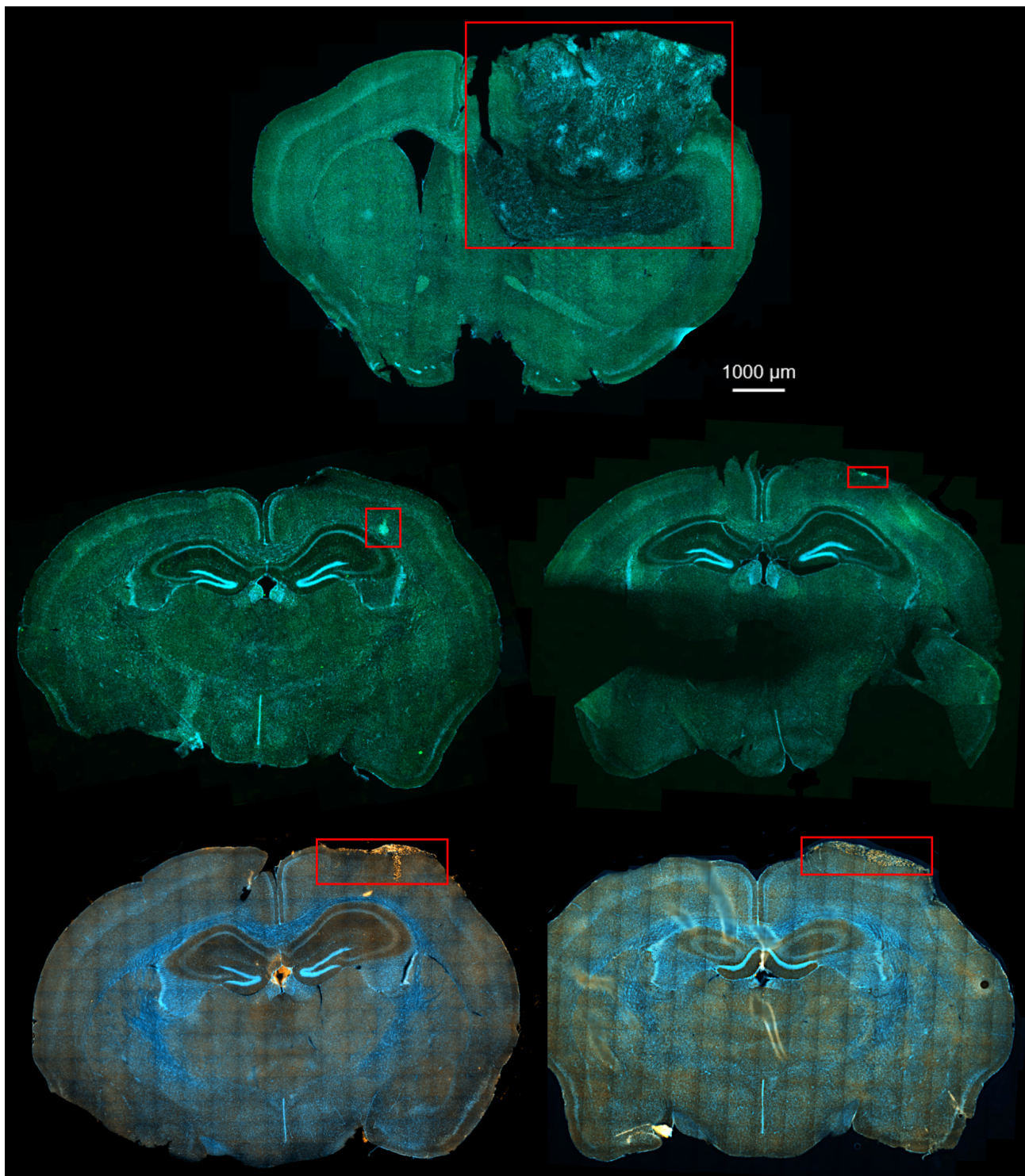


Figure 4.7: Representative slide scanner images of stained $40\mu\text{m}$ thick coronal brain slices containing human grafts of both genotypes taken at 20X. Graft regions are contained within the red boxes. A scalebar is placed at the right bottom of the top image, representing $1000\mu\text{m}$. This scalebar is indicative of the size of all brain slices in the image. Top: A representative slice from the Ds21 control graft AT60 used in the Real *et al.* [39] Science paper. Stained with DAPI+ (blue) and hNu+ (green). Graft area appears brighter. Middle: Representative slices containing grafts using the current (Nizetic) protocol and an injection of 20,000 cells. These are stained for DAPI+ (blue) and hNu+ (green). Small bright green graft regions are visible in the cortex on the right side of both slices. Left: Ds21 graft slice; Right: Ts21 graft slice. Bottom: Representative slices containing grafts using the current (Nizetic) protocol and an injection of 100,000 cells. Slices are stained for DAPI+ (blue) and hNu+ (orange). Graft regions are visible in bright orange in the top right cortical region of both slices. Left: Ds21 graft slice; Right: Ts21 graft slice.

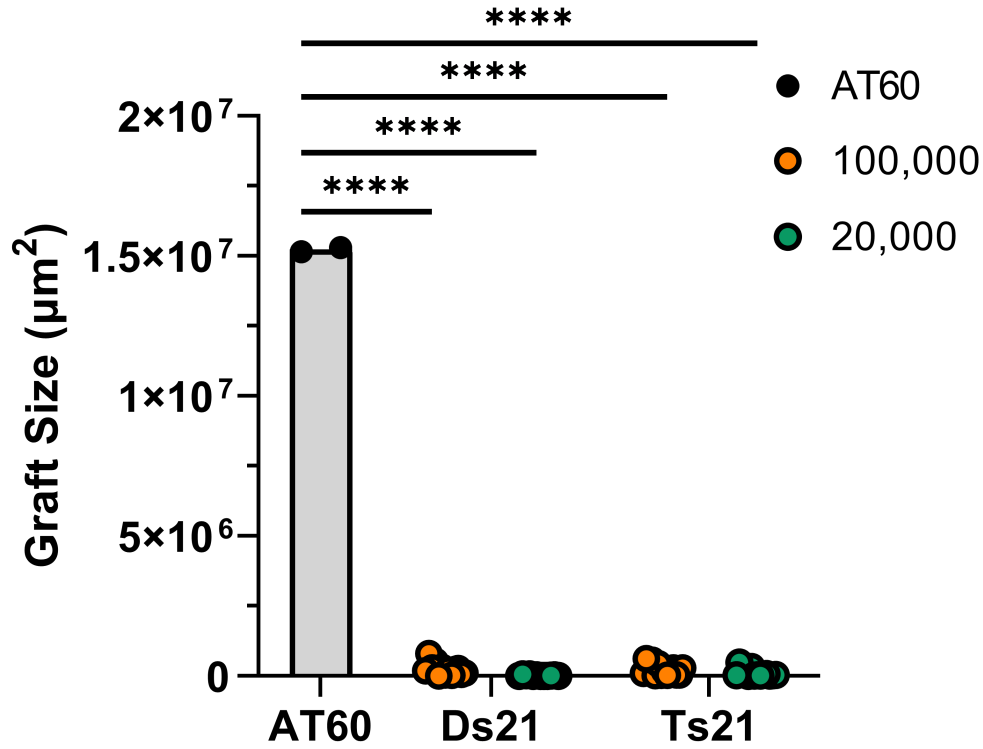


Figure 4.8: Size of current Ds21 and Ts21 grafts (in cubic micrometers) with injections of either 20,000 cells (green) or 100,000 cells (orange) compared to central large slices from a Ds21 brain called AT60, which utilized cells differentiated with the Shi *et al.*, 2012 protocol [85] and was used for the Real *et al.*, 2018 Science publication [39]. Statistical analyses were performed, but it should be noted that they are only a possible indication of size differences, given that each point represents one slice from the same brain, hence each group actually has an n-value of 1. Shapiro-Wilk normality test indicated that these data are not normally distributed (non-parametric). Statistical analysis was performed using 2-way ANOVA with a column mean multiple comparison Dunnett test, comparing all groups against AT60 as the control. Overall column factor was significant ($p < 0.0001$) and Dunnett's multiple comparisons indicated significant differences between AT60 and all other groups. **** $p < 0.0001$.

grafts, but no significant difference was found in graft size between the two Ts21 cell injection volumes ($p=0.15$).

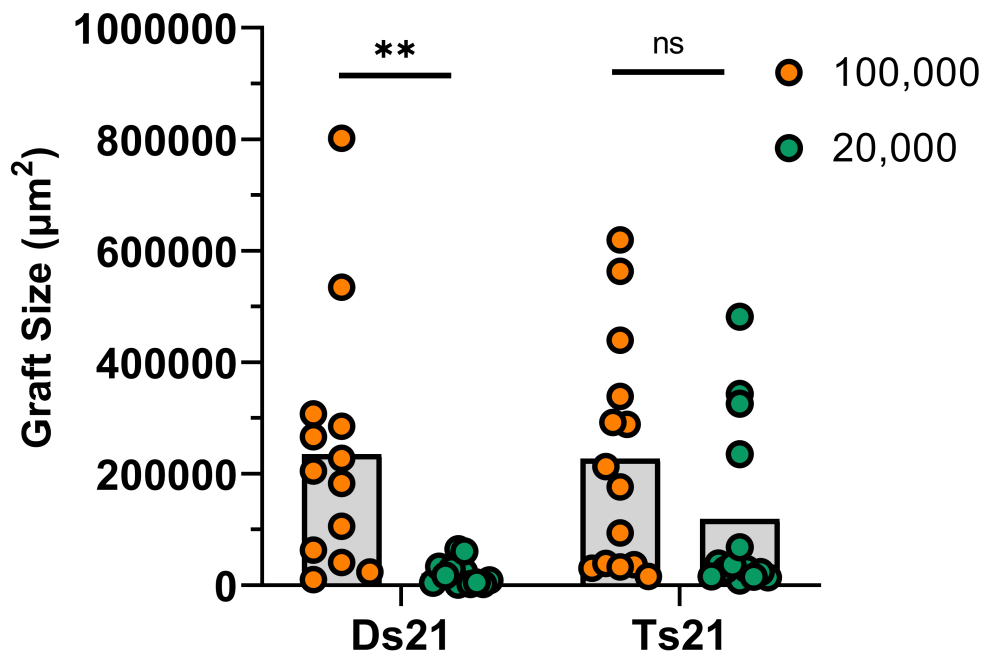


Figure 4.9: Comparison of size (in cubic micrometers) of 20,000 cell injection grafts versus 100,000 cell injection grafts in both Ds21 and Ts21. In this graph, each point represents the size of one slice from an animal. All Ds21 slices come from the same animal, and all Ts21 slices come from the same animal. Statistical analyses were performed as a general indication of variation in size, despite an $n=1$ animal per group sample size. Shapiro-Wilk normality test indicated that these data are not normally distributed (non-parametric). Statistical analyses were performed using separate unpaired Kolmogorov-Smirnov tests (non-parametric) for Ds21 and Ts21. Ds21 comparisons were found to be significantly different ($p=0.005$). Ts21 comparisons were not found to be significantly different ($p=0.15$).

Genotypic changes were also compared between injection volumes of the same size (see Figure 4.10). In grafts with 20,000 cells injected and 100,000 cells injected, there is no significant difference in size between Ds21 and Ts21 grafts ($p=0.19$; $p=0.94$).

Comparisons of the size of current grafts indicates that there is no significant difference in graft size between genotypes when the same amount of cells was injected (see Figure 4.10). When a higher number of cells was injected (100,000 cells compared to 20,000 cells, both in $1\mu\text{L}$ total volume) the size of the graft was increased (see Figure 4.9).

In summary, the new differentiation protocol used for current grafts has created grafts that are significantly smaller than previous grafts from Real *et al.* [39]. The size of grafts is consistent

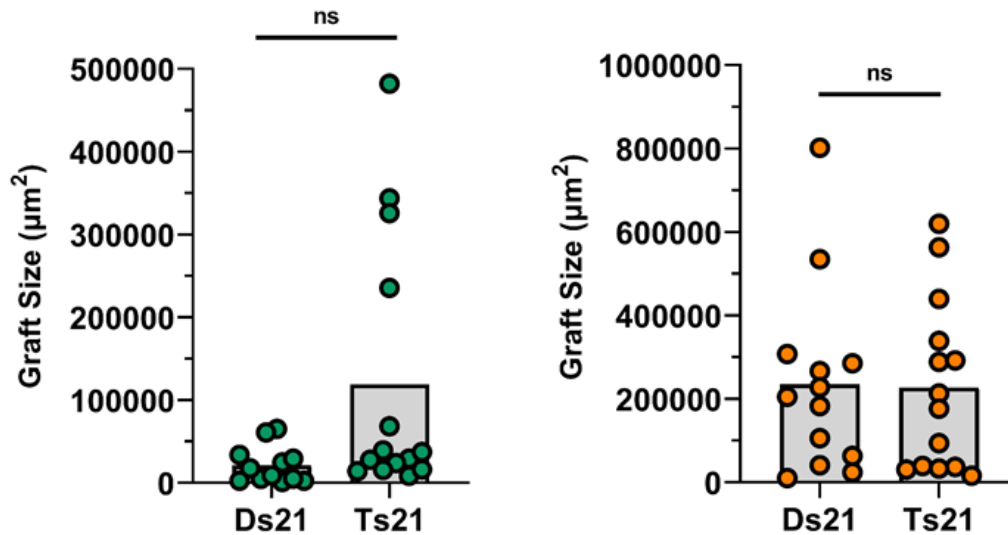


Figure 4.10: Comparison of Ds21 and Ts21 20,000 (left; green) and 100,000 (right; orange) cell injection grafts by size (in cubic micrometers). In this graph, each point represents the size of one slice from an animal. All Ds21 slices come from the same animal, and all Ts21 slices come from the same animal. Therefore, all groups have $n=1$ animal. Statistical analyses were performed regardless to provide a general idea of size variation. Shapiro-Wilk normality tests indicated that these data are not normally distributed (non-parametric). Statistical analyses were performed using a non-parametric unpaired Kolmogorov-Smirnov test, which found no significant difference ($p=0.19$ (left); $p=0.94$ (right)).

across both genotypes (see Figure 4.9) and increases when a greater number of cells is injected (see Figure 4.10). However, even with 100,000 cells injected, the size of current grafts is smaller than previous grafts (see Figure 4.7) by approximately two factors of ten (see Figure 4.8). This size change was not initially expected, but is important as it indicates a lower level of cell proliferation post-injection and is likely related to synaptic stability and calcium activity differences compared to Real *et al.* [39], which will be expanded on in the following sections.

4.2 Synaptic Structural Stability of Axons

Characterization and size analysis of the new grafts provided the baseline information upon which to proceed with the next project aims. Namely, the synaptic structural stability aim (Aim 3). My goal was to evaluate how these new grafts would differ in synaptic structural stability between Ts21 and isogenic controls. I also compared my findings to previously published work using a different cell differentiation protocol and graft type [39]. My initial hypotheses had

been that a significant difference in synaptic structural stability would be present in a similar degree to previous grafts, presenting as an increased EPB survival rate (Fr) and a decreased turnover rate (TOR). However, the changes in graft size and cell distribution made it more likely that these grafts would behave differently, altering my hypothesis to an expectation of minimal or no difference across genotypes. In order to determine whether this was the case, I utilised multiphoton imaging to investigate axonal EPB stability.

Synaptic structural stability of axons was determined using images of axonal EPBs at 3mpt and 5mpt in Ds21 and Ts21 grafts. Figure 4.11 depicts an axon from a 5mpt Ts21 graft imaged three times over the course of five days. Most EPBs are stable and can be seen clearly in all three images (see blue arrows in Figure 4.11). However, examples of EPB gain (see green arrow) and loss (see red arrow) are also visible. The amount of gains, losses, and stable EPBs were quantified and are shown in the remainder of this section.

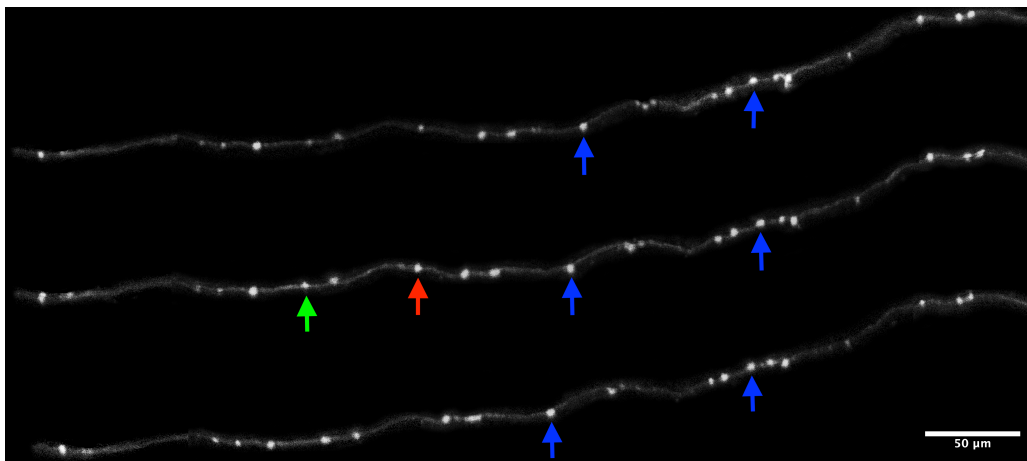


Figure 4.11: A representative set of 2-photon images of an axon with EPBs at 5mpt from a Ts21 graft. The figure depicts the same length of axon imaged three times: at Day 0 (top), at Day 2 (middle), and at Day 4 (bottom). These images have been filtered and background has been removed. The green arrow marks an example of a gain, where the bit resembling an EPB at that region in the previous day did not meet the criteria (1.7X the backbone intensity) to be classified as an EPB. The red arrow marks an example of a lost EPB, as in the image following no EPB is present at that location. The blue arrows mark the locations of two examples of stable EPBs.

Before analyzing EPB TOR and Fr outputs, I wanted to be sure that the Ds21 and Ts21 data was comparable. One way in which they may differ is the EPB density on the axons selected for imaging. Axons were selected at random and were imaged if boutons were present. Significant changes to the density of EPBs on the selected axons would indicate a difference in

their maturity level. Any significant maturity differences across genotypes would make them less comparable. For this reason, I quantified the density of imaged axonal EPBs in Ds21 and Ts21 grafts at 3mpt and 5mpt. I expected no significant difference in EPB density. Figure 4.12 presents these data both per animal (top) and per axon (bottom). For both timepoints per animal, statistical differences were evaluated using unpaired t-tests with Welch's correction. No significant differences were found between Ds21 and Ts21 EPB density at Day 0 ($p=0.23$), Day 2 ($p=0.51$), or Day 4 ($p=0.53$) of the 3mpt dataset. At 5mpt, Day 2 densities were found to be not statistically different ($p=0.08$), but Days 0 and 4 were found to have significant differences ($p=0.001$ and $p=0.03$, respectively). In both cases, Ts21 animals had significantly higher EPB densities. The same dataset evaluated by axon was analyzed using unpaired t-tests (Kolmogorov-Smirnov). No significant differences were found at any imaging Day for 3mpt ($p=0.10, 0.74, 0.17$, respectively). The only day of imaging with significant difference in EPB density at 5mpt was Day 0 ($p=0.04$, while Days 2 and 4 were not statistically significant (both $p=0.09$). The significant difference at 5mpt indicated a higher EPB density in Ts21 axons. An additional analysis was performed to expand on the statistical significance found and to determine its relevance.

Since significant differences were found in the EPB density dataset above, I wanted to analyze the data in an additional way to have a better idea of whether the maturity of the cells would be affected. I combined the EPB density data by animal for all three days of imaging (see Figure 4.13) and evaluated density averages. A one-way ANOVA with multiple comparisons was performed to test for significant differences across Ds21 and Ts21 datasets at both 3mpt and 5mpt, including changes from 3mpt to 5mpt within each genotype to check for axonal maturation. Figure 4.13 depicts these findings and the statistical analyses performed on them.

