Electrophysiological study of dopamine neurons in the dorsal raphe nucleus & ventrolateral periaqueductal grey

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Thesis submitted for the degree of Doctor of Philosophy

Declaration of originality

The experiments described within this thesis are my own unaided work, except where explicitly stated. No part of this thesis has been submitted for any other degree or professional qualification.

Declaration of publications

The majority of the work presented in chapters 2-4 of this thesis has been published in the European Journal of Neuroscience:

Dougalis, A. G., Matthews, G. A., Bishop, M. W., Brischoux, F., Kobayashi, K. and Ungless, M. A. (2012) Functional properties of dopamine neurons and co-expression of vasoactive intestinal polypeptide in the dorsal raphe nucleus and ventro-lateral periaqueductal grey. Eur J Neurosci *36*, 3322-3332

Abstract

The midbrain dopamine system plays a fundamental conserved role in regulating behaviour, and its dysfunction is associated with several neuropsychiatric disorders including addiction, schizophrenia, and Parkinson's Disease. Midbrain dopamine neurons display considerable heterogeneity in their neurochemical, electrophysiological, and functional properties, and project to many cortical and subcortical structures. The majority of these neurons reside within the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), but a less well-known, and understudied, dopamine population are housed within the dorsal raphe nucleus (DRN) and ventrolateral periaqueductal grey (vlPAG). These neurons provide the majority of the dopaminergic input to the central extended amygdala and have been implicated in the sleep-wake cycle and mediating the effects of opiates. However, their electrophysiological properties have not been examined. I have studied these neurons in mice using immunohistochemistry and electrophysiology in an acute brain slice preparation *ex vivo*. I found that they display similar properties to some VTA dopamine neurons, and noted co-expression of vasoactive intestinal peptide in a subset of this population. A characteristic feature of VTA dopamine neurons is the potentiation observed at glutamatergic synapses following a single dose of addictive drug or an acute stress experience. I investigated whether DRN/vlPAG dopamine neurons would show cocaine-induced plasticity and found that glutamatergic synapses were potentiated not only by a single dose of cocaine, but also by acute social isolation. This potentiation was associated with a change in AMPAR transmission, which is similar to that observed following cocaine in the VTA. I also investigated the hypothesis that this plasticity is a result of acute anxiety using behavioural analysis and administration of anxiolytic compounds. These findings suggest another form of salient stimulus which can induce plasticity in dopamine neurons, and have relevance to several neuropsychiatric diseases including those modelled by social isolation.

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Chapter 1. Introduction

The midbrain dopamine system plays a fundamental, conserved role in regulating behaviour. It is centrally involved in a range of mammalian functions including motor control, reward, motivation, neuroendocrine regulation, cognition, and emotional processing (Wise et al., 2004; Björklund and Dunnett, 2007a; Fields et al., 2007). Dysfunction of this system is consequently associated with a variety of disorders including Parkinson's Disease, drug addiction, attention-deficit hyperactivity disorder (ADHD), and schizophrenia (Sesack and Carr, 2002; Viggiano et al., 2003; Liss and Roeper, 2007).

1.1. Midbrain dopamine system: anatomical organisation

Dopamine neurons form a continuous network extending throughout the mesencephalon and diencephalon in the mammalian brain, but can be conveniently divided into nine major groups, denoted A8-A16 (Dahlström and Fuxe, 1964; Felten and Sladek, 1983; Björklund and Lindvall, 1984; Hökfelt et al., 1984). The best described of these are the A8 (retrorubal field), A9 (substantia nigra, pars compacta; SNc) and A10 (ventral tegmental area; VTA) cell groups in the mesencephalon. This nomenclature has remained in use since these populations were originally described in the early 1960s, which allows for the fact that the dopaminergic cell groups are not generally confined to distinct anatomical structures, and also facilitates inter-species comparison, since the distribution of cell bodies within each group can vary significantly between species (Smeets and Gonzalez, 2000).

1.1.1. VTA subnuclei

The VTA itself can be anatomically divided into five distinct dopamine cell groups (Dahlström and Fuxe, 1964; Phillipson, 1979a; Oades and Halliday, 1987). Cytoarchitectural studies in rats and humans have defined these as: the paranigral nucleus (PN), interfascicular nucleus (IF),

parabrachial pigmented nucleus (PBP), caudal linear nucleus (CLi), and rostral linear nucleus (RLi) (Phillipson, 1979a; Swanson, 1982; Halliday and Tork, 1986; Oades and Halliday, 1987). In addition, a lesser known, and relatively understudied, population of dopamine neurons has also been described in the dorsal raphe nucleus (DRN) and ventrolateral periaqueductal grey (vlPAG; Fig. 1a). This population is often referred to as a dorsocaudal extension of the A10 group (A10dc; Lindvall and Björklund, 1974; Hökfelt et al., 1976; Ochi and Shimizu, 1978; Hökfelt et al., 1984; Trulson et al., 1985; Descarries et al., 1986; Oades and Halliday, 1987; Arsenault et al., 1988; Stratford and Wirtshafter, 1990; Charara and Parent, 1998), and has been reported in rats (Hökfelt et al., 1976; Ochi and Shimizu, 1978; Nagatsu et al., 1979; Hökfelt et al., 1984; Köhler and Goldstein, 1984; Descarries et al., 1986; Stratford and Wirtshafter, 1990), cats (Miachon et al., 1984), monkeys (Arsenault et al., 1988; Charara and Parent, 1998), and humans (Saper and Petito, 1982; Pearson et al., 1983).

These neurons in the DRN/vlPAG have been confirmed as dopaminergic, as they express tyrosine hydroxylase (TH; the rate-limiting enzyme in dopamine synthesis; Levitt et al., 1965) and aromatic L-amino acid decarboxylase (AADC; the enzyme that catalyses conversion of L-dihydroxyphenylalanine, the product of TH, to dopamine; Hökfelt et al., 1976; Hökfelt et al., 1984; Köhler and Goldstein, 1984; Descarries et al., 1986; Stratford and Wirtshafter, 1990; Lu et al., 2006). However, they lack the enzyme dopamine-β-hydroxylase (DBH; expressed by noradrenergic neurons; Swanson and Hartman, 1975; Nagatsu et al., 1979; Miachon et al., 1984; Flores et al., 2004), and do not express 5-hydroxytryptamine (5-HT), suggesting they are dopaminergic and neurochemically distinct from the population of serotonin neurons in this region (Descarries et al., 1986; Stratford and Wirtshafter, 1990; Fu et al., 2010). Expression of the vesicular monoamine transporter (VMAT2) and the dopamine transporter (DAT), which are important in vesicular packaging and re-uptake of dopamine respectively, have yet to be

examined in this population. The DRN/vlPAG dopamine neurons reside in the midline, beginning in the ventral PAG, and extending to overlap with the rostral portion of the 5-HT neurons in the DRN, with only scattered dopaminergic neurons at more caudal levels, mostly occupying the region directly below the aqueduct of Sylvius (Hökfelt et al., 1984; Charara and Parent, 1998). There are few estimates of precise cell numbers in the DRN/vlPAG, and even those that exist set different boundaries, making accurate comparison difficult. Estimates restricted to the DRN suggest that there are around 1,000 dopamine neurons in the rat (Descarries et al., 1986), while in mice there are reported to be around 2,200 dopamine neurons in the DRN/vlPAG and RLi combined (Flores et al., 2004; Flores et al., 2006). This compares with around 40,000 and 11,000 dopamine neurons in the VTA of rats and mice, respectively (Nelson et al., 1996; Nair-Roberts et al., 2008).

In spite of their identification along with other midbrain dopamine groups, nearly 40 years ago, very little attention has been paid to this dopaminergic subpopulation in comparison with other components of the midbrain A10 group. This is most likely because they are less numerous than the dopamine neurons residing in the VTA, and because they are housed within a large serotonin population, which makes them difficult to target *in vitro* or *in vivo*. However, as I will describe, although these neurons appear similar to the VTA population in several respects, a few studies have indicated that they possess distinct anatomical and functional properties (e.g. Hasue and Shammah-Lagnado, 2002; Lu et al., 2006). The DRN/vlPAG population therefore represent an intriguing, understudied, dopaminergic group which warrant further investigation, particularly in light of the emerging functional heterogeneity of midbrain dopamine neurons.

1.1.2. Efferent projections of the midbrain dopamine neurons

1.1.2.1. VTA and SNc

Based on the major projection targets of the midbrain dopamine neurons, they have classically been divided into the nigrostriatal and mesocorticolimbic systems, originating in the SNc and VTA respectively. The nigrostriatal pathway projects to the dorsal striatum, (caudate-putamen in humans), while the mesocorticolimbic pathway projects to limbic structures including the ventral striatum (nucleus accumbens (NAc) and olfactory tubercle), amygdala, hippocampus, and hypothalamus, as well as limbic cortical regions including the prefrontal, orbitofrontal, cingulate, and entorhinal corticies (Anden et al., 1964; Anden et al., 1966; Ungerstedt, 1971; Fallon et al., 1978; Fallon and Moore, 1978a; Simon et al., 1979; Grecksch and Matties, 1981; Porrino and Goldman-Rakic, 1982; Albanese and Minciacchi, 1983; Björklund and Lindvall, 1984; Williams and Goldman-Rakic, 1998; Brinley-Reed and McDonald, 1999; Haber et al., 2000; Hasue and Shammah-Lagnado, 2002).

However, while the projection pathways emanating from the midbrain are largely anatomically and functionally distinct (with very few collaterals), their cells of origin within the dopaminergic groups are partly intermixed (Fallon and Moore, 1978b; Fallon and Loughlin, 1982; Swanson, 1982; Loughlin and Fallon, 1984). It has therefore been noted that more accurate terminology would be ‘mesostriatal’ rather than ‘nigrostriatal’ (Björklund and Dunnett, 2007b). The widespread nature of these projections allows dopamine to regulate neural processing and plasticity in many brain regions, and hence influence many forms of learning and behaviour. Furthermore, dense and complex arborization of dopaminergic terminals within target structures allows a single neuron to form synaptic contacts with a huge number of target cells (estimated as ~370,000 per SNc dopamine neuron; Arbuthnott and Wickens, 2007; Matsuda et al., 2009). Additionally, each target cell receives input from ~1000 dopaminergic terminals

(Doucet et al., 1986), so the activity of dopamine neurons can be highly influential in target regions.

1.1.2.2. DRN/vlPAG

Many regions receive dopaminergic input from the DRN/vlPAG population, including the NAc, caudate-putamen, central extended amygdala (central amygdaloid nucleus (CeA) and bed nucleus of the stria terminalis (BNST)), lateral septum, medial prefrontal cortex (mPFC), lateral habenula, hippocampus, midline and intralaminar thalamus, substantia innominata and horizontal limb of the diagonal band nucleus (HDB), ventrolateral preoptic nucleus (VLPO), perifornical region of the lateral hypothalamus, laterodorsal tegmental nucleus, and locus coeruleus (Pohle et al., 1984; Descarries et al., 1986; Ornstein et al., 1987; Grove, 1988; Semba et al., 1988; Yoshida et al., 1989; Stratford and Wirtshafter, 1990; Takada et al., 1990; Li et al., 1993; Freeman et al., 2001; Hasue and Shammah-Lagnado, 2002; Lu et al., 2006; Shin et al., 2008). There are also many TH-positive fibres with synaptic varicosities observed within the PAG, suggesting the presence of a local dopaminergic circuit (Buma et al., 1992; Peyron et al., 1995; Flores et al., 2004), as well as evidence of a small projection to the VTA (Omelchenko and Sesack, 2010).

Within the DRN/vlPAG, striatal-projecting neurons lie ventrally within the median subnucleus of the DRN, NAc-projecting neurons lie just below the aqueduct of Sylvius, and lateral septum and mPFC-projecting neurons are scattered more ventrally (Descarries et al., 1986; Yoshida et al., 1989; Stratford and Wirtshafter, 1990). There is also a crude mediolateral topographical organization of the central extended amygdala projection (Hasue and Shammah-Lagnado, 2002). This topographical organisation suggests there is very limited collateralization of DRN/vlPAG dopamine neurons, similar to those originating in the VTA (Hasue and Shammah-Lagnado, 2002).

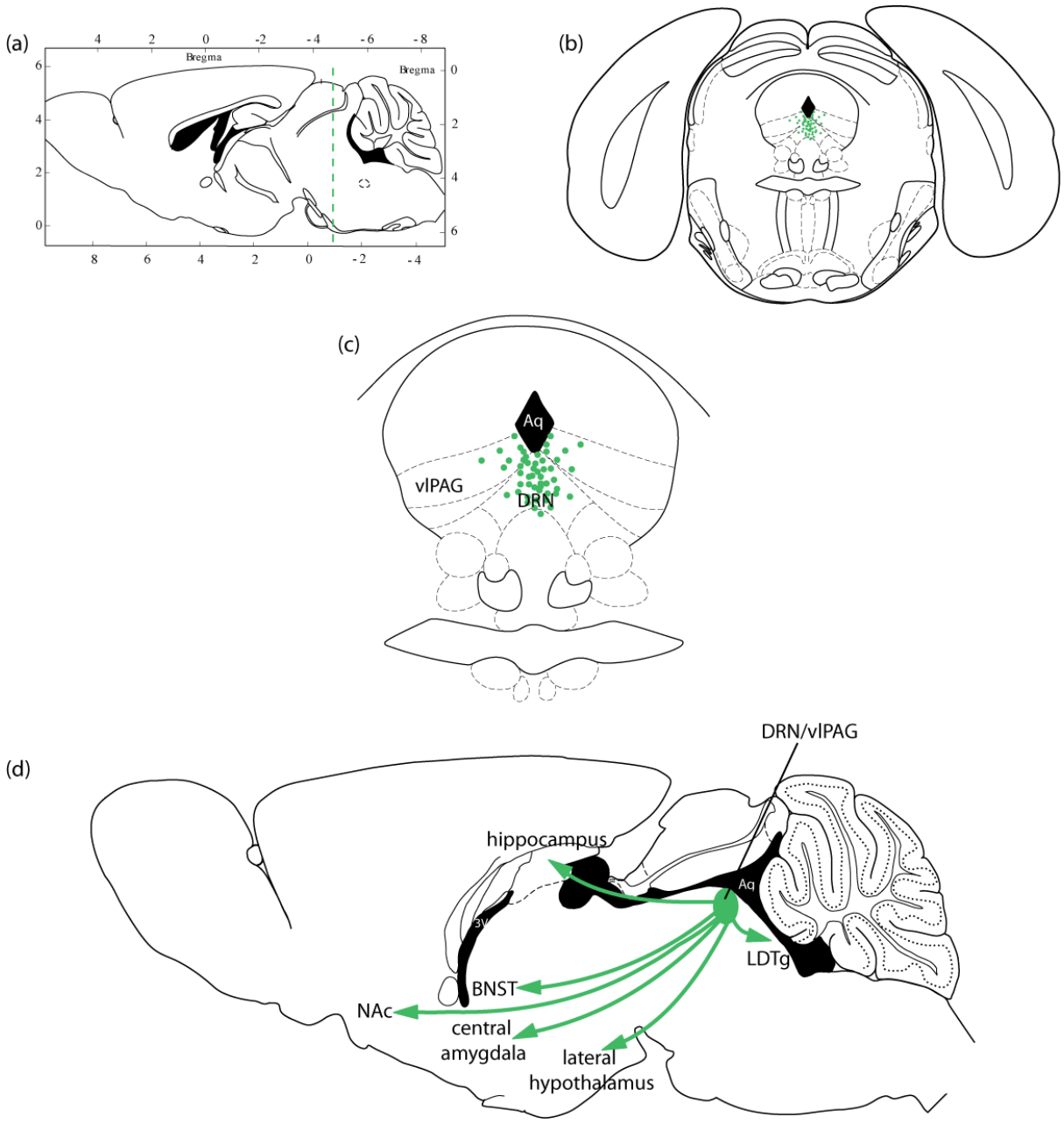


Illustration of the anatomical location of the DRN/vlPAG dopamine neurons and some of their major projections. (a) Sagittal view of the mouse brain indicating the rostro-caudal position of the DRN/vlPAG dopamine neurons, (b) corresponding coronal section, and (c) close up of the DRN/vlPAG showing the general distribution of the dopamine neurons (green dots). (d) Horizontal view of the mouse brain indicating some of the major projection targets of DRN/vlPAG dopamine neurons. Aq = aqueduct. Adapted from sagittal and coronal illustrations taken from the Mouse Brain Atlas in Stereotaxic Coordinates (Paxinos and Franklin, 2001)

1.1.3. Afferent inputs to midbrain dopamine neurons

1.1.3.1. VTA and SNc

The midbrain dopamine neurons receive afferent input from glutamatergic, GABAergic, cholinergic, serotonergic, orexigenic, and noradrenergic fibers, which influences their firing activity, and ultimately the release of dopamine in terminal projection sites (Grillner and Mercuri, 2002). The major excitatory drive to the midbrain dopamine neurons arises from glutamatergic neurons in the prefrontal cortex, subthalamic nucleus, laterodorsal- (LDTg) and pedunculopontine (PPTg) tegmental nuclei, lateral hypothamalus, superior colliculus, BNST, PAG, and lateral habenula (Herkenham and Nauta, 1979; Phillipson, 1979b; Kita and Kitai, 1987; Araki et al., 1988; Sesack and Pickel, 1992; Murase et al., 1993; Lavoie and Parent, 1994a,b; Charara et al., 1996; Tong et al., 1996a; Iribe et al., 1999; Lokwan et al., 2000; Fudge and Haber, 2001; Georges and Aston-Jones, 2002; Comoli et al., 2003; Floresco et al., 2003; Geisler and Zahm, 2005; Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Omelchenko and Sesack, 2006; Geisler et al., 2007; Omelchenko and Sesack, 2007; May et al., 2009; Omenchenko et al., 2009; Watabe-Uchida et al., 2012), as well as from local glutamatergic neurons within the VTA (Omelchenko and Sesack, 2009; Dobi et al., 2010).

Inhibitory GABAergic afferents to the midbrain dopamine neurons originate in the dorsal striatum (targeting mostly SNc), ventral striatum (targeting mostly VTA), rostromedial tegmental nucleus (RMTg), ventral pallidum, and amygdala (Conrad and Pfaff, 1976; Walaas and Fonnum, 1980; Price and Amaral, 1981; Zahm, 1989; Bolam and Smith, 1990; Zahm and Heimer, 1990; Smith and Bolam, 1990; Kalivas et al., 1993; Celada et al., 1999; Jhou et al., 2009; Kaufling et al., 2009; Sesack and Grace, 2010; Watabe-Uchida et al., 2012). However, there is some disagreement over whether striatal neurons form functional GABAergic synapses onto midbrain dopamine neurons, which has yet to be resolved (Chuhma et al., 2011; Xia et al., 2011; Watabe-

Uchida et al., 2012). Additionally, local GABAergic interneurons within the VTA and the neighbouring substantia nigra pars reticulata (SNr) provide inhibitory input to midbrain dopamine neurons (Di Chiara et al., 1979; Phillipson, 1979b; Grofova et al., 1982; Tepper et al., 1995; Celada et al., 1999; Omelchenko and Sesack, 2009; Dobi et al., 2010).

Midbrain dopamine neurons also receive serotonergic innervation from the DRN and median raphe nucleus, which is generally inhibitory (Herve et al., 1987; Wirtshafter et al., 1987; Corvaja et al., 1993; Broderick et al., 1997; Vertes et al., 1999; Gervais and Rouillard, 2000), cholinergic innervation from the PPTg (mainly to the SNpc) and LDTg (mainly to the VTA; Beninato and Spencer, 1987; Clarke et al., 1987; Cornwall et al., 1990; Bolam et al., 1991; Semba and Fibiger, 1992; Oakman et al., 1995; Blaha et al., 1996; Yeomans and Baptista, 1997; Omelchenko and Sesack 2005; Mena-Segovia et al., 2008), excitatory noradrenergic afferents from the locus coeruleus (Phillipson, 1979b; Herve et al., 1982; Grenhoff et al., 1993; Grenhoff et al., 1995; Mejías-Aponte et al., 2009) and orexigenic afferents from the lateral hypothalamus (Geisler and Zahm, 2005). Several peptide neurotransmitters and neuromodulators also contribute to modulating the activity of midbrain dopamine neurons (Kalivas, 1985), while axodendritic synapses (Wilson et al., 1977; Bayer and Pickel, 1990) and somatodendritic release of dopamine (Bjorklund and Lindvall, 1975; Geffen et al., 1976; Cheramy et al., 1981; Jaffe et al., 1998) regulates their excitability via feedback inhibition (Lacey et al., 1987; Beckstead et al., 2004).

1.1.3.2. DRN/vlPAG

Tracing studies have identified afferent inputs to the DRN/vlPAG dopamine neurons from the LDTg, lateral hypothalamus, locus coeruleus, mPFC, VLPO, infralimbic cortex, and nucleus of the solitary tract (Kalen et al., 1988; Hurley et al., 1991; Herbert, 1992; Herbert and Saper, 1992; Lu et al., 2006).

1.1.4. Neural connectivity comparison between the VTA and DRN/vlPAG

While many of the projection targets and afferent inputs of the DRN/vlPAG dopamine neurons overlap with those of the VTA, the relative composition of their dopaminergic efferents varies considerably. Retrograde tracing in rats revealed that the DRN/vlPAG dopaminergic projections to the NAc, lateral septum, mPFC, and lateral habenula are relatively sparse in comparison to the substantial input from the VTA (Stratford and Wirtshafter, 1990; Hasue and Shammah-Lagnado, 2002). However, DRN/vlPAG dopamine neurons have been shown to constitute around 44% of the dopaminergic cells projecting to the lateral BNST and nearly 60% of those projecting to the CeA, compared with around 40% and 35% from the VTA to the BNST and CeA, respectively (Hasue and Shammah-Lagnado, 2002). More recently, one study examining restricted retrograde tracer injection into the dorsolateral (dl)BNST, suggested that around 66% of dopamine cells projecting to this region originate in the DRN/vlPAG (Meloni et al., 2006). Similarly, the vast majority of dopamine neurons innervating the ventrolateral (vl)BNST are located in the DRN/vlPAG (Shin et al., 2008), which highlights the importance of this population for the dopamine input to the central extended amygdala. Additionally, the DRN/vlPAG receives input from, and projects to, many regions involved in regulating the sleep-wake cycle, including the VLPO (Lu et al., 2006). However, the VTA lacks a VLPO projection (Chou et al., 2002), indicating another distinct feature of the DRN/vlPAG population.

1.1.5. Comparison between rodent and primate DRN/vlPAG dopamine neurons

Evidence from primates indicates that dopamine neurons in the DRN are less numerous than in rodents (Charara and Parent, 1998). Moreover, the primate amygdala (specifically the basolateral amygdala; BLA) and extended amygdala receives broad dopaminergic input, mostly from the SNc and retrorubal field, with a less prominent input from the VTA and almost none from the DRN/vlPAG dopamine neurons (Cho and Fudge, 2010). This clearly differs from the rat

projection system, in which the medial VTA and DRN/vlPAG are the major sources of dopamine to the BLA and central extended amygdala, respectively (Fallon et al., 1978; Swanson, 1982; Hasue and Shammah-Lagnado, 2002). However, this species difference should be treated with caution for two reasons: firstly, the BNST was not included in these primate tracing studies, and secondly, it has been previously noted that the midbrain dopamine system is considerably expanded in primates, including humans, and displays a different organisation (Björklund and Dunnett, 2007b). There are around 70,000 dopaminergic neurons within the A8-A10 groups in the rat midbrain, with around 55% of these residing within the VTA, and 35% in the SNc (Nair-Roberts et al., 2008). This is increased to 160-320,000 dopaminergic neurons in the monkey, and 400-600,000 in humans, with more than 70% located in the SNc, and just 15% in the VTA (Hirsch et al., 1992; Francois et al., 1999). However, this may partly be an artefact relating to the anatomical definition of the VTA in primates compared to rodents.

1.2. Midbrain dopamine system: electrophysiological characteristics

1.2.1. Electrophysiological identification of dopamine neurons

Although the VTA is considered to be largely dopaminergic (Lacey et al., 1989; Yung et al., 1991; Johnson and North, 1992a), more recent estimates suggest that dopamine neurons only account for about 55-65% of neurons in this region, with the remainder consisting of mostly GABAergic neurons (35-45%) with a few glutamatergic neurons (2-3%; Kawano et al., 2006; Margolis et al., 2006a; Olson and Nestler, 2007; Yamaguchi et al., 2007; Nair-Roberts et al., 2008). Early electrophysiological studies performed in the SNc developed criteria for identifying dopamine neurons based on their electrophysiological properties (Guyenet and Aghajanian, 1978; Grace and Bunney, 1980; Grace and Bunney, 1983; Grace and Onn, 1989; Lacey et al., 1989). Specifically, dopamine neurons exhibit slow regular firing, broad action potentials with a large afterhyperpolarisation, and a prominent hyperpolarisation-activated cation current (I_h; Grace

and Bunney, 1980; Grace and Bunney, 1983; Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991; Johnson and North, 1992a). They are also hyperpolarised by a D_2-receptor agonist but not by a μ-opioid agonist (Bunney et al., 1973a,b; Groves et al., 1975; Aghajanian and Bunney, 1977; Lacey et al., 1987; Lacey et al., 1989; Johnson and North, 1992a,b; Chieng et al., 2011). In contrast, GABAergic neurons display high frequency firing, have short duration action potentials with a small afterpolarisation, do not exhibit an I_h, and are hyperpolarised by a μ-opioid agonist, but not by a D_2-receptor agonist (Grace and Onn, 1989; Johnson and North, 1992a,b; Cameron et al., 1997; Steffensen et al., 1998; Chieng et al., 2011).