These data indicate that there is no significant difference in EPB density at 3mpt, thus the selected and imaged axons at 3mpt are at comparable levels of maturity. EPB density does, however, increase significantly in Ts21 axons at 5mpt compared to Ds21 axons at the same timepoint and at earlier timepoints. This fits with density data per day from Figure 4.12 where significant differences were found in Days 0 and 4 of imaging per animal and Day 0 of imaging per axon. This indicates that EPB density may be a confounding factor at 5mpt, but that

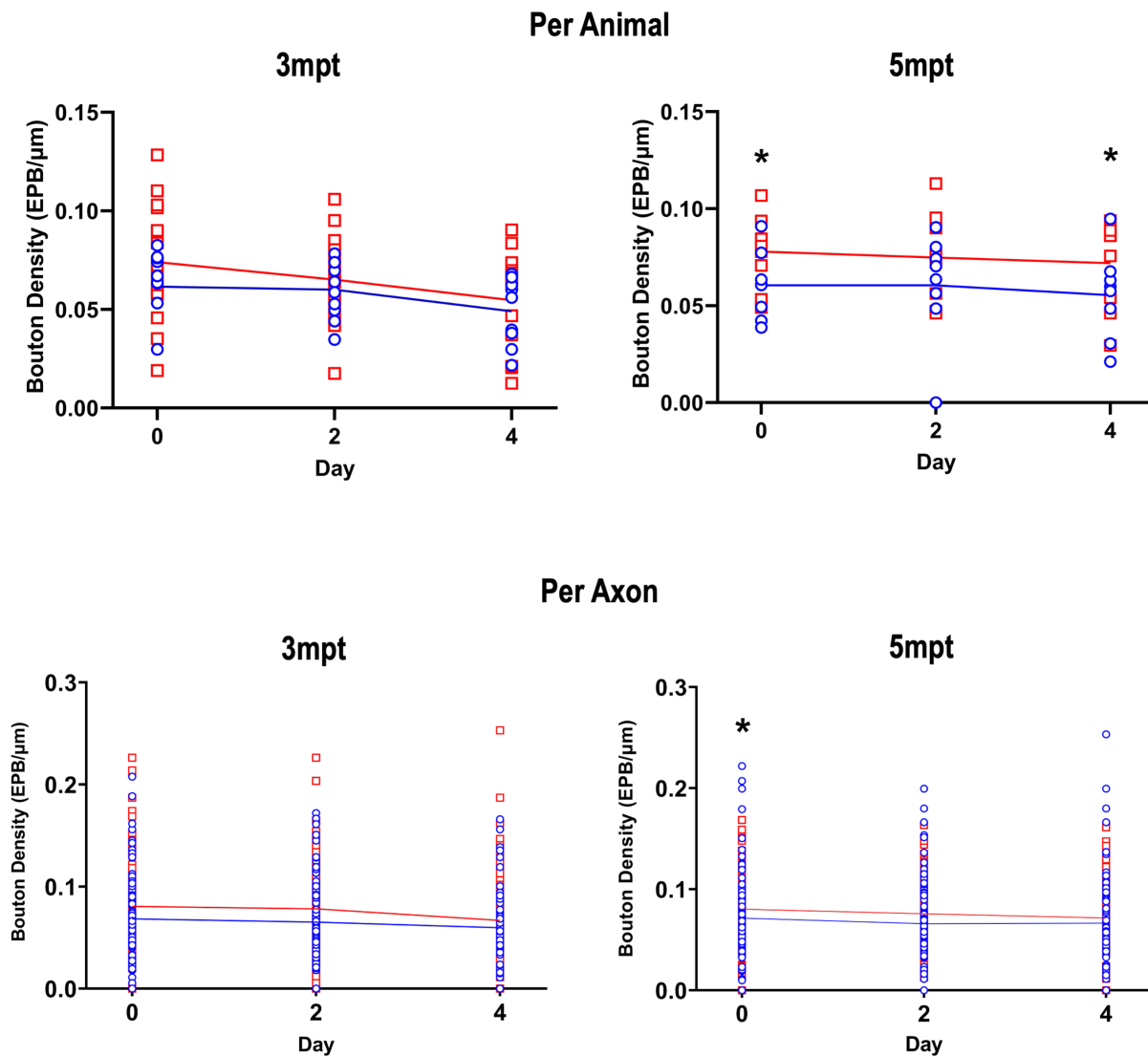


Figure 4.12: The density of axonal en-passant boutons as a measure of boutons per micrometer in grafts at 3mpt and 5mpt. Density is evaluated at each of the 3 imaging session days (Days 0, 2, and 4). Blue circles = Ds21; red squares = Ts21. Shapiro-Wilk normality tests indicate that all per animal data are normally distributed, but per axon data is not normally distributed. Per animal data was analyzed using unpaired parametric t-tests with Welch's correction. All 3mpt per animal days were not significantly different ($p=0.23$, 0.51 , 0.53 respectively). Significant differences were found between genotypes at 5mpt comparing per animal at Day 0 ($p=0.001$) and Day 4 ($p=0.03$), but not at Day 2 ($p=0.08$). When comparing per axon, non-parametric unpaired t-tests (Kolmogorov-Smirnov) were used. All 3mpt Days were not significantly different ($p=0.10$, 0.74 , 0.17 respectively). Only Day 0 from 5mpt was significantly different ($p=0.04$), while Days 2 and 4 were not (both are $p=0.09$). Top: Each point represents one animal. Bottom: Same dataset as the top, but with each point representing one axon. Error bars indicate SEM. * $p<0.05$.

axons are comparable at 3mpt. However, a significant difference in EPB density at 5mpt does not necessarily mean that these axons are at significantly different maturity levels. To evaluate this further, I decided to look into the strength of the synaptic connections being made at the

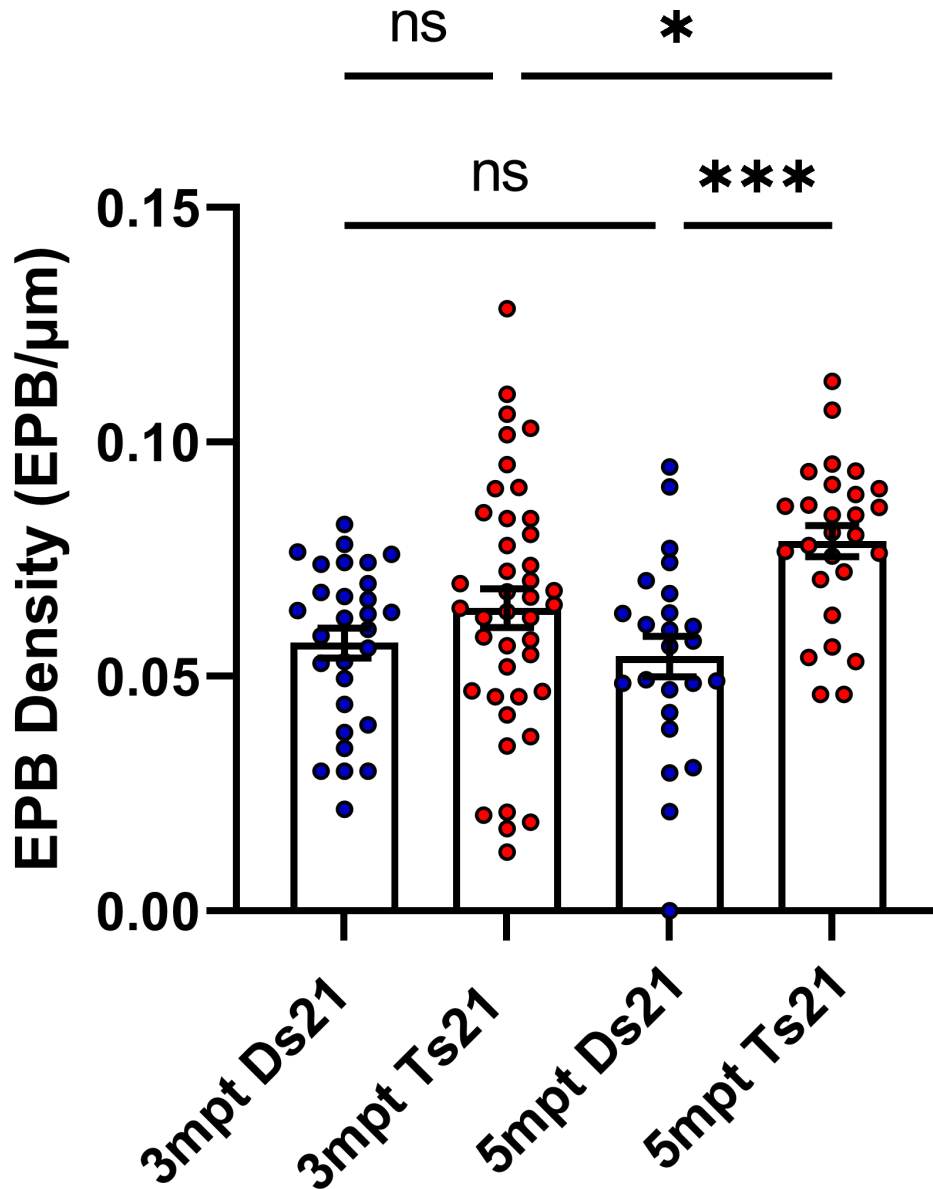


Figure 4.13: Measurement of density of axonal en-passant boutons (as a measure of boutons per micrometer) in grafts at 3mpt and 5mpt. All Days of imaging are amalgamated, with each point representing the average from one day of imaging from one animal. Blue circles = Ds21; red circles = Ts21. Shapiro-Wilk normality tests indicate that all per animal data are normally distributed. Data were analysed using one-way parametric ANOVA with Sidak's multiple comparisons. ANOVA test found a significant difference ($p=0.0003$), therefore Sidak's multiple comparisons were performed. Multiple comparisons indicate that there is no significant difference (ns) between 3mpt Ds21 and 5mpt Ds21 ($p=0.98$), or between 3mpt Ds21 and 3mpt Ts21 ($p=0.50$). Significant differences were found between 3mpt Ts21 and 5mpt Ts21 ($p=0.03$), and between 5mpt Ds21 and 5mpt Ts21 ($p=0.0004$). Error bars indicate the standard error of the mean (SEM). * $p<0.05$; *** $p<0.005$.

EPBs. Based on the literature [41], this analysis can be performed by evaluating the fluorescent intensity of the boutons.

The fluorescent intensity of imaged axonal EPBs was measured, as these data are directly proportional to the size of an EPB and therefore the strength of the synapse [41]. My aim was to determine whether synaptic strength differed, as this provides information about the level of maturity of the selected axons and about the effect of Ts21. EPB intensity was quantified in relation to the axonal backbone intensity, such that a fluorescent intensity of 2.0 would mean the intensity was double the axonal fluorescent intensity. Figure 4.14 presents the intensity data of EPBs imaged at both 3mpt and 5mpt in both genotypes. All data are not normally distributed as determined by Shapiro-Wilk normality tests. Statistical analyses were performed in each case using non-parametric 1-way ANOVAs with Dunn's multiple comparisons. No significant differences were found per animal ($p=0.1615$), however significant differences were found per axon ($p=0.0056$) and per bouton ($p<0.0001$). Dunn's multiple comparison per axon found significance only between Ds21 3mpt and Ds21 5mpt ($p=0.0038$). Per bouton, multiple comparison significance was found between Ds21 3mpt and both Ds21 5mpt ($p=0.0002$) and Ts21 5mpt ($p<0.0001$), as well as between 3mpt Ts21 and 5mpt Ds21 ($p=0.0034$).

The intensity of the EPBs is crucial to understanding synapse strength as it is a measure of synapse size [41]. As such, fluctuations in size are relevant to this project as they indicate changes to the synapse stability. In Figure 4.14 I determined that synapse size and strength were increasing over time from 3mpt to 5mpt. I therefore also wanted to know if synaptic size and strength changes were occurring over shorter intervals. Specifically, over the three days of imaging that are counted as the same timepoint (eg: 3mpt). To do this, I took the EPB intensity data and created intensity ratios. Initially, this was done in a manner that I term non-directional, meaning that it is measuring any size fluctuation regardless of whether it is an increase or decrease over time. Following this I performed what I have termed directional intensity ratio calculations, which take into account the direction of change (larger or smaller over time). In the next paragraphs I explain how these were calculated and the intensity ratio outcomes.

Ratios were first produced in a non-directional manner. This is calculated by dividing the larger of the values over the smaller of the values, regardless of the order of data acquisition (see Figure 4.15). These data are normally distributed (Shapiro-Wilk test), except for the Days 0 and 2 ratio at 5mpt, which is not normally distributed. All parametric groups were analyzed using parametric t-tests with Welch's correction, while the non-parametric group was analyzed using a non-parametric paired t-test (Mann-Whitney). No significant differences were found between Ds21 and Ts21 in any ratio at both 3mpt and 5mpt (3mpt Days 0&2: $p=0.28$; 3mpt Days 2&4: $p=0.33$; 5mpt Days 0&2: $p=0.09$; 5mpt Days 2&4: $p=0.25$).

EPB fluorescent intensity ratios were also evaluated in a directional manner. This means that the intensity of EPBs imaged at Day 2 were always divided by those at Day 0, and the intensity of EPBs imaged at Day 4 were always divided by those at Day 2, regardless of their values. The rationale behind this is that, since no differences were found in the non-directional data, I wanted to know if taking the direction of size change into account would reveal significant changes. If there had been significant non-directional changes, I would expect those to be more prominent when evaluating directionally. Figure 4.16 details directional fluorescent intensity ratios at 3mpt. Disomic data is in blue and trisomic data is in red. The Days of imaging are labelled as A (Day 0), B (Day 2), and C (Day 4). These data are normally distributed (Shapiro-Wilk), and no significant differences were found between genotypes in both ratios (B/A or Day2/Day0: $p=0.80$; C/B or Day4/Day2: $p=0.46$).

Directional ratio analyses were also calculated at 5mpt (see Figure 4.17). These data are normally distributed (Shapiro-Wilk) and Days 0, 2, and 4 are once again labelled as timepoints A, B, and C respectively. No significant differences were found between the genotypes in either ratio when evaluated with parametric t-tests with Welch's correction (For B/A or Day2/Day0: $p=0.97$; For C/B or Day4/Day2: $p=0.63$).

As no significant difference was found in the intensity ratios of the EPBs, I concluded that the size and strength of the boutons does not significantly change within a week of imaging. This is important, as significant fluctuations in synaptic stability over the five days needed to collect one timepoint of data would mean that further analyses may have required separate

evaluation of the different imaging days. I did not expect that size and strength would fluctuate significantly within a week, and since this is indeed the case, I could continue with the remaining synaptic stability analyses with the knowledge that Days 0, 2, and 4 in a timepoint can be amalgamated into larger datasets as needed. Furthermore, the comparable EPB intensities make it more likely that axons are at comparable levels of maturation across genotypes, despite the significant differences found in EPB density at 5mpt.

In summary, I evaluated both the density and fluorescent intensity of axonal EPBs at 3mpt and 5mpt. This was to ensure that imaged axons of both genotypes are at comparable levels of maturity. Since there is no significant difference in EPB density at 3mpt or in fluorescent intensity or intensity ratio (both directional and non-directional) at 3mpt and 5mpt across genotypes, I am confident that these axons are at comparable stages of maturity, particularly at 3mpt. Since there was a significant difference found at 5mpt in axonal EPB density, these data must be viewed with the caveat that some difference in maturity level may be present, creating confounding factors. However, the lack of difference in fluorescent intensity and change in fluorescent intensity of these boutons makes it likely that the axons are still comparable. Furthermore, fluorescent intensity changes indicate that while bouton strength does not change over a week of imaging, maturation increases over time in both genotypes. Therefore, normal maturation is occurring in both Ds21 and Ts21 synapses.

The information above is necessary in order to elucidate the level of graft axon maturity, functionality, and to what degree the axons from both genotypes can be compared. Given that they are comparable in the majority of outcomes, I was able to proceed with the analysis of the next main outcome; synaptic stability (aim 3). The following work describes the outcomes for synaptic stability of axonal EPBs by measurement of EPB TOR and Fr.

To study changes in synaptic structural stability in Ts21, I imaged axonal EPBs longitudinally and quantified the outcomes normalized to axonal backbone signal. EPB TOR was calculated (see equation 3.1) by animal and by axon across two major developmental timepoints; 3mpt and 5mpt. TOR data per animal data was analyzed using parametric t-tests with Welch's correction and was found to be not statistically significant across genotypes (3mpt: $p=0.47$;

5mpt: $p=0.51$). Per axon data was not found to be significant across genotypes, when evaluated using unpaired t-tests (Mann-Whitney) (3mpt: $p=0.06$; 5mpt: $p=0.25$).

The lack of significant difference found in EPB TOR is incredibly interesting, indicating that the additional chromosome 21 in Ts21 grafts is not have an effect on synaptic structure stability at early stages. This hypothesis of a lack of effect from the presence of Ts21 was confirmed with survival rate analysis.

Survival rates (Fr) were calculated with EPB imaging data using equation 3.2. These data indicate the number of EPBs present in Day 0 that remain in the following two imaging sessions (Days 2 and 4). Figure 4.19 visualizes these outputs at 3mpt and 5mpt, both by animal (top) and by axon (bottom). Blue points are Ds21 and blue lines are the Ds21 average values. Red points are Ts21 and red lines are the Ts21 average values. Parametric per animal datasets were statistically evaluated using t-tests with Welch's correction and were found to be not statistically significant (3mpt Day 2: $p=0.67$; 3mpt Day 4: $p=0.95$; 5mpt Day 2: $p=0.23$; 5mpt Day 4: $p=0.16$). Per axon data was statistically analyzed using unpaired t-tests (Mann-Whitney) and were found to be not statistically significant (3mpt Day 2: $p=0.16$; 3mpt Day 4: $p=0.09$; 5mpt Day 2: $p=0.97$; 5mpt Day 4: $p=0.54$).

These findings indicate that EPB Fr also shows no significant difference between genotypes. This was expected, given the similar lack of significant difference found in EPB TOR. This lack of significant difference is very different from the decreased EPB TOR and an increased EPB Fr found in Real *et al.* [39] paper grafts. Current grafts, creating using the Nizetic protocol, have comparable EPB TOR and Fr and therefore comparable synaptic structural stability in both genotypes. As such, the variation in Ts21 synaptic stability has been normalized in these grafts. The importance of this outcome will be addressed in the discussion section of this thesis. Once these findings were unveiled, I wanted to know whether a similar normalization had occurred in calcium activity. In the following section I detail the results from the evaluation of spontaneous and oscillatory activity in current grafts.

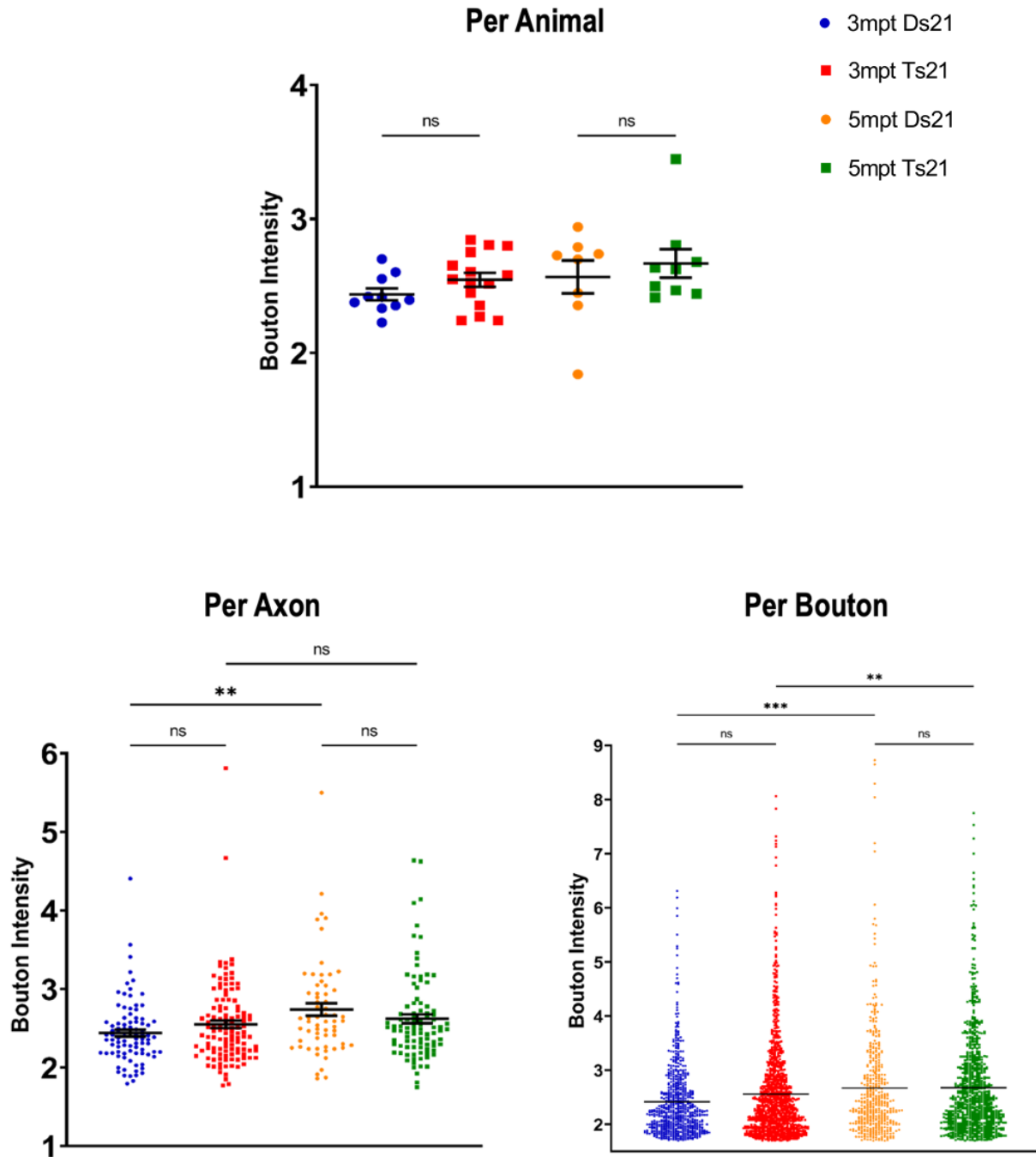


Figure 4.14: Intensity of fluorescence of axonal en-passant boutons measured by EPBscore in relation to the axonal fluorescence. An intensity 1.7X higher than the backbone intensity (1.7 on graph) is the minimum intensity for a point along the axon to be considered a bouton. Intensity is presented by animal (top), by axon (bottom left) and by individual bouton (bottom right). All data are not normally distributed (Shapiro-Wilk normality test). Analyses were performed using non-parametric 1-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparison test. Per animal, no significant differences were found in the ANOVA ($p=0.16$), however significant differences in the ANOVA test were found at the level of the axon ($p=0.0056$) and the bouton ($p<0.0001$). Dunn's multiple comparison per axon found significance only between Ds21 3mpt and Ds21 5mpt ($p=0.0038$). Per bouton, multiple comparison significance was found between Ds21 3mpt and both Ds21 5mpt ($p=0.0002$) and Ts21 5mpt ($p<0.0001$), as well as between 3mpt Ts21 and 5mpt Ds21 ($p=0.0034$). Error bars indicate SEM. ** $p<0.01$; *** $p<0.001$.

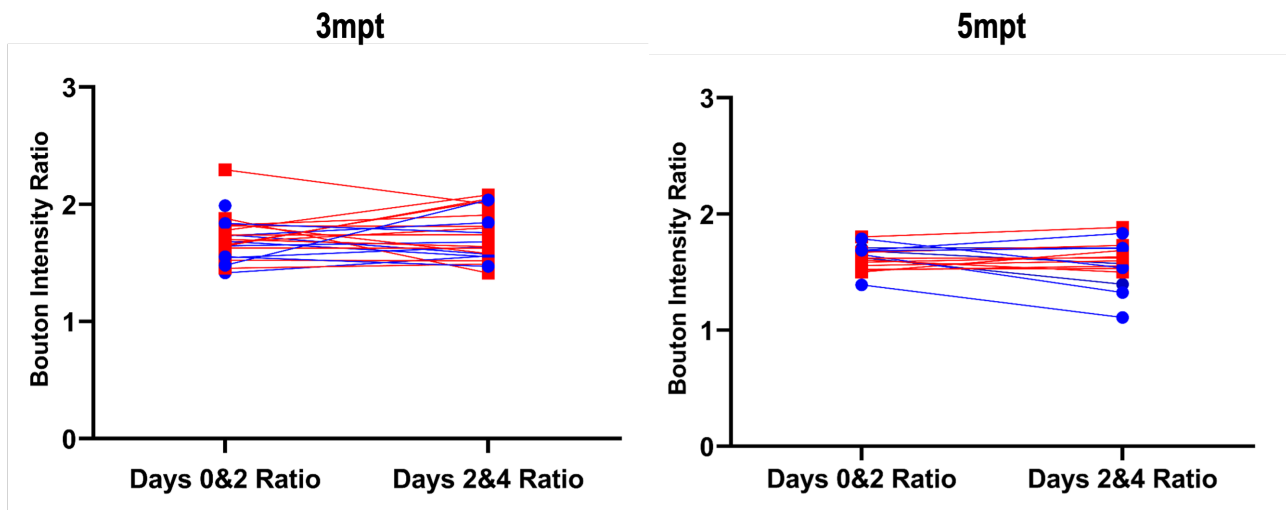


Figure 4.15: The ratio of EPB intensities across adjacent imaging sessions at both 3mpt and 5mpt. Blue circles = Ds21; red squares = Ts21. Ratios are not directional, meaning that ratios are calculated as the largest value over the smallest value of the two Days in question. Shapiro-Wilk normality test indicated that all 3mpt ratios and Days 2&4 Ratio from 5mpt are normally distributed, but Days 0&2 Ratio from 5mpt is not normally distributed. These data were analyzed for statistical significance using parametric t-tests with Welch's correction for all normally distributed groups, and using a non-parametric unpaired t-test (Mann-Whitney) for the not normally distributed 5mpt Days 0&2 Ratio. Error bars indicate SEM. No significant difference between Ds21 and Ts21 (3mpt Days 0&2: $p=0.28$; 3mpt Days 2&4: $p=0.33$; 5mpt Days 0&2: $p=0.09$; 5mpt Days 2&4: $p=0.25$.)

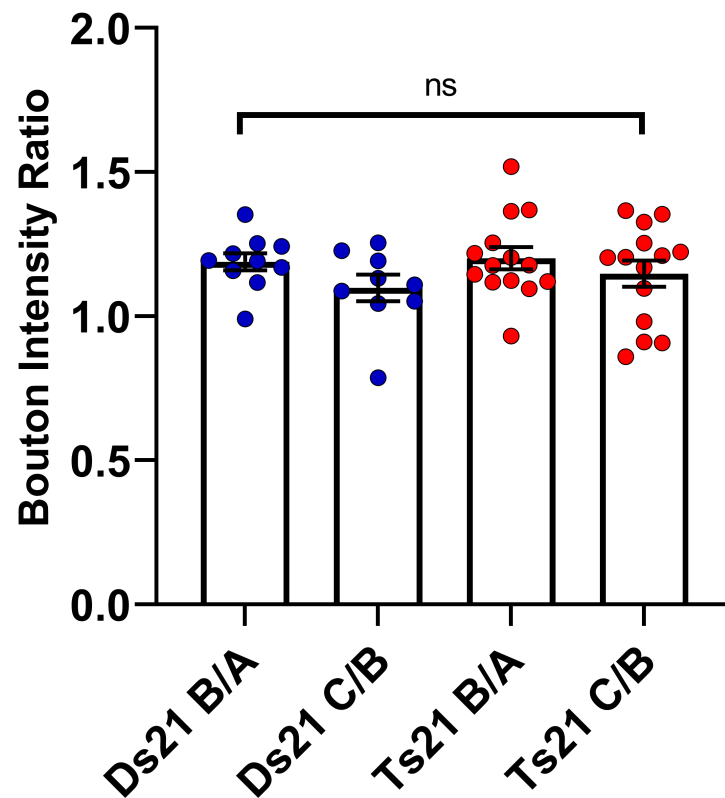


Figure 4.16: The ratio of EPB intensities across adjacent imaging sessions at 3mpt. These ratios are directional, meaning that ratios are calculated as Day 2 over Day 0 (B/A) and Day 4 over Day 2 (C/B), regardless of value. Blue circles = Ds21; red circles = Ts21. Shapiro-Wilk normality test indicated that these data are normally distributed. Using parametric t-test with Welch's correction, no significant difference was found between the genotypes at either timepoint (For B/A: $p=0.80$, For C/B: $p=0.46$. Error bars indicate SEM).

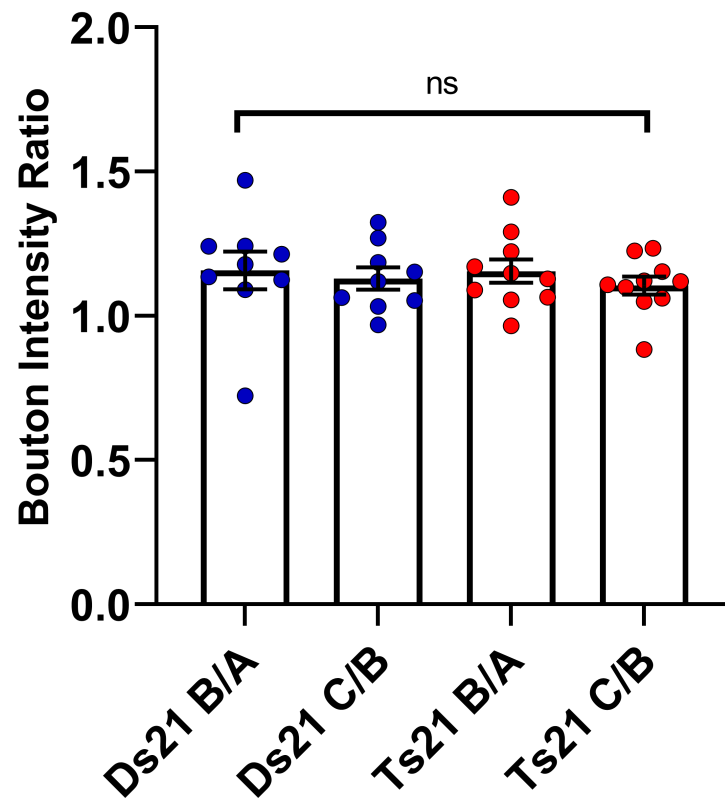


Figure 4.17: The ratio of EPB intensities across adjacent imaging sessions at 5mpt. These ratios are directional, meaning that ratios are calculated as Day 2 over Day 0 (B/A) and Day 4 over Day 2 (C/B), regardless of value. Blue circles = Ds21; red circles = Ts21. Shapiro-Wilk normality test indicated that these data are normally distributed. Using parametric t-test with Welch's correction, no significant difference was found between the genotypes at either timepoint (For B/A: $p=0.97$; For C/B: $p=0.63$). Error bars indicate SEM.

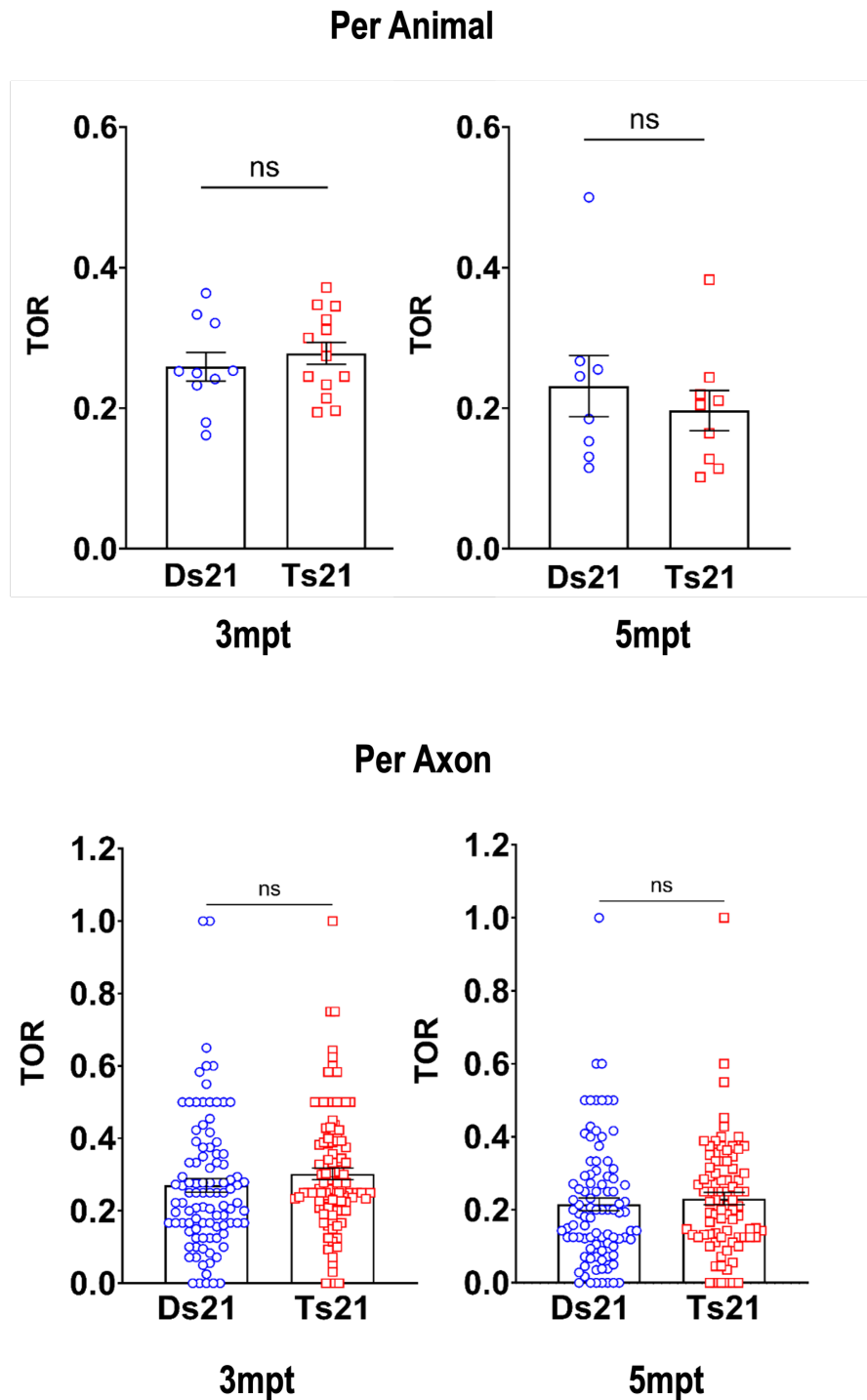


Figure 4.18: Turnover rate (TOR) of axonal en-passant boutons within grafts at 3mpt and 5mpt. Shapiro-Wilk normality tests indicated that per animal data are normally distributed and per axon data are not normally distributed. For each timepoint per animal, no significant difference was found when comparing Ds21 and Ts21 using parametric t-tests with Welch's correction (3mpt: $p=0.47$; 5mpt: $p=0.51$). For each timepoint per axon, no significant difference was found between genotypes using non-parametric unpaired t-test (Mann-Whitney) (3mpt: $p=0.06$; 5mpt: $p=0.25$). Error bars indicate SEM.

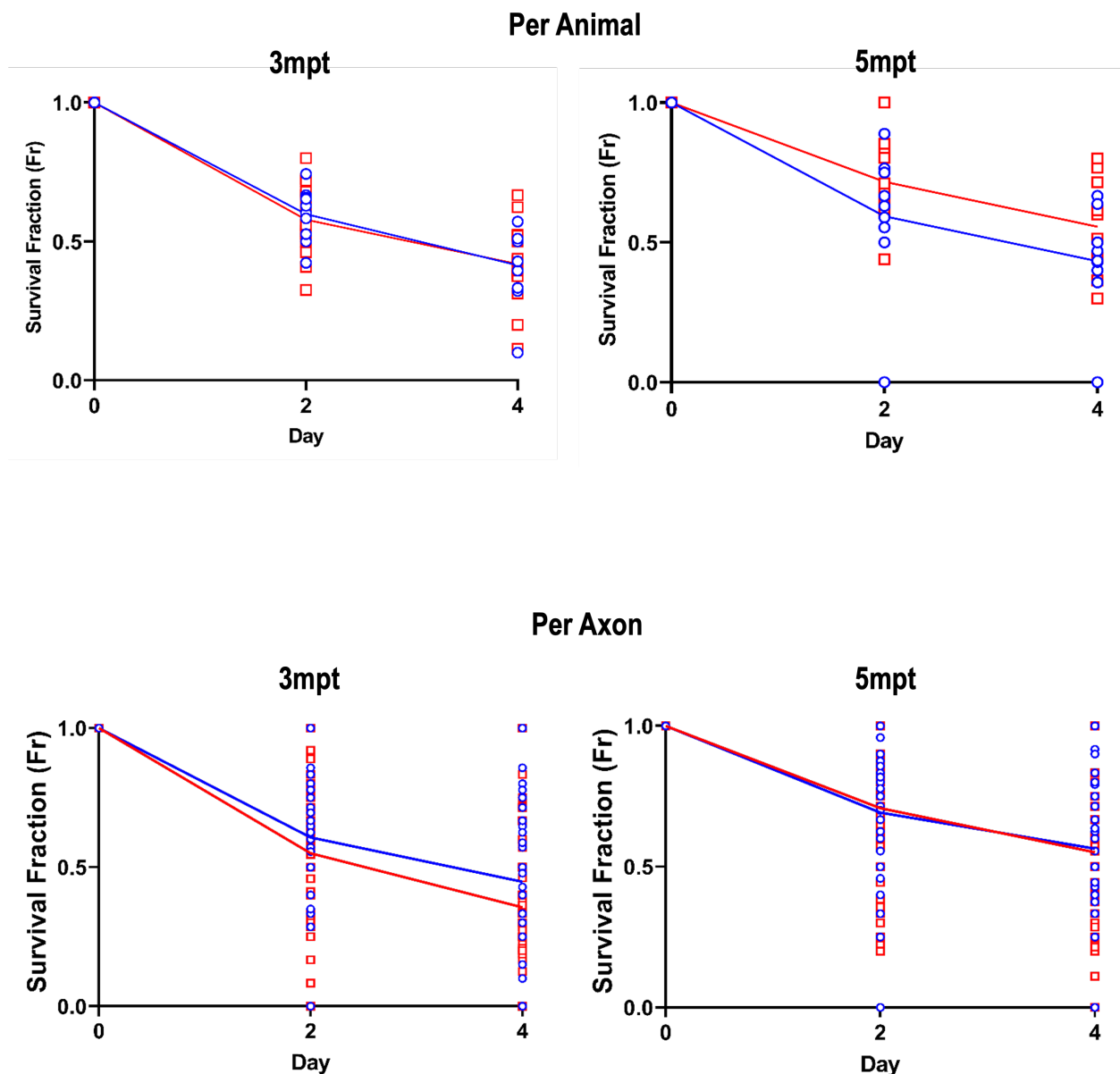


Figure 4.19: Survival rate (Fr) of axonal en-passant boutons within grafts at 3mpt and 5mpt. Blue circles = Ds21; Red squares = Ts21. In all cases, Ds21 and Ts21 were found to be not significantly different. Shapiro-Wilk normality tests indicated that these data per animal are normally distributed, but per axon are not. For each timepoint and imaging session per animal, no significant difference was found when comparing Ds21 and Ts21 using parametric t-tests with Welch's correction (3mpt Day 2: $p=0.67$; 3mpt Day 4: $p=0.95$; 5mpt Day 2: $p=0.23$; 5mpt Day 4: $p=0.16$). Per axon, no significant differences were found using non-parametric unpaired t-tests (Mann-Whitney) (3mpt Day 2: $p=0.16$; 3mpt Day 4: $p=0.09$; 5mpt Day 2: $p=0.97$; 5mpt Day 4: $p=0.54$). Error bars indicate SEM. Top: Each point represents one animal. Bottom: The same dataset as the top graphs, but with each point representing one axon.

4.3 Calcium Activity

4.3.1 Spontaneous Somatic Calcium Activity

At this point in the project, it had been determined that my current grafts were significantly smaller than past grafts, with normalized amounts of each cell types across the genotypes and comparable synaptic strengths. I quantified synaptic structural stability by analyzing the TOR and Fr of axonal EPBs and determined that there is no significant difference between Ds21 and Ts21 grafts. My next goal was to proceed with addressing the fourth project aim: evaluating both spontaneous and oscillatory calcium activity in the graft cells. I expected that there would be no difference in calcium activity across genotypes, given that there were no significant differences in synaptic stability. This section will outline the major findings from my multiphoton analysis of calcium activity, starting with spontaneous activity and then moving on to oscillatory activity.

The spontaneous calcium activity of graft cell somas was analyzed at 1mpt, 3mpt, 5mpt, and 7-8mpt. In order to perform this analysis, it was crucial to isolate a threshold activity level. Optimization of the threshold level was performed by evaluating spontaneous somatic calcium activity integrals at 1mpt, 3mpt, and 5mpt in both Ds21 and Ts21 grafts with the following thresholds (in $\Delta F/F_0$): 1, 5, 10, 15, 20, 30, and 50 (see Figure 4.20). These data were found to be not normally distributed (Shapiro-Wilk) and statistical analyses of the difference in integral outputs using different threshold levels were evaluated by 1-way non-parametric repeated measures ANOVA (Friedman) with Dunn's multiple comparisons ($n=3-6$ animals/group). Statistically significant changes are marked in Figure 4.20 as *, **, ***, or **** (representing $p<0.05$, 0.01, 0.001, and 0.0001 respectively). In all cases, the amount of activity (number of represented points) decreases as the threshold increases, as does the average integral ($\Delta F/F_0$). Based on these data, a threshold of $30\Delta F/F_0$ was selected for all further analysis. At $30\Delta F/F_0$, the change in integral between thresholds is no longer significantly different, indicating that the noise has been removed. A threshold of $50\Delta F/F_0$ was deemed to be too high, as removing too much data would result in a loss of all low-level activity within the dataset.

Equation 3.4 was used to calculate the change in fluorescent intensity based on background signal intensity and using a threshold of $30\Delta F/F_0$. Figure 4.21 illustrates the calcium activity of a Ts21 graft region at 3mpt. Figure 4.21A depicts the calcium fluctuations (GCaMP6) of two somas every 5 seconds for two minutes. Figure 4.21B shows the tdTomato structural image of the graft region used to identify the somas before GCaMP6 imaging was performed. Figure 4.21C is a zoomed in section of the image from Figure 4.21B which depicts the location of the two somas from Figure 4.21A when imaged structurally. These two somas are marked with red arrows.

A MATLAB script was used to extrapolate the integral ($\Delta F/F_0$), frequency (Hz), and amplitude ($\Delta F/F_0$) of all somas (see code in Chapter 3). Integral, amplitude, and frequency outputs are represented in Figure 4.22, with each point representing the average of one animal. Blue points are Ds21 and red points are Ts21. An additional graph in the top left of Figure 4.22 outlines the percentage of active somas per animal. All data was tested for normality using Shapiro-Wilk tests. The data for percentage of active somas, amplitude, and frequency is all normally distributed. Integral data was found to be not normally distributed. All parametric data were analyzed using unpaired parametric ANOVAs with Tukey's multiple comparisons, while integral data was analyzed using 1-way non-parametric ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons. No significant differences were found in any of the ANOVA tests (Percentage of Active Somas: $p=0.80$; Frequency: $p=0.90$; Amplitude: $p=0.65$; Integral: $p=0.92$; $n=3-6$ animals per group in all datasets).

Integral, amplitude, and frequency datasets were also analyzed by soma (see Figure 4.23). Analysis was performed in the same manner as Figure 4.22, except all data were found to be not normally distributed and were therefore analyzed using Kruskal-Wallis tests. In addition to frequency (top left), amplitude (top right), and integral (bottom left) graphs, an additional integral graph (bottom right) has been included which depicts the same data as the bottom left integral graph but using a 10-90% whisker plots. This graphic was included as the per soma integral dataset has a large degree of variability, making the means cluster towards the bottom of the graph. The whisker plot allows for easier visualization of the trends as it does not include the highest and lowest points. Statistically significant multiple comparisons are shown

on the graphs (see Figure 4.23). Importantly, no significant differences were found between Ts21 and Ds21 at the same timepoints. Differences across timepoints are present, indicating the possibility of activity decreases over time, but this trend is not visible when evaluated per animal (see Figure 4.22).