However, while these properties are sufficient for accurately concluding dopaminergic identity in the SNc (e.g., Brown et al., 2009), dopamine neurons of the VTA exhibit considerable heterogeneity in their physiological and pharmacological properties (Neuhoff et al., 2002; Ford et al., 2006; Margolis et al., 2006a,b; Lammel et al., 2008; Margolis et al., 2008). This warrants greater caution when utilising these electrophysiological criteria to establish dopaminergic phenotype in this population. One frequently used dopaminergic marker is the presence of a large I_h (Lacey et al., 1989; Johnson and North, 1992a; Ungless et al., 2001; Gutlerner et al., 2002; Saal et al., 2003; Borgland et al., 2004; Faleiro et al., 2004; Bellone and Lüscher, 2006; Hommel et al., 2006; Margolis et al., 2006a; Argilli et al., 2008; Stuber et al., 2008; Zweifel et al., 2008). However, it has been shown that this does not reliably predict dopaminergic phenotype, because not only can some dopamine neurons lack a prominent I_h (Jones and Kauer, 1999; Neuhoff et al., 2002; Liss et al., 2005; Ford et al., 2006; Lammel et al., 2008; Chieng et al., 2011; Lammel et al., 2011), but many non-dopamine neurons can express a large I_h (Johnson and North, 1992a; Cameron et al., 1997; Jones and Kauer, 1999; Margolis et al., 2003; Ford et al., 2006; Margolis et al., 2006a; Margolis et al., 2008; Zhang et al., 2010a, Chieng et al., 2011). Other properties, which have often been used to identify dopamine neurons, are also found in a

significant proportion of non-dopamine neurons, including response to a D_2-receptor or μ-opioid agonist (Yim and Mogenson, 1980; Johnson and North, 1992a,b; Cameron et al., 1997; Margolis et al., 2003), and action potential shape, duration, and firing rate (Ungless et al., 2004; Margolis et al., 2006a; Luo et al., 2008; Chieng et al., 2011). Therefore, previous *in vivo* and *ex vivo* studies which employed these criteria, may have not only overrepresented certain dopamine subpopulations while neglecting others, but also included a proportion of non-dopamine neurons (Lammel et al., 2008; Margolis et al., 2008).

Due to the variability in the electrophysiological properties of VTA dopamine neurons and the different neuronal types housed within this nucleus, many studies now utilise transgenic mice which express green fluorescent protein (GFP) selectively in dopamine neurons, in order to more efficiently target this population (e.g. Labouebe et al., 2007). Two mouse lines, which have been successfully employed in electrophysiology studies are Pitx3-GFP (Zhao et al., 2004) and TH-GFP mice (Matsushita et al., 2002). Pitx3 is a transcription factor required for the development and maintenance of midbrain dopamine neurons (van den Munckhof et al., 2003) and is expressed exclusively in this population (Smidt et al., 1997). Pitx3-directed GFP expression is therefore confined to midbrain dopamine neurons in Pitx3-GFP mice (Zhao et al., 2004). TH is required for neurotransmitter synthesis in all catecholaminergic neurons, and therefore, under the control of the TH promoter, GFP is expressed in dopaminergic, noradrenergic, and adrenergic cell groups in TH-GFP mice (Matsushita et al., 2002). The GFP signal in both these lines of transgenic mouse provides a useful guide for efficient targeting of dopaminergic neurons (discussed in more detail in 2.1.1).

1.2.2. Projecting target-related heterogeneity in midbrain dopamine neurons

Several studies have associated the variability in electrophysiological and neurochemical properties to axonal projection target (Ford et al*.,* 2006; Margolis et al*.,* 2006b; Lammel et al*.,*

2008; Margolis et al., 2008), since VTA dopamine neurons almost exclusively project to a single forebrain structure (Deniau et al*.,* 1980; Fallon, 1981; Swanson, 1982). However, it should be noted that these studies were performed in both mice and rats, and they reveal considerable inter-species differences in the properties of VTA dopamine neurons, as described below.

1.2.2.1. Rat studies

In rats, amygdala-projecting dopamine neurons exhibit the shortest action potential duration, followed by mPFC-projecting neurons and NAc-projecting neurons (Margolis et al*.,* 2008). This correlates with D_2-receptor inhibition, whereby amygdala-projecting dopamine neurons are unaffected by a D_2-receptor agonist, while most mPFC- and NAc-projecting dopamine neurons respond with significant hyperpolarisation (Margolis et al*.,* 2008). Additionally, a κ-opioid agonist selectively inhibits dopamine neurons projecting to the mPFC and amygdala, with no effect on NAc-projecting dopamine neurons or non-dopamine neurons (Margolis et al*.,* 2006b; Margolis et al., 2008). A range of I_h amplitudes are present in both dopamine and non-dopamine neurons, but the absence of an I_h is thought to reliably predict a non-dopamine neuron (Margolis et al*.,* 2006a).

1.2.2.2. Mice studies

In mice, it has been suggested that midbrain dopamine neurons consist of two anatomically and functionally distinct neuronal subtypes: 'conventional' dopamine neurons projecting to the NAc lateral shell and dorsolateral striatum, and 'unconventional' dopamine neurons projecting to the NAc medial shell, NAc core, mPFC, and BLA (Lammel et al*.,* 2008). Conventional dopamine neurons have relatively short duration action potentials with a large afterhyperpolarisation, exhibit a prominent I_h, and have maximal firing rates of <10 Hz. In contrast, unconventional dopamine neurons have long duration action potentials with a small afterhyperpolarisation,

exhibit little or no I_h, and can maintain firing frequencies >10 Hz (Chiodo et al., 1984; Gariano et al., 1989; Neuhoff et al*.,* 2002; Ford et al*.,* 2006; Lammel et al*.,* 2008). Interestingly, mPFC-projecting dopamine neurons can be distinguished by their lack of D_2-receptor inhibition (Lammel et al*.,* 2008), while most NAc-projecting neurons, but few amygdala-projecting neurons, are inhibited by a κ-opioid agonist (Ford et al*.,* 2006).

Midbrain dopamine neurons also display quantitative molecular differences, with conventional and unconventional dopamine neurons exhibiting high and low DAT/TH ratios, respectively (Lammel et al*.,* 2008). This is attributed mainly to significantly higher expression of DAT in conventional neurons, implying a greater capacity for dopamine re-uptake, relative to synthesis, which may impact on the decay kinetics of extracellular dopamine (Lammel et al*.,* 2008).

These studies highlight the neurochemical and electrophysiological heterogeneity of midbrain dopamine neurons, and also caution against generalising between different species in terms of projection-specific properties (Margolis et al*.,* 2008). Furthermore, these findings indicate that the temporal profile of dopamine release in target regions may differ depending on the intrinsic properties of the dopamine neuron (Lammel et al*.,* 2008). For example, excitation of mPFC-projecting dopamine neurons, which can maintain high frequency firing, lack somatodendritic D_2-receptor inhibition, and have a low DAT/TH ratio, is likely to result in a sustained dopamine release pattern in the mPFC (Lammel et al*.,* 2008), which is consistent with *in vivo* studies (Yavich et al., 2007).

1.2.3. Heterogeneity within DRN/vlPAG dopamine neurons

Two major subtypes of dopamine neuron have been observed within the DRN/vlPAG, differing in their size, morphology, and anatomical distribution (Hökfelt et al*.,* 1976; Stratford and Wirtshafter, 1990; Charara and Parent, 1998). Many small, rounded cells (10-15 µm in

diameter), with bipolar morphology, reside on the dorsal border of the rostral DRN, forming a thin band which lines the ventral surface of the aqueduct of Sylvius (Stratford and Wirtshafter, 1990; Flores et al*.,* 2004; Flores et al., 2006). Large (30-50 µm diameter) multipolar cells, exhibiting abundant arborisation, lie in the medial DRN, extending dorsocaudally from the ventro-rostral border of the DRN, (where the neurons are continuous with the dopamine neurons of the CLi (Stratford and Wirtshafter, 1990), and into the vlPAG (Flores et al*.,* 2004).

Interestingly, 6-hydroxydopamine (6-OHDA) treatment of this region selectively affects the large cells of the DRN/vlPAG (Flores et al*.,* 2004). This corresponds to about an 80% reduction in the dopamine peak, as measured by *in vitro* voltammetry, suggesting that these large cells (although lower in number) contribute to the majority of the local dopamine release (Flores et al., 2004). Additionally, it has been noted that the small cells, located close to the aqueduct, show lower levels of TH immunoreactivity compared to other midbrain dopamine populations (Hökfelt et al., 1976; Rogers, 1992). These findings have led to the suggestion that the small periaqueductal cells are an extension of the periventricular dopamine system (Lindvall et al, 1974; Lindvall and Björklund, 1974; Moore and Bloom, 1978), which lacks DAT and is immune to 6-OHDA, whereas the larger dopamine neurons are an extension of the VTA, which expresses DAT and is therefore vulnerable to 6-OHDA (Kilty et al., 1991; Shimada et al., 1992; Fujita et al., 1993; Hasue and Shammah-Lagnado, 2002).

1.2.4. Firing modes of midbrain dopamine neurons

Midbrain dopamine neurons fire in two distinct modes: tonic and phasic (Grace and Bunney, 1984a,b; Grace, 1991; Grace et al*.,* 2007). Tonic, pacemaker-like firing (mean frequency ~4 Hz) maintains steady, dopamine release in downstream target structures, which is essential for the normal functioning of these neural circuits (Grace and Bunney, 1984a; Sanghera et al., 1984; Schultz, 2007). Phasic firing refers to a rapid change in firing rate (for 100-500 ms), which results

in a substantial increase or decrease in dopamine release in target structures lasting for a few seconds (Grace, 1991; Floresco et al., 2001; Schultz, 2007; Schultz, 2008; Sombers et al., 2009). Phasic excitation in dopamine neurons manifests as 'bursts', which are short periods of high frequency firing (starting with an interspike interval (ISI) of <80 ms and finishing with an ISI >160 ms; Grace and Bunney, 1984b).

Synchronous phasic bursts in dopamine neurons can be initiated by an unexpected reward or reward-predicting cue (Schultz et al., 1997; Phillips et al., 2003; Cheer et al., 2007; Joshua et al., 2009). This is thought to be functionally relevant for signalling salient information about the environment, and is believed to facilitate associative learning and motivation in a distinct manner from tonic activity (Grace, 1991; Overton and Clark, 1992; Berridge and Robinson, 1998; Schultz, 1998; Cooper, 2002; Grace et al., 2007; Lapish et al., 2007; Schultz, 2007). This is mainly attributable to the supralinear increase in extracellular dopamine resulting from burst firing (Gonon and Buda, 1985; Gonon, 1988; Bean and Roth, 1991; Chergui et al., 1994; Floresco et al., 2003; Venton et al., 2003), which is thought to be important for influencing neural firing activity and modifying synaptic connections in the projection site (Surmeier et al., 2007; Robbins and Arnsten, 2009). It can also promote long-term plasticity and trigger re-wiring of neural circuits (Reynolds et al., 2001; Bromberg-Martin et al., 2010). By exploiting the temporal and spatial resolution offered by optogenetics, it has been confirmed that phasic bursting activity in VTA dopamine neurons is sufficient to establish behavioural conditioning (Tsai et al., 2009). Interestingly, however, phasic activation of VTA dopamine neurons in the absence of a reward does not drive 'self-stimulation'-like behaviour, suggesting that activation of these dopamine neurons alone is not rewarding (Adamantidis et al., 2011). Phasic activation is, however, sufficient to re-activate previously extinguished reward-seeking behaviour, in the absence of a

reward or cues, which underscores its positive-reinforcing properties (Wise, 2004; Berridge, 2007; Schultz, 2007; Koob and Volkow, 2010; Adamantidis et al*.,* 2011).

1.3. Functional diversity in the midbrain dopamine system

1.3.1. Reward learning

The traditional theory of midbrain dopamine neurons proposes that they encode positive motivational value (Berridge and Robinson, 1998; Schultz, 1998; Wise, 2004). This was originally based on findings that animals would repeatedly press a lever for self-stimulation in the VTA and its projection sites, or delivery of dopamine agonists into these regions (Olds and Milner, 1954; Phillips and Fibiger, 1978; Yokel and Wise, 1978; Carr and White, 1983; Gallistel and Karras, 1984; Fiorino et al., 1993; Carlezon and Wise, 1996; McBride et al., 1999; Wise, 2004; Cheer et al., 2007). Reward-related stimuli increase dopamine release in the NAc, while aversive stimuli inhibit release (Roitman et al., 2008), and this correlates with an increase and decrease in dopamine neuron firing, respectively (Phillips and Olds, 1969; Schultz and Romo, 1987; Mantz et al., 1989; Mirenowicz and Schultz, 1996; Guarraci and Kapp, 1999; Ungless et al*.,* 2004). Furthermore, reinforcing actions are potentiated by drugs which increase dopamine release, but attenuated by dopamine receptor antagonists (Fouriezos and Wise, 1976; Franklin, 1978; Gallistel and Karras, 1984; Wise, 2004).

A defined role for the midbrain dopamine neurons in reward signalling was first clearly demonstrated through the pioneering work of Schultz et al*.,* with the observation that dopamine neurons were phasically excited on presentation of an unexpected reward, or a reward-predicting cue, but were inhibited on reward omission (Schultz et al*.,* 1997). This led to the hypothesis that dopamine neurons encode a reward-prediction-error signal, which is essential in reinforcement learning (Montague et al*.,* 1996; Berridge and Robinson, 1998;

Schultz, 1998; Hyland et al., 2002; Bayer and Glimcher, 2005; Roesch et al., 2007; Schultz, 2007; D'Ardenne et al., 2008; Dayan and Niv, 2008; Schultz, 2010) and goal-directed behaviour (Wise, 2004; Wise, 2005) by driving the formation of an appetitive associative memory (Montague et al., 1996; Schultz, 1998; Schultz, 2007).

1.3.2. Aversive learning

In an apparent contradiction to this established reward-coding role for dopamine neurons, many studies report that aversive stimuli can increase dopamine release in certain target sites (particularly the mPFC and NAc; Abercrombie et al., 1989; Kalivas and Duffy, 1995; Klitenick et al., 1996; Rougé-Pont et al., 1998; Bassareo et al., 2002; Giorgi et al., 2003; Joseph et al., 2003; Young, 2004; Amato et al., 2011; Budygin et al., 2012), while behavioural aversion is correlated with increased dopamine levels (Salamone, 1994; Joseph et al., 2003; Ventura et al., 2007; Anstrom et al., 2009; Barr et al., 2009; Fadok et al., 2009) and phasic activity in some putative dopamine neurons (Zweifel et al., 2009). Furthermore, there are inconsistencies in the literature regarding the response of putative dopamine neurons to aversive stimuli, with some studies reporting both inhibitions and excitations (Chiodo et al., 1980; Maeda and Mogenson, 1982; Trulson and Preussler, 1984; Schultz and Romo, 1987; Mantz et al., 1989; Gao et al., 1990; Mirenowicz and Schultz, 1996; Guarraci et al., 1999; Guarraci and Kapp, 1999; Coizet et al., 2006; Wang and Tsien, 2011; Zweifel et al., 2011). This led to the alternative hypothesis that dopamine neurons signal salient, motivationally-relevant events (Berridge and Robinson, 1998; Redgrave et al., 1999; Horvitz, 2000; Di Chiara, 2002; Joseph et al., 2003; Kapur, 2003; Pezze and Feldon, 2004; Redgrave and Gurney, 2006; Anstrom et al., 2009). However, a role for dopamine in aversive signalling remained controversial, as these studies inferred dopaminergic identity using purely electrophysiological and pharmacological characteristics.

Subsequently, however, it was shown that a population of immunohistochemically-identified dopamine neurons in the ventral VTA of rats were phasically excited in response to noxious footshocks *in vivo* (Brischoux et al*.,* 2009). Furthermore, a study in nonhuman primates, confirmed that some dorsolateral SNc putative dopamine neurons were excited by an unpredictable aversive stimulus, and also by cues predicting that aversive stimulus (Matsumoto and Hikosaka, 2009). These *in vivo* studies therefore presented a solution to the long-standing debate over information coding in dopamine neurons, by suggesting that there could be multiple anatomically and functionally distinct populations responding to different types of salient stimuli (Brischoux et al., 2009).

1.3.3. Response to addictive drugs

Midbrain dopamine neurons are known to be integral to the brain's natural reward circuitry, and are activated by numerous naturally rewarding stimuli. In addition, it is widely documented that the mesolimbic dopamine system is critically involved in the response to drugs of abuse (Koob and Bloom, 1988; Self and Nestler, 1995; Wise, 2005). In spite of their diverse molecular targets, nearly all drugs of abuse, including cocaine, amphetamine, nicotine, ethanol, marijuana, and opiates, increase extracellular dopamine in the projection targets of the mesocorticolimbic system, most notably the NAc (Ungerstedt and Pycock, 1974; Heikkila et al., 1975; Adams, 1976; Plotsky et al., 1977; Di Chiara and Imperato, 1985; Di Chiara and Imperato, 1986; Imperato et al., 1986a,b; Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Self and Nestler, 1995; Bardo, 1998; Koob et al., 1998; Volkow et al*.,* 1999; Drevets et al*.,* 2001; Boileau et al*.,* 2003; Nestler, 2005; Bossong et al*.,* 2009; Brody et al*.,* 2009), which is thought to mediate their rewarding or euphoric properties (Wise and Bozarth, 1987; Corrigall et al., 1992; McBride et al., 1999; Ikemoto and Wise, 2004). Contrastingly, drugs without addictive properties do not increase synaptic dopamine release (Di Chiara and Imperato, 1988). Furthermore, animals will self-

administer addictive drugs into specific dopaminergic regions, but this is blocked by delivery of dopamine receptor antagonists, or lesion of dopaminergic terminals (De Wit and Wise, 1977; Gardner, 2000; Bressan and Crippa, 2005). Consistent with animal work, SPECT studies in humans have revealed that amphetamine-mediated dopamine release is correlated with positive emotional responses in subjects, described as 'happiness' and 'energy' (Abi-Dargham et al., 2003). The drug-induced rapid increase in dopamine release is thought to mimic the increased dopamine triggered by a naturally rewarding or salient stimulus (Schultz, 2010). In this way, addictive drugs are able to 'hijack' the brain's natural reward circuitry to initiate maladaptive learning and behaviour (Hyman and Malenka, 2001; Jones and Bonci, 2005).

Psychomotor stimulants, such as cocaine, are particularly dependent on dopamine to elicit their rewarding effects (DiChiara and Imperato, 1988). Cocaine elevates extracellular dopamine levels by inhibiting DAT (Heikkila et al*.,* 1975; Ritz et al*.,* 1987). DAT-deficient mice, however, still show cocaine-induced increase in dopamine levels and can still learn drug self-administration, which is thought to be due to blockade of dopamine re-uptake via serotonin and noradrenaline transporters (Rocha et al., 1998; Rocha, 2003). However, this is likely to be a compensatory mechanism in these knockout mice, since mice expressing a cocaine-insensitive form of DAT do not show cocaine-induced increase in extracellular dopamine, do not develop cocaine reward (Chen et al*.,* 2006) or self-administration (Thomsen et al*.,* 2009), and do not express cocaine-induced synaptic plasticity (Brown et al*.,* 2010). This suggests that blockade of DAT is cocaine's principle mechanism of action. Contrastingly, amphetamine and methamphetamine increase dopamine release predominantly by reversing DAT and causing vesicular release of dopamine by acting as a substrate for VMAT2 (Ritz et al., 1987; Schuldiner et al*.,* 1993; Pifl et al*.,* 1995; Self and Nestler, 1995; Jones et al*.,* 1998; Schmitz et al*.,* 2001; Sulzer et al., 2005).

1.4. Functional studies on DRN/vlPAG dopamine neurons

Very few studies have sought to identify a functional role for the DRN/vlPAG dopamine neurons. However, selective 6-OHDA lesions of this region have implicated this population in a number of distinct processes.

1.4.1. Promoting arousal in the sleep-wake cycle

The DRN/vlPAG dopamine neurons have been suggested to play a role in maintaining wakefulness in the sleep-wake cycle (Lu et al*.,* 2006). These neurons were shown to express c-Fos exclusively during wakefulness, and selective 6-OHDA lesion caused a 20% increase in total sleep (Lu et al*.,* 2006), which is substantial when compared to the effect of lesions in other brain regions (Wenk et al*.,* 1994; Hara et al*.,* 2001). Contrastingly, the firing activity (Trulson et al*.,* 1981; Miller et al*.,* 1983; Trulson and Preussler, 1984) and c-Fos expression (Lu et al*.,* 2006) of VTA dopamine neurons does not correlate with the sleep-wake cycle. VTA dopamine neurons also lack a projection to the VLPO (a region which is critically involved in sleep regulation; Chou et al*.,* 2002), and selective VTA lesion causes a decrease, not an increase, in total sleep time (Lai et al*.,* 1999; Lu et al*.,* 2006).

These results indicate a distinct role for DRN/vlPAG dopamine neurons, based on both connectivity and function. This view, however, is still the subject of controversy, as conflicting results have been obtained for the VTA and DRN/vlPAG, and there is also evidence that midbrain dopamine neurons participate in the regulation of paradoxical/REM sleep (Monti and Monti, 2007). Specifically, Leger et al. did not observe a substantial increase in c-Fos expression in DRN/vlPAG or VTA dopamine neurons following a manipulation that increased wakefulness or paradoxical sleep deprivation (Leger et al*.,* 2010). Additionally, others propose that VTA dopamine neurons switch to a prominent burst firing pattern during paradoxical sleep (Dahan et

al., 2007), which is associated with increased dopamine release in the NAc and mPFC (Lena et al., 2005).

1.4.2. Opiate-induced antinociception and reward

PAG dopamine neurons have been implicated in mediating the supraspinal nociceptive properties of opiates (Flores et al., 2004; Meyer et al., 2009). 6-OHDA treatment, which selectively affects large DRN/vlPAG dopamine neurons and depletes PAG dopamine by ~80%, reduces opiate-induced analgesia as measured using the hot-plate test (Flores et al., 2004). Infusion of a D_1-, but not a D_2-receptor antagonist into the PAG attenuates opiate-induced analgesia, suggesting that this antinociceptive effect is mediated by dopamine acting on local D_1-receptors (Flores et al., 2004). Furthermore, these effects are specific to the PAG, as a D_1-receptor antagonist had no effect on antinociception when infused outside the PAG (Flores et al., 2004). Basal nociception, however, is unaffected by lesion of the PAG dopamine neurons, suggesting direct modulation of these neurons by exogenous opiates (Flores et al., 2004; Meyer et al., 2009).

Another study examining the PAG dopamine neurons found that 6-OHDA lesion of these neurons (and the neighbouring RLi) significantly attenuated the rewarding and sensitizing properties of heroin, shown by a lack of conditioned place preference (CPP; a behavioural task in which an animal learns to associate a physical environment with a drug experience) or locomotor sensitisation on repeated exposure (Flores et al., 2006). Repeated heroin administration also upregulated TH levels in PAG dopamine neurons (Flores et al., 2006), similar to the VTA (Beitner-Johnson and Nestler, 1991). In contrast to the effects of antinociception, a D_2- but not a D_1-receptor antagonist in the PAG/RLi, blocked heroin-induced reward, suggesting a different mechanism of action for PAG-mediated reward-related behaviour (Flores et al., 2006). This function is reminiscent of the VTA, in terms of mediating the effects of addictive drugs, which suggests the possibility of some functional overlap between the two populations.

1.5. Drug addiction and neuroplasticity in the midbrain dopamine system

1.5.1. The burden of drug addiction

Drug addiction is a chronic neuropsychiatric disorder, characterised by compulsive drug use despite aversive consequences and a propensity to relapse during abstinence (Mendelson and Mello, 1996; American Psychiatric Association [DSM-IV], 2000). It represents a substantial global public health problem and a huge burden to society, with the total cost exceeding $600 billion annually in the United States, based on medical costs, criminal damage, incarceration, accidents, and loss of employment (Uhl and Grow, 2004; Volkow and Li, 2005; Robison and Nestler, 2011; Volkow et al*.,* 2011). This includes ~$280 billion for illegal drugs, ~$185 billion for alcohol, and ~$158 billion for nicotine (Volkow and Li, 2005). More recently, European estimates in 2010 covering addiction to alcohol, opioid drugs, and cannabis, suggest a total annual cost of €65,684 million across 30 European countries (Gustavsson et al*.,* 2011). Although the economic cost is astounding, it does not reflect the huge emotional cost of drug addiction on the individual, their family, friends, and wider society. Moreover, these figures do not include the cost of non-drug addictions, such as pathological gambling and food addiction, which have recently been recognised as addictive disorders, with similar neurobiological substrates, and physiological and behavioural manifestations to those of addictive drugs (Potenza, 2008; Frascella et al., 2010; Grant et al., 2010; Holden, 2010; Kenny, 2011). The huge social and economic burden of drug addiction is not least attributable to the high proportion of the population that are classified as drug dependent or abusing psychoactive substances, estimated at 9.1% of the total population over 12 years old in the US (Wright et al., 2007).

The high relapse rate among drug abusers is a major clinical problem, and represents the main challenge to the management and treatment of drug addiction (Aguilar et al*.,* 2009). Intense craving and relapse is frequently observed in addicts, even after decades of abstinence, often

triggered by exposure to drug-related environmental stimuli or stress (O'Brien, 1997; el-Guebaly and Hodgins, 1998; De Wit, 2000). While there are a number of types of anti-craving medication currently in use (e.g., naltrexone for alcoholism, bupropion for nicotine, and methadone for heroin; van den Brink and van Ree, 2003) these treatments are limited in their efficacy and have significant side-effects. Additionally, at present, there are no effective medications available for the treatment of psychostimulant addiction (Gorelick and Gardner, 2004; Vocci and Ling, 2005; Karila et al*.,* 2008). The substantial disruption to normal behaviour in addicted individuals is due to the impact of chronic drug intake on multiple neuronal circuits, and is suggestive of long-term changes in neural circuitry (Nestler, 2004). This emphasises the definition of addiction as a chronic relapsing disease (Volkow et al*.,* 2011), and highlights the need for a deeper understanding of the underlying neural actions of addictive drugs, in order to identify novel targets for treating addictive disorders (O'Brien and Gardner, 2005; Robison and Nestler, 2011).