At the same time as these calcium activity data were being collected, a small subset of animals were aged further to address Aim 5: the development of a proof of concept for long-term grafts (7-8 months old). This pilot dataset of graft cells that are the oldest analyzed to date using this model. Figure 4.24 details the amplitude, frequency, and integral of one Ds21 animal (blue) and three Ts21 animals (red), shown by animal (left-hand column) and by soma (right-hand column). These data were not able to be statistically analyzed due to the Ds21 n-value of 1 animal. No striking trend differences are visible at this time. Optimization of long-term graft maintenance will allow for this dataset to be expanded by other lab members in the future. These findings confirmed that my grafts can be maintained and imaged at 7-8 months post-transplantation, the latest timepoint to date with this model.

In summary, spontaneous somatic calcium activity was analyzed using an optimized $\Delta F/F_0$ threshold of 30 (see Figure 4.20). No significant differences were found in the level of spontaneous calcium activity between genotypes at 1mpt, 3mpt, 5mpt or in the pilot group at 7-8mpt when analyzed by animal (see Figures 4.22 and 4.24) and by soma (see Figures 4.23 and 4.24). These findings proved a new timepoint at which these grafts can be imaged, meeting Aim 5 criteria, while also proving our Hypothesis number 4 to be true. The lack of genotypic difference here mirrors that of the synaptic structural stability dataset. In these grafts, Ts21 is not sufficient to alter either outcome. The next section will investigate whether this holds true for oscillatory activity by performing additional analyses on the same dataset.

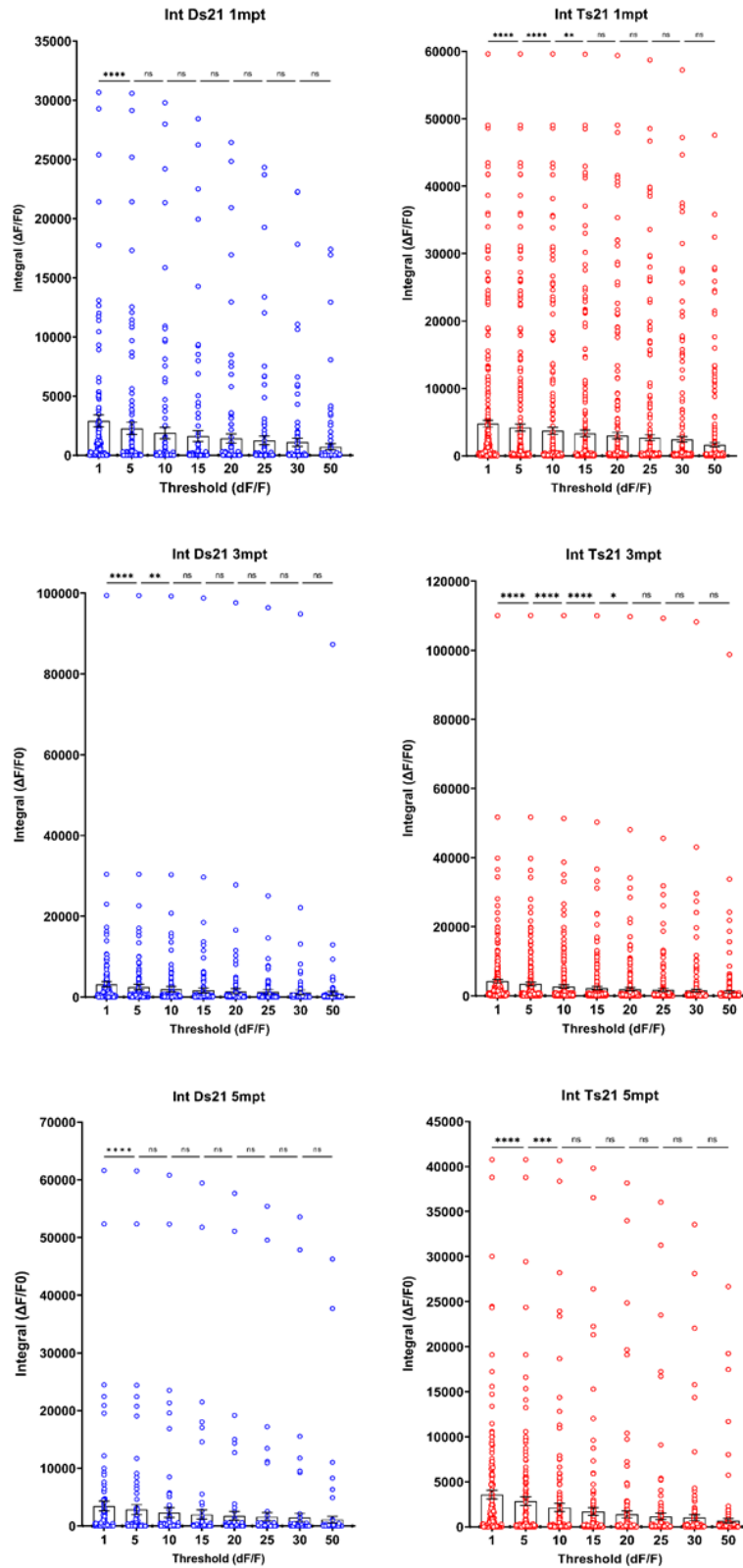


Figure 4.20: Threshold optimization for spontaneous somatic calcium activity. Integral outputs ($\Delta F/F0$) are displayed using thresholds from 1 to 50. Each point is an ROI ($n=3-6$ animals per group). Data is not normally distributed as determined by Shapiro-Wilk tests. Statistical analyses were performed using 1-way non-parametric repeated measured (Friedman) ANOVA with Dunn's multiple corrections. Error bars=SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

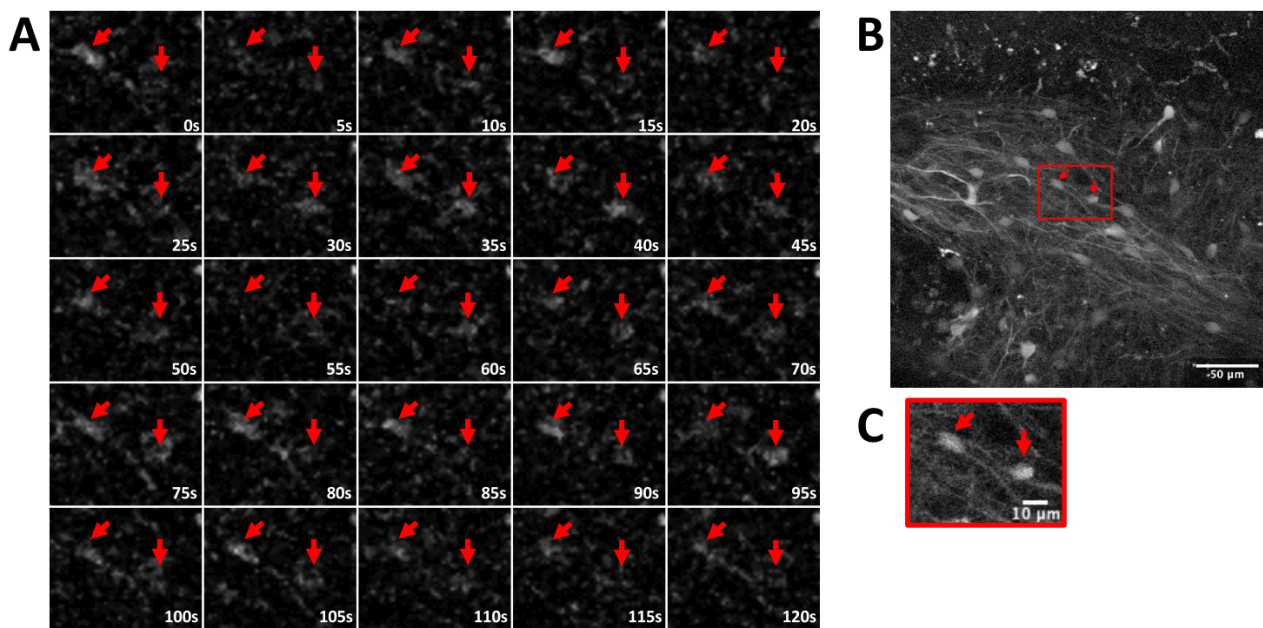


Figure 4.21: Example of calcium activity imaging at 3mpt in a Ts21 graft. A: GCaMP6+ images of two somas (marked by red arrows) taken over two minutes. Every 5 seconds of the t-series is shown. B: The tdTomato image depicting the structure of the entire graft region imaged with the 2P microscope at 20X (zoom 2). C: Zoomed in view of the image from B, showing the same area as the video images in A. The two somas depicted in A are shown here with tdTomato structural signal.

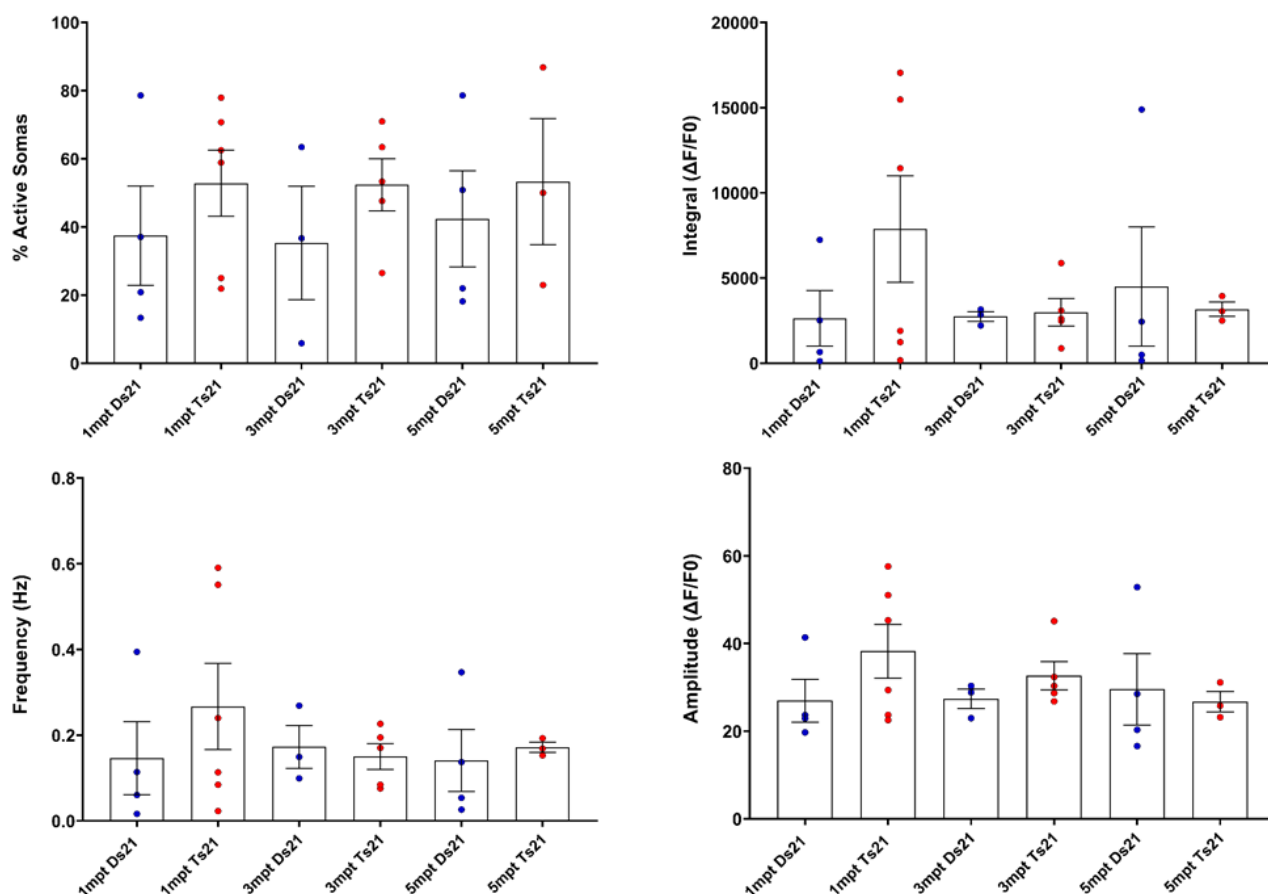


Figure 4.22: Spontaneous somatic calcium activity outputs per animal from Ds21 grafts (blue) and Ts21 grafts (red) at 1mpt, 3mpt, and 5mpt with a threshold of $30\Delta F/F_0$. Outputs of integral ($\Delta F/F_0$), amplitude ($\Delta F/F_0$), frequency (Hz) and percentage of active somas are displayed. All data are normally distributed as determined by Shapiro-Wilk tests, except for Integral which is not normally distributed. Statistical analyses were performed using 1-way parametric unpaired ANOVAs with Tukey's multiple corrections. For Integral, 1-way non-parametric ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons was used. No significant differences were found in any ANOVA test (% Active Somas: $p=0.80$; Integral: $p=0.92$; Frequency: $p=0.90$; Amplitude: $p=0.65$). $n = 3-6$ animals per group. Error bars indicate SEM.

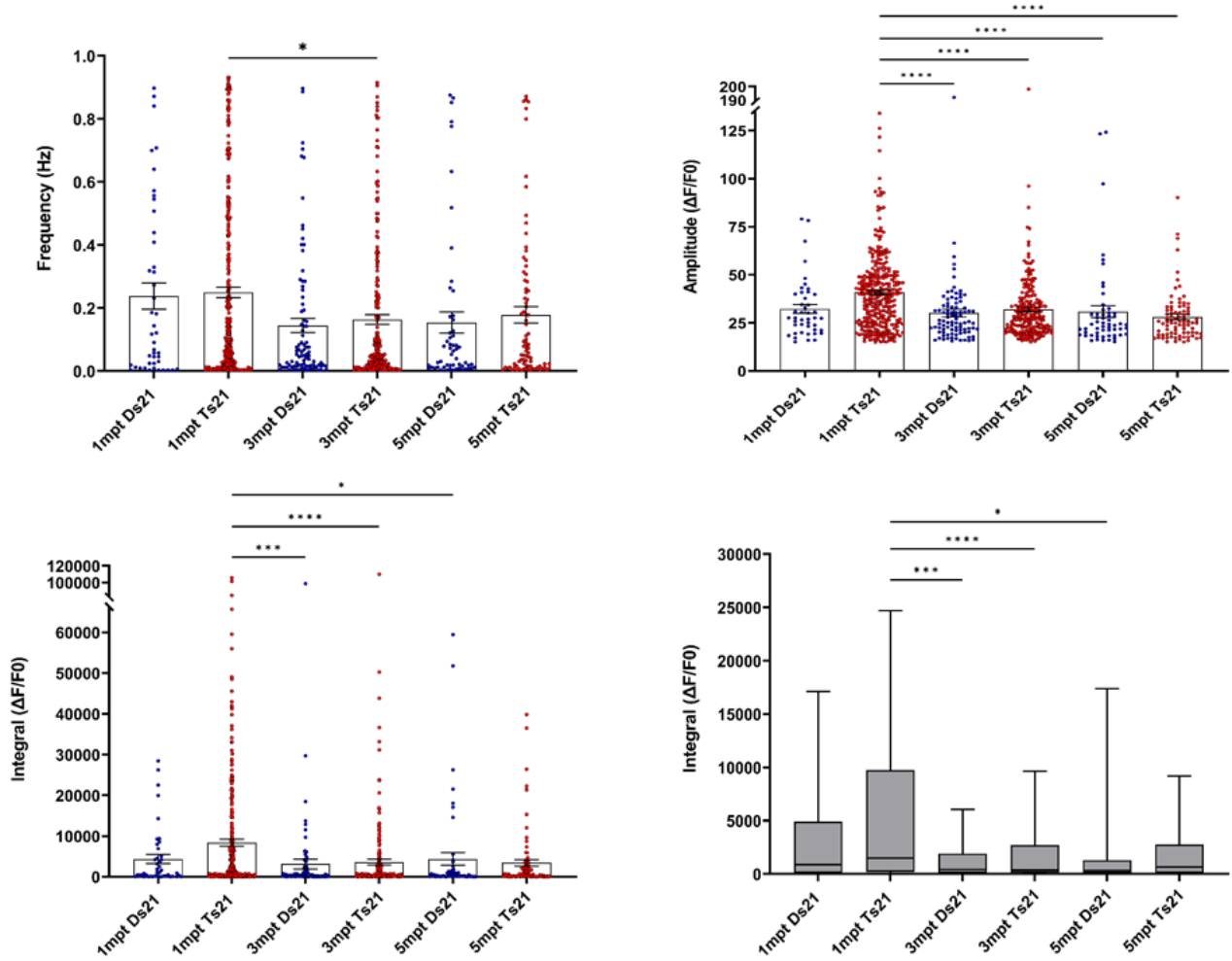


Figure 4.23: Spontaneous somatic calcium activity outputs per soma from Ds21 grafts (blue) and Ts21 grafts (red) at 1mpt, 3mpt, and 5mpt with a threshold of $30\Delta F/F_0$. Outputs of integral ($\Delta F/F_0$), amplitude ($\Delta F/F_0$), and frequency (Hz) are displayed. Additionally, a second figure displaying the integral outputs as 10%-90% box and whisker plots is shown (bottom right) to illustrate averages, as the integral dataset has high variability. All data are not normally distributed as determined by Shapiro-Wilk tests. Statistical analyses were performed using 1-way non-parametric unpaired (Kruskal-Wallis) ANOVAs with Dunn's multiple corrections. $n = 3-6$ animals per group. Error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

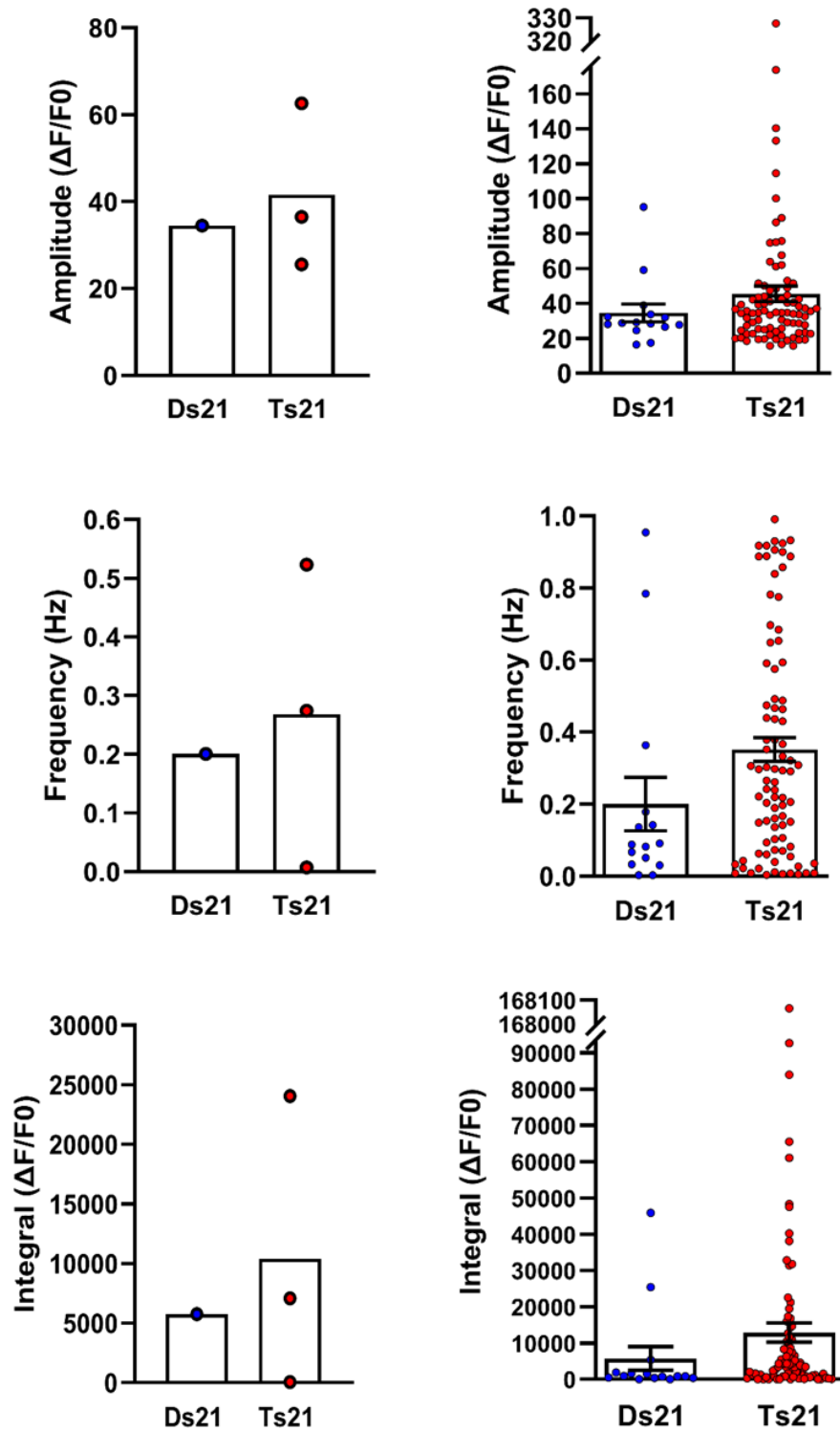


Figure 4.24: 7-8mpt spontaneous somatic calcium activity outputs from Ds21 (blue) and Ts21 (red) grafts with a threshold of $30\Delta F/F_0$. Outputs of integral ($\Delta F/F_0$), amplitude ($\Delta F/F_0$), and frequency (Hz) are displayed, both per animal (left column: $n=1$ for Ds21; $n=3$ for Ts21) and per soma (right column: $n=15$ for Ds21; $n=86$ for Ts21). These data are considered a pilot study due to low n -values. Error bars in right-hand column figures indicate SEM of per soma datasets. As there is only one Ds21 animal, no statistical analyses could be performed.

4.3.2 Oscillatory Calcium Activity

As stated above, the work in this section details the second half of Aim 4 with the goal of investigating whether the presence of a third copy of chromosome 21 is sufficient to create an effect on oscillatory activity. Given the lack of significant difference between Ds21 and Ts21 grafts in both synaptic structural stability and spontaneous somatic calcium activity, I hypothesized that oscillatory activity would also reveal no significant difference between genotypes. The spontaneous calcium activity dataset from 1mpt, 3mpt, 5mpt, and the pilot dataset from 7-8mpt were analyzed by ROI to determine the level of network oscillatory calcium activity. The same threshold as used for the spontaneous activity ($30\Delta F/F_0$) was used for these analyses.

Using spontaneous activity data I determined the number of ROIs that contained activity, and of those the number that contained 2 or more active somas. The purpose of this soma classification in terms of oscillatory activity is to eliminate ROIs where oscillation is not possible, as there are no active somas or only 1 active soma. Figure 4.25 displays this data for each genotype and timepoint. The presence of no active somas or only 1 active soma in an ROI was deemed exclusion criteria for the oscillatory activity dataset. No statistical analyses were performed, as these data are simply an indication of inclusion/exclusion criteria. However, as the number of ROIs included in the oscillatory network activity dataset can be as low as approximately 16% (see Figure 4.25, Ds21 3mpt) it is crucial to note that the dataset reflected in the remainder of this section is smaller than the spontaneous activity dataset.

Once exclusion criteria had been applied, oscillatory network activity data was extracted for all timepoints and both genotypes. Figure 4.26 illustrates the oscillatory activity levels of each individual ROI for all timepoints and genotypes using heatmaps. The heatmaps plot the percentage of coactive cells at each individual frame of the t-series. T-series lengths range from 800-1200 frames, with two ROIs extending to 1800 frames (see Figure 4.26, Ds21 7-8mpt ROI A and Ts21 7-8mpt ROI F). The frame number is shown on the x-axis while the y-axis is the ROI number, represented with a letter label. The legends details the range of values present on the heatmap to its left. Overall, the percentage of coactivity at all timepoints and genotypes is fairly low. Heatmaps that depict percentages of coactivity that are high (over 50% coactivity)

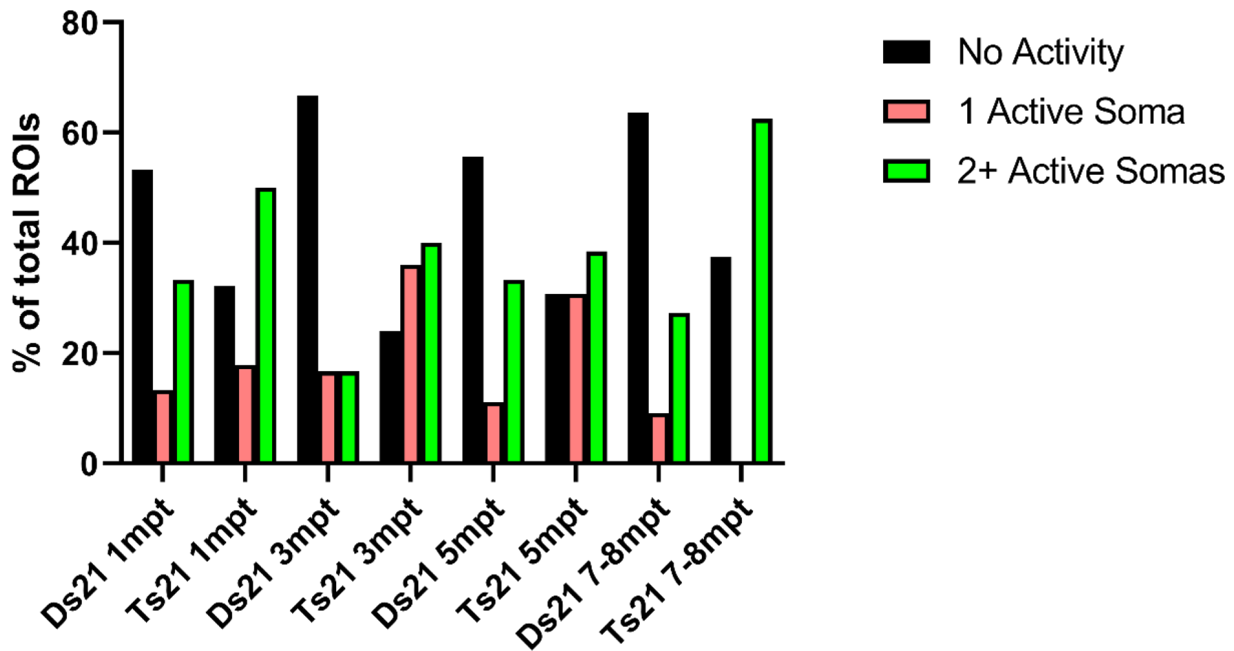


Figure 4.25: Graph depicting the number of t-series ROIs containing no activity (black), only 1 active soma (pink), or 2+ active somas (green) at 1mpt, 3mpt, 5mpt, and 7-8mpt in both Ds21 and Ts21 grafts. At least 15% of captured ROIs for all genotypes and timepoints contain 2+ somas, with most groups maintaining over 30%. The black and pink bars are ROIs that meet the exclusion criteria for oscillatory activity analysis. Only the ROIs represented by the green bars were included in the oscillatory analysis that follows.

are regions with fewer somas in the ROI, rather than any one ROI having a larger amount of coactivity.

These coactivity datasets were averaged in order to create a heatmap displaying the average percentage of coactivity at each frame for all genotype and timepoint combinations (see Figure 4.27). Once averaged, the point made above becomes clear. The maximum coactivity percentages in Figure 4.27 are just over 30%. However, the fact that number of somas per ROI is not normalized in these data makes comparisons across genotypes difficult. Therefore, I proceeded with further analyses to depict these data more clearly.

Percentage of coactivity data was correlated to the number of somas per ROI. My hypothesis was that there would be a linear relationship between the number of somas per ROI and the level of coactivity. In this case, genotypic differences could be analyzed by evaluating the slope of each correlational trendline. However, no significant correlation was found in either Ds21

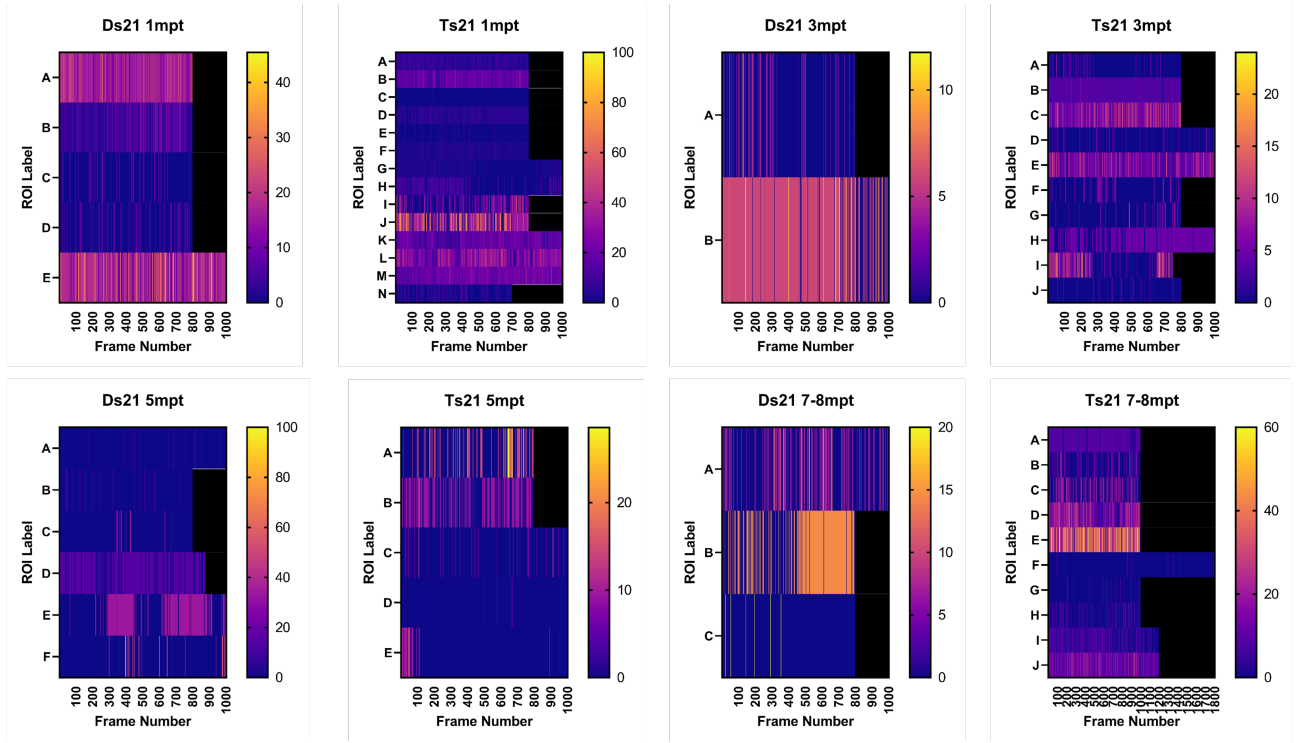


Figure 4.26: Heatmaps for Ds21 and Ts21 at 1mpt, 3mpt, 5mpt, and 7-8mpt depicting the percentage of coactive somas at each frame of each ROI (t-series location). The y-axis indicates the different ROIs, with each labelled as a letter. The x-axis indicates the frame number. The number of frame imaged in the t-series vary across ROIs (most are 800, 1000, or 1200 frames with some being 1800 frames). Black regions indicate areas where there is no data. The legends for each graph are on the left y-axis.

or Ts21 datasets (see Figure 4.28). Figure 4.28 shows all disomic ROIs (left) and all trisomic ROIs (right) from 1mpt, 3mpt, 5mpt, and 7-8mpt as a correlation between the number of somas in that ROI and the average percentage of coactivity of that ROI. Each dot represents one ROI and the lines are the linear trendlines. All data was found to be non-parametric using a normality test. In both Ds21 and Ts21 grafts, no significant correlation was found between the number of somas in the ROI and the average percentage of coactivity of the ROI using Spearman (non-parametric) correlation (Ds21: $r = -0.038$, $p = 0.89$; Ts21: $r = -0.07$, $p = 0.67$).

Since there is no correlation between the number of somas per ROI and the coactivity level of that ROI, I had to find another way to statistically analyze the oscillatory activity while normalizing soma number. To do this, I evaluated the average percentage of coactivity per genotype and timepoint against a number of different thresholds. In this manner, I could

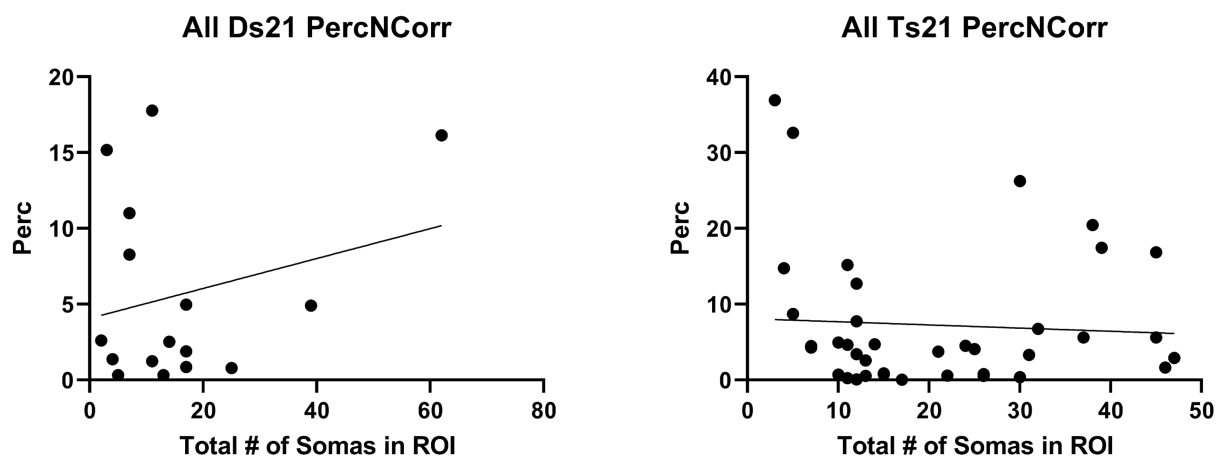


Figure 4.28: Correlative analysis between the average percentage of coactivity of an ROI and the number of somas in that ROI. All timepoints (1mpt, 3mpt, 5mpt, and 7-8mpt) were included together in these analyses. Normality tests determined that all data was non-parametric. Correlations were evaluated using Spearman non-parametric correlation tests. Left: Ds21 correlative analyses. $r = -0.038$, $p = 0.89$. Right: Ts21 correlative analyses. $r = -0.07$, $p = 0.67$. Abbreviations: Perc = average percentage of coactivity in the ROI; PercNCCorr = perc/n-value correlation.

increases. Since that activity is hypothesized to be background noise, it makes sense that this noise would be universal across the ROI and appear “coactive”. Normality tests determined that all data was non-parametric. The 1mpt, 3mpt, 5mpt, and 7-8mpt curves of both genotypes were all compared using simple linear regression analysis in GraphPad Prism. No significant difference was found in the slope ($p > 0.9999$), or in the elevation and Y-intercept ($p = 0.94$) of any of the curves, indicating that there is no significant difference in the percentage of coactive somas across both genotypes over all timepoints.

In addition to evaluating the percentage of coactive somas at each frame and averaging per ROI/timepoint, I also evaluated the average strength of significantly coactive somas by correlative analysis. Strength is a correlation coefficient (r) measurement in the positive (0-1) direction only. Only significantly coactive somas are included in this dataset ($p < 0.05$). In Figure 4.30 the strength measurements for each timepoint and genotype are depicted, with each dot representing one soma and its degree of correlation to others, averaged (see Methods section for code). Normality tests determined that all data are non-parametric. An unpaired nonparametric one-way ANOVA (Kruskal-Wallis) test was performed with Dunn’s multiple comparisons. The Kruskal-Wallis test produced an overall statistically significant outcome

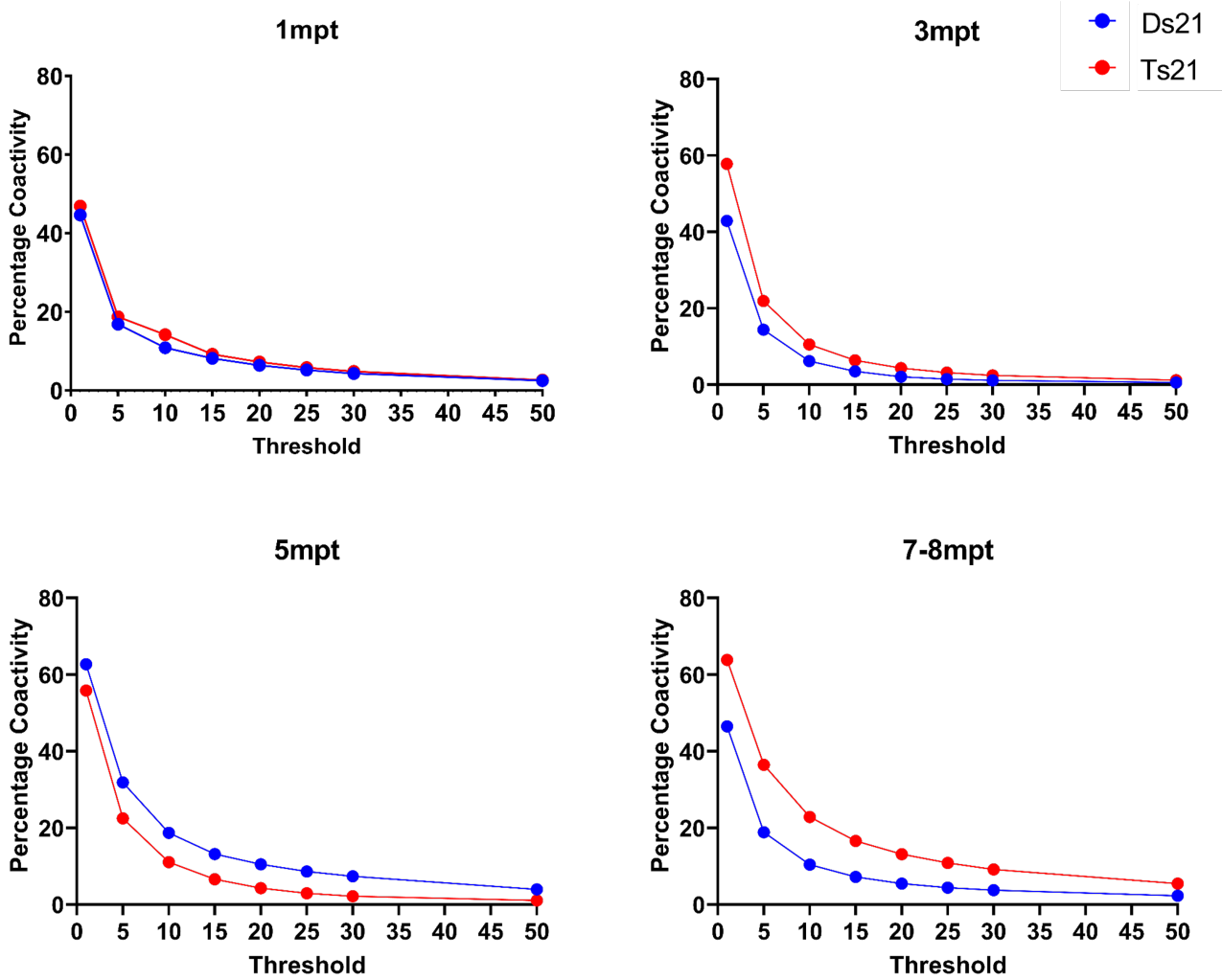


Figure 4.29: Average percentage of ROI coactivity for Ds21 and Ts21 grafts at 1mpt, 3mpt, 5mpt, and 7-8mpt over a range of thresholds. Averages are presented for all genotypes and timepoints using thresholds of 1, 5, 10, 15, 20, 25, 30, and 50 $\Delta F/F_0$. Red points/lines are Ts21 and blue points/lines are Ds21. Percentage of coactivity (y-axis) is an average of all ROIs at that timepoint (above graphic) and threshold (x-axis). All data was non-parametric. No significant differences were found in slope, or elevation and y-intercept across all genotypes and timepoints using simple linear regression analysis in GraphPad Prism.

($p=0.0002$). Dunn's multiple comparisons determined that there was a significant increase in the strength of Ts21 at 1mpt when compared to Ds21 1mpt ($p=0.0016$). All other genotype comparisons within each timepoint were found to be not statistically significant (3mpt Ds21 vs Ts21: $p=0.20$; 5mpt Ds21 vs Ts21: $p=0.84$; 7-8mpt Ds21 vs Ts21: $p>0.99$).