1.5.2. Drug-induced synaptic plasticity

Synapses represent the fundamental information-processing unit in the brain and are diverse in shape and properties. Synaptic dysfunction is implicated in nearly all neuropsychiatric disorders, therefore examining the role of synapses in disease is an essential focus for research and drug development (Sudhof and Malenka, 2008).

Synapses can display activity-dependent plasticity, which is revealed as strengthening (long-term potentiation; LTP) or weakening (long-term depression; LTD) of a synaptic connection (Sudhof and Malenka, 2008). This plasticity is a ubiquitous, basic property of excitatory synapses in the brain, which is essential for learning and memory as well as other brain functions (Malenka and Bear, 2004). Drugs of abuse are able to hijack synaptic plasticity mechanisms in the reward circuitry of the brain, and this is thought to create powerful and enduring memories relating to the drug experience, which forms the neural basis of addiction (Hyman et al*.,* 2006;

Kauer and Malenka, 2007). Numerous and complex neuroadaptations occur in response to drugs of abuse, but glutamatergic synapses onto dopamine neurons in the VTA have been particularly intensively studied (Kauer, 2004; Thomas et al*.,* 2008).

Glutamatergic afferents are the major source of fast excitatory input to VTA dopamine neurons, via glutamate acting on postsynaptic ionotrophic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors located on neuronal cell bodies and dendrites (Mereu et al., 1991; Albin et al*.,* 1992; Martin et al*.,* 1993; Wang and French, 1993a,b; Wang and French, 1995; Paquet et al*.,* 1997). Activation of AMPA or NMDARs in the VTA increases neuronal firing (Grace and Bunney, 1984b; Johnson et al., 1992; Suaud-Chagny et al*.,* 1992; Chergui et al., 1993; Murase et al., 1993; Wang and French, 1993a,b) and therefore increases dopamine release in target regions (Grenhoff et al., 1988; Suaud-Chagny et al*.,* 1992; Karreman et al*.,* 1996; Westerink et al*.,* 1996; Westerink et al., 1998, Floresco et al., 2003). AMPARs are permeable to Na^+ and K^+ ions, and contribute to the majority of excitatory current when the postsynaptic neuron is near its resting membrane potential. In contrast, NMDARs are also permeable to Ca^{2+} and are voltage-dependent, requiring sufficient depolarisation of the postsynaptic membrane to remove the Mg^{2+} ion which blocks the ion pore at rest (Mayer et al., 1984; Nowak et al., 1984). NMDAR activation is particularly important in the switch from tonic to phasic burst firing *in vivo* (Grenhoff et al*.,* 1988; Svensson and Tung, 1989; Charléty et al*.,* 1991; Johnson et al., 1992; Chergui et al., 1993; Tong et al., 1996b; White, 1996; Overton and Clark, 1997; Lokwan et al., 2000; Mathon et al., 2003; Kuznetsov et al., 2006; Lodge and Grace, 2006; Zweifel et al*.,* 2009), which results in a significant increase in dopamine release (Forster and Blaha, 2000; Sombers et al., 2009). NMDARs are also thought to be crucially involved in some forms of synaptic plasticity (Malenka and Nicoll, 1999) including those that occur in response to drugs of abuse (Kauer, 2004).

1.5.3. Cocaine-induced plasticity at glutamatergic synapses in the VTA

Following a single dose of cocaine, there is potentiation of AMPAR transmission at glutamatergic synapses onto VTA dopamine neurons (Ungless et al*.,* 2001). This occurs within 2-3 hours of drug exposure (Argilli et al*.,* 2008) and is prevented by co-administration of an NMDAR antagonist (Ungless et al*.,* 2001), which is reminiscent of NMDAR-dependent LTP (Nicoll et al*.,* 1988; Malenka 1991; Malenka and Nicoll, 1993; Bonci and Malenka, 1999; Liu et al*.,* 2005; Luu and Malenka, 2008). The potentiation persists for at least 5, but no more than 10, days following an acute drug dose (Ungless et al*.,* 2001), even after multiple noncontingent injections (Borgland et al., 2004). Importantly, LTP-like potentiation at these glutamatergic synapses can also be induced by other addictive drugs including nicotine, alcohol, amphetamine, benzodiazepines, and morphine (Saal et al*.,* 2003; Faleiro et al*.,* 2004; Heikkinen et al*.,* 2009; Tan et al*.,* 2010), but not by non-addictive psychoactive compounds, such as fluoxetine or carbamazepine, suggesting that this synaptic potentiation is related to the addictive qualities of the drug (Saal et al*.,* 2003). Additionally, acute stress, such as cold water swim or formalin injection, has been shown to potentiate these synapses (Saal et al., 2003; Lammel et al., 2011).

Recently, it was demonstrated by Lammel et al. that this potentiation is projection-specific. In particular, cocaine-induced potentiation only occurs at synapses onto dopamine neurons projecting to the NAc lateral and medial shell, but not those projecting to the dorsal striatum or mPFC (Lammel et al*.,* 2011). Contrastingly, an aversive stimulus selectively potentiates synapses onto mPFC- and NAc lateral shell-projecting dopamine neurons, without affecting those projecting to the NAc medial shell or dorsal striatum (Lammel et al*.,* 2011). Furthermore, dopamine neurons projecting to the NAc medial shell, showed more enduring cocaine-induced potentiation, lasting for at least 21 days after the single cocaine dose (Lammel et al*.,* 2011). This selective synaptic modification indicates that separate subsets of dopamine neurons, integrated

into distinct neural circuits, are involved in processing different types of motivationally-relevant cue.

1.5.3.1. Induction

Initially, cocaine transiently enhances NMDAR transmission due to increased extracellular dopamine acting on postsynaptic D_5-receptors and activating the PKA pathway, which causes increased insertion of NMDARs onto the postsynaptic membrane (Schilstrom et al., 2006; Argilli et al., 2008). The activation of postsynaptic NMDARs, and subsequent Ca^{2+} entry, is then essential to activate appropriate downstream signalling cascades in the dopamine neuron, which involves several protein kinases and results in protein synthesis (Bellone and Lüscher, 2006; Argilli et al., 2008). Activation of VTA dopamine neurons alone is sufficient to induce this plasticity, when paired with spontaneous activity, as demonstrated by bath application of cocaine to VTA slices (Argilli et al., 2008) and selective optogenetic stimulation of burst firing in dopamine neurons (Brown et al., 2010).

This strengthening of AMPAR transmission is indicative of synapses in a potentiated state, which does not only occlude further induction of activity-dependent LTP (Ungless et al., 2001; Liu et al., 2005; Argilli et al., 2008; Luu and Malenka, 2008), but actually changes the rules governing induction of plasticity at these synapses (Mameli et al., 2011). Specifically, a spike-timing dependent protocol, which involves depolarisation of dopamine neurons, is no longer able to induce NMDAR-dependent LTP in slices from cocaine-treated animals (Ungless et al., 2001; Argilli et al., 2008; Luu and Malenka, 2008). However, a protocol combining afferent stimulation with hyperpolarisation of the dopamine neuron elicits significant LTP in cocaine-treated, but not saline-treated mice (Mameli et al., 2011).

1.5.3.2. Postsynaptic expression

Cocaine-induced AMPAR potentiation is associated with increased insertion of GluR2-lacking AMPARs, which display a characteristic inwardly rectifying I-V relationship (i.e. passing less current at positive potentials than at the corresponding negative potentials; Bellone and Lüscher, 2006; Isaac et al., 2007; Liu and Zukin, 2007; Mameli et al*.,* 2007; Argilli et al*.,* 2008; Brown et al*.,* 2010; Good and Lupica, 2010). AMPARs which lack the GluR2 subunit have greater single channel conductance than GluR2-containing AMPARs (Liu and Zukin, 2007) and are Ca^{2+}-permeable, which allows entry of Ca^{2+} into the neuron, and initiation of Ca^{2+}-dependent intracellular signalling cascades (Kauer and Malenka, 2007). AMPAR trafficking in response to cocaine is thought to result in an exchange of GluR2-containing receptors for GluR2-lacking receptors, without a significant change in receptor number (Lüscher and Malenka, 2011), and hence causes a rapid change in the quality of synaptic transmission at these synapses. However, not all studies have reported this drug-induced change in AMPAR rectification (e.g., Faleiro et al., 2004; Dong et al., 2004), which may be due to methodological differences.

Cocaine-induced synaptic potentiation was first clearly demonstrated by an increase in the ratio of evoked postsynaptic AMPAR- to NMDAR-mediated current, known as the AMPAR:NMDAR ratio (Ungless et al*.,* 2001). Since bath application of exogenous AMPA, but not NMDA, increased the evoked current in animals that had received cocaine, this was interpreted solely as a change in AMPAR transmission (Ungless et al*.,* 2001). However, by measuring unitary EPSCs from single synapses, using 2-photon laser photolysis of caged glutamate, it has recently been demonstrated that there is a concomitant reduction in NMDAR-transmission (Mameli et al*.,* 2011). This occurs at the same synapses which show an increase in the rectification of the AMPAR current, suggesting synapse-specific modulation by cocaine (Mameli et al*.,* 2011). A reduction in NMDAR-mediated current would account for the observation that there is an

increase in the AMPAR:NMDAR ratio in dopamine neurons from cocaine-treated animals, even though AMPARs lacking the GluR2 subunit pass relatively little current at positive potentials.

1.5.3.3. Reversal

It has been demonstrated that activation of metabotropic (m)GluR1-receptors in the VTA can induce LTD (Bellone and Lüscher, 2005), which is necessary for the reversal of cocaine-induced potentiation about a week following exposure (Bellone and Lüscher, 2006; Mameli et al., 2009). This mGluR-dependent LTD requires GluR2-lacking AMPARs at the synapse, and causes replacement of these AMPARs with GluR2-containing receptors, thereby de-potentiating the synapse back to its basal state (Bellone and Lüscher, 2006; Mameli et al., 2007; Good and Lupica, 2010).

1.5.4. Other reward-related stimuli induce plasticity at glutamatergic synapses in the VTA

Interestingly, natural reward-related learning has also been shown to induce NMDAR-dependent potentiation of AMPAR transmission in VTA dopamine neurons (Stuber et al., 2008), which is similarly persistent to that of an acute dose of cocaine (Chen et al., 2008; Stuber et al., 2008). This supports the notion that addictive drugs hijack the neural circuitry that processes motivationally-relevant cues (Bowers et al., 2010). Conversely, however, voluntary cocaine self-administration, rather than passive administration, can induce LTP-like potentiation at glutamatergic synapses that persists for up to 3 months following the final drug exposure (Chen et al., 2008). This potentiation, therefore, is possibly mediated not just by the pharmacological actions of the drug, but also by an active learning mechanism related to cocaine intake in animals which show learned associative behaviour (Chen et al., 2008; Bowers et al., 2010). Furthermore, potentiation following self-administration persists even after drug-seeking behaviour is extinguished (Chen et al., 2008), but this is not a feature of potentiation in response

to natural rewards, which is extinguished following acquisition of these behaviours (Pan et al., 2008). This resilient cocaine-induced synaptic potentiation may underlie the enduring 'memory' associated with drug use, which can facilitate reinstatement of drug-seeking behaviour, often seen following long periods of abstinence (Nestler, 2001). It may also promote maladaptive behaviour, through aberrant assignment of value to drug-related cues, encouraging behaviours associated with drug-use, and attenuating response and behaviours related to other salient stimuli (Kalivas and O'Brien, 2008).

1.5.5. Importance of drug-induced plasticity in VTA dopamine neurons

Drug-induced synaptic plasticity in the VTA may represent a transient, early neuroadaptation in the response to addictive drugs, but it is thought to be important for CPP and behavioural sensitization (Kalivas and Alesdatter, 1993; Wolf and Jeziorski, 1993; Vezina and Queen, 2000; Harris and Aston-Jones, 2003; Borgland et al., 2004). Given the importance of glutamatergic afferents onto dopamine neurons in the control over phasic burst firing, strengthening this excitatory input will have consequences for the firing activity of dopamine neurons, and hence dopamine release in target regions. Consistent with this, cocaine-induced potentiation is essential for the induction of plasticity at glutamatergic synapses in the downstream NAc, which is known to express more enduring drug-induced plasticity following chronic exposure (Boudreau and Wolf, 2005; Kourrich et al., 2007; Conrad et al., 2008; Mameli et al., 2009). These changes are thought to be critical in mediating the long-term behavioural changes seen in drug addiction (Kauer and Malenka, 2007; Thomas et al., 2008) and may underlie the development of persistent memories associated with the reinforcing properties of the drug experience (Hyman et al., 2006; Kauer and Malenka, 2007). Hence, drug-induced strengthening of glutamatergic synapses onto VTA dopamine neurons is an early critical step, which may ultimately trigger remodelling of the brain reward circuitry and the development of addiction (Mameli et al., 2009).

While the mesocorticolimbic dopamine system is essential for the induction of addiction, many chronic aspects of addiction are thought to be dopamine-independent (Wolf, 1998). Nevertheless, understanding the early stages in the process of addiction is important for developing targeted therapeutic interventions. Furthermore, dopamine is also thought to play a key role in drug-induced relapse (Kalivas and McFarland, 2003; Phillips et al*.,* 2003), which is an important stage in the treatment of drug addiction. Several pharmacological agents, which manipulate dopamine transmission, either by acting on dopamine receptors or the dopamine transporter, have yielded promising results in treating psychostimulant addiction (Xi and Gardner, 2008). This indicates that the dopamine system could be a key target for pharmacotherapies to treat addiction-related behaviours, and highlights the importance of fully understanding this system and the changes it undergoes following exposure to drugs of abuse.

1.6. Project overview

Midbrain dopamine neurons display considerable heterogeneity with regard to their molecular, electrophysiological, and functional properties. Only relatively recently, with much wider sampling of the VTA population, have these differences clearly been revealed. The DRN/vlPAG dopamine neurons are an understudied component of the midbrain dopamine system. This is likely to be because of their small size and number, relative to the VTA population, and also because they are housed within a large serotonergic population. This has hindered the targeting of these neurons *in vitro* and *in vivo*, and therefore no studies have reported their electrophysiological properties. However, these neurons provide the majority of the dopaminergic input to the central amygdala and BNST, and they have been implicated in promoting arousal and mediating the effects of opiates. Therefore, they are likely to be an important functional subgroup. I sought to characterise the neurochemical and electrophysiological properties of the DRN/vlPAG dopamine neurons, and examine the ability of cocaine to induce synaptic plasticity.

To overcome the difficulty in targeting these neurons, I utilised two lines of transgenic mice expressing GFP in dopamine neurons: TH-GFP and Pitx3-GFP. In **chapter 2** I have described the neurochemical features of the DRN/vlPAG dopamine neurons using immunohistochemistry, and compared the suitability of the GFP signal in TH-GFP and Pitx3-GFP mice for designating this population. I found that the majority of GFP+ neurons are TH+ in both mouse models, and additionally noted co-expression of vasoactive intestinal peptide (VIP) in the small periaqueductal subset of dopamine neurons. In **chapter 3** I explored the electrophysiological properties of these dopamine neurons, using whole-cell patch-clamp in an acute brain slice preparation *ex vivo*. I focused, in particular, on their firing characteristics in current clamp, and their response to depolarisation and hyperpolarisation. The DRN/vlPAG dopamine neurons displayed similar properties to 'unconventional' VTA dopamine neurons, and post-hoc identification of recorded

neurons revealed that VIP+ neurons fire in a more irregular pattern and exhibit a larger I_h than VIP- neurons. Importantly, however, I found no differences between identified TH+ and TH- GFP+ neurons, or between neurons recorded in TH-GFP and Pitx3-GFP mice. I next recorded spontaneous and evoked synaptic currents, which are described in **chapter 4**. Specifically, I compared glutamatergic and GABAergic currents in DRN/vlPAG dopamine neurons identified as VIP+ or VIP- and TH+ and TH-. In **chapter 5**, I studied the effect of a single dose of cocaine on the AMPAR:NMDAR ratio. Unexpectedly, I found that a single dose of cocaine, or just 24 hours of social isolation, causes an increase in the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons. I examined the characteristics of this plasticity in **chapter 6**, focusing particularly on changes in AMPAR transmission. I found that both cocaine- and social isolation-induced plasticity appear to be associated with a change in the AMPAR subunit composition, similar to cocaine-induced plasticity in the VTA. Finally, in **chapter 7**, I addressed the hypothesis that this may be a form of anxiety-induced plasticity, by assaying behaviour in the open field test and examining the effect of diazepam on synaptic strength. I also tested whether diazepam or ketamine could prevent social isolation-induced potentiation in DRN/vlPAG dopamine neurons. These findings are discussed in **chapter 8**, in relation to the functional implications of this plasticity for downstream projection targets, the significance of VIP co-expression, the clinical relevance of the results, and future experiments that would complement this work.

Chapter 2. Characterisation of DRN/vlPAG dopamine neurons: immunohistochemistry

2.1. Introduction

Dopamine neurons in the DRN/vlPAG are scattered in amongst many other neuronal types (Kohler and Steinbusch, 1982; Jacobs and Azmitia, 1992; Fu et al., 2010). They are also not the most abundant population, with estimates suggesting that around two-thirds of all neurons in the DRN are serotonergic (Molliver, 1987; Jacobs and Azmitia, 1992). This suggests that unguided patching within this region is unlikely to yield a high number of dopaminergic neurons. Indeed, in spite of wide sampling in the DRN/vlPAG (e.g. Allers and Sharp, 2003), only one confirmed dopamine neuron has been recorded *in vivo* (Schweimer and Ungless, 2010). Therefore utilising mice which express Green Fluorescent Protein (GFP; Chalfie et al., 1994) selectively in dopamine neurons is likely to be the most efficient method for targeting the dopaminergic population in the DRN/vlPAG. GFP mice are now frequently used in an attempt to improve the efficiency of electrophysiological recordings, and they have proved useful for targeted electrophysiological recordings in the VTA (e.g. Labouebe et al., 2007; Mameli et al., 2009).

2.1.1. GFP mouse models

Two lines of transgenic mice, in particular – Pitx3-GFP and TH-GFP – have been generated to selectively express GFP in dopamine neurons, and have both been successfully used to conduct targeted recordings from the VTA in brain slices (e.g., Labouebe et al., 2007; Zhang et al., 2010). This suggests that they may also be suitable for targeting the DRN/vlPAG population. Preliminary work by Antonios Dougalis (a former postdoc in Mark Ungless's laboratory) in Pitx3-

GFP mice found that the GFP signal is relatively weak, and he showed with a small sample of recorded DRN/vlPAG neurons that most, but not all, GFP+ neurons were TH+. This indicated that a more extensive quantitative and qualitative analysis was required to determine the suitability of Pitx3-GFP mice for targeting these dopamine neurons, along with the use of TH-GFP mice for comparison.

2.1.1.1.Pitx3-GFP mice

In Pitx3-GFP mice, GFP is inserted into the Pitx3 locus, resulting in Pitx3-directed GFP expression (Zhao et al., 2004). Pitx3 is a paired-like homeobox transcription factor which is functionally required for dopamine neuron development and maintenance (Semina et al., 2000; Rieger et al., 2001; Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003), and is expressed exclusively in midbrain dopamine neurons (Smidt et al., 1997). In Pitx3-GFP mice there is almost complete overlap between GFP and TH expression in the VTA and SNc (Zhao et al., 2004). Not only are the vast majority of GFP+ cells TH+ (99% in the SNc, 95% in the VTA), but also nearly all TH+ cells in this region are GFP+ (98% in the SNc and 95% in the VTA; Zhao et al., 2004), indicating high expression efficiency. It is noted that Pitx3 (Smidt et al., 1997), and therefore Pitx3-directed GFP expression (Zhao et al., 2004), is restricted to dopaminergic neurons of the ventral midbrain (VTA and SNc), and is not expressed by other dopaminergic populations. The DRN/vlPAG was not examined by Zhao et al. and therefore it remains to be established whether Pitx3 is expressed throughout this population, and whether the Pitx3-GFP mouse line is an appropriate tool for the purposes of this study.

2.1.1.2.TH-GFP mice

In TH-GFP mice, GFP is under the control of the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002). As TH is the rate-limiting enzyme in catecholamine synthesis (Levitt et

al., 1965) this results in GFP expression in midbrain and non-midbrain dopamine populations, as well as noradrenergic and adrenergic cell groups in the hindbrain and posterior hindbrain (Sawamoto et al., 2001; Matsushita et al., 2002). Although the proportions are slightly lower than in Pitx3-GFP mice, the majority of GFP+ cells in the midbrain are TH+ in these mice (93% in the SNc, 92% in the VTA; Matsushita et al., 2002). Similarly, most TH+ neurons express GFP (94% in the SNc, 85% in the VTA; Matsushita et al., 2002), demonstrating high expression efficiency in these transgenic mice. However, as in Pitx3-GFP mice, the overlap in the DRN/vlPAG population has not been specifically examined.

Even though both of these mouse models display impressive overlap between GFP and TH in the VTA/SNc, and have both been used to target dopamine neurons in these regions in brain slices (e.g., Mameli et al., 2009; Zhang et al., 2010), it is unknown whether this also applies to dopamine neurons in the DRN/vlPAG. This will be essential for accurate targeting of this dopamine population, and so quantification of GFP/TH co-expression in the DRN/vlPAG is required.

2.1.2. Molecular markers of dopamine neurons

2.1.2.1. TH expression

The most consistently used marker for identifying midbrain dopamine neurons is the presence of TH, the rate-limiting enzyme in catecholamine synthesis (Levitt et al., 1965). In the VTA/SNc, TH-expressing neurons lack the enzyme dopamine-β-hydroxylase (DBH), so cannot synthesise noradrenaline, and therefore the presence of TH can reliably identify dopamine neurons in this region. DRN/vlPAG dopamine neurons are similarly TH+ and DBH-, indicating their dopaminergic rather than noradrenergic phenotype (Swanson and Hartman, 1975; Nagatsu et al., 1979; Miachon et al., 1984; Flores et al., 2004). In some brain regions, however, there are reports of

TH+ neurons which do not appear to produce catecholamines (Meredith et al., 1999; Weihe et al., 2006). Conversely, the absence of TH is not always a definitive indicator of a non-dopamine neuron, as TH levels can be dynamically regulated within neurons (Liu and Baker, 1999; Chevalier et al., 2008; Akiba et al., 2009), while certain intracellular solutions can cause 'wash-out' of intracellular contents during recordings, preventing detection of TH (Margolis et al., 2010; Zhang et al., 2010). Additionally, it has been suggested that TH is efficiently transported to the terminals of some dopamine neurons, rendering TH levels in the somatodendritic regions undetectable with immunohistochemistry (Frain and Leviel, 1998). Therefore TH expression should be interpreted with caution, but it is currently the best indicator of dopaminergic phenotype in the ventral midbrain (Björklund and Dunnett, 2007b).

2.1.2.2.AADC and DAT expression

In order to assess the suitability of these GFP mouse models, the primary marker I have used is TH expression in the DRN/vlPAG. However, in addition to this I have examined two other molecular markers of dopamine neurons: the enzyme aromatic L-amino acid decarboxylase (AADC) and the plasma membrane dopamine transporter (DAT).

Although TH is the rate-limiting enzyme in dopamine synthesis, AADC is required to catalyse the conversion of L-DOPA (the product of the TH reaction) into dopamine. Therefore the presence of both proteins indicates that the neuron is fully capable of enzymatically producing dopamine. This is important (as mentioned above), since in some regions TH+/AADC- neurons can be found, which are not thought to be enzymatically capable of producing dopamine. However, as AADC is also required to convert 5-hydroxytryptophan into serotonin, it is not useful as a selective marker in regions with a serotonergic population, such as the DRN/vlPAG.

DAT mediates uptake of dopamine back into the cell, and its expression is restricted to dopamine neurons, particularly those in the ventral midbrain (Augood et al., 1993). DAT expression has been used to identify dopamine neurons in brain slices (e.g. Good and Lupica, 2010). However, the expression pattern of DAT has been shown to vary significantly between different subpopulations of VTA dopamine neurons (Lammel et al., 2008). Dopamine neurons projecting to the NAc core, NAc medial shell, BLA, and mPFC show significantly lower DAT/TH ratio than NAc lateral shell-projecting neurons, which is attributed to lower levels of DAT in these neurons (Lammel et al., 2008). Moreover, only 33% of these neurons with a low ratio showed levels of DAT that were significantly higher than background (Lammel et al., 2008). This suggests that even though DAT has been previously used to identify dopamine neurons and, when quantified, it can be a useful indicator of the dopamine uptake capacity of the neuron (Lammel et al., 2008), it is likely to yield a considerable number of false negatives if used as a dopaminergic marker alone.

In summary, TH is the most widely accepted molecular marker for dopamine neurons, and (as long as it is used in a region that does not contain noradrenergic neurons) provides a selective indicator of dopaminergic identity. Other dopaminergic markers, can provide additional useful information about the neurons, but are not necessarily the most appropriate indicators of neurotransmitter content. For this reason I have used a combination of immunohistochemistry for TH, AADC, and DAT, along with markers for other cell types in the DRN/vlPAG, in order to provide a well-rounded characterisation of these dopamine neurons, and establish the suitability of GFP mice for targeting this population.