In summary, network oscillatory calcium activity was measured in two ways: by the percentage of coactive somas per frame within an ROI (excluding ROIs with 1 soma or fewer), and by the strength of significant coactive pairings as determined by Pearson Correlation coefficient. In

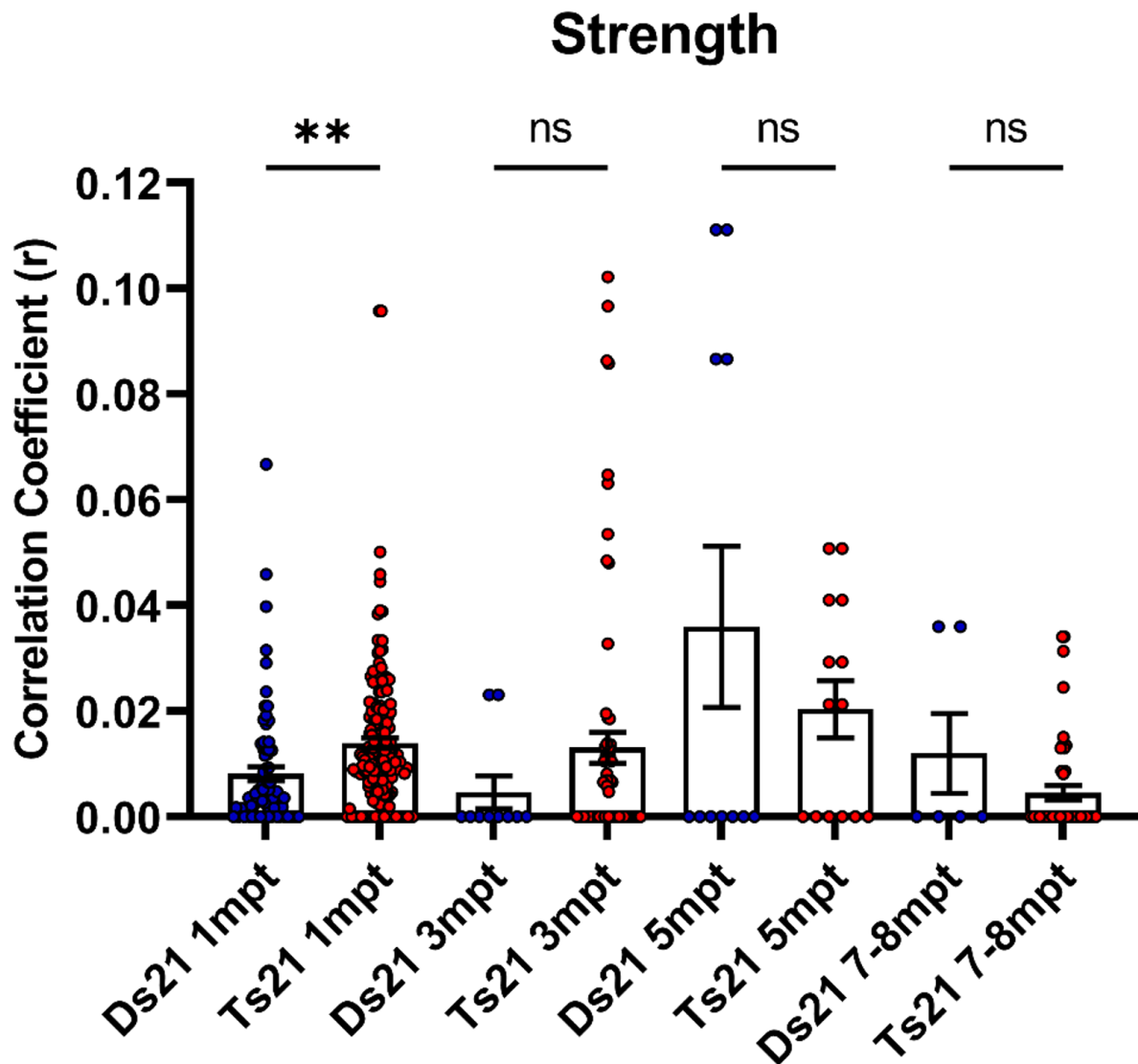


Figure 4.30: Graph of the strength of significant coactivity as determined by correlation (r). Each point represents the average strength of significant correlations for a soma (as calculated using the MATLAB code in the Methods section). Only significant correlations ($p < 0.05$) were included. All data is non-parametric. Analyses were performed with Kruskal-Wallis test (unpaired one-way ANOVA) with Dunn's multiple corrections. The overall ANOVA outcome was statistically significant ($p = 0.0002$). Ds21 and Ts21 comparison at 1mpt was found to be statistically significant ($p = 0.0016$), while Ds21 and Ts21 comparisons at 3mpt ($p = 0.20$), 5mpt ($p = 0.84$), and 7-8mpt ($p < 0.99$) were not statistically significant. ** $p < 0.01$.

both measures, no significant differences were found between Ds21 and Ts21 at 3mpt, 5mpt, or in the Aim 5 pilot study at 7-8mpt. A significant difference was found at 1mpt between Ds21 and Ts21, but this is gone by 3mpt. Network activity was found to be present in all groups and at all timepoints, but the levels of network activity were low. Average percentage

of coactivity was under 30% (see Figure 4.27) and average strength was under $r=0.04$, indicating weak correlation (see Figure 4.30).

4.4 Correlative Analysis: GFAP+ and SATB2+ Graft Cells

In tandem with the major aims of this project (Aims 3 and 4), I expanded my data analysis of synaptic stability and spontaneous/oscillatory calcium activity to attempt to isolate potential mechanisms involved in my outcomes. My outcomes from this graft analysis created many additional questions. Why is the presence of an additional human chromosome 21 not sufficient to create alterations to synaptic structural stability and calcium activity in early development? Why are the current grafts results different from the ones found in previous work from our group [39]? Answering these questions is not a simple task, and it is one that is being explored in greater depth by other members of my team who have received funding for comparative transcriptomic and genomic work. My goal, as a part of Aims 3 and 4, was to utilise the data at my disposal to identify mechanisms of interest for future research. To do this, I performed correlational analyses between my major outcomes (EPB TOR, Calcium Activity Integral) and the amount of different cell types within the graft. Namely, I focused on SATB2+ upper layer excitatory cortical neurons and hGFAP+ human astrocytes, as these two cell types were normalized in the current graft. Both cell types were present in significantly different numbers between Ds21 and Ts21 grafts in Real *et al.* [39]. My hypothesis was that a significant correlation between the amount of SATB2+ or hGFAP+ cells and EPB TOR or Calcium Integral would isolate a major effect contributor. This would affect the targets of future transcriptomic and genomic analyses.

The relationship between SATB2+ excitatory upper layer cortical neuron concentrations within the grafts and the main thesis outputs for synaptic structural stability and spontaneous calcium activity were evaluated by Pearson's correlation. The percentage of SATB2+ cells in the graft was correlated to both EPB TOR and the calcium activity integral. Correlations were per-

formed within the same animal, by comparing immunohistochemistry staining counts at 5mpt *ex vivo* to 2-photon *in vivo* imaging at 5mpt prior to perfusion. No significant correlation was found between the percentage of SATB2+ cells in the graft and EPB TOR ($p=0.75$; $r=0.40$; $n=4$ animals) (see Figure 4.31). No significant correlation was found between the percentage of SATB2+ cells in the graft and spontaneous calcium integral outputs ($p>0.99$; $r=0.50$; $n=3$ animals) (see Figure 4.31). These data include both Ds21 and Ts21 animals, as there is no significant difference in TOR (see Figure 4.18) or in the integral of spontaneous calcium activity (see Figure 4.22) and since correlated values are always within the same animal.

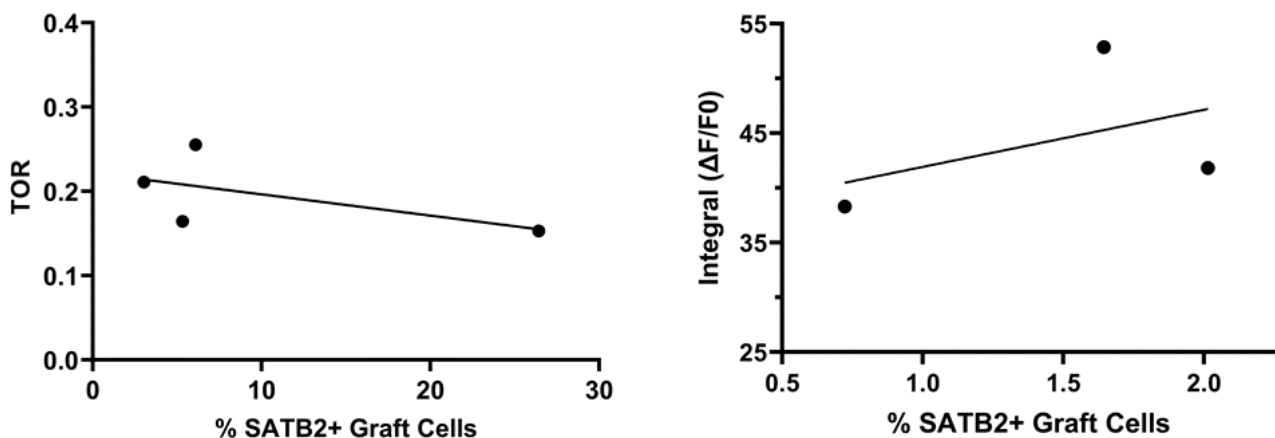


Figure 4.31: Analysis of the correlation between the percentage of graft cells that are SATB2+ upper layer neurons from 5mpt *post-mortem* graft slices and the EPB turnover rate (TOR; left) and calcium activity integral ($\Delta F/F0$; right) measurements from the same animals with *in vivo* 2-photon microscopy at 5mpt. Correlative analyses were performed using Pearson's correlation, with statistical significance set at $p<0.05$. No significant correlation was found between number of SATB2+ graft cells and EPB TOR ($p=0.75$; $r=-0.40$; $n=4$ animals) or integral ($p>0.99$; $r=0.50$; $n=3$ animals).

The same type of correlative analysis was performed with the amount of hGFAP+ astrocytes in the graft (represented as number of cells per cubic micron). Pearson's correlation was used again to compare the amount of astrocytes per cubic micrometer to both EPB TOR and spontaneous calcium activity integral in the same animal, with both Ds21 and Ts21 animals being included together. Importantly, a significant correlation ($p=0.03$; $r=0.9968$; $n=3$ animals) was found between the 5mpt *ex vivo* number of astrocytes per cubic micrometer and the EPB TOR of the same animals at 5mpt (see Figure 4.32). Post-hoc power calculations were performed using G*Power for this correlation. With an r -value of 0.9968 and significance set at $p<0.05$, an n -value of 3 animals produces a power of 0.8899944. As the power is above 0.8, we can conclude

that in this instance, an n of 3 adequately powers this finding. No significant correlation was found between the number of astrocytes per cubic micrometer and the spontaneous calcium activity (integral) at 5mpt ($p=0.08$; $r=0.28$; $n=3$ animals) (see Figure 4.32).

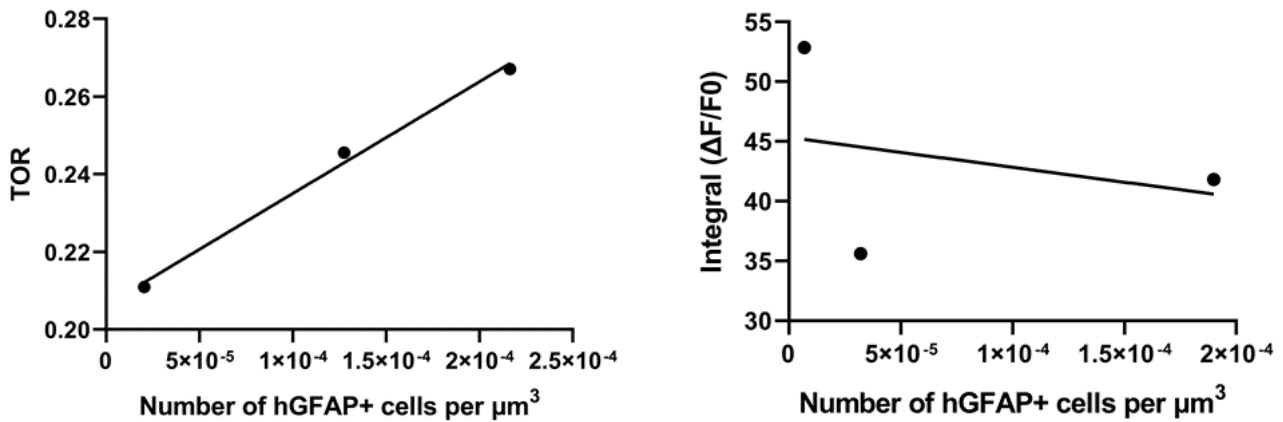


Figure 4.32: Analysis of the correlation between the percentage of graft cells that are hGFAP+ human astrocytes from 5mpt *post-mortem* graft slices and the EPB turnover rate (TOR; left) and calcium activity integral ($\Delta F/F_0$; right) measurements from the same animals with *in vivo* 2-photon microscopy at 5mpt. Correlative analyses were performed using Pearson's correlation, with statistical significance set at $p < 0.05$. No significant correlation was found between number of human astrocytes and integral output ($p=0.08$; $r=-0.28$; $n=3$ animals), but a significant positive correlation was found between the number of human astrocytes in the graft and the EPB TOR of the same animal ($p=0.03$; $r=0.9968$; $n=3$ animals).

In summary, correlational analyses were performed between the number of SATB2+ cells in the graft and the EPB TOR and calcium integral outcomes for those grafts. The same correlational analyses were performed with the number of hGFAP+ astrocytes per cubic micrometer. The goal of this work was to determine whether the density of SATB2+ and/or GFAP+ cells might regulate synaptic structural stability and neural network activity. The correlations between SATB2+ cell number (as a percentage of graft cells) and EPB TOR and calcium integral were both not significant. The correlation between the number of hGFAP+ graft cells per cubic micrometer and calcium integral was also not significantly different. However, I did find a significant positive correlation between the number of hGFAP+ cells per cubic micrometer and the EPB TOR of the same grafts. This indicates that SATB2+ cell levels in current grafts less correlated with current outcomes than hGFAP+ astrocyte levels. The implications of these findings, and the rest of the findings from the Results section, will be dissected in the Discussion section.

Chapter 5

Discussion

5.1 Graft Characterization & Graft Size

Characterization of *ex vivo* 5mpt stained graft slices indicates that grafts of both genotypes present with dividing cells (Ki67+), migrating cells (DCX+), neuronal progenitor cells (Pax6+, Nestin+), deep layer excitatory cortical neurons (TBR1+, CTIP2+), upper layer excitatory cortical neurons (SATB2+), and astrocytes (hGFAP+) (see Figures 4.1, 4.2, 4.3, 4.4, and 4.5). No inhibitory interneurons were found in either genotype, as determined by GAD67+ staining (see Figures 4.1 and 4.6). There is no significant difference in percentage of each cell type between Ds21 and Ts21 grafts (see Figure 4.1).

These grafts are distinct in their presentation when compared to past grafts derived from the Shi *et al.* [85] protocol [39] (see Figure 1.8). Differences include a significant increase in the amount of Ts21 astrocytes (hGFAP+) compared to controls in Real *et al.* [39] grafts (see Figure 1.8) which is normalized in current grafts. Real *et al.* [39] grafts were also found to have significantly lower SATB2+ upper layer neuron levels in Ts21 compared to controls [244], which is also normalized in current grafts. Genotypic differences in SATB2+ neurons and hGFAP+ astrocytes have been normalized using the Nizetic differentiation protocol. Despite these major differences, there are also a number of similarities between the grafts including the presence of TBR1+ deep layer neurons, Pax6+ progenitor neurons, Ki67+ dividing progenitors, and

hGFAP+ astrocytes. Real *et al.* [39] past grafts contained no significant difference in the percentage of Pax6+, Ki67+, and TBR1+ graft cells across both genotypes, similar to current grafts.

Alterations to the distribution of graft cell types created by switching to the Nizetic protocol significantly affected graft size (see Figure 4.8). The mean size of representative graft slices from the Ds21 graft AT60 (from Real *et al.* [39]) is approximately $1.5 \times 10^7 \mu m^2$. In contrast, the mean graft size of current grafts with 100,000 cell injections is just over $2.0 \times 10^5 \mu m^2$ based on the example slices from Figures 4.10 and 4.9. Graft sizes are even lower for 20,000 cell injections of the current graft, with mean grafts sizes of $1.0 \times 10^5 \mu m^2$ or less (see Figures 4.10 and 4.9).

Comparison between current grafts and alternative grafts created by past researchers [39] is difficult considering the sheer number of variables present involved. Changes to the level of cell types, in particular SATB2+ cells, is a major variable. The mean percentage of SATB2+ graft cells in Real *et al.* [39] Ds21 grafts at 5mpt is approximately 70%. In the current grafts, SATB2+ cells make up approximately 20% of the graft cells (see Figure 4.1). Another significant variable is the change in differentiation protocol. Differences between the protocols are not fully accessible due to product licensing. Products used in both protocols (namely media) do not have publicly available ingredient lists. This eliminates our ability to compare the components of both protocols accurately.

The variables introduced by cell type and protocol differences are some of the limitations of this study. Despite these limitations, we can still draw conclusions about DS neurodevelopment from the grafts we have derived. Importantly, the only mature neuronal subtypes present in these grafts are SATB2+, CTIP2+, and TBR1+ excitatory cortical neurons. These are the cell types most likely to be imaged and subsequently represented in EPB and activity data. The logic behind that statement is that the *in vivo* grafts are labelled using a human synapsin I promoter tag, and imaged cells are selected if they have neuronal morphology and are mature (eg: in the EPB dataset, the axons must have boutons in order to be selected for imaging, indicating a degree of maturation). If this applies, then we can hypothesize that the analysis

performed on current grafts includes both upper and deep layer excitatory cortical neurons. In contrast, the majority of the 5mpt data from Real *et al.* [39] likely comes from upper layer neurons due to the high percentage (approx. 70% of graft) of SATB2+ cells.

In the following discussion sections, I will demonstrate that I am able to draw important conclusions from my data. I will detail comparisons between my Ts21 and Ds21 isogenic control grafts. I will also compare my outcomes to those found when using a different graft differentiation protocol from the Real *et al* [39] paper, as this comparison may aid in the understanding of mechanistic changes. Graft characterization and graft size data provide the foundation upon which the remaining data will be evaluated.

5.2 Synaptic Structural Stability of Axons

The synaptic structural stability of human axons was quantified to investigate whether current grafts display differences between Ts21 and isogenic controls in early development of synaptic connections. In order to make an accurate comparison between genotypes, I first analyzed axonal EPB density and fluorescent intensity. This data allowed me to infer whether imaged axons were at comparable levels of maturity and development. From there, synaptic structural stability was compared across genotypes by EPB TOR and Fr. The following paragraphs describe the outputs for each of these measurements and compare them to previous findings from the Real *et al.* [39] grafts.

2P imaging data of axonal EPBs at 3mpt and 5mpt indicates that the density of EPBs is comparable along each selected axon. No significant density differences were found between genotypes at 3mpt (see Figure 4.12), thus these axons are comparable. Some significant differences were found in 5mpt EPB densities (see Figure 4.12), specifically days 0 and 4 when evaluated per animal and day 0 when evaluated per axon. In all cases of significant difference, Ts21 axons had higher EPB densities than Ds21 axons. I hypothesize that the difference in EPB density across genotypes is not sufficient to indicate variation in the maturation of imaged axons between Ts21 and Ds21 grafts. The reason for this is two-fold. First, not all days of imaging show significant differences in EPB density, despite all three imaging days being taken in the same axons and within a week of each other. Second, in the cases where the Ts21 axons have significantly higher EPB density on average, the axons with the highest individual densities are all Ds21. Therefore, Ds21 axons with high densities are still represented in the dataset.

EPB size provides an indication of synaptic strength [41], as a larger EPB equates to a stronger synaptic connection. The fluorescent intensity of EPBs is a direct measure of EPB size [41]. My findings indicate that there is no significant difference in the size of EPBs at both 3mpt and 5mpt. I can infer from the data in Figure 4.14 that the strength of synaptic connections is comparable in Ds21 and Ts21 graft excitatory cortical neurons at both 3mpt and 5mpt. Some significant differences were found across timepoints (see Figure 4.14 per axon and per bouton),

indicating that over time EPB size and therefore synaptic strength increases. This is indicative of normal axonal and synaptic maturation. Per bouton data indicates significant differences within both genotypes across the timepoints. This data allowed me to conclude that average synaptic strength was increasing over time.

To determine whether EPB size and strength changes differ between genotypes, intensity ratios were calculated. These intensity ratios also provide data on the plasticity of the human synapses. Changes in EPB size and strength over time may indicate synaptic strengthening or weakening indicative of plasticity created by environmental cues or neuronal activity. Figure 4.15 displays the non-directional ratio of EPB fluorescent intensity at both 3mpt and 5mpt. A Day 0 and Day 2 ratio and a Day 2 and Day 4 ratio was calculated. In both cases, the larger value was divided by the smaller value, regardless of which one came first. This provides a generalized idea of EPB size changes. No changes were found over the course of the three imaging sessions when evaluated non-directionally. Directional ratio calculations were then performed (see Figures 4.16 and 4.17) to determine whether there was any difference in EPB strengthening or weakening between genotypes. In this case, Day 2 (marked as B) is divided by Day 0 (marked as A) and Day 4 (marked as C) is divided by Day 2 (marked as B) regardless of which is the larger value. In Ds21 and Ts21 at both 3mpt (see Figure 4.16) and 5mpt (see Figure 4.17) no significant difference was found for any ratio and/or any genotype. This corroborates the non-directional data, indicating that EPB size and synaptic strength does not fluctuate significantly over the course of the 5 days of imaging. The only significant EPB size and strength fluctuations are due to normal synaptic development within the genotypes, seen between 3mpt and 5mpt (see Figure 4.14).

As I concluded that EPB density, size, and synaptic strength are relatively comparable across genotypes, I inferred that EPB turnover rate (TOR) and survival rate (Fr) would not differ across genotypes due to effects from these variables. Knowing this, I proceeded with analysis of EPB TOR and Fr. Figure 4.18 displays the outcomes for EPB TOR per animal and per axon at both 3mpt and 5mpt. In all cases, no significant difference was found between Ds21 and Ts21 within the same timepoint (n=10 animals (Ds21) and 14 animals (Ts21) at 3mpt and n=8 animals (Ds21) and 9 animals (Ts21) at 5mpt). Furthermore, the range and mean of Ds21 and

Ts21 values is similar (see mean and SEM bars in Figure 4.18, with no visible non-significant trends). This finding is further corroborated by the lack of significant difference in EPB Fr at both 3mpt and 5mpt displayed in Figure 4.19. These findings indicate an incredibly important finding: that I have developed a graft model within which the presence of an additional copy of chromosome 21 does not affect synaptic structural stability or calcium activity. These findings vary from the alternate grafts created by Real *et al.* [39], which found significantly decreased Ts21 TOR and a trend increase in Ts21 Fr compared to Ds21 controls. This indicates that the use of a novel differentiation protocol and subsequent normalization of cell-type distributions/decrease in graft size across the genotypes has created grafts with normalized EPB TOR and Fr outcomes at 3mpt and 5mpt. It is worth noting that the significant decrease in Ts21 EPB TOR from previous work [39] was only found in the Ts21 cell line (Ts21-2) that was not an isogenic match to the control line being used. The lack of paired isogenic controls is a limitation of the previous work that was addressed in this project. However, this may indicate that the decrease in Ts21 TOR should be evaluated as a trend decrease rather than a statistically significant one (as was the case with the Ts21 line that matched the control isogenically (Ts21-1)). Regardless, previous data found a decrease in Ts21 TOR and an increase in Ts21 Fr that was at least a visible trend, which has been completely eliminated in the current grafts.