2.2. Materials and methods

2.2.1. Animals and breeding

Male heterozygous TH-GFP (B6.Cg-Tg(TH-GFP)21-31/C57B6; Matsushita et al., 2002) and Pitx3-GFP (Zhao et al., 2004) mice, aged 1-4 months, were housed under a 12 hour light/dark schedule with food and water available *ad libitum*. TH-GFP mice were originally obtained from Kazuto Kobayashi (Department of Molecular Genetics, Fukushima Medical University, Japan) and Pitx3-GFP mice from Meng Li (MRC Clinical Sciences Centre, Imperial College London, UK). All breeding and experimental procedures were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom, UK). TH-GFP mice were bred from heterozygous x wildtype (C57BL/6) breeding pairs, generating both heterozygous and wildtype offspring. The genotype of TH-GFP mice was confirmed by PCR using primers 5'-CCTGTGACAGTGGATGCAATTG-3', 5'-CTTGTACAGCTCGTCCATGCCGAG-3', 5'-TGAGCGAGCTCATCAAGATAATCAGGT-3', and 5'-GTTAGCATTGAGCTGCAAGCGCCGTCT-3', which yielded an internal control fragment of 550 bp in wildtype mice, and an additional fragment of 1,660 bp in heterozygous mice. Pitx3-GFP mice were produced from homozygous x wildtype (C57BL/6) breeding pairs, whose offspring were all heterozygous and therefore did not require genotyping.

2.2.2. Perfusion-fixation and cryostat sectioning

Mice were given a lethal intraperitoneal (IP) injection of ketamine (KETASET, Willows Francis, UK) and xylazine (ROMPUN, Bayer, Germany), then transcardially perfused with 50 ml phosphate buffered saline (PBS; 0.1 M, pH 7.4) followed by 80 ml of 4% weight/volume (w/v) paraformaldehyde (PFA; Sigma, UK) in PBS. Brains were subsequently removed and post-fixed for 1-5 hours in 4% PFA, at 4 °C, then cryoprotected for 24 hours in 30% sucrose in PBS, at 4 °C.

They were then embedded in Optimal Cutting Temperature (OCT) medium and frozen in isopentane at -50 °C before cryostat sectioning (Leica CM1800, Leica Microsystems, Germany). Coronal sections (30 µm thickness) were prepared from the region containing the DRN/vlPAG, and also the VTA for comparison.

2.2.3. Brain slice preparation and fixation

Mice were sacrificed by isoflurane anaesthesia followed by decapitation. The brain was then rapidly removed out of the cranial cavity and submersed in ice-cold (0-4 °C) artificial cerebrospinal fluid (aCSF, composition in mM, NaCl 120, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, Glucose 10, $MgCl_2$ 1, $CaCl_2$ 2) fully equilibrated with carbogen gas (95% oxygen, 5% carbon dioxide). Two or three coronal brain slices (220 µm thickness) encompassing the DRN/vlPAG were obtained using a vibratome (Leica VT1000S, Leica Microsystems, Germany) and immediately transferred to 4% PFA for 1 hour at room temperature.

2.2.4. Immunohistochemistry

Free-floating 30 µm sections, or 220 µm brain slices, were washed in PBS to remove OCT medium or fixative respectively, then blocked in PBS containing 0.2% Triton X-100 (PBS-T) with 6% normal donkey serum (NDS; Jackson ImmunoResearch, USA) for 30-60 min at room temperature. They were then incubated overnight at room temperature in 0.2% PBS-T with 2% NDS and appropriate primary antibodies: anti-TH monoclonal chicken antibody (1:1000, Abcam, USA), anti-serotonin (5-HT) polyclonal rabbit antibody (1:1000, Immunostar, USA), anti-DAT rabbit polyclonal antibody (1:500, Millipore, MA, USA), anti-AADC rabbit antibody (1:1000, Millipore, USA), anti-VIP (vasoactive intestinal polypeptide) rabbit antibody (1:500, Immunostar, USA). Sections or brain slices were then washed in 0.2% PBS-T and incubated for 1.5-2 hours at room temperature in 0.2% PBS-T containing 2% NDS and corresponding secondary antibodies:

Alexa 633-conjugated anti-chicken (1:1000, Invitrogen, USA), Cy3-conjugated anti-rabbit (1:1000, Jackson ImmunoResearch, USA). Lastly, sections or brain slices were rinsed with 0.2% PBS-T, followed by PBS alone, and then mounted onto glass microscope slides. Once dry, coverslips were placed over the sections using VectaShield mounting medium (Vector Laboratories, USA), for visualisation using confocal microscopy.

2.2.5. Confocal microscopy

A Leica SP5 II confocal laser scanning microscope (Leica Microsystems, Germany) was used to view the sections, and images were captured using Leica Application Suite software (Leica Microsystems, Germany). Sections were viewed through a 20x / 0.7 numerical aperture dry HC Plan-Apochromat CS DIC objective, with 1.5x digital zoom applied during image capture (30x total magnification). GFP was excited by a 488 nm line of an Argon laser, Cy3 by a 561 nm line of a DPSS laser, and AlexaFluor633 by a 633 nm line of a HeNe laser. The pinhole size was set to 1 Airy unit and an optical slice thickness of 2 µm was used. The signal-to-noise ratio was improved by averaging at least six scans for each *z*-section. Images were taken at a resolution of 1024 x 1024 and were processed with general brightness and contrast curve adjustments in Adobe Photoshop CS5 (Adobe Systems Incorporated).

2.2.6. Cell counting and statistics

To quantify overlap between GFP and TH in 30 µm sections, 9 sections containing the DRN/vlPAG were counted (3 each from 3 animals) from TH-GFP and Pitx3-GFP mice. For 220 µm brain slices, 6 brain slices were counted (1 each from 6 animals) from TH-GFP and Pitx3-GFP mice. Overlap between GFP and AADC was quantified in 30 µm sections from TH-GFP mice by counts from 9 sections (3 each from 3 animals). Statistical comparisons on the proportion of

double-labelled neurons were performed using a chi-squared test in Prism 5.04 (Graphpad, CA, USA).

2.3. Results

2.3.1. Co-expression of GFP and TH in Pitx3-GFP and TH-GFP mice

The pattern of expression of TH+ neurons within the DRN/vlPAG of both mouse models was identical to that described in previously reports (Hökfelt et al., 1984; Hasue and Shammah-Lagnado, 2002; Flores et al., 2004; Flores et al., 2006; Lu et al., 2006). I observed both large multipolar and small rounded neurons in this region, with the large neurons generally located more ventrally and laterally, compared to the small rounded neurons, which were mostly found in the midline and close to the ventral surface of the aqueduct of Sylvius. Rostrally, the DRN/vlPAG TH+ neurons were continuous with the TH+ neurons of the rostral and caudal linear nuclei (RLi and CLi), and extended caudally throughout the vlPAG/DRN, generally in the medial portion of this nucleus and ventral to the aqueduct of Sylvius, but with some larger neurons residing more laterally, and some smaller neurons spreading up the lateral surface of the aqueduct. Most caudally in the DRN, the number of large ventral TH+ neurons diminished, and only small periaqueductal neurons remained.

2.3.1.1. Perfused-fixed sections

In 30 µm sections from perfused-fixed TH-GFP and Pitx3-GFP mice, the majority of DRN/vlPAG GFP+ neurons were TH+ (Fig. 1a,b). I quantified this with cell counts from 9 sections (3 animals, 3 sections per animal) for each GFP mouse model. This revealed that both models had similar proportions of GFP+/TH+ cells (TH-GFP: 74%, 606/823 cells; Pitx3-GFP: 71%, 274/386 cells; chi-squared = 0.93, p = 0.33). The GFP+/TH- neurons were typically small periaqueductal neurons in both Pitx3-GFP and TH-GFP mice. However, given that the GFP signal in TH-GFP mice is under

the control of the TH promoter, it is likely that these small, periaqueductal GFP+/TH- neurons are dopaminergic, but TH may be below the detection threshold for immunohistochemistry. Consistent with this, lower TH levels in the small, periaqueductal dopamine neurons has been previously reported (Hökfelt et al., 1976; Rogers, 1992). Additionally, I observed no TH immunostaining if either primary or secondary antibodies were omitted.

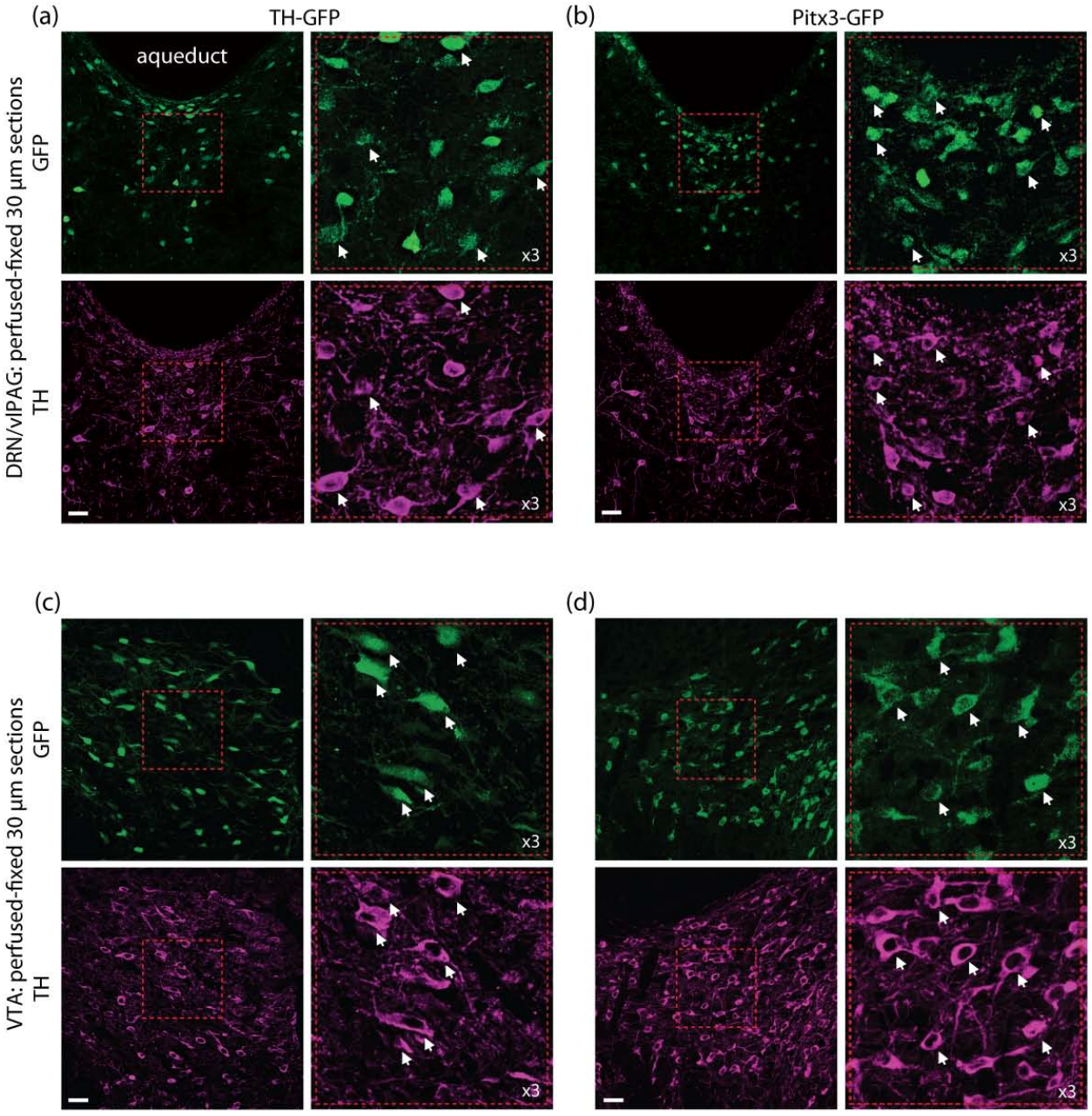
The GFP signal was considerably stronger in TH-GFP compared to Pitx3-GFP mice, with significantly higher laser power required during microscopy to visualise the neurons in Pitx3-GFP mice. Additionally, although GFP expression in TH-GFP mice was relatively uniform throughout the DRN/vlPAG population, in Pitx3-GFP mice expression was particularly strong in small, periaqueductal neurons, with weaker, and sometimes absent, expression in the large, more ventral and laterally located TH+ neurons. Specifically, in Pitx3-GFP mice, cell counting revealed that 40% of TH+ neurons in the DRN/vlPAG were GFP- (183/457 cells) compared with just 10% in TH-GFP mice (70/676 cells; chi-squared = 138.6, $p < 0.0001$). As in the case of the GFP+/TH- neurons, this could be a detection issue, particularly given the weaker GFP signal observed in Pitx3-GFP mice. However, this does indicate that the GFP expression efficiency is higher in TH-GFP mice, compared to Pitx3-GFP mice, and the GFP signal appears to be more evenly spread across the dopamine population. GFP and TH showed a high rate of co-expression in the VTA (Fig. 1c,d) of both mouse models, as previously described (Matsushita et al., 2002; Zhao et al., 2004), with few GFP+/TH- neurons, or TH+/GFP- neurons.

2.3.1.2. Brain slices

I examined the overlap between GFP and TH in 220 µm brain slices containing the DRN/vlPAG. These were prepared in the same way as brain slices made for *ex vivo* electrophysiology, in order to confirm that the GFP signal is strong enough in this preparation for visually-guided patching, and that TH is still detectable in this region using immunohistochemistry. GFP and TH

showed a similar pattern of co-expression in brain slices compared to perfused-fixed sections, in both Pitx3-GFP and TH-GFP mice (Fig. 1e,f). Interestingly, a higher proportion of GFP+ neurons in the DRN/vlPAG co-expressed TH in brain slices compared to perfused-fixed sections: 83% in TH-GFP mice (403/485 cells) and 76% in Pitx3-GFP mice (247/325 cells; total counts from 6 brain slices for each mouse model, 1 section per animal from 6 animals). This could either be because the GFP+/TH- neurons are less likely to survive brainslicing, or because their low levels of TH (undetectable in perfusion-fixed sections) are at detection level in brain slices. Similarly, there was a lower proportion of TH+/GFP- neurons in both mouse lines, with 9**%** in TH-GFP (942/445 cells) and 36**%** in Pitx3-GFP mice (140/387 cells). This could also be due to the improved ability to detect a weak GFP signal in the large TH+ neurons in brain slices, compared to perfused-fixed sections.

In summary, these results indicate that the GFP signal in both mouse models can be used as an effective means to target dopamine neurons in the DRN/vlPAG, with TH-GFP mice providing a stronger, and perhaps more uniformly spread, GFP signal.


(a)
TH-GFP
aqueduct
(b)
Pitx3-GFP
DRN/vlPAG: perfused-fixed 30 µm sections
GFP
TH
x3
(c)
(d)
VTA: perfused-fixed 30 µm sections
GFP
TH
x3

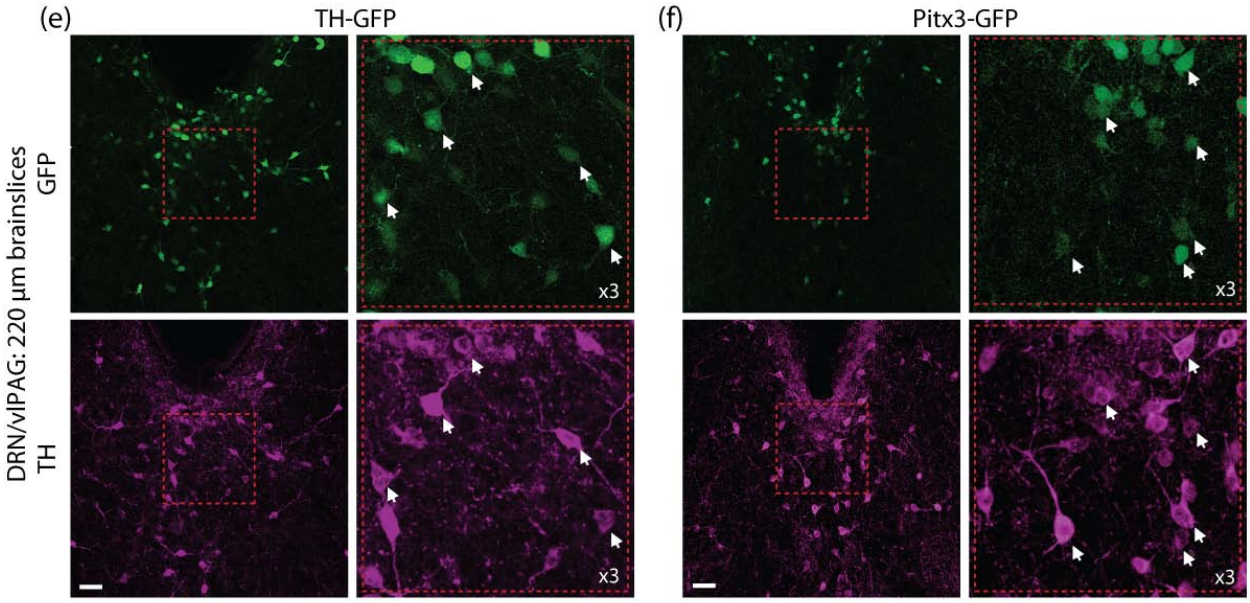


Figure 1. Colocalisation of GFP with tyrosine hydroxylase (TH). Examples of 30 µm perfused-fixed sections from (a,c) a TH-GFP and (b,d) a Pitx3-GFP mouse showing the overlap between GFP and TH in the DRN/vlPAG and VTA. Examples of 220 µm brain slices from (e) a TH-GFP and (f) a Pitx3-GFP mouse showing the overlap between GFP and TH in the DRN/vlPAG. The GFP signal is shown in green and TH in magenta, with selected double-labelled neurons indicated by the white arrows. Images shown are single 2 µm thick optical sections captured on a confocal microscope through a 20x objective with 1.5x digital zoom applied during image capture. The red dotted box marks the region of 3x zoom. Scale bars = 30 µm.

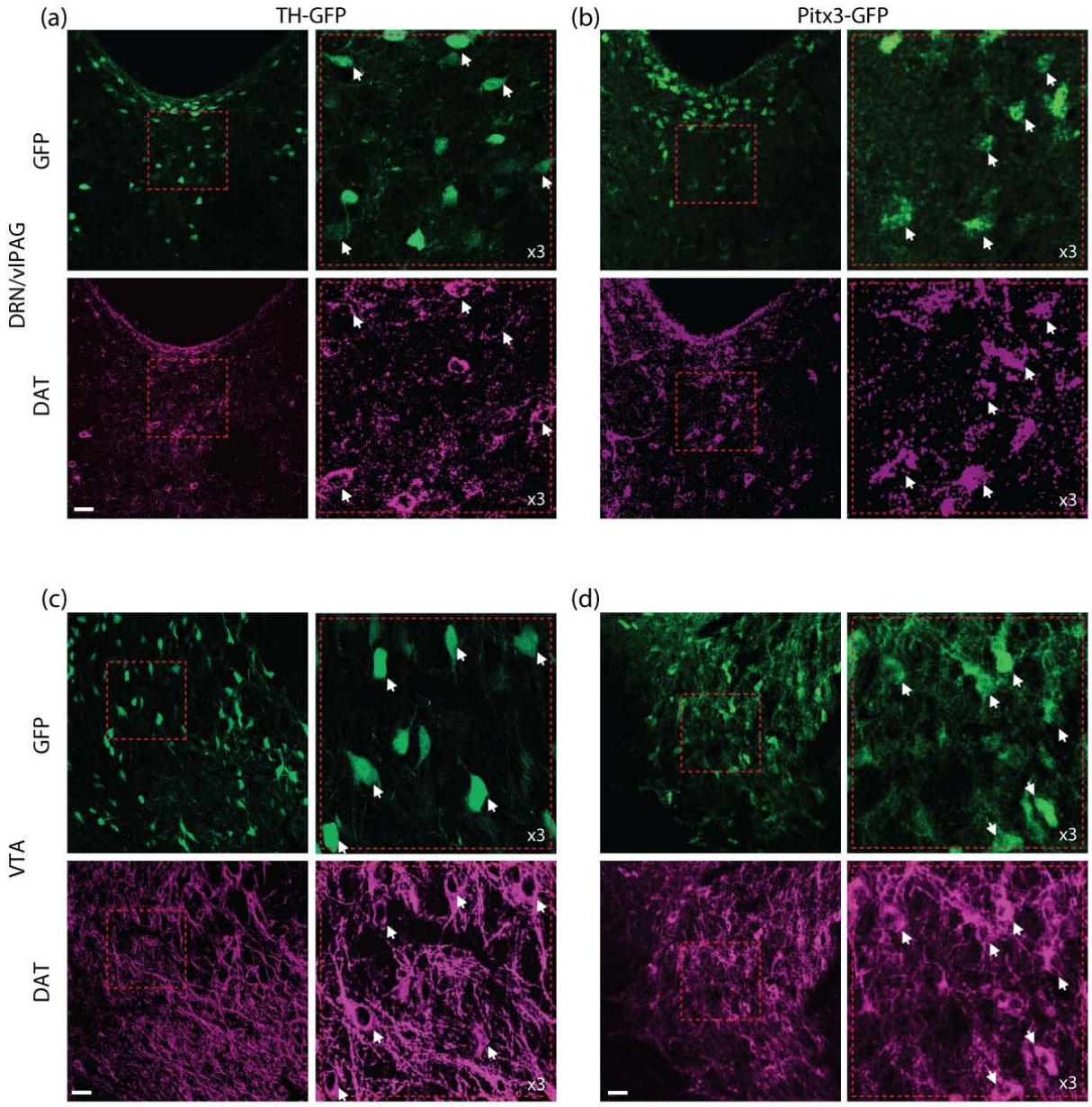
2.3.2. Co-expression of GFP with other dopaminergic molecular markers

2.3.2.1. DAT

In 30 µm sections from both TH-GFP and Pitx3-GFP mice, DAT was expressed in most large, multipolar GFP+ neurons, generally localised to the cell membrane and along neuronal processes (Fig. 2a,b). It was not usually observed in the small periaqueductal neurons, which is consistent with the observation that the large multipolar dopamine neurons are sensitive to 6-OHDA, whereas the small periaqueductal neurons are relatively spared following administration of this neurotoxin (Flores et al., 2004; Flores et al.,2006; Lu et al., 2006). DAT staining was also present in TH+ neurons of the VTA (Fig. 2c,d), suggesting that the lack of expression in small periaqueductal DRN/vlPAG neurons, and the generally weaker staining in this region, was not a result of the antibody used.

2.3.2.2. AADC

I found that in sections from perfused-fixed TH-GFP mice, 72% of GFP+ neurons in the DRN/vlPAG co-expressed the enzyme AADC (Fig. 2e,f; TH-GFP mice: 510/704 cells; 3 animals, 3 sections per animal counted). This is a similar proportion to those which were TH+, suggesting that these enzymes are immunohistochemically-detectable in a similar number of GFP+ neurons. In addition, as with TH expression, the GFP+/AADC- neurons were generally small periaqueductal neurons. AADC is also required for synthesis of 5-HT, therefore I also observed many large AADC+, GFP- neurons in the DRN/vlPAG located more ventrally, which are likely to be serotonergic (Fig. 2e,f). I also found almost complete co-expression of GFP and AADC in the VTA (Fig. 2g,h), confirming the specificity of this antibody. Furthermore, omission of either DAT or AADC primary or secondary antibodies resulted in no detectable immunostaining.


(a)
TH-GFP
(b)
Pitx3-GFP
DRN/vlPAG
GFP
DAT
x3
(c)
(d)
VTA

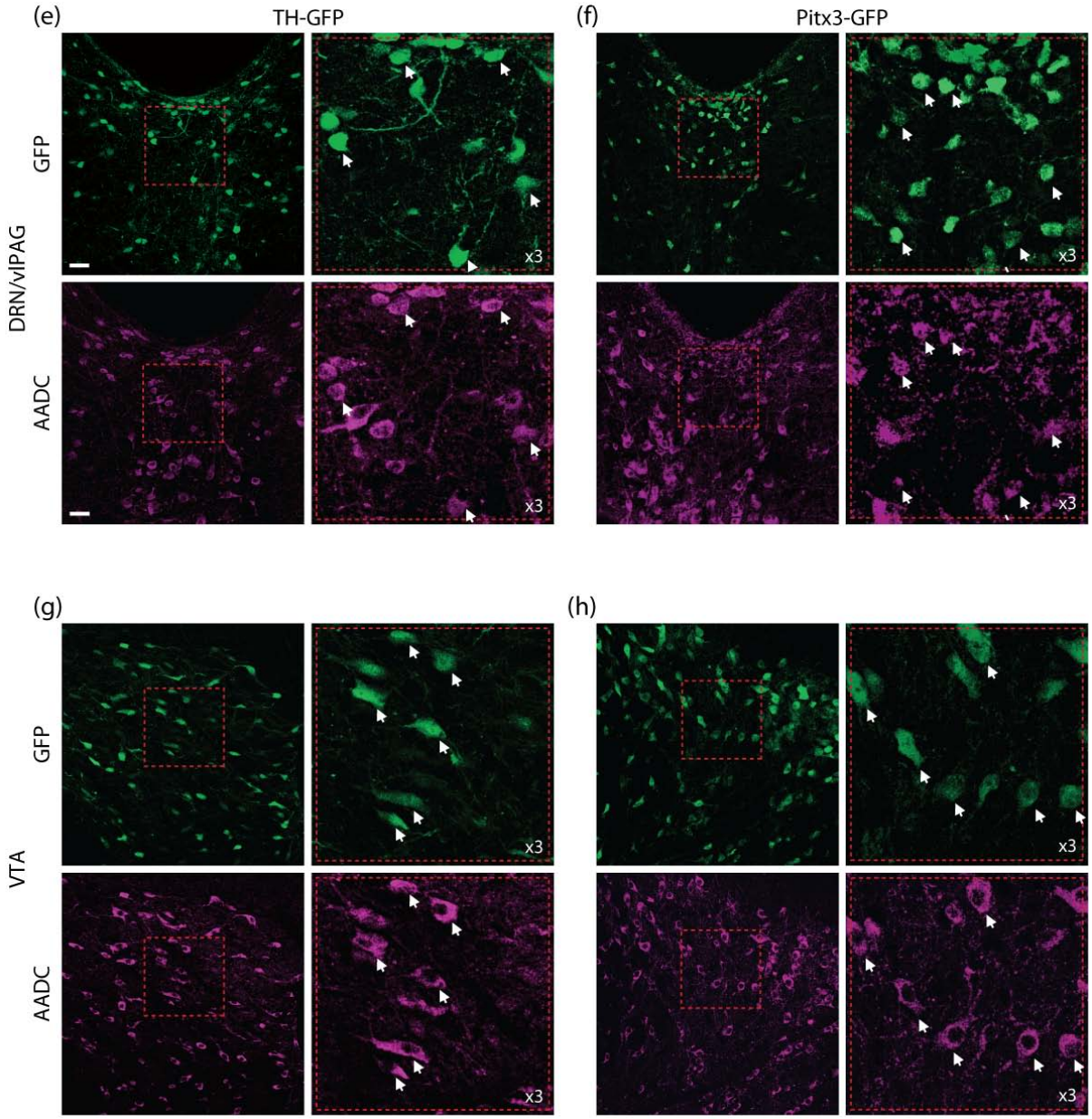


Figure 2. Colocalisation of GFP with other dopaminergic markers. Examples sections of the DRN/vlPAG from (a) a TH-GFP and (b) a Pitx3-GFP mouse showing co-expression of DAT in some GFP+ neurons. Example sections of the VTA from (c) a TH-GFP and (d) a Pitx3-GFP mouse showing DAT co-expression in the majority of GFP+ neurons. Example sections from (e,g) a TH-GFP and (f,h) a Pitx3-GFP mouse showing that most GFP+ neurons co-express AADC in the DRN/vlPAG and VTA. The GFP signal is shown in green and DAT or AADC in magenta, with selected double-labelled neurons indicated by the white arrows. Images shown are single 2 µm thick optical sections captured on a confocal microscope through a 20x objective with 1.5x digital zoom applied during image capture. The red dotted box marks the region of 3x zoom. Scale bars = 30 µm.

2.3.3. Co-expression of GFP and TH with other cell types in the DRN/vlPAG

2.3.3.1.5-HT

Consistent with previous reports, numerous large 5-HT+ neurons were present in the DRN, overlapping with TH+ neurons mainly in the rostral DRN/vlPAG. 5-HT+ neurons were most abundant at the more caudal levels of the DRN, occupying the majority of the medial and ventral portion of the nucleus, with many cells also located in the lateral wings. More rostrally, 5-HT+ neurons were scattered ventrally, overlapping with the large TH+ neurons in this region, but less with the small periaqueductal neurons. I found no co-expression of GFP or TH with 5-HT, at any level of the DRN/vlPAG (Fig. 3a), as previously reported (Fu et al., 2010), and staining was absent in the VTA (Fig. 3b).

2.3.3.2.Vasoactive intestinal peptide (VIP)

Through studying the immunohistochemical work in the DRN detailed in Fu et al. (2010) I noticed that there was a striking similarity between the expression pattern of GFP close to the aqueduct and that of the neuropeptide transmitter vasoactive intestinal peptide (VIP; Fu et al., 2010). I therefore used an antibody against VIP to determine whether this neuropeptide was co-localised with GFP and TH. I found that all VIP+ neurons in the DRN/vlPAG were GFP+, in both mouse models (Fig. 3c), with the majority also co-expressing TH (TH-GFP: 75%, 246/330 cells, Pitx3-GFP: 76% 171/224 cells; 3 animals, 3 sections per animal counted). Interestingly, I observed increased colocalisation of VIP and TH in 220 µm brain slices (TH-GFP: 87%, 248/286 cells; Pitx3-GFP: 88%, 203/231 cells), similar to that noted for GFP and TH described previously.

The distribution of these VIP+ neurons matched that of the small, rounded GFP+ neurons (i.e. around the ventral and lateral surface of the aqueduct, and spreading ventrally in midline), and was identical to previous descriptions of the expression pattern of this neuropeptide (Sims et al.,

1980; Marley et al., 1981; Hökfelt et al., 1982; Moss and Basbaum, 1983; Petit et al., 1995; Fu et al., 2010). Additionally, I noted co-expression in some GFP+/TH+ neurons in the CLi, but found no VIP+ cells in the VTA (Fig. 3d), which suggests that this neuropeptide co-expression is specific to more caudal dopaminergic populations. VIP+ neurons constituted 40% of GFP+ neurons in TH-GFP mice (330/823 cells) and 58% of GFP+ neurons in Pitx3-GFP mice (224/386 cells; chi squared = 34.0, $p < 0.0001$). This is consistent with the observation that the GFP signal was somewhat biased towards the small periaqueductal neurons in Pitx3-GFP mice, with weaker, or sometimes absent, expression in the larger more ventral neurons, which are typically VIP-. This indicates that GFP-guided patching in Pitx3-GFP mice may yield a slightly greater proportion of VIP+ neurons than patching in TH-GFP mice, although both cell types are present.

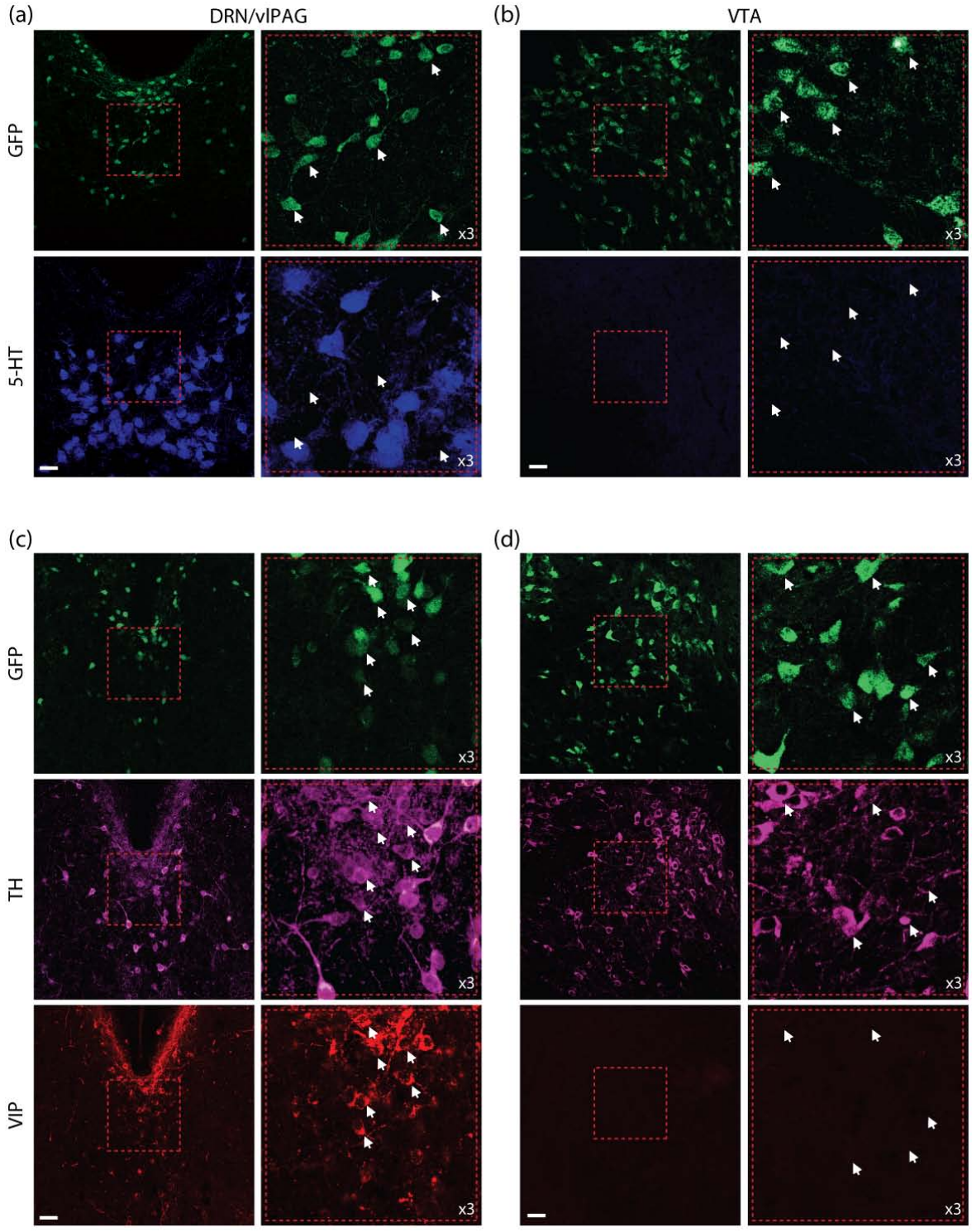

(a)
DRN/vlPAG
(b)
VTA
GFP
5-HT
x3
(c)
(d)
TH
VIP

Figure 3. Colocalisation of GFP with markers for other cell types. Example sections of (a) the DRN/vlPAG and (b) the VTA, from a TH-GFP mouse, showing 5-HT+ neurons in the DRN/vlPAG, but no co-expression with GFP. The GFP signal is shown in green and 5-HT in blue, with selected GFP+ neurons indicated by the white arrows. Example sections from (c) the DRN/vlPAG and (d) the VTA showing co-expression of VIP in the small periaqueductal GFP+/TH+ neurons, but not in the large ventral/lateral neurons of the DRN/vlPAG, or in the VTA. The GFP signal is shown in green, TH in magenta, and VIP in red, with selected triple-labelled neurons indicated by the white arrows. Images shown are single 2 µm thick optical sections captured on a confocal microscope through a 20x objective with 1.5x digital zoom applied during image capture. The red dotted box marks the region of 3x zoom. Scale bars = 30 µm.

2.4. Conclusion

I have used immunohistochemistry to examine the expression of GFP and TH in the DRN/vlPAG of two lines of transgenic mice, which were generated to selectively express GFP in dopamine neurons. Quantification of GFP/TH co-expression in 30 µm sections indicated that TH colocalises with the large majority of GFP+ neurons in the DRN/vlPAG of both mouse models, but with somewhat lower co-expression than that reported for the VTA (Matsushita et al., 2002; Zhao et al., 2004). Furthermore, I found a higher degree of co-expression in 220 µm brain slices, which is the primary means by which I will be examining these dopamine neurons. The reason for this is not clear. However, it has been observed that TH can be transported out to neuronal terminals (Frain and Leviel, 1998), so the severing of these processes during brain slice preparation may result in increased accumulation of TH in the soma, resulting in levels detectable with immunohistochemistry.

The GFP signal in TH-GFP mice was more evenly distributed throughout the TH+ population in the DRN/vlPAG, whereas there was some bias towards GFP expression in the small, periaqueductal TH+ neurons in Pitx3-GFP mice. However, this might be a result of the weak GFP expression in this mouse line, because improved overlap was observed in brain slices compared to sections. As long as care is taken to sample widely throughout this region, both small periaqueductal and large ventral GFP+/TH+ neurons can be located in both mouse models. Therefore, although colocalisation of TH and GFP in the DRN/vlPAG was not complete (which is a common feature of most GFP mouse models), these results suggest that the GFP signal in these transgenic mice can be used to target dopamine neurons in this region for electrophysiological recordings, in combination with post-hoc immunohistochemistry.

Analysis of other dopaminergic molecular markers revealed that AADC colocalised with GFP to a similar extent as TH in 30 µm sections. Serotonergic neurons in this region also express AADC, and

the signal intensity was often much stronger in these neurons (possibly due to their larger size), which often obscured the signal in smaller GFP+ neurons. AADC is not, therefore, the most useful indicator of dopaminergic phenotype in this region, although it does suggest that these TH+ neurons are enzymatically capable of producing dopamine. DAT expression was generally weaker in the DRN/vlPAG dopamine neurons, compared to the VTA, and on the whole was only expressed by the large multipolar neurons, with very little observed in the small, rounded neurons. This is consistent with previous reports indicating that these two cell types differ in their sensitivity to the neurotoxin 6-OHDA, which selectively lesions the large, ventral neurons (Flores et al., 2004; Flores et al., 2006; Lu et al., 2006). Based on the pattern of DAT expression I observed, this is likely to be due to the lack of the transporter in the small, periaqueductal neurons, which would prevent uptake of this neurotoxin (Jonsson and Sachs, 1971; Kostrzewa and Jacobowitz, 1974; Glinka et al., 1997). Additionally, this may have implications for the decay kinetics of extracellular dopamine, since DAT-mediated reuptake is one method of regulating extracellular dopamine levels (Horn, 1990; Giros et al., 1996), although diffusion of dopamine also plays a major role in this process (Cragg and Rice, 2004).

Previous immunohistochemical studies have confirmed that TH+ neurons in the DRN/vlPAG are DBH- (and therefore do not synthesise noradrenaline; Nagatsu et al., 1979; Miachon et al., 1984; Lu et al., 2006), and also do not co-express 5-HT (Fu et al., 2010) suggesting that they are a distinct population. These findings confirm that 5-HT is not co-expressed in TH+ neurons in this region, nor in GFP+ neurons in either mouse model. Furthermore, I have shown that the two cell types previously described in the DRN/vlPAG can be distinguished based on the expression of VIP. The presence of VIP+ neurons within the DRN/vlPAG has been reported (Sims et al., 1980; Marley et al., 1981; Hökfelt et al., 1982; Moss and Basbaum, 1983; Smith et al., 1994), and it has been shown that these neurons are neither serotonergic, nor GABAergic (Fu et al., 2010). It has

also been previously noted that peptide histidine isoleucine (PHI; a related peptide)/VIP and CCK are co-expressed with a limited number of TH+ neurons in periventricular regions and around the aqueduct (Seroogy et al., 1988a), but the data presented here suggests significant co-expression of VIP with DRN/vlPAG dopamine neurons, which may delineate a distinct subpopulation. The functional significance of this co-expression remains to be determined, although the presumed co-release of dopamine and VIP may have implications for downstream projection targets.

Chapter 3. Characterisation of DRN/vlPAG dopamine neurons: electrophysiology

3.1. Introduction

The electrophysiological properties of VTA/SNc dopamine neurons have been widely documented both *in vivo* and *ex vivo*. Electrophysiology has been proposed to be sufficient for reliably identifying SNc, and the majority of VTA, dopamine neurons *in vivo*, by employing criteria including action potential waveform, firing rate, and pattern (Ungless and Grace, 2012). *Ex vivo*, however, different criteria can be utilised, partly because most afferent inputs are severed in the slice preparation, resulting in a more regular firing pattern (Grace and Onn, 1989), and additional currents can be assayed using the whole-cell configuration. As discussed in chapter 1, there is some inter-species variation in the expression of many of these classically-used electrophysiological markers, but for the purposes of this study I will focus on the properties reported in mice.

3.1.1. Electrophysiological markers of dopamine neurons

One frequently used indicator of dopaminergic phenotype is the presence of a prominent hyperpolarisation-activated cation current (I_h; Lacey et al., 1989; Johnson and North, 1992a; Ungless et al., 2001; Gutlerner et al., 2002; Saal et al., 2003; Borgland et al., 2004; Faleiro et al., 2004; Bellone and Lüscher, 2006; Margolis et al., 2006a; Argilli et al., 2008; Stuber et al., 2008; Zweifel et al., 2008; McCutcheon et al., 2012). However, considerable variation has been observed across the VTA dopamine population in terms of the size of the I_h, and it is even absent in certain subpopulations (Johnson and North, 1992a; Neuhoff et al., 2002; Liss et al., 2005; Ford et al., 2006; Sarti et al., 2007; Lammel et al., 2008; Zhang et al., 2010; Lammel et al., 2011).

Other characteristic features of dopamine neuron electrophysiology include their broad action potential width, slow firing rate, and prominent afterhyperpolarisation (Sanghera et al., 1984; Trulson and Trulson, 1987; Johnson and North, 1992a). However, similar to the I_h, it has been shown that dopamine neurons in fact exhibit a broader range of these measures than previously thought (Lammel et al., 2008), which emphasises the heterogeneity of this population. Equally, the spike-frequency adaptation seen in response to depolarisation, thought to be a consistent property of midbrain dopamine neurons, has been shown to vary in different dopamine neuron subpopulations (Zhang et al., 2010; Lammel et al., 2011).

3.1.2. Projection-specific electrophysiological properties

Differences in the I_h and action potential properties have been associated with projection target (Ford et al., 2006; Lammel et al., 2008). Specifically, VTA and SNc dopamine neurons projecting to the NAc lateral shell and dorsolateral striatum exhibit a large I_h, low frequency firing, (~1-3 Hz), short duration action potential (~3 ms), large afterhyperpolarisation, and do not sustain firing above ~10 Hz in response to depolarisation (Lammel et al., 2008). In contrast, VTA dopamine neurons projecting to the NAc core, NAc medial shell, BLA, and mPFC exhibit a considerably smaller I_h (or lack this current), fire, on average, at higher frequencies (~5 Hz), possess a wider action potential (~4.5-7 ms), smaller afterhyperpolarisation, and display significantly higher maximal firing frequencies in response to depolarisation (~20-30 Hz; Lammel et al., 2008; Lammel et al., 2011). Therefore, while 'classic' dopamine properties may be found in certain subpopulations, there is considerable variability across the whole dopamine population.

Preliminary work, conducted by Antonios Dougalis, in Pitx3-GFP mice suggested that GFP+ neurons in the DRN/vlPAG exhibit similar electrophysiological properties to VTA dopamine neurons, but distinct from those of putative serotonergic neurons within the same nucleus

(Dougalis et al., 2012). I have therefore examined these same properties, but instead focused on comparing DRN/vlPAG GFP+ neurons from TH-GFP and Pitx3-GFP mice, as well as neurochemically-identified TH+ or TH-, and VIP+ or VIP- neurons. The electrophysiological properties assayed include those which have frequently been attributed to midbrain dopamine neurons, including action potential waveform, firing pattern, and the I_h.

3.2. Materials and methods

3.2.1. Brain slice preparation

Coronal 220 µm brain slices containing the DRN/vlPAG were prepared from TH-GFP and Pitx3-GFP mice (aged 1-2.5 months) at 10.00 am, as described in chapter 2. The slices were transferred to a custom-made holding bath containing artificial cerebrospinal fluid (aCSF; composition in mM, NaCl 120, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, Glucose 10, $MgCl_2$ 1, $CaCl_2$ 2), saturated with carbogen gas (95% oxygen, 5% carbon dioxide), at room temperature. Slices were allowed to recover in the holding bath for at least one hour before being transferred to the recording chamber for electrophysiology. Once in the recording chamber, slices were continuously perfused at a rate of 2-4 ml/min with fully oxygenated aCSF at 32 °C. Neurons were visualised under an upright microscope (BXWI 51; Olympus, Tokyo, Japan), equipped with infra-red (IR) differential interference contrast (DIC) optics and a charge coupled device (CCD) video camera (Hamamatsu Photonics, Germany), through a 40x immersion objective. GFP+ neurons were identified in the slice, prior to recording (Fig. 4a), using brief fluorescence illumination (X-cite 120Q, EXFO, UK) through a GFP excitation filter. PatchVision software (Scientifica, UK) was used to view IR-DIC and fluorescence images on a standard desktop computer.

3.2.2. Whole-cell patch-clamp electrophysiology

Whole-cell patch-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, CA, USA) and Clampex 10.2 software (Molecular Devices, CA, USA). Records were low-pass filtered at 1 kHz and digitised at 3-5 kHz. Electrodes were pulled from thin-walled borosilicate glass capillary tubing (GC150F-10, Harvard Apparatus, UK) using a two-stage vertical puller (model PC-10, Narashige). They had resistances of 5-7 MΩ when filled with internal solution (composition in mM: potassium gluconate 140, NaCl 5, $MgCl_2$ 1, EGTA 1, HEPES 10, Mg-ATP 2, Li-GTP 0.5, and 0.1% neurobiotin, with pH 7.3 and osmolarity 280-290 mosmol/l.) Series resistance (R_s; typical values 15-30 MΩ, compensated by 60-70% in the majority of experiments) and input resistance (R_{in}) were monitored frequently throughout experiments, via a 10 mV, 250 ms hyperpolarizing step in voltage-clamp or a 10-100 pA current injection in current-clamp. Recordings were terminated if R_s exceeded 35 MΩ, if R_{in} changed more than 20% after achieving whole-cell configuration, or if there were any large changes in holding current or noise characteristics. Membrane capacitance (C_m) was measured in pClamp (Molecular Devices, CA, USA), from the change in membrane charge taken from the integrated capacity transients, using a 10 mV, 250 ms step in voltage-clamp at -50 mV. All values quoted here have not been corrected for liquid junction potential (estimated as 12.8 mV using the pClamp calculator; Neher, 1992).

3.2.2.1. Current-clamp recordings

Once whole-cell configuration had been achieved, neurons were recorded in current-clamp mode for at least 1-2 min, in order to establish their resting membrane potential (V_m) and action potential firing properties. In spontaneously active neurons, the basal action potential firing frequency and inter-spike interval (ISI) were determined across the initial 60-120 s of activity. The coefficient of variation of the ISI (CV-ISI) was calculated by dividing the standard deviation

of the ISI by the mean ISI. Average action potential waveforms were produced using Spike2 software (Cambridge Electronic Design, UK), from which the action potential threshold (defined as a rate of change in membrane potential > 10 mV/ms), amplitude (threshold to peak), width (at the level of the threshold), afterhyperpolarisation (AHP) maximum (absolute membrane potential at the peak of the AHP), and AHP repolarisation rate (over the first 10 ms following the AHP peak) were calculated. Depolarising current steps, (20-100 pA, 5 s duration) were delivered every 20 s and the instantaneous and steady-state firing frequencies were calculated over the first 100 ms and last 2 s of the current step, respectively. Hyperpolarising current steps (120 pA, 1 s duration), were delivered every 10 s to activate the I_h.

3.2.2.2. Voltage-clamp recordings

Neurons were held at -50 mV in voltage-clamp mode, and a series of 10 mV incremental hyperpolarising steps (1 s duration) were delivered up to -120 mV, in order to activate the I_h. The amplitude of the I_h was measured as the difference between the peak instantaneous and the steady-state current (see Fig. 7b), with the final step to -120 mV producing near complete activation of the I_h. The instantaneous current values from this protocol were also used to calculate the R_{in} of the neuron. The peak of the residual current, following termination of the -120 mV step, was also measured.

3.2.3. Immunohistochemistry and confocal microscopy

Following recording, slices were transferred to 4% PFA at room temperature for 1 hour, washed in PBS to remove fixative, and blocked in 0.2% Triton PBS-T with 6% NDS (Jackson ImmunoResearch, USA) for 30-60 min at room temperature. They were then incubated overnight at room temperature in 0.2% PBS-T with 2% NDS and primary antibodies: anti-TH monoclonal chicken antibody (1:1000, Abcam, USA) and anti-VIP rabbit antibody (1:500,

Immunostar, USA). Brain slices were washed in 0.2% PBS-T and incubated for 1.5-2 hours at room temperature in 0.2% PBS-T containing 2% NDS and secondary antibodies: Alexa 633-conjugated anti-chicken (1:1000, Invitrogen, USA), Cy3-conjugated anti-rabbit (1:1000, Jackson ImmunoResearch, USA), and AMCA-conjugated streptavidin (1:1000, Jackson ImmunoResearch, USA) to reveal neurobiotin labelling. Lastly, brain slices were rinsed with 0.2% PBS-T, followed by PBS alone, and then mounted onto glass microscope slides. Once dry, coverslips were placed over the sections using VectaShield mounting medium (Vector Laboratories, USA), for visualisation using confocal microscopy.

A Leica SP5 II confocal laser scanning microscope (Leica Microsystems, Germany) was used to view the brain slices, and images were captured using Leica Application Suite software (Leica Microsystems, Germany). Slices were viewed through a 20x / 0.7 numerical aperture dry HC Plan-Apochromat CS DIC objective, with 2x digital zoom applied during image capture (40x total magnification). AMCA was excited by a 405 nm line of a diode laser, GFP by a 488 nm line of an Argon laser, Cy3 by a 561 nm line of a DPSS laser, and AlexaFluor633 by a 633 nm line of a HeNe laser. The pinhole size was set to 1 Airy unit and an optical slice thickness of 2 µm was used. The signal-to-noise ratio was improved by averaging at least six scans for each *z*-section. Images were taken at a resolution of 1024 x 1024 and were processed with general brightness and contrast curve adjustments in Adobe Photoshop CS5 (Adobe Systems Incorporated).

3.2.4. Analysis and statistics

Recordings were analysed using Clampfit 10.2 (Molecular Devices, CA, USA) unless otherwise stated, and values are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 5.04 (Graphpad, CA, USA). Unpaired t-tests (non-directional), one-way ANOVA with Newman-Keuls post-hoc tests, two-way ANOVA with Bonferroni post-hoc tests, and chi-squared tests were employed where appropriate.