The major points to take away from the synaptic structural stability data are as follows:

1. **Comparable Bouton Densities:** Data collected on EPB density per μm of axon indicates no significant difference in EPB density at 3mpt and a hypothesized lack of significant difference at 5mpt based on the majority of the imaging days having no significant difference between genotypes.
2. **Comparable Bouton Size & Strength:** Analysis of fluorescent intensity of EPBs in relation to axonal backbone fluorescence indicates no significant alterations in EPB size and strength over the course of the imaging week or between genotypes at either timepoint.
3. **Normalization of EPB TOR and Fr:** No significant differences were found between Ds21 and Ts21 EPB TOR and Fr at 3mpt or 5mpt, indicating a lack of effect from the

additional copy of chromosome 21 in Ts21 grafts.

How is it that EPB TOR and Fr are comparable across genotypes, when one genotype has an extra copy of an entire chromosome? A number of variables are at play that could contribute to this outcome. The lack of human inhibitory interneurons (GAD67+) in the graft is a possible factor. Interneurons are known to be significant players in DS cortical alterations and developmental deficiencies. Excitation/inhibition (E/I) imbalance remains at the core of DS research [186]. Many studies have demonstrated the effect of over-inhibition by Ts21 GABAergic parvalbumin positive and somatostatin positive interneurons in mouse models of DS, linking inhibition to cognitive, intellectual and memory deficits [183], [186], [189], [188], [187], [248]. It is possible that the absence of trisomic inhibitory interneurons created Ts21 grafts with a decreased severity of impairment, thus making genotypic differences easier to normalize. However, the lack of human interneurons is present in both genotypes. Furthermore, the Real *et al.* [39] grafts revealed TOR and Fr abnormalities in Ts21 despite an equivalent absence of inhibitory interneurons. I must therefore conclude that the lack of inhibitory interneurons may have primed the grafts for TOR and Fr normalization, as the synaptic phenotype may be less severe without them, but this cannot be the sole causal factor.

Therefore, I hypothesize that the following factors may contribute to the normalization of EPB TOR and Fr in current grafts, in addition to the lack of interneurons:

1. **Increase in Astrocyte Levels:** No significant difference in astrocyte level was found in current grafts. Additionally, astrocyte levels were significantly higher in my graft in both genotypes when compared to grafts that use cells differentiated with the Shi *et al.* protocol [85], [39].
2. **Decrease in Graft Size:** The significant decrease in graft size of current grafts compared to those from Real *et al.* [39] (see Figure 4.8).
3. **Altered Levels of Specific Cell Types:** Changes to the levels of each cell type in both genotypes, caused by altering the differentiation protocol (eg: approx. 20% of current graft cells are SATB2+, compared to approx. 70% in Real *et al.* [39]).

I will discuss how these hypotheses may contribute to the outcomes found in each section of my results, starting with EPB synaptic stability.

Astrocytes play a significant role in Ts21 and affect the development, synaptic strength, and activity levels of cortical neurons [190], [112]. For example, Mizuno *et al.* [190] demonstrated using iPSC-derived cultures *in vitro* that Ts21 astrocytes produce an elevated frequency of spontaneous calcium currents, leading to deficits in adjacent neuronal calcium fluctuations. I postulate that the significant increase in astrocyte levels in current grafts (both genotypes) compared to past grafts made using the Shi *et al.* [85] protocol would create variability in development and EPB strength. Since astroglial release of glypicans and thrombospondins promotes the development of the pre- and post-synapse respectively [21], [33], [34], increased astrocyte number within the graft would increase the amount of secreted pro-synaptic factors in the graft microenvironment. I hypothesize that increased release of glypicans and thrombospondins could increase the number of new EPBs being produced in current grafts of both genotypes. Increasing the number of new EPBs would increase TOR, thus creating an opposing effect to that found in Real *et al.* [39]. This hypothesis could be null if synapse elimination was able to compensate. However, at early stages of differentiation, it is possible that compensation by synapse elimination has not yet been introduced at the level necessary to leave TOR unchanged. Not only are the levels of astrocytes normalized across genotypes in our grafts, allowing for increased comparative accuracy, but the levels of astrocytes are significantly higher than past grafts, which may have reversed the EPB TOR effects of the Ts21 simply by secreting pro-synaptogenic factors in elevated concentrations. Furthermore, previous literature has identified that WT neurons develop a significantly higher number of synapses when co-cultured with Ts21 astrocytes [190]. Co-culture studies have demonstrated the effects of Ts21 astrocytes on surrounding neurons, including that they affect synaptogenesis and synaptic plasticity by altering activation of the mammalian target of rapamycin (mTOR) pathway [195].

Graft size is the second factor that I hypothesize is at play in the normalization of EPB TOR and Fr. Current control grafts are significantly smaller than grafts from Real *et al.* [39] (see Figure 4.8). Grafts are isolated islands of human cells surrounded by mouse brain, as shown by connectivity data in Real *et al.* [39]. Therefore, smaller grafts have more contact with the mouse

brain. This is visible in Figure 4.7. A higher degree of interaction with the non-trisomic mouse brain and its microenvironment would have a significant impact on graft development. Morrow *et al.* [249] published a paper which highlights that environment can drastically change the way that cells develop, including cross-species neuronal environments. They found that the mouse cells plated on E18 rat slices differentiated mostly into neurons, with less than 10% becoming glial. When plated onto rat slices from P15, over 90% of the mouse progenitors mature into glial cells [249]. I hypothesize that the reduced size of current grafts has significantly contributed to the normalization of EPB TOR and Fr by means of providing a higher degree of disomic microenvironmental factors than those present in larger grafts. It is important to note that unpublished data from my group demonstrates that Ts21 grafts were significantly smaller than control grafts using the previous protocol. Therefore, the control grafts from the previous protocol had less exposure to the mouse microenvironment than their Ts21 counterparts. This also points to graft size and the degree of interaction with the mouse microenvironment playing a significant part in the normalization of Ts21 synaptic structural phenotypes.

Between the current and past protocol grafts, there is a significant change in the density of each cell type. This is the third factor that I hypothesize contributes to normalization of EPB TOR and Fr. The largest cell type distribution difference is in SATB2+ upper layer neurons. Current grafts contain approximately 20% SATB2+ cells, while past grafts contained approximately 70% SATB2+ cells at 5mpt [39]. I initially hypothesized that fluctuations in SATB2+ levels across protocols could be a result of the protocol favouring specific cell types, or could be due to differences in the degree of neuron maturation across protocols. As stated previously, comparative analysis of the differentiation protocols is not possible due to lack of detailed product information. As such, I cannot hypothesize accurately on the possibility of one protocol favoring upper layer neurons more than the other. However, it is possible that the current grafted neurons may be at an earlier developmental stage than those from the Real *et al* [39] paper. As shown in Figures 1.1 and 1.2, upper layer neurons develop after deep layer neurons in the neurogenic process. Such a significant decrease in SATB2+ cells in the current grafts compared to past grafts may indicate that most cells have yet to reach upper layer neuronal maturation stages. This hypothesis fits with the levels of CTIP2+ deep-layer neurons,

which are lower in current grafts (under 10%) than in previous grafts (approx. 20%) [39], and with Pax6+ neuronal progenitor levels, which trend higher in current grafts (Current:25-38%; Real *et al.*:15-25% [39]). TBR1+ deep layer neurons cell levels are comparable, as are Ki67+ cell levels (see Figure 4.1).

While SATB2+ and CTIP2+ cell levels make it appear like current grafts are less mature, astrocyte data indicates otherwise. Current hGFAP+ characterization indicates astrocytes contribute approximately 30-35% of the graft (see Figure 4.1). This is much higher than the approximately 5% graft contribution from astrocytes in the Real *et al.* [39] paper. Figure 1.1 shows that astrocytes develop at the latest stages of corticogenesis. This contradicts the previous hypothesis of current grafts being at earlier stages of maturation, as the number of astrocytes is significantly higher. Differences in neuronal maturation wouldn't create this pattern of differentiation, so what would? The answer lies in the ratio between SATB2+ upper layer cortical neurons and hGFAP+ astrocytes in the two protocols. The current grafts have a lower level of SATB2+ upper layer neurons and a higher levels of hGFAP+ astrocytes than previous grafts from the Real *et al.* [39] paper at 5mpt. My hypothesis is that the current graft differentiation protocol (from Nizetic Lab) provides an environment within which the switch from neurogenesis to gliogenesis is more likely to occur.

There are a number of factors that increase gliogenesis and decrease neurogenesis. The IL-6 cytokines leukemia inhibitor factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) are all pro-gliogenic via their binding to the co-receptors LIF β and glycoprotein gp130 [250]. The binding of LIF β and gp130 activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, which is gliogenic as STATs bind to the promoter regions of S100 β and GFAP, respectively, inducing their transcriptional activation. Transcriptional activation occurs via the formation of the a complex containing STAT3, Smad, and a coactivator family called p300/CBP containing histone acetyltransferase p300 (p300) and CREB-binding protein (CBP). The Smads from this complex are signalled by the pro-gliogenic binding of bone morphogenic protein 2 (BMP2). An additional function of the CT-1 ligand is that it activates nuclear receptor corepressor (NCoR) relocation from the nucleus into the cytoplasm, thus allowing for the Notch-signalled recombination binding protein for immunoglobulin

kappa J region (RBP-J) to be able to bind to the GFAP promoter and induce transcription [250]. Inhibition of neurogenesis also occurs from binding of the neurogenin 1 (ngn1)/inhibitor of DNA binding (Id) complex on alpha-tubulin promoters. During neurogenic phases, Smad and p300/CBP form a pro-neurogenic complex with ngn1 instead of STAT3 [250]. When the pro-gliogenic STAT3 complex is formed, ngn1 binds with Id and becomes anti-neurogenic. Nuclear factor 1 (NF-1) is an additional pro-gliogenic/pro-astrocytic factor which promotes GFAP transcription and has been found to separately inhibit neurogenesis [250].

Pro-gliogenic factors which increase astrocyte differentiation and decrease neuronal differentiation could be more highly expressed in current grafts, leading to the altered distribution of cell types compared to Real *et al.* [39]. This includes NFI, JAK, STAT3, BMP2, Notch, RBP-J, NCoR, Smad (in particular Smads 1, 5, and 8 which are signalled by BMP2), p300/CBP, CNTF, and LIF [250], [249]. While Miller *et al.* [250] outline multiple pro-gliogenic pathways, these are far from the only ones. Morrow *et al.* [249] have demonstrated that fibroblast growth factor 2 (FGF2) can induce glial fates. They grew E15 extracted mouse progenitors on top of rat cortical slices from various stages of pre- and post-natal development. My hypothesis is that this would include secreted factors such as LIF, CT-1, BMP2, Notch, FGF2, and CNTF, thus increasing pro-gliogenic maturation in the grafts.

As previously stated, an increase in astrogliogenesis and thereby an increase in astrocyte number in the graft would elevate levels of secreted cholesterol, glypicans, and thrombospondins [21], [33], [34]. These pro-synaptogenic factors may elevate EPB TOR by inducing the development of many new EPBs in a shorter time-span. Additionally, with increased astrogliogenesis comes decreased neurogenesis, as exemplified by the decrease in the levels of SATB2+ neurons in current grafts compared to previous [39] grafts. The decreased levels of SATB2+ upper layer neurons in current grafts would increase the likelihood of other excitatory cortical neuron types (CTIP2+, TBR1+) being selected for imaging along with SATB2+ cells. Given that Real *et al.* [39] grafts were made up of approximately 70% SATB2+ cells, we can assume that the majority of imaged cells were upper layer neurons. In contrast, SATB2+ cells make up only approximately 20% of current grafts, thus diminishing the number of upper layer neurons likely to make up the EPB TOR and Fr dataset. This shift in cortical neuron type selection is

important. As stated in the introduction, EPB-rich axons are more likely to be found in upper cortical layers, while TB-rich axons are more likely to be found in deep cortical layers [38], [37]. This means that past data was mainly composed of SATB2+ cells with EPB-rich axons, while current data contains fewer EPB-rich axons and more TB-rich axons from deep layer neurons. EPB-rich axons have been found to have increased synaptic stability [41], thus by changing the cell type distribution in current grafts, we may have inadvertently decreased synaptic stability. This ties the cell-type composition of the grafts directly to the graft axonal EPB TOR.

Now that I had confirmed a lack of significant difference in synaptic structural stability across genotypes in the current grafts, the next step was to identify whether differences in spontaneous and oscillatory calcium activity were also normalized across genotypes. My hypothesis was that I would find no significant difference in activity as I had found no significant difference in EPB TOR, Fr, size, and strength. Additionally, I hoped to use the data collected to narrow down some of the mechanisms behind this normalization; a daunting task considering the number of variables involved. The next sections describe my work with neural activity and some correlational analyses performed to narrow down DS dysfunction mechanistic factors. I will include discussion of the potential mechanisms involved in current findings, as well as discussion on how these data compare to the grafts from the Real *et al.* [39] paper.

5.3 Calcium Activity: Spontaneous Somatic Activity & Oscillatory Network Activity

Calcium fluctuations were evaluated using 2P microscopy by measuring GCaMP6+ signal during t-series imaging at 1mpt, 3mpt, 5mpt, and in a small pilot population at 7-8mpt. After t-series processing including motion correction and 3D filtering, the change in fluorescent intensity ($\Delta F/F_0$) was extracted. At this point, a threshold $\Delta F/F_0$ value was needed in order to remove background noise while maintaining as much low-level activity as possible. Spontaneous somatic activity data were analyzed for integral ($\Delta F/F_0$) outcomes at a variety of thresholds (see Figure 4.20) using 1mpt, 3mpt, and 5mpt data from both genotypes. Based on these data

I selected a threshold of $30\Delta F/F_0$ to be used for all proceeding analyses. I believe that this threshold eliminates the majority of the background noise from the signal while maintaining the highest degree of low-level GCaMP6+ activity (see Figure 4.20).

Spontaneous somatic activity data were analyzed to determine the amplitude ($\Delta F/F_0$), frequency (Hz), and integral ($\Delta F/F_0$) of the activity from each soma independently. Figure 4.21 depicts what the tdTomato structural imaging and GCaMP6+ calcium imaging look like in current grafts. No significant differences were found between Ds21 and Ts21 animal in the percentage of active somas, the integral, the frequency, or the amplitude of signals (see Figure 4.22). This includes both within each timepoint and across all timepoints for 1mpt, 3mpt, and 5mpt. When analyzed per axon, the same data again shows no significant differences between Ds21 and Ts21 at 1mpt, 3mpt, and 5mpt (see Figure 4.23). I did find significant differences in all outcomes when evaluating differences from one timepoint to the next. On average, the frequency, amplitude, and integral appears to decrease from 1mpt to 3mpt in Ts21 somas. Since this significance is lost at the per animal level, this can only be viewed as a trend, but it may also point to cell death between 1mpt and 3mpt.

A few animals were imaged at 7-8mpt as part of the Aim 5 proof of concept. These were analyzed and compiled into a pilot study of the most mature grafts analyzed to date (see Figure 4.24). This data was evaluated per animal (Ds21 = 1 animal, Ts21 = 3 animals) and per axon (Ds21 = 15 axons, Ts21 = 86 axons). Statistical analyses could not be performed for per animal data due to $n=1$ for the Ds21 group, however the amplitude, frequency, and integral outcomes appear to have comparable means with no visible trend differences (see Figure 4.24). Statistical analyses on per axon data confirmed no significant differences between Ts21 and Ds21 in all three outcomes. This pilot data tentatively matched with the outcomes from 1mpt, 3mpt, and 5mpt showing no significant effect of the trisomy of chromosome 21 on somatic activity levels.

The next step in this project was to utilize the calcium fluctuation data to evaluate the level of activity at the network level. I wanted to understand whether the spontaneous activity previously quantified was occurring across multiple somas in synchronous network patterns,

known as oscillations. Oscillatory network activity data were extracted from the same t-series as the spontaneous somatic activity. Using a threshold of $30\Delta F/F_0$, active somas were identified. Subsequent analysis was performed to determine the percentage of imaged ROIs containing no activity, activity from only 1 soma, or activity from 2 or more somas (see Figure 4.25). This data provides an indication of the number of ROIs collected compared to those that were able to be included in the remainder of the oscillatory activity dataset. All ROIs containing no active somas or only 1 active soma were excluded from further oscillatory analysis. These data indicate that a significant portion (40-50% or more) of the ROIs imaged contain 1 or fewer active somas and were not able to be included in the oscillatory analysis dataset. This is a lower degree of coactivity than was present in past grafts, which found almost 100% of ROIs had population burst activity by 3mpt [39]. Subsequent figures (see Figures 4.26 and 4.27) show the percentage of coactive somas at all frames of each ROI (see Figure 4.26) and of the mean of all ROIs (see Figure 4.27). The mean values in Figure 4.27 indicate that the maximum average percentage of coactivity is approximately 35%. No significant correlation between the total number of somas per ROI and the percentage of coactivity in the same ROI was found (see Figure 4.28), therefore percentage of coactivity was evaluated by threshold to have a better idea of comparative values (see Figure 4.29). The curves are comparable between Ds21 and Ts21 at all timepoints, particularly 1mpt and 3mpt. The later timepoints (5mpt and 7-8mpt) have slight variation in distribution (approx 5% variation in both cases at $30\Delta F/F_0$). However, this trend is not consistent, flipping from lower coactivity in Ts21 at 5mpt to higher coactivity in Ts21 at 7-8mpt. By a threshold of $50\Delta F/F_0$, all data are comparable and almost no coactivity is present at all timepoints. I therefore conclude that oscillatory network activity is comparable between Ds21 and Ts21 at all timepoints, with regards to percentage of coactivity per frame.

I also evaluated oscillatory activity in terms of strength, a measure of somatic correlation by synchronous calcium firing. This analysis takes the somas with significant ($p < 0.05$) coactivity and evaluates the correlation coefficient (r) of that coactivity relationship. Figure 4.30 illustrates the outcomes of this strength analysis, indicating that while there is a significant increase in oscillatory activity strength in Ts21 compared to Ds21 at 1mpt, this significance is gone by 3mpt. No significant difference between genotypes is found at 3mpt, 5mpt, or in the pilot

dataset at 7-8mpt.

The lack of significant differences found in spontaneous and oscillatory calcium activity outcomes agrees with the hypotheses derived from EPB TOR and Fr results. Like the synaptic structures, the spontaneous and network activity of the somas has also been normalized across genotypes, with the exception of oscillatory strength at 1mpt. In both synaptic stability and calcium activity measures, the presence of a third chromosome 21 is insufficient to create a significant phenotype. Why is this the case? Let us return once again to the hypotheses listed in the previous section, which were determined by comparison with the past grafts from Real *et al.* [39].

Hypotheses for Factors Affecting Normalization of Synaptic Structural Stability and Neural Network Activity:

1. **Lack of Inhibitory Interneurons:** Current grafts lack inhibitory GAD67+ interneurons in both genotypes. While this cannot be the only factor at play, the lack of Ts21 interneurons likely decreases the severity of the Ts21 phenotype, thus priming the graft for normalization by other factors.
2. **Decrease in Graft Size:** The significant decrease in graft size of current graft compared to those from Real *et al.* [39]. This decrease in graft size translates to increased effect of and interaction with the surrounding mouse brain.
3. **Altered Levels of Cell Types:** When comparing past and current grafts, certain cell types contribute significantly different amounts to the total graft volume. The most stark example is SATB2+ cells, which make up approx. 20% of current graft cells and approx. 70% of graft cells from Real *et al.* [39].
4. **Increased Astrogliogenesis/Increased Astrocyte Levels:** Due to the decrease in SATB2+ cells and increase in hGFAP+ astrocytes in the current graft (compared to Real *et al.* [39] grafts), I hypothesize that increased astrogliogenesis could play a part in the normalization of synaptic stability and calcium activity outcomes. Altering protocols may have altered expression levels of pro-gliogenic factors such as LIF, CNTF, CT-1, STAT3,

or others. More astrocytes lead to an increase in secreted pro-synaptogenic factors such as glypicans and thrombospondins.

In the context of spontaneous and oscillatory activity, a lack of trisomic GAD67+ interneurons would eliminate expected over-inhibition of excitatory cells [251]. This over-inhibition by Ts21 GABAergic neurons has been demonstrated to decrease spontaneous and oscillatory activity of excitatory pyramidal neurons [251]. Therefore, the lack of inhibitory interneurons in these grafts would eliminate the over-inhibition aspect of DS from our grafts, making Ds21 and Ts21 graft activity more comparable. As such, the lack of significant differences in our spontaneous and oscillatory activity levels agree with the hypothesis that the lack of inhibitory neurons creates an environment whereby Ds21 and Ts21 grafts are more comparable. However, past research from my group has shown that excitatory Ts21 pyramidal cortical neurons have significantly decreased activity levels in the absence of inhibitory interneurons, both *in vitro* (Peter, Livesey, and DePaola, unpublished) and *in vivo* [39]. The lack of inhibitory interneurons cannot be the only factor affecting the normalization of activity across our genotypes.

Based on changes to astrocyte and upper layer neuron levels across genotypes in current grafts compared to grafts developed with alternative differentiation protocols [39], I hypothesize that and increased level of gliogenesis could be occurring in my grafts due to the use of the Nizetic protocol. Increased astrogliogenesis and decreased neurogenesis both affect excitatory neuronal activity, as the roles of both neurons and astrocytes are crucial in spontaneous and oscillatory activity processes [39], [183], [56], [190], [195]. A decrease in neurogenesis results in fewer excitatory cortical neurons in the graft. If, as in Real *et al.* [39], over 90% of the graft synapses are human to human, fewer excitatory neurons would mean less available connections and fewer developed human circuits. This may also be affected by or be a causal factor for the decrease in graft size in current grafts compared to previous grafts. With smaller grafts and fewer developed activity circuits, less calcium activity would be present, particularly at the oscillatory activity level. The effect of Ts21 on spontaneous and oscillatory activity would therefore be limited. It is possible that the combination of smaller graft size and fewer developed circuits slowed the development of these cells.

I have hypothesized that current grafts have increased astrogliogenesis in comparison to the Real *et al.* [39] grafts. But how would this affect graft calcium activity? DS astrocytes have been shown to decrease the excitatory activity of neighbouring WT neurons, both human [190] and rat [195]. DS astrocytes themselves demonstrate 7-34X higher spontaneous calcium fluctuations compared to control astrocytes [190]. Therefore, the increased calcium fluctuations in Ts21 astrocytes create activity deficits in co-cultured neurons. There are many mechanisms that affect the astrocyte/neuron synaptic relationship and their calcium activity levels. One such mechanism hypothesized to play a part is the mTOR pathway, which is hyperactivated in DS neurons [195]. The mTOR pathway is a known contributor to synaptic development, synaptic plasticity, long-term plasticity (LTP) and the learning process in development [252]. DS astrocytes have an additive effect on mTOR hyperactivity, contributing increases to the phosphorylation levels of mTOR and other mTOR pathway proteins independently of co-cultured neuronal-derived mTOR hyperactivation [195].

If hyperactivation of mTOR via Ts21 astrocytic activity alters neuronal synaptogenesis and creates deficits in WT neuronal activity levels, then why is there no significant difference between Ds21 and Ts21 current grafts in spontaneous or oscillatory calcium activity measurements? To answer this query, I attempted to find a direct comparison between past and current graft activity levels. Oscillatory calcium activity can be compared with available data by using the burst activity in from Real *et al.* [39]. Figure 5.1 shows the figure from the Real *et al.* [39] which details the percentage of ROIs containing burst activity, the frequency of the burst activity, and the level of global oscillatory activity as defined by the integral output. Global activity level values are not comparable to the current dataset given that the level of background noise was significantly different and therefore the threshold utilized was also different. However, the trends can be compared. A significant deficit was found in Ts21 global oscillatory activity (integral) compared to controls in both pairs of cell lines. Likewise, Ts21 neurons demonstrates significantly reduced activity frequencies (Hz) compared to controls and the number of ROIs containing burst activity was significantly lower in Ts21 compared to control.

Figure 4.25 provides the an indication of the percentage of ROIs with bursts in current grafts. The green bars (2+ active somas) in Figure 4.25 are the maximum percentage of ROIs that

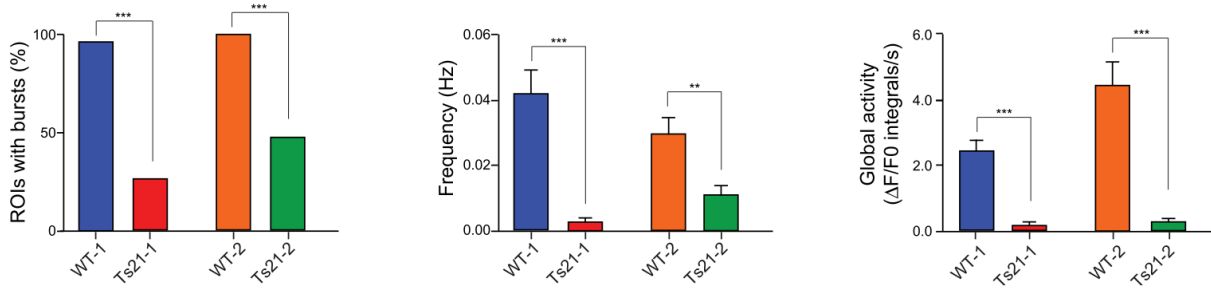


Figure 5.1: Burst calcium activity data from control (WT) and Ts21 lines. Burst activity refers to oscillatory calcium activity. This figure shows the percentage of ROIs with burst activity (left), the frequency of burst activity (middle, in Hz), and the level of global oscillatory activity (right, integral). From Real *et al.*, 2018 [39]. Reprinted with permissions from AAAS. For permissions, see Appendix Table 2.

could have burst activity. Where previous control graft ROIs almost all contain burst oscillatory activity (see Figure 5.1, left), current grafts could have no more than 60% of ROIs with burst activity, with some timepoints containing a maximum of 20% of ROIs with bursts (see Figure 4.25). Therefore, it is clear that burst activity is present in significantly lower amounts in current grafts. Many imaged ROIs containing neurons did not present with significant calcium fluctuations within the imaging period, whereas nearly all ROIs had burst activity in past control grafts. Interestingly, Ts21 past grafts had a significantly lower percentage of ROIs with bursts than control lines (see Figure 5.1, left). Ts21 lines contained on average approximately 25% and 48% of ROIs with bursts respectively. This is more similar to current grafts. Overall, this indicates that past control grafts had much more burst activity than current grafts, but Ts21 lines from past grafts may be more similar. This may point to a difference caused by the unpublished decrease in size of previous Ts21 grafts compared to previous controls, stated in the synaptic structural stability section of this discussion. That means that past control grafts had a decreased effect from the mouse brain than Ts21 grafts, which may have influenced the amount of oscillatory activity present. Current grafts are small and therefore follow a similar deficit in the percentage of ROIs with burst activity as the past Ts21 grafts. Additionally, the frequency and global activity integral of oscillatory activity bursts (see Figure 5.1) can be compared to the same outcomes from spontaneous activity (see Figures 4.22, 4.23, and 4.24). The analysis methodologies varied for current and past graft oscillatory analysis due to a larger degree of background noise in current graft recordings, therefore I do not have these exact

outcomes for oscillatory activity, only for spontaneous activity. Nonetheless, this provides us with some information. Namely, past grafts had significant deficits in frequency and integral of burst activity in Ts21 grafts compared to controls, which was not present in current grafts. Thus, if I assume that the difference between Ds21 and Ts21 is comparable across spontaneous and oscillatory activity, even if the values are different, then current grafts have normalized activity levels across genotypes where past grafts had a large difference. Again, the presence of a higher mouse brain influence in past Ts21 grafts compared to controls could be influencing this normalization. Therefore, graft size as a measure of mouse brain interaction is solidified as a likely mechanistic contributor to the change in calcium activity phenotype seen between current and past grafts.

Current Ds21 and Ts21 grafts have comparable astrocyte levels, thus activity differences are lessened. The decreased graft size of current control grafts (see Figure 4.8) is another likely contributor to the lack of difference across current graft genotype activity levels. Araujo *et al.* [195] not only demonstrate that astrocytic secretions affect neuronal activity, but also that WT astrocytes can minimize Ts21 neuron activity changes. Thus more exposure to the WT mouse brain neurons and astrocytes likely diminishes the effect of the Ts21 astrocytes in our current grafts. As stated previously, Morrow *et al.* [249] also describe the significant effect of cross-species microenvironments on developing neuronal progenitor fates. Microenvironmental factors secreted by the mouse brain could be changing the levels of certain cell types [250], [249], contributing to the higher level of astrocytes and lower level of SATB2+ neurons in current grafts compared to past grafts that were significantly larger. Upon inspection of gene expression profile research in the literature, LIF, CNTF, FGF2, and BMP2 are all not significantly changed in their expression levels in DS. However, the HSA21 gene $S100\beta$ is significantly overexpressed in DS [253], [190] and is directly affected by the JAK/STAT pathway binding caused by the binding of LIF/ β and gp130 [250]. $S100\beta$ expression is also significantly tied to astrocyte activity in DS [190]. Gene expression is something my group will be expanding on in the next few years in an in-depth analysis of the Shi *et al.* [85] and Nizetic [39] protocols. Current data only provides room for hypotheses when it comes to gene expression and its effects. It is important to note that a major confounding factor in comparisons across these two protocols is the differences

found in the DS genotype across individuals. Since current experiments utilise cells taken from the fibroblasts of a different individual to those of Real *et al.* [39], the gene expression experiments planned and ongoing by others in my group will be crucial. While I could not go into detailed transcriptomic analysis in this thesis, I expect that future analyses will test the hypotheses laid out in this thesis.

5.4 Correlative Analysis: GFAP+ and SATB2+ Graft Cells

Correlative analyses were performed to investigate the relationship between the number of astrocytes and upper layer excitatory neurons present in current grafts and the other experimental outputs collected from those same grafts: synaptic structural stability and calcium activity. Correlations were performed for SATB2+ upper layer neurons and hGFAP+ astrocytes as these are the two cells types that have been normalized across genotypes in current grafts, but were present in significantly different amounts across genotypes in previous grafts [39]. Specifically, Real *et al.* [39] grafts had significantly lower levels of SATB2+ cells and significantly higher levels of hGFAP+ cells in Ts21 grafts compared to controls. In current grafts, these levels are not significantly different across Ds21 and Ts21 (see Figure 4.1). Given that astrocyte levels are much higher in my grafts, both Ds21 and Ts21 grafts produced for this thesis have increased above the levels of the Real *et al.* [39] paper grafts and have evened out, becoming comparable across the two genotypes.

The correlation between the number of SATB2+ upper layer excitatory neurons and the synaptic structural stability (EPB TOR) of the same grafts was not significant at 5mpt ($r=0.4000$; $p=0.7500$; $n=4$ animals) (see Figure 4.31). The correlation between SATB2+ cell number and the spontaneous calcium activity integral in the same grafts was also not significantly different ($r=0.5000$; $p>0.9999$; $n=3$ animals) (see Figure 4.31). Note that these correlations are from a mixture of Ds21 and Ts21 animals as no significant difference was found between Ds21 and Ts21 in EPB TOR (see Figure 4.18) or calcium activity integral (see Figure 4.22) at 5mpt.

The correlations with hGFAP+ astrocytes were calculated in the same way as SATB2+ cell level correlations. The correlation between the number of hGFAP+ astrocytes per cubic micrometer and the spontaneous activity integral was not significant ($r=0.2842$; $p=0.08077$; $n=3$ animals) (see Figure 4.32). However, the correlation between the number of astrocytes per cubic micrometer and the EPB TOR of the same grafts at 5mpt was significant ($r=0.9968$; $p=0.0253$; $n=3$ animals) (see Figure 4.32). This significant correlation is sufficiently powered (see Power Calculations in Methods Section) and is a positive correlation, indicating that the TOR of EPBs increases as the number of human astrocytes per cubic micrometer increases.

These data expand on the hypothesis that changes to cell type amounts in current vs past grafts contribute to the normalization of synaptic structural stability and neural network activity outcomes. Since no significant correlations were found between EPB TOR or Integral and SATB2+ cell levels but significant correlation was found between hGFAP+ cell levels and TOR, it is likely that SATB2+ upper layer excitatory neurons are contributing less to the normalization than astrocytes. Current grafts have a higher number of astrocytes than upper layer SATB2+ neurons, so it is logical that astrocytes contribute to the phenotype to a larger degree. But what of the positive trend between astrocyte number and TOR? And why is there no significant trend between astrocyte number and spontaneous activity integral?

A positive correlation between EPB TOR and astrocyte number means that when human astrocyte number (per cubic micron) increases, so does the TOR of axonal EPBs. An increase in EPB TOR means that boutons are being formed and lost more quickly, not remaining stable or growing in strength. Synaptic pruning, a naturally occurring process in early neuronal development, may be a contributing factor to EPB loss. Pruning would increase EPB TOR, but should maintain or even strengthen the activity from the remaining synapses as this allows for energy to be diverted to strengthening commonly used synaptic connections. This could explain why there is a significant trend between hGFAP number and TOR but not between hGFAP number and activity integral. Furthermore, astrocytes play a large part in the formation and activity of the synapse. It is their roles in the synaptic cleft that has led to the use of the term “tri-partite synapse”. Relationships between astrocytes and synaptic structural stability are key to neuronal function. But while their relationship is without question, the positive

correlation here may be simply correlation and not causation due to the number of variables that influence in tri-partite synapse in its development. The lack of significant correlation with integral to match the TOR/hGFAP correlation decreases the likelihood of the TOR/hGFAP correlation being causative. Furthermore, the GFAP/TOR correlation found in current grafts is positive, indicating an increase in TOR when the number of GFAP positive cells is higher. However, previous grafts found the opposite effect. This correlation was not analysed in their paper, however it appears that TOR increased with greater numbers of GFAP. This further increases the likelihood that the GFAP/TOR correlation seen in the current grafts is created by multiple factors. Further experimentation would be needed to determine exactly why this correlation seems to have switched from negative in Real *et al.* [39] grafts to positive in current grafts.

So what does this correlational analysis provide? Ultimately, few concrete conclusions can be taken from these data. These are correlations that need to be researched further to confirm or deny their validity. Future and ongoing work in my group will address this, particularly by examining genomic and transcriptomic datasets comparing current and past differentiation methods. However, these data do indicate that astrocytes are important in the current dataset grafts. These data will influence where genomic and transcriptomic experimentation should be focused. I hypothesize that they will find variance in astrocytic function caused by alterations to expression of significant factors like S100 β between the two differentiation protocols. Overall, the lack of significant differences found in current grafts are a crucial step in our understanding of early DS development. Isolation of the factors that eliminate the effect of Ts21 on synaptic stability and neural network activity in current grafts compared to previous grafts may be the key to advancing DS understanding and treatment.

Chapter 6

Conclusion

6.1 Summary of Thesis Achievements

In this thesis, I successfully developed a novel graft type for studying DS using *in vivo* xenotransplantation and longitudinal multiphoton imaging. This novel graft type was developed by the differentiation of neuronal progenitors *in vitro* from fibroblast-derived iPSCs using a differentiation protocol which I have termed the Nizetic protocol. This protocol limits experimenter bias, and develops neuronal progenitors that are able to be injected into the somatosensory cortex of an NSG mouse and continue to develop into a range of functional, active cortical neurons, glial cells, and progenitors *in vivo*.

The main finding of this study is that there are no significant differences in synaptic structural stability or spontaneous and oscillatory calcium activity were found between Ts21 and isogenic control lines. **Therefore, the presence of a third copy of chromosome 21 is insufficient to create a significant alteration to the synaptic structural stability of calcium activity (both spontaneous and oscillatory) in these grafts at early stages of development.** This was determined by evaluating EPB TOR at 3mpt and 5mpt in both genotypes and by analyzing spontaneous and oscillatory somatic calcium fluctuations at 1mpt, 3mpt, 5mpt, and in a pilot study at 7-8mpt.

Additionally, this study demonstrates that grafts of this type are able to be maintained and imaged as long as 8 months post-transplantation, with graft cells remaining active. This is the longest that grafts of this type have been maintained in adult mice to date.

Finally, the correlative analyses performed between EPB TOR and calcium Integral and the number of astrocytes per cubic micrometer indicate that GFAP+ graft astrocytes may be a main mechanistic contributor to the lack of change in the synaptic structural stability and neural network activity in Ts21 grafts. Additional experimentation is required to isolate the degree of contribution to the current phenotype provided by Ts21 astrocytes and other specific cell types. These additional experiments are addressed in the Future Work section below.

6.2 Applications

The findings from this project are beneficial and practical for scientific understanding, research applications, and medical advancement. My findings have increased our scientific understanding of DS, neurodevelopment, synaptic stability, calcium activity, and the development of neurons from induced pluripotent stem cells. The successful use of the xenotransplantation model developed by Real *et al.* [39] provides a second source of confirmation of the usefulness and applicability of that model in neurodevelopmental disease research. This work also uses a differentiation protocol with fewer detailed and/or qualitative steps, making it more reproducible and reducing experimenter bias. Additionally, this thesis provides the first proof of successful graft imaging at 8 months post-transplantation into an adult mouse brain, indicating the extent to which this model can be used for developmental research. Research applications are also aided by the findings from this project. Another grant is already ongoing in my group to follow up on this dataset (see Future Work section below for details), and I am confident that this work will propel forward research from other labs once published. This project identifies a differentiation protocol (Nizetic Protocol) which can be used to develop neuronal cultures with a significant number of astrocytes, thus providing a novel astrocyte model that can be used by the international research community to study astrocyte generation and function in

disease. Finally, this project provides information necessary for medical advancements for DS. The lack of significant difference in synaptic structural stability and neural network activity between genotypes found in current grafts, despite the presence of Ts21, means that we have achieved a model where Ts21 is not sufficient to create early developmental changes in EPB stability or network activity. Comparative analyses between current and past grafts will allow for the mechanisms behind the lack of significant changes to synaptic structural stability and spontaneous/oscillatory neuronal activity from current grafts that were present in past grafts to be elucidated, with a focus on the role of astrocytes based on my correlative analysis findings.

6.3 Future Work

Future works will aim to identify specific mechanisms underlying the current normalization of Ts21 synaptic structural stability and neural network activity compared to isogenic controls. To do this, my group will develop the current graft type (Nizetic protocol) side-by-side with grafts developed using the Shi *et al.* protocol [85] used in the Real *et al.* [39] grafts. This will allow for direct comparison and the removal of experimenter and lab-related variables. Once the grafts have been developed and grown *in vivo* inside a mouse brain, the graft cells will be extracted and used for transcriptomic analyses. In this way, the transcriptome of Ds21 and Ts21 paired grafts from both protocols will be compared and significant changes in expression levels can be identified. We hypothesize that this will isolate specific contributors to the change in DS phenotype seen across the differentiation protocols. Funding has been successfully attained for this project and a post-doc (Dr. Michael Lattke) has begun work on this project. Additionally, this grant includes access to fetal DS tissue, which will also be processed for transcriptomic analysis and compared to the data from both protocols, thereby allowing us to determine how similar these grafts are to what would be seen in the human fetal DS brain.

In addition to the project outlined above, other future works may be produced from this project including the use of the current graft type as a model for astrocytic roles in DS and/or other neurodevelopmental conditions. This could include studying astrocyte activity or development

in an *in vivo* environment, a task often limited by the time and environmental factors necessary to maintain healthy astrocytic cultures *in vitro* for extended periods and to view their relationship with other glial cells or with neurons. The proof that this model can be used for up to 8 month post-transplantation opens up a plethora of opportunities for future longitudinal developmental research in DS and other neurodevelopmental diseases or injury.

Overall, this project has accomplished the development of a novel graft type, capable of developing *in vivo* for up to 8 months post-transplantation in adult NSG mice, in which the presence of a trisomy of chromosome 21 is insufficient to create alterations to synaptic structural stability and spontaneous and oscillatory calcium activity at early developmental stages. This graft type could be used as an astrocyte research model, given the significant number of astrocytes present. The lack of Ts21 effect provides a fascinating source of information for the DS research community and the ability to investigate the mechanisms behind the early stages of neurodevelopment of DS more closely. The fact that these grafts are so astrocyte-heavy and can last for 8 months post-transplant also means that Ds21 control cells from this thesis could be used to study a wide variety of neurological functions as a model for early fetal neurodevelopment. This would be a fantastic model and graft type for researching neurodevelopmental disorders with a significant astrocytic component. The possibilities for this graft type and this model are endless, and I believe that this research significantly contributes to our understanding of DS and our ability to research early neuronal dysfunction in DS as a scientific community.

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Appendix

Table 1: Appendix: List of Primary Antibodies

Antibody	Host Species	Dilution	Reference
Doublecortin	Rabbit	1 in 400	Cell Signaling, 4604
Human Nuclei	Mouse	1 in 200	Millipore, MAB1281
Pax-6	Rabbit	1 in 300	Biolegend, 901301
Human NuMA	Rabbit	1 in 200	Abcam, ab97585
Human GFAP	Mouse	1 in 500	Stem123, AB-123-U-050
GFAP	Rabbit	1 in 1000	DAKO, Z0334
GAD67	Rabbit	1 in 500	Abcam, ab97739
TBR1	Rabbit	1 in 200	Abcam, ab31940
SATB2	Mouse	1 in 200	Abcam, ab51502
CTIP2	Rat	1 in 300	Abcam, ab18465
Ki67	Rat	1 in 500	Abcam, ab156956
Human Nestin	Mouse	1 in 1000	R&D Systems, MAB1259

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31	Figure	Fig 1.7	Lamotte et al., 2018 Citation: [174]	© 2018 Lamotte, Martinez, Devillard, Hograindleur, Satre, Coutton, Harbuz, Amblard, Lespinasse, Benchaib, Bessonnat, Brouillet, Hennebicq.	N/A	yes	Licensed with a Creative Commons Attribution 4.0 International (CC-BY 4.0)
41, 128	Figures	Fig 1.8 and Fig 5.1	Real et al., 2018 Citation: [39]	© 2018 Real, Peter, Tralbalza, Khan, Smith, Dopp, Barnes, Momoh, Strano, Volpi, Knott, Livesey, De Paola, licensee is the AAAS	Email sent to author on August 28, 2023. Confirmation of permission received on Sept 1, 2023.	yes	Permissions granted from author. This thesis meets all necessary criteria for the use of this figure, as detailed on the AAAS website.
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35	Table	Table 1.3	Bartasaghi et al., 2022 Citation: [180]	© 2022 by Annual Reviews. All rights reserved	August 15, 2023 via CCC Marketplace	yes	Order Licence ID: 1386420-1