3.3. Results

3.3.1. Single-cell labelling in brain slices

In these experiments a total of 78 GFP+ neurons were recorded and analysed in the DRN/vlPAG, with 59 of these successfully processed with immunohistochemistry for TH. This revealed that a similar proportion of recorded GFP+ neurons were TH+ in each mouse line, with 83.3% in TH-GFP mice (30/36 cells) and 82.6% in Pitx3-GFP mice (19/23 cells; chi-squared = 0.005, p = 0.94). These proportions are also consistent with those I obtained when quantifying the overlap between GFP and TH in non-recorded cells in brain slices (chapter 2). Additionally, of the neurobiotin-filled GFP+ neurons processed for VIP immunolabelling (Fig. 4b), 40.5% were VIP+ in TH-GFP mice (15/37 cells) and 52.2% in Pitx3-GFP mice (12/23 cells; chi-squared = 0.78, p = 0.38). This is again consistent with my counts in brain slices, which suggests that a slightly higher proportion of GFP+ neurons co-express VIP in Pitx3-GFP compared with TH-GFP mice.

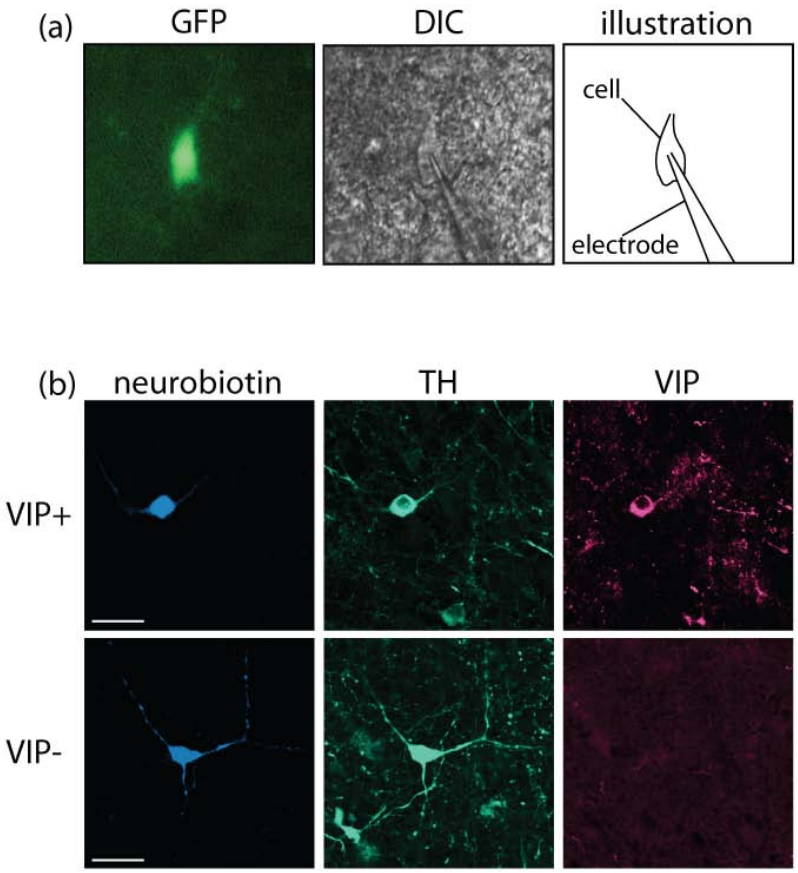


Figure 4. Whole-cell patch-clamp electrophysiology in an *ex vivo* brain slice preparation. (a) GFP signal, DIC image, and diagrammatic illustration of a neuron targeted with a recording electrode. Brain slices were subsequently processed with immunohistochemistry to reveal the neurochemical identity of the recorded neuron. (b) Examples of neurobiotin-filled neurons confirmed as TH+/VIP+ (upper panels) and TH+/VIP- (lower panels). Neurobiotin is shown in blue, TH in green, and VIP in magenta. Images shown are single 2 µm thick optical sections captured on a confocal microscope through a 20x objective with 2x digital zoom applied during image capture. Scale bars = 30 µm.

3.3.2. Action potential properties

In current-clamp recordings I found that the majority of GFP+ neurons in the DRN/vlPAG fired spontaneous action potentials (TH-GFP: 71.4%, Pitx3-GFP: 73.7%; Table 1), with an average firing frequency of ~4 Hz, which is within the range of values reported for VTA dopamine neurons (e.g. Johnson and North, 1992a; Neuhoff et al., 2002; Wolfart et al., 2001; Ford et al., 2006; Lammel et al., 2008; Zhang et al., 2010; Chieng et al., 2011). Neither the proportion of spontaneously active cells, nor the average firing frequency differed between TH-GFP and Pitx3-GFP mice (TH-GFP: 25/35 active; 4.2 ± 0.6 Hz; Pitx3-GFP: 14/19 active, 3.9 ± 0.6 Hz; chi-squared = 0.03, p = 0.86; t_{37} = 0.36, p = 0.72), or between TH+ and TH- neurons (TH+: 27/38 firing; 4.3 ± 0.6 Hz; TH-: 5/7 firing, 3.6 ± 1.1 Hz; chi-squared = 0.0004, p = 0.98; t_{30} = 0.45, p = 0.67; Table 1).

The firing pattern, as indicated by the CV-ISI, was somewhat higher in DRN/vlPAG GFP+ neurons (0.48 ± 0.07, n = 39) than values typically reported in the VTA suggesting greater variability in the ISI between action potentials, which results in a more irregular firing pattern. There were no differences between neurons recorded in TH-GFP and Pitx3-GFP mice (TH-GFP: 0.47 ± 0.06, n = 25; Pitx3-GFP: 0.48 ± 0.14, n = 14; t_{37} = 0.08, p = 0.93), or between TH+ and TH- (TH+: 0.48 ± 0.09, n = 27; TH-: 0.55 ± 0.20, n = 5; t_{30} = 0.31, p = 0.76) neurons in terms of firing pattern. However, I did observe that firing in VIP+ neurons was more irregular than in VIP- neurons, as shown by a greater CV-ISI (VIP+: 0.67 $\pm$ 0.13, n = 15; VIP- 0.33 $\pm$ 0.07, n = 18; t_{31} = 2.24 p < 0.05; Fig. 5a,c, Table 1). This was independent of a difference in the proportion of active cells (VIP+: 68%, 15/22 cells, VIP-: 75%, 18/24 cells, chi-squared = 0.26, p = 0.61) or the firing frequency (VIP+: 3.7 $\pm$ 0.8 Hz, n = 15; VIP-: 4.5 $\pm$ 0.65 Hz, n = 18; t_{31} = 0.80, p = 0.43). Differences in the CV-ISI could be a result of differences in the synaptic inputs of these neurons, or differences in their intrinsic excitability due to ion channel expression (Overton and Clark, 1997).

An average action potential waveform was generated and analysed for each cell, with the different properties shown in Table 1. DRN/vlPAG GFP+ neurons generally fired broad action potentials (4.2 ± 0.2 ms, n = 39), with a relatively depolarised threshold (-26.0 ± 1.1 mV), and a prominent afterhyperpolarisation (-51.0 ± 1.1 mV, n = 39), which is within the range of most VTA dopamine neurons (e.g. Lammel et al., 2008). I found no differences in waveform properties between neurons recorded in TH-GFP and Pitx3-GFP mice, between TH+ and TH- neurons, or between VIP+ and VIP- neurons (Fig. 5b, Table 1).

Property	TH-GFP	Pitx3-GFP	TH+	TH-	VIP+	VIP-
Current-clamp						
Resting potential (mV)	-45.94 ± 1.64	-41.47 ± 2.20	-44.37 ± 1.61	-43.11 ± 2.22	-44.32 ± 2.04	-43.87 ± 1.88
% spontaneously active	71.4% (25/35)	73.7% (14/19)	71.1% (27/38)	71.4% (5/7)	68.1% (15/22)	75.0% (18/24)
Firing rate (Hz)	4.2 ± 0.6	3.9 ± 0.6	4.3 ± 0.6	3.6 ± 1.1	3.7 ± 0.8	4.6 ± 0.7
CV-ISI	0.47 ± 0.6	0.48 ± 0.14	0.48 ± 0.09	0.55 ± 0.20	**0.67 ± 0.14**	**0.33 ± 0.07***
AP threshold (mV)	-27.04 ± 1.93	-23.27 ± 1.96	-24.33 ± 1.35	-26.42 ± 0.80	-23.31 ± 1.98	-25.86 ± 1.27
AP amplitude (mV)	56.57 ± 1.69	58.39 ± 1.74	57.88 ± 1.55	58.66 ± 4.22	57.14 ± 2.31	58.76 ± 1.82
AP width (ms)	3.94 ± 0.21	4.34 ± 0.21	4.03 ± 0.16	4.04 ± 0.52	4.03 ± 0.25	4.03 ± 0.19
TTP-AHP (ms)	17.01 ± 0.842	17.93 ± 0.69	17.59 ± 0.77	15.69 ± 0.60	16.67 ± 0.72	17.84 ± 1.09
AHP maximum (mV)	-53.03 ± 1.85	-49.00 ± 2.06	-50.76 ± 1.47	-50.50 ± 1.52	-50.65 ± 2.10	-50.79 ± 1.54
Repolarisation rate (mV/ms)	0.132 ± 0.010	0.106 ± 0.009	0.121 ± 0.010	0.125 ± 0.014	0.137 ± 0.013	0.110 ± 0.011
n	35, 25 firing	19, 14 firing	38, 27 firing	7, 5 firing	22,15 firing	24, 18 firing
Voltage-clamp						
Capacitance (pF)	12.01 ± 0.53	12.25 ± 0.97	12.39 ± 0.66	12.46 ± 0.96	**10.87 ± 0.56**	**13.66 ± 0.88***
Holding current at -50mV (pA)	-12.78 ± 3.67	-9.49 ± 7.81	-6.87 ± 4.59	-22.96 ± 9.05	-13.41 ± 5.75	-7.14 ± 5.75
Input resistance (MΩ)	1640.46 ± 256.63	1531.50 ± 264.64	1750.96 ± 267.75	1015.50 ± 130.60	1745.16 ± 410.70	1522.59 ± 226.69
I_h amplitude (pA)	12.07 ± 1.64	10.98 ± 2.10	10.89 ± 1.66	15.53 ± 4.50	**19.12 ± 2.80**	**5.74 ± 0.74***
I_h residual amplitude (pA)	192.25 ± 22.25	137.60 ± 22.66	177.05 ± 19.00	130.46 ± 24.69	164.28 ± 25.12	172.95 ± 21.08
n	52	26	49	10	27	33

Table 1: Electrophysiological properties of identified TH+, TH-, VIP+, and VIP- neurons in the DRN/vlPAG of TH-GFP and Pitx3-GFP mice. Values quoted are mean ± standard error of the mean (SEM). Statistically significant comparisons ($p < 0.05$) are shown in bold and marked with an *. The mean age for each experimental group was 7.5 weeks (VIP+), 7.0 weeks (VIP-), 7.2 weeks (TH+), 8.0 weeks (TH-), 8.5 weeks (TH-GFP), and 5.6 weeks (Pitx3-GFP).

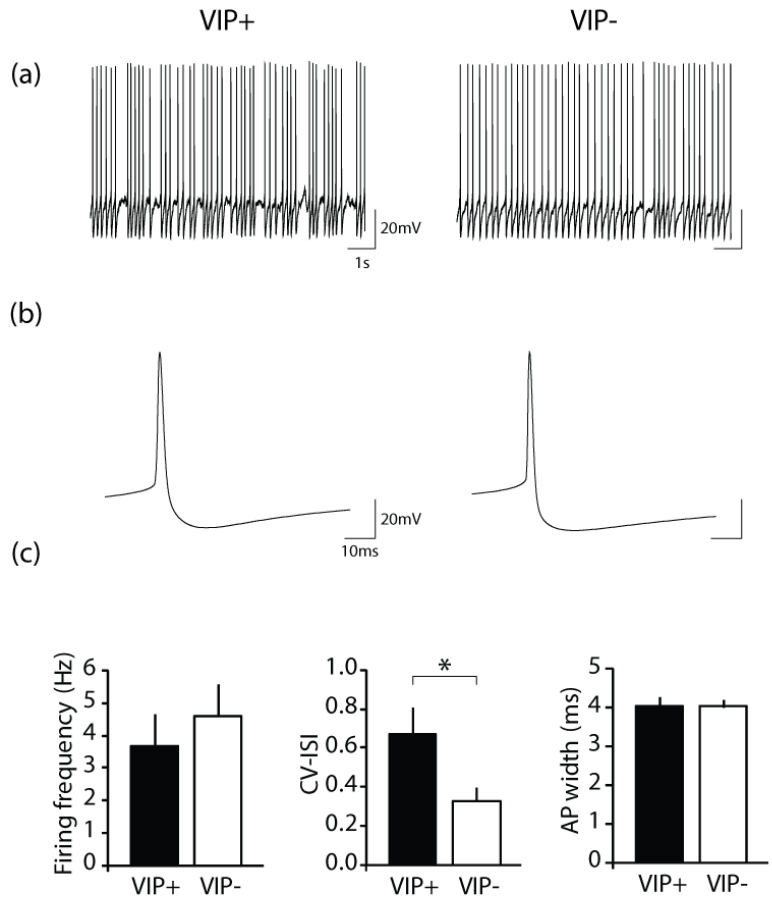


Figure 5. VIP+ and VIP- neurons displayed similar firing properties, apart from a greater CV-ISI in VIP+ neurons. Representative examples of (a) spontaneous firing activity and (b) average action potential waveform for an identified VIP+ and VIP- neuron. (c) Bar charts showing the mean (+ SEM) firing frequency, CV-ISI, and action potential (AP) width for VIP+ and VIP- neurons. * indicates $p < 0.05$.

3.3.3. Response to depolarisation and hyperpolarisation

3.3.3.1. Depolarisation

The response to depolarisation was assessed by applying positive steps in current-clamp, in 20 pA increments up to 100 pA (Fig. 6a). DRN/vlPAG GFP+ neurons fired rapidly throughout the pulse, with a higher discharge rate immediately following onset of the pulse (instantaneous firing frequency) compared to the end of the pulse (steady state firing frequency), i.e. demonstrating spike-frequency adaptation. In response to the larger depolarisations (≥60 pA), some neurons began to enter depolarisation block during steady-state firing, with the majority (80%, 24/30 cells) showing depolarisation block during the final 100 pA step.

There was no difference in the firing response observed between TH-GFP and Pitx3-GFP mice (n = 24 and 10; instantaneous: interaction between mouse model and depolarising step, $F_{4,160}$ = 0.02, p = 0.9995; main effect of group, $F_{1,160}$ = 0.30, p = 0.86; main effect of pulse amplitude, $F_{4,160}$ = 10.35, p < 0.0001; steady-state: interaction between mouse model and depolarising step, $F_{4,160}$ = 0.17, p = 0.95; main effect of group, $F_{1,160}$ = 0.14, p = 0.71; main effect of pulse amplitude, $F_{4,160}$ = 14.08, p < 0.0001), or between TH+ and TH- neurons (n = 26 and 6; instantaneous: interaction between cell type and depolarising step, $F_{4,150}$ = 0.15, p = 0.96; main effect of group, $F_{1,150}$ = 0.02, p = 0.88; main effect of pulse amplitude, $F_{4,150}$ = 7.70, p < 0.0001; steady-state: interaction between cell type and depolarising step, $F_{4,150}$ = 0.54, p = 0.71; main effect of group, $F_{1,150}$ = 1.02, p = 0.31; main effect of pulse amplitude, $F_{4,150}$ = 9.20, p < 0.0001). However, identified VIP+ neurons tended to fire at lower instantaneous frequencies, with each current injection, compared to VIP- neurons, which was confirmed by a main effect of cell type in a two-way ANOVA (VIP+: n = 13, VIP-: n = 20; interaction between cell type and depolarising step, $F_{4,155}$ = 0.83, p = 0.51; main effect of group, $F_{1,155}$ = 11.81, p < 0.001; main effect of pulse amplitude, $F_{4,155}$ = 11.26, p < 0.0001; Fig. 6b). The sustained firing frequency, however, did not differ

between VIP+ and VIP- neurons (interaction between cell type and depolarising step, $F_{4,155}$ = 0.07, p = 0.99; main effect of group, $F_{1,155}$ = 0.30, p = 0.59; main effect of pulse amplitude, $F_{4,155}$ = 14.28, p < 0.0001; Fig. 6c), suggesting that this difference is limited to the firing rates achievable immediately after the onset of the current injection.

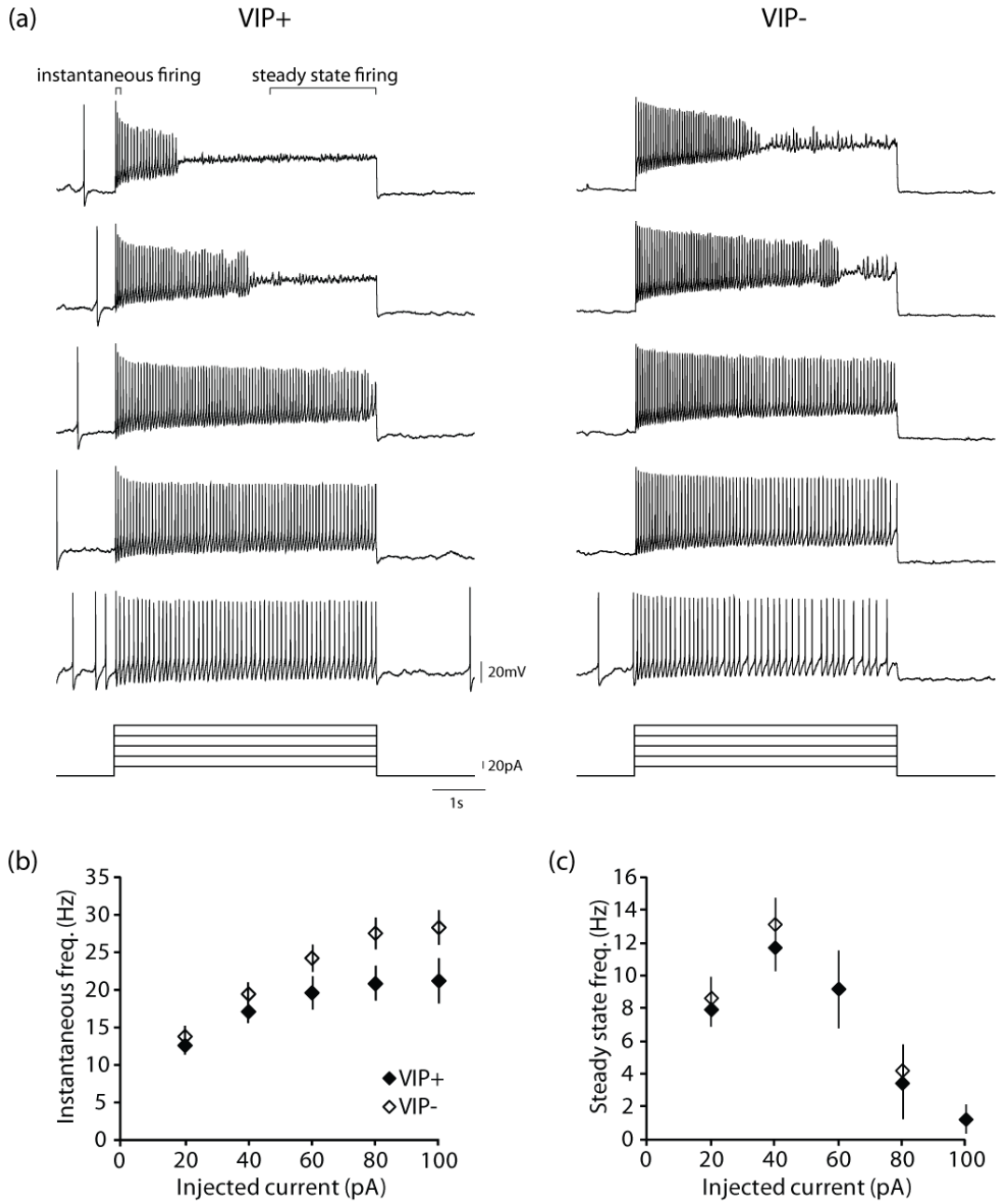


Figure 6. VIP+ neurons fired at lower instantaneous frequencies in response to depolarisation than VIP- neurons, but showed similar steady-state frequencies. (a) Representative examples of the firing response to depolarising steps, showing spike-frequency adaptation leading to depolarisation block, in an identified VIP+ and VIP- neuron. Graphs showing the mean (± SEM; b) instantaneous and (c) steady-state firing frequency in VIP+ and VIP- neurons. A two-way ANOVA revealed a significant main effect of cell type ($p < 0.05$) for the instantaneous, but not the steady-state, frequencies.

3.3.3.2. Hyperpolarisation

The I_h was activated in DRN/vlPAG GFP+ neurons by hyperpolarising steps delivered in both voltage- and current-clamp (Fig. 7a,b). Most GFP+ neurons exhibited a clear I_h (11.70 ± 1.24 pA, n = 78) that was within the range of many VTA dopamine neurons, but considerably smaller than SNc dopamine neurons (Liss et al., 2005; Neuhoff et al., 2002; Ford et al., 2006; Lammel et al., 2008; Zhang et al., 2010; Chieng et al., 2011; Lammel et al., 2011). The I_h amplitude was not significantly different between TH-GFP and Pitx3-GFP mice (TH-GFP: 12.07 ± 1.64 pA, n = 48; Pitx3-GFP: 10.98 ± 2.1 pA n = 24; t_{70} = 0.39, p = 0.70), or between identified TH+ and TH- neurons (TH+: 10.89 ± 1.66 pA, n = 46; TH-: 15.53 ± 4.50 pA, n = 10; t_{54} = 1.13, p = 0.26, Table 1). However, on average, the I_h was significantly larger in VIP+ compared to VIP- neurons (VIP+: 19.12 ± 2.80 pA, n = 25; VIP-: 5.74 ± 0.74 pA, n = 32; t_{55} = 5.14, p < 0.0001; Fig 7c). This difference could be observed with hyperpolarising steps ≥ -40 mV, in response to which VIP- neurons displayed very little or no I_h, in contrast to the overall larger current with each step in VIP+ neurons (Fig. 7d). This could also be seen in current-clamp by the lack of a prominent I_h sag in VIP- neurons, compared to VIP+ neurons (Fig. 7a).

Following removal of the hyperpolarising step, a large outward residual current was observed in voltage-clamp, which mediated the delayed repolarisation seen in current-clamp. This is thought to represent the I_A, an outward potassium current, and is observed in VTA dopamine neurons, but not SNc dopamine neurons (Neuhoff et al., 2002; Ford et al., 2006; Lammel et al., 2008; Lammel et al., 2011), The size of this current did not differ significantly between TH-GFP and Pitx3-GFP mice (TH-GFP: 192.25 ± 22.25 pA, n = 48; Pitx3-GFP: 137.60 ± 22.66 pA, n = 24; t_{70} = 1.55 p = 0.13), TH+ and TH- neurons (TH+: 177.05 ± 19.00 pA, n = 46; TH-: 130.46 ± 24.69 pA, n = 10; t_{54} = 1.10, p = 0.28), or between VIP+ and VIP- neurons (VIP+: 164.28 ± 25.12 pA, n = 25; VIP-: 172.95 ± 21.08 pA, n = 32; t_{55} = 0.27, p = 0.79; Table 1).

Hyperpolarising steps in voltage-clamp were also used to calculate the R_{in}, by measuring the instantaneous current (i.e. the inward leak current, mediated by background conductances), that occurs immediately after applying the step (Fig. 7b). When measured closest to the resting potential of the cell (i.e., a -10 mV step from holding potential of -50 mV), the R_{in} of DRN/vlPAG GFP+ cells was calculated as 1603 ± 190 MΩ (n = 55). This is considerably larger than values reported for the VTA (∼200-400 MΩ; Ford et al., 2006; Zhang et al., 2010), although not too dissimilar from NAc medial shell-projecting dopamine neurons (Lammel et al., 2011). This difference is likely to be due to the noticeably smaller size of the DRN/vlPAG dopamine neurons (as indicated by their smaller capacitance (C_m), ∼12 pA) compared to the VTA (typically ∼20-30 pA; Neuhoff et al., 2002; Ford et al., 2006). Neither R_{in}, (TH-GFP: 1640.46 ± 256.63 MΩ n = 36; Pitx3-GFP: 1531.50 ± 264.64 MΩ, n = 19, t_{53} = 0.27, p = 0.79) nor C_m was significantly different between TH-GFP and Pitx3-GFP mice (TH-GFP: 12.01 ± 0.53 pF, n = 52; Pitx3-GFP: 12.25 ± 0.97 pF, n = 26; t_{76} = 0.23, p = 0.82 (C_m)) or between TH+ and TH- neurons (TH+: 1750.96 ± 267.75 MΩ, n = 37, 12.39 ± 0.66 pF, n = 49; TH-: 1015.50 ± 130.60 MΩ, n = 8, 12.46 ± 0.96, n = 10; t_{43} = 1.26, p = 0.22 (R_{in}), t_{57} = 0.05, p = 0.96 (C_m); Table 1). However, VIP+ neurons had, on average, significantly smaller whole-cell capacitance compared with VIP- neurons (VIP+: 10.87 ± 0.56 pF, n = 27; VIP-: 13.66 ± 0.88 pF, n = 33; t_{58} = 2.55, p < 0.05), which is consistent with their observed smaller size, but this did not significantly affect the mean R_{in} (VIP+: 1745.16 ± 410.70 MΩ, n = 21; VIP-: 1522.59 ± 226.69 MΩ, n = 24; t_{43} = 0.49, p = 0.63).

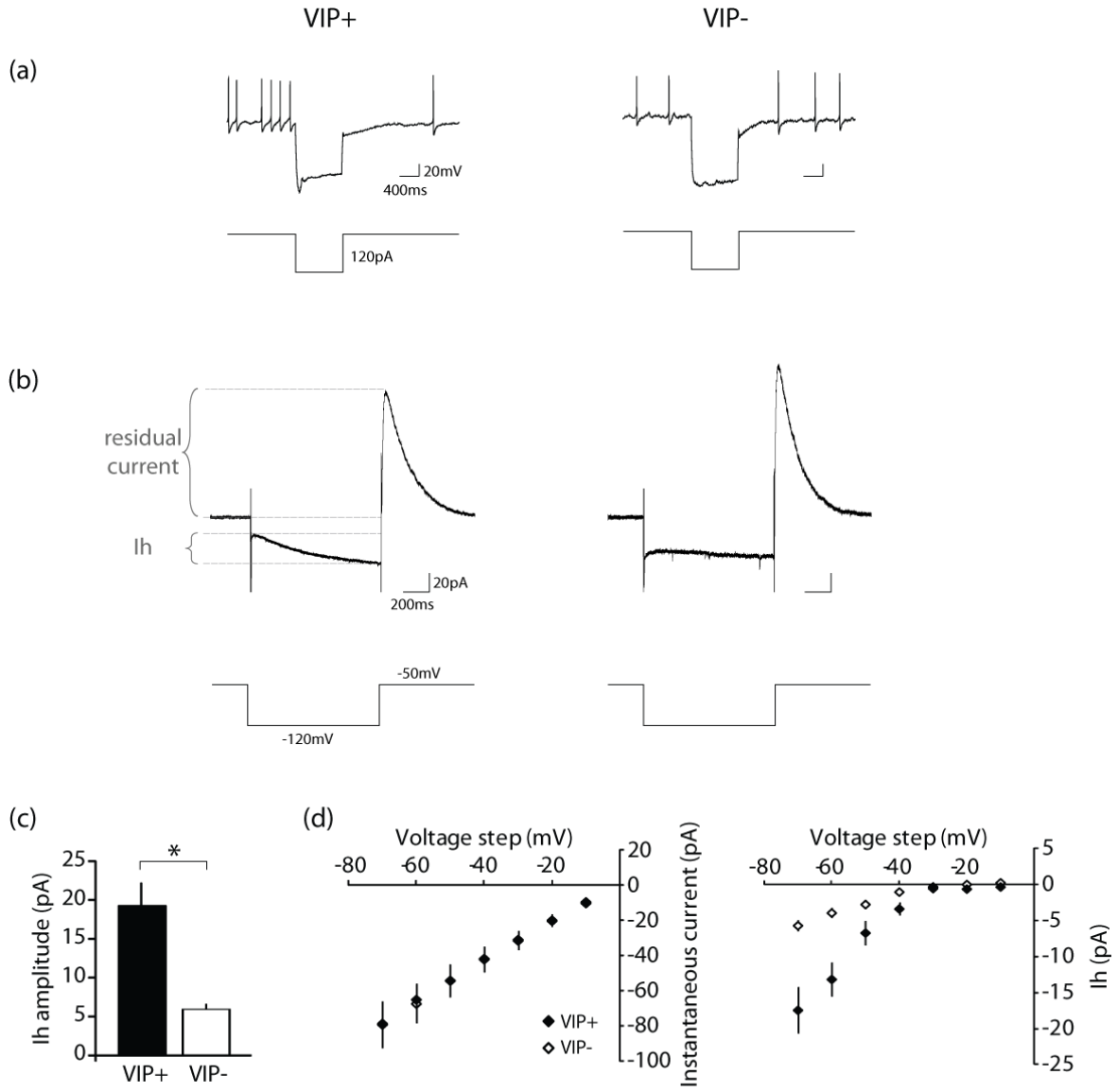


Figure 7. The average hyperpolarisation-activated cation current (I_h) was larger in identified VIP+ compared to VIP- neurons. Representative examples of the I_h measured in (a) current-clamp and (b) voltage-clamp from an identified VIP+ and VIP- neuron. (c) Bar chart showing mean (+ SEM) I_h amplitude and (d) graphs showing the mean (± SEM) instantaneous current and I_h at each hyperpolarising step in VIP+ and VIP- neurons. * indicates $p < 0.05$.

3.4. Conclusion

In this chapter, I have examined the basic electrophysiological properties of DRN/vlPAG dopamine neurons using whole-cell patch-clamp in acute brain slices, in combination with single-cell labelling and immunohistochemistry. Specifically, I analysed passive membrane properties, action potential firing, and the response to depolarisation/hyperpolarisation, which are well-documented characteristics of dopamine neurons in the VTA/SNc. Given that the basic features of unlabelled GFP+ DRN/vlPAG neurons in Pitx3-GFP mice had already been studied in comparison with putative serotonergic neurons (Dougalis et al., 2012), I focused on comparing GFP+ neurons in TH-GFP and Pitx3-GFP mice, as well as immunohistochemically identified TH+/TH- and VIP+/VIP- neurons, in order to provide a more thorough electrophysiological characterisation of this population.

I have shown that DRN/vlPAG dopamine neurons exhibit firing rates and patterns, and an I_h amplitude, that are similar to those displayed by some VTA dopamine neurons. Specifically, the range of firing rates and action potential widths in DRN/vlPAG dopamine neurons overlaps with the values cited for midbrain dopamine neurons projecting to the NAc core, NAc medial shell, BLA, and mPFC (which are calbindin-positive; Neuhoff et al., 2002), but not those projecting to the NAc lateral shell or dorsal striatum (Lammel et al., 2008; Lammel et al., 2011). Additionally, I noted some differences between DRN/vlPAG dopamine neurons expressing VIP, and those lacking this neuropeptide. In particular, VIP+ neurons fired in a more irregular manner than VIP- neurons, fired at lower maximal frequencies in response to depolarisation, and expressed a significantly larger I_h. The more irregular firing pattern may be a result of intrinsic neuronal differences in channel expression or function, and/or extrinsic differences in synaptic input. However, the differences in maximal firing frequency and I_h, are most likely to result from intrinsic differences in ion channel expression or function.

Given that, in the VTA, different maximal firing frequencies and I_h amplitudes are associated with dopamine neurons projecting to distinct target structures (Lammel et al., 2008; Lammel et al., 2011), it is tempting to speculate that these properties may also distinguish projection target in the DRN/vlPAG. Consistent with this, the VIP projection from the DRN/vlPAG is restricted to the dorsolateral BNST (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1998), in comparison with the multiple targets innervated by the rest of the DRN/vlPAG population. Therefore VIP may serve as a useful marker of this projection. However, VIP is not the first neuropeptide to be colocalised to dopamine neurons, either in the DRN/vlPAG (Seroogy et al., 1988a) or the VTA (Seroogy et al., 1988b; Seroogy et al., 1989; Jayaraman et al., 1990; Seroogy et al., 1994), and its functional significance remains to be determined.

Importantly, these results indicate that there are no significant differences, in any of the measures examined, between neurons recorded in TH-GFP and Pitx3-GFP mice. Therefore, even though a slightly greater proportion of VIP+ neurons may be sampled in Pitx3-GFP mice, this does not significantly affect the average properties of the sample. In addition, there were no significant differences between recorded GFP+ neurons that were TH+ and TH-. This is further evidence to support the view that these TH- neurons are dopaminergic, and may just express very low levels of TH, which are undetectable with standard immunohistochemical methods, or have experienced 'wash-out' of intracellular contents (Zhang et al., 2010). There is debate over whether this occurs very frequently with potassium gluconate-based internal solutions (Margolis et al., 2010), but these DRN/vlPAG dopamine neurons are considerably smaller than those in the VTA, which may suggest that wash-out would occur more rapidly in this population.

Chapter 4. Synaptic physiology of DRN/vlPAG dopamine neurons

4.1. Introduction

VTA dopamine neurons are highly innervated by glutamatergic and GABAergic afferents originating in many cortical and subcortical regions (Kalivas and Alesdatter, 1993; Carr and Sesack, 1999; Geisler et al., 2007; Jhou et al., 2009; Omelchenko and Sesack, 2009). The balance between this excitatory and inhibitory input is crucial in governing the neuronal firing pattern, which shapes dopamine release in target structures (White, 1996; Mathon et al., 2003; Marinelli et al., 2006; Morikawa and Paladini, 2011). Furthermore, diversity in the origin of these afferents allows dopamine neurons to integrate information arising from a wide range of brain regions, which are sensitive to different environmental stimuli (Morikawa and Paladini, 2011). DRN/vlPAG dopamine neurons also receive both glutamatergic and GABAergic afferents, and their activity is likely to be modulated by these inputs in a similar manner to VTA dopamine neurons.

4.1.1. Excitatory and inhibitory transmission in midbrain dopamine neurons

Fast excitatory transmission onto dopamine neurons is mediated mainly by glutamate activating ionotrophic AMPARs and NMDARs on the postsynaptic membrane, which results in depolarisation and an increase in neuronal firing (Christoffersen and Meltzer, 1995; Morikawa et al., 2003). However, NMDARs appear to be unique in their ability to generate burst firing in dopamine neurons, which cannot be initiated by AMPARs alone (Charlety et al., 1991; Overton and Clark, 1992; Chergui et al., 1993; Chergui et al., 1994; Christoffersen and Meltzer, 1995; Tong et al., 1996b; Deister et al., 2009; Zweifel et al., 2009). Additionally, metabotrophic glutamate receptors (mGluRs) can hyperpolarise dopamine neurons via mobilisation of

intracellular Ca^{2+}, and the subsequent opening of Ca^{2+}-sensitive K^+ (SK) channels (Fiorillo and Williams, 1998; Morikawa et al., 2003). The major inhibitory input to dopamine neurons arises from GABA acting on postsynaptic ionotrophic $GABA_A$ or metabotropic $GABA_B$ receptors, which can suppress burst firing (Bolam and Smith, 1990; Johnson and North, 1992a; Paladini et al., 1999; Erhardt et al., 2002; Brazhnik et al., 2008). Conversely, there is evidence that removal of this input (i.e., disinhibition) may contribute to the generation of phasic bursting in dopamine neurons *in vivo* (Paladini and Tepper, 1999; Jhou et al., 2009; Matsumoto and Hikosaka, 2007; Tepper and Lee, 2007; Lobb et al., 2010; Tan et al., 2010; Lobb et al., 2011). The nature of these glutamatergic and GABAergic synapses allows the firing pattern of dopamine neurons to be controlled on the order of milliseconds, which is essential for the tight association of environmental stimuli with behaviour.

4.1.2. Plasticity at synapses onto dopamine neurons

Excitatory and inhibitory inputs to dopamine neurons are important not only for short-term changes in neuronal firing and neurotransmitter release, but also long-term changes in neuronal activity. This is due to the ability of these synapses to exhibit plasticity, i.e. activity-dependent modulation of synaptic strength, which serves to strengthen (long-term potentiation; LTP) or weaken (long-term depression; LTD) the synaptic connection. Both glutamatergic and GABAergic synapses onto dopamine neurons have been shown to express several forms of LTP and LTD. These differ in the conditions for, and mechanism of, induction and expression, and can be mimicked in brain slices with specific stimulation protocols (Bonci and Malenka, 1999; Overton et al., 1999; Jones et al., 2000; Thomas et al., 2000; Liu et al., 2005; Bellone and Lüscher, 2006; Luu and Malenka, 2008; Nugent et al., 2008; Pan et al., 2008; Harnett et al., 2009; Mameli et al., 2011).

One important, well-characterised, form of plasticity is that which occurs at glutamatergic synapses onto VTA dopamine neurons in response to an acute dose of addictive drug (Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004; Dong et al., 2004; Faleiro et al., 2004; Liu et al., 2005; Bellone and Lüscher, 2006; Argilli et al., 2008; Engblom et al., 2008; Heikkinen et al., 2009). This potentiation has recently been shown to occur only at a subset of dopamine neurons projecting to the NAc lateral and medial shell, but not those projecting to the mPFC or dorsal striatum (Lammel et al., 2011). The DRN/vlPAG dopamine neurons have not been studied in relation to this form of plasticity or any others, but given that they have been implicated in drug-related learning (Flores et al., 2006), it is possible that they may show synaptic modifications in response to addictive drugs.

I ultimately wanted to test this directly, but it was first necessary to assay some basal measures of synaptic strength at these dopamine neurons, as these have not been previously reported. Recordings by Antonios Dougalis, from unlabelled GFP+ neurons in Pitx3-GFP mice, had shown that it was possible to measure spontaneous and evoked synaptic currents within the coronal brain slice preparation of the DRN/vlPAG (Dougalis et al., 2012). This was used for comparison with putative serotonergic neurons, but I wanted to broaden this analysis by examining synaptic currents in identified VIP+/VIP- dopamine neurons of the DRN/vlPAG. In order to directly compare to this previous work (Dougalis et al., 2012), I employed the same measures of synaptic strength: glutamatergic and GABAergic spontaneous synaptic currents and evoked paired-pulse ratios. I also assayed the AMPAR:NMDAR ratio, as this is a sensitive measure of synaptic strength and is frequently used to reveal changes following drug exposure. Additionally, I examined evoked glutamatergic currents in the VTA for comparison with the DRN/vlPAG dopamine neurons.

4.2. Materials and methods

4.2.1. Brain slice preparation

Coronal 220 µm brain slices containing the DRN/vlPAG were prepared and maintained as described in chapter 3. Additionally, in separate animals, horizontal 220 µm brain slices were prepared, which contained the VTA. Recordings were made from GFP+ cells in the region medial to the medial terminal nucleus of the accessory optic tract (MT) to target the VTA.

4.2.2. Electrophysiology

Whole-cell patch-clamp recordings were performed as described in chapter 3. In the recording chamber brain slices were continuously perfused at a rate of 2-4 ml/min with fully oxygenated aCSF (composition in mM, NaCl 120, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, Glucose 10, $MgCl_2$ 1, $CaCl_2$ 2) at 32 °C. A potassium gluconate-based internal solution (composition in mM: potassium gluconate 140, NaCl 5, $MgCl_2$ 1, EGTA 1, HEPES 10, Mg-ATP 2, Li-GTP 0.5, and 0.1% neurobiotin, with pH 7.3 and osmolarity 280-290 mosmol/l) was used for recording spontaneous postsynaptic currents and paired-pulse ratios when comparing VIP+ and VIP- neurons of the DRN/vlPAG (Fig. 8 and 9). A cesium-based internal solution (composition in mM: $CsCH_3SO_3$ 130**,** NaCl 2.8, HEPES 20, EGTA 0.4, TEA-Cl 5, MgATP 2, Li-GTP 0.5, and 0.1% neurobiotin; pH 7.3, osmolarity 280-290 mosmol/l) was used to record the AMPAR:NMDAR ratio at +40 mV, and for all protocols comparing DRN/vlPAG with VTA dopamine neurons (Fig. 10 and 11). The values cited here have not been corrected for liquid junction potential (estimated as 12.8 mV for the potassium-gluconate solution and 9.8 mV for the cesium-based solution using the pClamp calculator; Neher, 1992).

Following recording, brain slices were fixed in 4% PFA and processed with immunohistochemistry (as described in chapter 3) to reveal TH and VIP staining in recorded cells.

4.2.2.1. Spontaneous postsynaptic currents

Synaptic currents were recorded in voltage-clamp mode, using the potassium gluconate-based internal solution. The neuron was held at either -70 mV or 0 mV to selectively monitor spontaneous excitatory postsynaptic currents (sEPSCs) or inhibitory postsynaptic currents (sIPSCs), respectively. Spontaneous activity was recorded for 3-5 min at each holding potential, and the data was analysed using MiniAnalysis 6.0.7 (Synaptosoft, Fort Lee, NJ). The detection threshold was set at 7 pA for sEPSCs at -70 mV, and 15 pA for sIPSCs at 0 mV, which was suitable to distinguish events from the baseline noise. Events were subsequently verified by eye to confirm the presence of a fast rise time (<3 ms) and an exponential decay.

4.2.2.2.Evoked currents

In order to evoke postsynaptic currents, a bipolar stimulating electrode (FHC, USA) was placed 100-300 µm ventrolateral (in coronal slices) or rostral (in horizontal slices) to the recorded neuron, and used to stimulate afferents at 0.03 Hz. Stimulus intensity was controlled using an ISO-flex stimulus isolator (AMPI, Jerusalem, Israel) and adjusted to evoke monosynaptic events, typically using a stimulus intensity of 10-40 V, 500 µs duration. At least 12 stable sweeps were recorded for each protocol, with averaged responses calculated and analysed using Clampfit 10.2 (Molecular Devices, CA, USA).

To measure the paired-pulse ratio (PPR) the potassium gluconate-based internal solution was used and neurons were held at -70 mV or 0 mV to evoke EPSCs or IPSCs, respectively. Two afferent stimuli were delivered with an inter-stimulus interval of 50 ms, and the PPR was

calculated by dividing the average peak amplitude of the PSC generated by the second stimulus by the average peak amplitude generated by the first.

To determine the AMPAR:NMDAR ratio the cesium-based internal solution was used and the neuron was voltage-clamped at +40 mV. Picrotoxin (100 μm) was also included in the aCSF to block $GABA_A$ receptor-mediated synaptic currents. A stable mixed AMPAR- and NMDAR-mediated EPSC was recorded, following which the NMDAR antagonist d-AP5 (50 μM) was applied to the slice, and the pure AMPAR current recorded. This was then digitally subtracted from the mixed current, using ClampFit 10.2, to give the pure NMDAR current. The AMPAR:NMDAR ratio was calculated by dividing the peak amplitude of the average AMPAR-mediated EPSC by the peak amplitude of the NMDAR-mediated EPSC (Hsia et al., 1998).

The decay kinetics of the AMPAR-mediated and NMDAR-mediated current recorded at +40 mV were determined by fitting the decay phase of the current with an exponential curve using Spike 2 (Cambridge Electronic Design, UK). The AMPAR current was fitted with a single exponential function and the NMDAR-mediated current was fitted with a double exponential function (as shown in Fig. 10d). The following formula was used to calculate the weighted decay time constant (τ_W) of the NMDAR-mediated current:

$$\tau_W = [(A_1 \times \tau_1) + (A_2 \times \tau_2)]/[(A_1 + A_2)]$$

A_1 and A_2 are the amplitudes, and τ_1 and τ_2 the decay time constants of the fast and slow components, respectively (Lammel et al., 2011).

4.2.2.3. NMDA application

The neuron was voltage-clamped at +40 mV, using the cesium-based internal solution, and once the holding current had stabilised NMDA (10 μM) was applied to the slice for 30 s. The holding

current was sampled every 10 s before, during and after the drug washed on to the slice, and the amplitude of the response calculated by subtracting the baseline current from the peak current following NMDA application.

4.2.3. Statistics

Unpaired t-tests (non-directional) were used for statistical comparison with Prism 5.04 (Graphpad, CA, USA). Values are reported as mean ± standard error of the mean (SEM).

4.2.4. Drugs

Picrotoxin, NBQX, and NMDA were obtained from Sigma-Aldrich (Sigma, UK) and d-AP5 from Tocris Bioscience (Tocris, UK). Stock solutions of picrotoxin (400 mM) and NBQX (11.9 mM) were prepared in dimethylsulphoxide (DMSO), and NMDA (6.8 mM) and d-AP5 (50 mM) were prepared in dH_20. They were then divided into aliquots and stored at -20 °C. Individual aliquots were defrosted immediately before recording, and added to the aCSF to give a final DMSO concentration of <0.045%.

4.3. Results

4.3.1. Glutamatergic and GABAergic currents in DRN/vlPAG dopamine neurons

Synaptic physiology was sampled in 48 neurons from TH-GFP mice (aged 1-2months), 38 of which were successfully labelled and processed with immunohistochemistry for TH and VIP. 97% of these neurons (37/38 cells) were confirmed TH+, with 45% (17/38 cells) VIP+, and 55% (21/38 cells) VIP-, in line with my previous proportions of recorded and non-recorded neurons in TH-GFP brain slices. The mean age for each experimental group was 5.2 weeks (VIP+) and 5.0 weeks (VIP-).

4.3.1.1. Glutamatergic currents

Spontaneous events (sEPSCs) recorded at -70 mV were blocked with the AMPAR antagonist NBQX (5 µM, n = 6), indicating that they are AMPAR-mediated glutamatergic events. The average amplitude of sEPSCs was smaller in VIP+ compared to VIP- neurons (VIP+: 15.0 ± 1.6 pA, n = 9; VIP-: 21.5 ± 1.5 pA, n = 17; $t_{24} = 2.73$, $p < 0.05$), as was the frequency of events (VIP+: 0.46 ± 0.19 Hz, n = 9, VIP-: 1.43 ± 0.25 Hz, n = 17; $t_{24} = 2.62$, $p < 0.05$; Fig. 8a)

Postsynaptic currents were evoked in the slice by means of a bipolar stimulating electrode placed on the brain slice, ventrolateral to the recorded neuron. When afferent fibres are stimulated twice in quick succession, the paired-pulse ratio (PPR) can be measured, which is the amplitude ratio of the second postsynaptic response to the first (Zucker and Regehr, 2002). The degree of facilitation or depression of the response is one method used to assess the probability of neurotransmitter release (P_r) at the synapse (Katz and Miledi, 1968; Citri and Malenka, 2008; Branco and Staras, 2009). Synapses with a high P_r tend to depress their response to the second stimulus whereas synapses with a low P_r normally exhibit an increase in their response to the second stimulus (Dobrunz and Stevens, 1997). Paired-pulse recordings made at -70 mV, to stimulate AMPAR-mediated currents, showed that VIP+ neurons exhibited slight paired-pulse depression (i.e., exhibiting a PPR less than 1; PPR: 0.97, n = 8), whereas VIP- neurons tended towards facilitation (PPR: 1.35, n = 10; $t_{16} = 2.28$, $p < 0.05$; Fig. 8b). Differences in the PPR are often interpreted as presynaptic differences in the P_r, although there are alternative explanations involving postsynaptic alterations (e.g. Savtchouk and Liu, 2011).

I recorded AMPAR:NMDAR ratios at +40 mV using a cesium-based internal solution and with picrotoxin present in the aCSF. This ratio is a frequently used measure of the relative contribution of glutamatergic receptors at a synapse, and it allows comparisons between cells since it is independent of factors such as the position of the stimulating electrode and the

number of afferent fibres stimulated (Kauer and Malenka, 2007; Thomas et al., 2008). The average AMPAR:NMDAR ratio was 1.23 ± 0.13 (n = 15) for GFP+ DRN/vlPAG neurons, and was not significantly different between identified VIP+ and VIP- neurons (VIP+: 1.16 ± 0.19, n = 6; VIP-: 1.34 ± 0.15, n = 8; $t_{12} = 0.75$, p = 0.47; Fig. 8c).

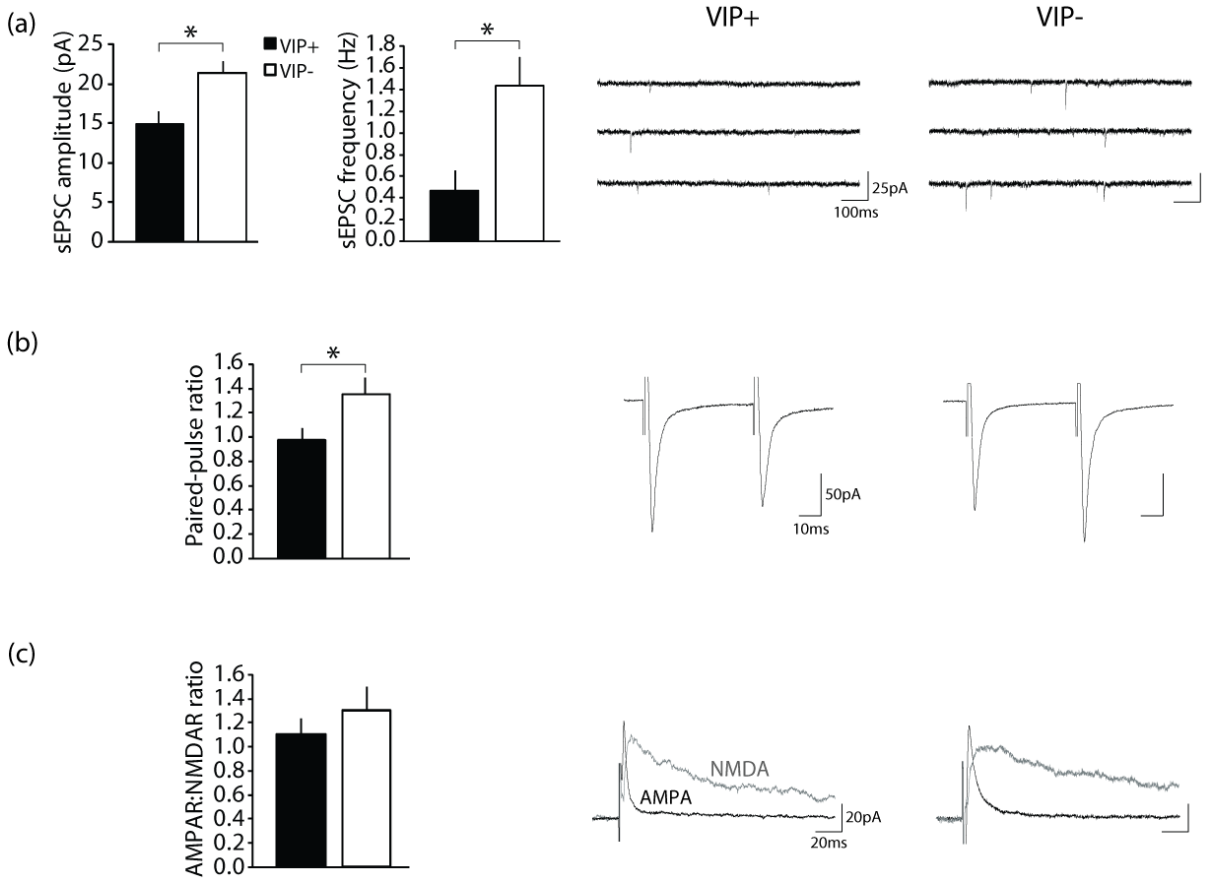


Figure 8. Spontaneous and evoked glutamatergic currents in VIP+ and VIP- neurons. Bar charts (mean + SEM) and representative examples from an identified VIP+ and VIP- neuron showing (a) sEPSC amplitude and frequency, (b) the PPR, and (c) the AMPAR:NMDAR ratio. * indicates $p < 0.05$.

4.3.1.2. GABAergic currents

Spontaneous events recorded at 0 mV (sIPSCs) were abolished by the $GABA_A$-receptor antagonist picrotoxin (100 µM, n = 5) indicating that they are $GABA_A$-mediated inhibitory events. There was considerable variability in the frequency of events in VIP+ neurons, ranging from 0.05 Hz to 3.15 Hz (standard deviation: 1.18). There appeared to be a bimodal distribution with 7/9 cells displaying a frequency of <0.5 and 2/9 cells displaying a frequency of >2 Hz. More data would be useful to determine whether there are two distinct groups within the VIP+ population, which differ in the nature of their GABAergic input. In contrast, I only observed low sIPSC frequency in VIP- neurons (<0.3 Hz; standard deviation: 0.05). The average frequency was therefore quite different between VIP+ and VIP- (VIP+: 0.74 ± 0.39 Hz, n = 9; VIP-: 0.09 ± 0.01 Hz, n = 13; Fig. 9a), but this did not reach statistical significance (t_{20} = 2.00, p = 0.06) probably due to the high variability in VIP+ neurons. The average amplitude of sIPSCs was similar between VIP+ and VIP- neurons (VIP+: 25.5 ± 2.8 pA, n = 9, VIP-: 22.1 ± 0.9 pA, n = 13; t_{20} = 1.31, p = 0.20), but again there was greater variability within the VIP+ population (standard deviation - VIP+: 8.5pA, VIP-: 3.3pA). Interestingly, less GABAergic input onto VIP- neurons may explain the more regular firing observed in this subpopulation in chapter 3 (Fig. 5). Reducing $GABA_A$-mediated inhibition has been shown to reduce the CV-ISI, and hence improves spike timing (Caillard, 2011). Therefore less $GABA_A$-mediated inhibition may underlie the smaller CV-ISI in VIP- compared to VIP+ neurons.

Paired-pulse stimulation at 0 mV, to evoke GABAergic currents, produced paired-pulse facilitation in both VIP+ (PPR: 1.61 ± 0.28, n = 6) and VIP- neurons (PPR: 1.64 ± 0.25, n = 9), with no significant difference between the two cell types (t_{13} = 0.07, p = 0.95, Fig. 9b).

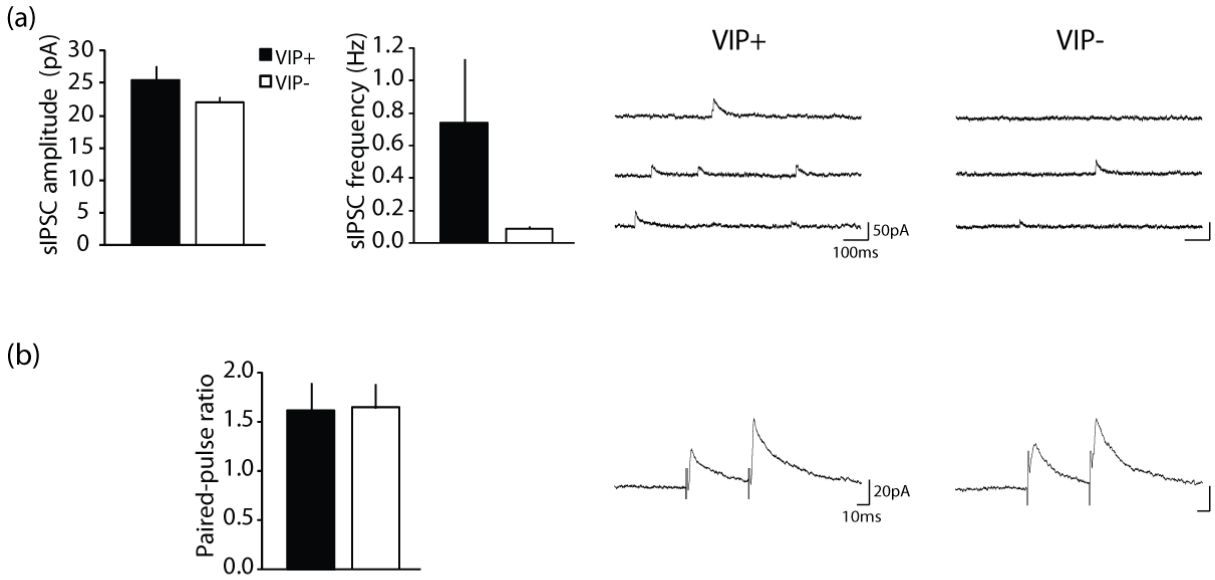


Figure 9. Spontaneous and evoked GABAergic currents in VIP+ and VIP- neurons. Bar charts (mean + SEM) and representative examples from an identified VIP+ and VIP- neuron showing (a) sEPSC amplitude and frequency and (b) the PPR.

4.3.2. Glutamatergic synapses onto DRN/vlPAG and VTA dopamine neurons

Both TH-GFP and Pitx3-GFP mice (aged 1-3 months) were used to target DRN/vlPAG and VTA dopamine neurons, in coronal and horizontal brain slices, respectively. The mean age for each experimental group was 8 weeks (DRN/vlPAG) and 8.1 weeks (VTA). There was no difference in any of the measures examined between TH+ and TH- neurons, therefore all GFP+ neurons are reported here. A cesium-based solution was used for all recordings with picrotoxin included in the aCSF, in order to sample only glutamatergic currents.

I found that DRN/vlPAG and VTA dopamine neurons differed in their passive properties (Fig. 10a). Whole-cell capacitance (C_m) was significantly larger in VTA compared to DRN/vlPAG dopamine neurons (VTA: 37.18 ± 1.61 pF, n = 107; DRN/vlPAG: 13.60 ± 0.25 pF, n = 449; t_{554} = 25.18 $p < 0.0001$), which is consistent with their generally larger size. In line with this, the R_{in} of VTA dopamine neurons was considerably smaller than DRN/vlPAG dopamine neurons (VTA: 658.3 ± 88.9 MΩ, n = 32; DRN/vlPAG: 1821.4 ± 72.1 MΩ, n = 192; t_{222} = 6.45, $p < 0.0001$), which is likely to be related to the difference in cell size. (Note that the R_{in} here was calculated from a 10 mV hyperpolarising step at -70 mV, with a cesium-based internal solution, rather than at -50 mV with a potassium gluconate-based solution, as described in chapter 3). Additionally, I found that significantly more negative current was required to hold the cell at -50 mV in the VTA compared to DRN/vlPAG dopamine neurons (VTA: -57.08 ± 3.78 pA, n = 96; DRN/vlPAG: -25.04 ± 0.89 pA, n = 398; t_{492} = 12.39, $p < 00001$; Fig. 10a). This again may be a result of the difference in cell size, or differences in the intrinsic properties and/or extrinsic inputs to these different dopamine populations.

The PPR of the AMPAR-mediated current was smaller in VTA compared to DRN/vlPAG dopamine neurons (VTA: 0.81 ± 0.05, n = 12; DRN/vlPAG: 1.04 ± 0.07, n = 22; t_{32} = 2.17, $p < 0.05$; Fig. 10b). VTA dopamine neurons generally showed slight paired-pulse depression (consistent with

previous reports (e.g., Bonci and Malenka 1999; Lammel et al., 2011), but DRN/vlPAG neurons showed a mixture of both slight facilitation and depression in their responses, resulting in an average PPR around 1. I recorded AMPAR:NMDAR ratios in VTA dopamine neurons with values similar to those previously reported (0.48 ± 0.05, n = 8; e.g. Ungless et al., 2001; Saal et al., 2003; Argilli et al., 2008, Lammel et al., 2011), but this was significantly smaller than the ratios recorded in DRN/vlPAG dopamine neurons (1.23 ± 0.11, n = 13; $t_{19} = 5.07$, $p < 0.0001$; Fig. 10c). This higher ratio observed in DRN/vlPAG dopamine neurons could be a result of a larger AMPAR-mediated component, or a smaller NMDAR-mediated component, or both.

The decay kinetics of the evoked AMPAR- and NMDAR-mediated current at +40 mV were analysed by fitting the decay phase with one and two exponential functions, respectively. I observed no difference between the VTA and DRN/vlPAG in the weighted decay time constant (τ_W) of the NMDAR-mediated current (VTA: 132.6 ± 39.4 ms, n = 8; DRN/vlPAG: 110.8 ± 16.7 ms, n = 12; $t_{19} = 0.56$, $p = 0.58$; Fig. 10d) or the decay time constant of the AMPAR-mediated current (VTA: 3.03 ± 0.53 ms, n = 7; DRN/vlPAG: 3.31 ± 0.22 ms, n = 24; $t_{29} = 0.57$, $p = 0.57$; Fig. 10e). To further examine the NMDAR current I bath applied 10 µM NMDA for 30 s, while holding the neuron at +40 mV (to relieve Mg^{2+} block of the channel). The opening of NMDARs causes influx of Na^+, K^+ and Ca^{2+} ions, which depolarises the cell, and so more positive current is required to voltage-clamp the cell at +40 mV. The amplitude of the current elicited by NMDA application was significantly greater in VTA (258.9 ± 31.2 pA, n = 7) compared to DRN/vlPAG (40.2 ± 9.4 pA, n = 7; $t_{12} = 6.70$, $p < 0.0001$; Fig. 10f) dopamine neurons, which may (in part) explain the difference in the AMPAR:NMDAR ratio. However, exogenous application of NMDA will indiscriminately activate both synaptic and extrasynaptic receptors (Kullmann et al., 1999), which can preclude detection of a specific change in synaptic receptors (Mameli and Lüscher, 2011), and therefore this is crude method for evaluating receptor differences at the synapse.

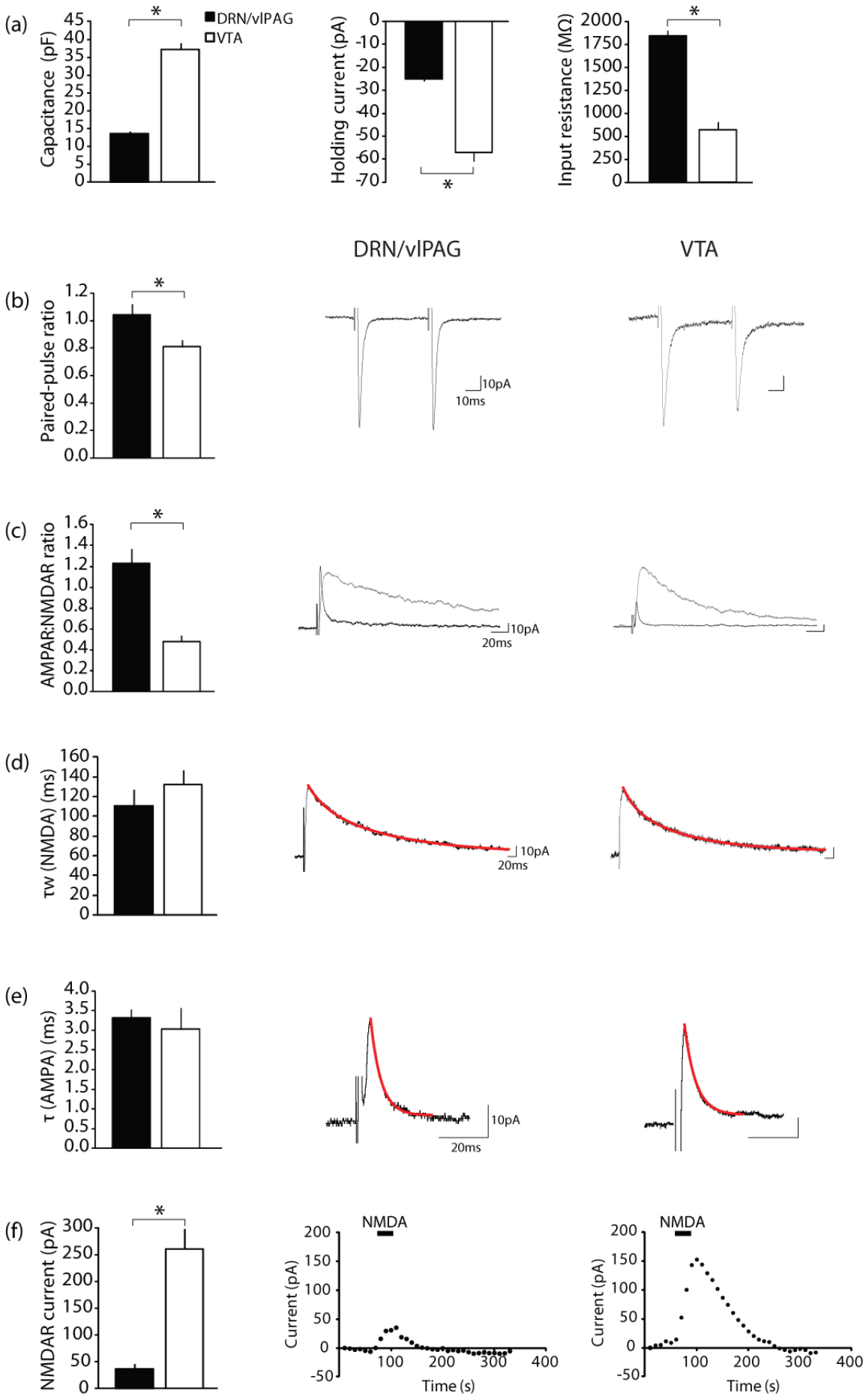

(a)
Capacitance (pF)
DRN/vlPAG
VTA
Holding current (pA)
Input resistance (MΩ)
DRN/vlPAG
VTA
(b)
Paired-pulse ratio
10pA
10ms
(c)
AMPAR:NMDAR ratio
10pA
20ms
(d)
τw (NMDA) (ms)
10pA
20ms
(e)
τ (AMPA) (ms)
10pA
20ms
(f)
NMDAR current (pA)
NMDA
Current (pA)
Time (s)
NMDA
Current (pA)
Time (s)

Figure 10. Passive membrane properties and glutamatergic currents in DRN/vlPAG and VTA dopamine neurons. (a) Bar charts (mean + SEM) showing membrane capacitance (C_m), holding current at -50 mV, and input resistance (R_{in}) of recorded DRN/vlPAG and VTA dopamine neurons. Bar charts and representative examples of (b) the PPR, (c) the AMPAR:NMDAR ratio, (d) the weighted decay time constant of NMDAR-mediated current,(e) the decay time constant of AMPAR-mediated current, and (f) the response to NMDA application. The red line indicates the exponential fit of the decay phase of the current.* indicates $p < 0.05$.

4.4. Conclusion

I have recorded glutamatergic and GABAergic currents in identified VIP+ and VIP- DRN/vlPAG dopamine neurons in order to broaden the synaptic analysis of this population. Spontaneous glutamatergic currents in VIP+ neurons displayed smaller amplitude and frequency than in VIP- neurons, and showed paired-pulse depression in response to paired stimuli. Contrastingly, there was no difference in the PPR of GABAergic current between VIP+ and VIP- neurons, but there was a trend for less frequent sIPSCs in VIP- neurons, compared to the greater range of frequencies observed in VIP+ neurons. Additionally, there was no difference in the AMPAR:NMDAR ratio between VIP+ and VIP- dopamine neurons, suggesting a similar contribution by the two glutamatergic receptor-mediated currents at these synapses. Therefore, both VIP+ and VIP- neurons within the DRN/vlPAG receive glutamatergic and GABAergic input, but their relative proportion of excitatory and inhibitory input may vary.

I also recorded glutamatergic currents in VTA dopamine neurons in order to compare with the DRN/vlPAG population. I found that the two populations differed in their passive membrane properties, including C_m, R_{in}, and holding current, which is likely to be attributable to the larger size of VTA dopamine neurons. Additionally, I found that DRN/vlPAG dopamine neurons had a larger AMPAR:NMDAR ratio and a higher PPR than VTA dopamine neurons. This suggests that glutamatergic synapses may have distinct characteristics in the VTA and DRN/vlPAG, which may reflect pre- and postsynaptic differences. It is important to note, however, that there is considerable variability in many of these properties in VTA dopamine neurons, which corresponds to projection target (Lammel et al., 2008; Lammel et al., 2011). In particular, it has been recently demonstrated that there is a significant difference in the AMPAR:NMDAR ratio between dopamine neurons projecting to the NAc lateral shell, and those projecting to the NAc medial shell or mPFC (Lammel et al., 2011). Therefore a closer inspection of the DRN/vlPAG may

reveal differences in the properties of glutamatergic synapses relating to neuronal projection target.

One limitation of this comparison between the VTA and DRN/vlPAG is the use of different orientations of slice preparation. Coronal preparations are rarely used for studying synaptic physiology in the VTA, since currents are difficult to evoke. However, this is the most appropriate orientation for targeting the DRN/vlPAG population. In practice, it was initially difficult to evoke adequate-sized synaptic currents in the DRN/vlPAG dopamine neurons, particularly the small, periaqueductal population. This may have been due to the orientation of the slice preparation and/or the small cell size (as evoking currents in serotonergic neurons was not as challenging). With practice, I found that positioning the bipolar stimulating electrode ventrolateral to the recorded DRN/vlPAG neuron was the most efficient way of evoking currents. However, it is possible that different brain slice orientations may result in a different degree of preservation of glutamatergic afferents from different regions, while the position of the stimulating electrode may result in stimulation of different glutamatergic afferents. This may be the cause of some of the differences noted here, but without neuronal tracing, the origin of these fibres cannot be determined with any certainty in either preparation.

Chapter 5. Cocaine- and social isolation-induced plasticity in DRN/vlPAG dopamine neurons

5.1. Introduction

In spite of their diverse chemical structures, molecular targets, and associated behavioural and psychological effects, addictive drugs converge on the mesolimbic dopamine system to elicit their rewarding and reinforcing effects (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Corrigall et al., 1992; Kalivas, 1995; Wolf, 1998; McBride et al; 1999; Overton et al., 1999; Ikemoto and Wise, 2004; Nestler, 2005). One crucial locus of modification following exposure to drugs of abuse occurs at excitatory synapses onto dopamine neurons in the VTA. Nearly all addictive drugs, including amphetamine, nicotine, morphine, alcohol, and diazepam, are able to induce LTP-like potentiation of glutamatergic synapses onto VTA dopamine neurons (Ungless et al., 2001; Faleiro et al., 2003; Saal et al., 2003; Heikkinen et al*.,* 2009; Tan et al*.,* 2010). Additionally, potentiation at these same synapses can be induced by an aversive stimulus, but not by psychoactive, non-addictive substances such as carbamazepine or fluoxetine (Saal et al., 2003). Cocaine-induced potentiation occurs within a few hours of a single exposure (Faleiro et al., 2004; Argilli et al., 2008) and lasts for about a week (Ungless et al., 2001), even following multiple noncontingent injections (Borgland et al., 2004).

Recently, it has been demonstrated that plasticity at these synapses is projection-specific. Cocaine-induced potentiation only occurs at synapses onto dopamine neurons projecting to the NAc lateral and medial shell, but not dopamine neurons projecting to the mPFC, or SNc neurons projecting to the dorsal striatum (Lammel et al., 2011). Contrastingly, an aversive stimulus selectively potentiates synapses onto mPFC- and NAc lateral shell-projecting dopamine neurons,

without affecting VTA neurons projecting to the NAc medial shell, or SNc neurons projecting to the dorsal striatum (Lammel et al., 2011). Therefore dopamine neurons of the VTA are functionally heterogeneous in their response to salient stimuli, which is related to their projection target.

5.1.1. Significance of drug-induced plasticity in the VTA

Synaptic potentiation in the VTA induced by passive cocaine administration was thought to be required for the early induction of behavioural sensitization and CPP (Kalivas and Alesdatter, 1993; Vezina, 1996; Wolf, 1998; Vezina and Queen, 2000; Carlezon and Nestler, 2002; Harris and Aston-Jones, 2003; Borgland et al., 2004; Harris et al., 2004; Wolf et al., 2004; Dunn et al., 2005; Mead et al., 2005; Borgland et al., 2006; Zweifel et al., 2008). However, this idea was challenged by the demonstration that disruption of glutamatergic signalling selectively in dopamine neurons, which abolishes cocaine-induced plasticity, does not alter behavioural sensitisation to cocaine (Dong et al., 2004) or CPP (Engblom et al., 2008; Luo et al., 2010). Drug-evoked plasticity in the VTA has also been implicated in cue- and drug-induced reinstatement during withdrawal (Engblom et al., 2008; Mameli et al., 2009), although contradictory results also exist (Zweifel et al., 2008). Therefore, establishing a causal relationship between drug-evoked synaptic plasticity and behaviour remains a major challenge in the field.

In addition to possibly mediating some of the behavioural effects of addictive drugs, plasticity in VTA dopamine neurons is required for neural adaptations in downstream targets (Mameli et al., 2009). It was demonstrated that abolishing drug-induced plasticity in the VTA, by inducible deletion of the obligatory NR1 subunit of the NMDAR selectively in dopamine neurons (Engblom et al., 2008), prevented plasticity in the NAc (Mameli et al., 2009). Furthermore, disrupting mGluR1 function selectively in VTA dopamine neurons, which prevents the reversal of cocaine-induced plasticity in the VTA (Bellone and Lüscher, 2006), is itself sufficient to trigger plasticity in

the NAc, even after a single cocaine injection (Mameli et al., 2009). Therefore the increase in dopamine neuron firing and dopamine release in target regions, which would be expected to accompany an increase in excitatory synaptic strength, is likely to influence activity and plasticity in these downstream structures of the reward circuit, resulting in multiple, complex neuroadaptations (Hyman and Malenka, 2001; Kalivas and Volkow, 2005). Hence, in spite of its transient nature, drug-induced plasticity in VTA dopamine neurons may be important not only for the behavioural response to addictive drugs, but also for promoting long-term alterations in other regions of the reward circuit.

Interestingly, the context and mode of drug administration appears to be important in the induction of plasticity. For example, the potentiation induced by prolonged self-administration (rather than passive injections) can last for around 3 months (Chen et al., 2008), and cues predicting a natural reward also potentiate these excitatory synapses following classical conditioning (Stuber et al., 2008). This indicates that this plasticity can involve an associative learning mechanism, which may be important in determining drug-related behaviour in the early development of addiction.

Given that synaptic plasticity is thought to represent the cellular correlate of learning and memory (Hebb, 1949; Eccles 1964; Alkon and Nelson, 1990; Kandel, 1997; Malenka and Bear, 2004), it is widely considered that addiction represents an aberrant form of learning in neural circuits evolutionarily conserved for the processing and reinforcement of natural rewards (Kelley, 2004; Kalivas and Volkow, 2005; Hyman et al., 2006; Kauer and Malenka, 2007). This is thought to facilitate the development of maladaptive behaviours during chronic drug exposure (Robinson and Berridge, 1993; Robinson and Berridge, 2000; Hyman and Malenka, 2001; Kelly and Berridge, 2002; Robinson and Berridge, 2003; Kauer, 2004; Vezina, 2004; Kalivas, 2009), which ultimately results in compulsive drug consumption, despite negative consequences, which

is the hallmark of addiction (Hyman and Malenka, 2001; Ikemoto and Wise, 2004; Jones and Bonci, 2005; Nestler, 2005).

5.1.2. The AMPAR:NMDAR ratio as a measure of synaptic strength

Cocaine-induced potentiation of glutamatergic synapses onto VTA dopamine neurons was first directly demonstrated by measuring the AMPAR:NMDAR ratio following a single dose of cocaine in mice (Ungless et al., 2001). The AMPAR:NMDAR ratio is a sensitive measure used to assay changes in synaptic strength, defined as the peak AMPAR-mediated current, relative to the peak of the NMDAR-mediated current. It conveniently facilitates inter-cell, and inter-brain slice, comparison since it is independent of factors such as the relative position of the stimulating and recording electrodes, and the number of afferent fibres stimulated within the brain slice, so is routinely used to identify changes in synaptic efficacy (Hsia et al., 1998; Kauer and Malenka, 2007; Thomas et al*.,* 2008). This measure was initially used because cocaine was hypothesised to induced synaptic changes that closely resembled those of NMDAR-dependent LTP (Karler et al., 1991; Schenk and Snow, 1994; Wolf, 1998; Bonci and Malenka, 1999; Overton et al., 1999). This is thought to involve an increase in AMPAR transmission, without a significant change in NMDAR transmission (Malenka and Nicoll, 1999), and so the AMPAR:NMDAR ratio was an appropriate tool for measuring such a change.

5.1.3. Does cocaine potentiate synapses onto DRN/vlPAG dopamine neurons?

Spontaneous and evoked glutamatergic currents can be recorded in DRN/vlPAG dopamine neurons in a coronal brain slice preparation *ex vivo* (Dougalis et al., 2012). Cocaine-induced potentiation is present at glutamatergic synapses onto a subset of VTA dopamine neurons, but the DRN/vlPAG population have yet to be explored. I therefore assayed the AMPAR:NMDAR ratio in these dopamine neurons, following a single dose of cocaine, in the same way as

previously reported for the VTA (Ungless et al., 2001). Although there is evidence that the DRN/vlPAG population may mediate some of the effects of opiates (Flores et al., 2004; Flores et al., 2006; Meyer et al., 2009), drug-induced plasticity in the DRN/vlPAG dopamine neurons has not been specifically studied. However, given that these neurons appear to provide a greater input to the central amygdala and BNST compared to the VTA (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006; Shin et al., 2008), a change in synaptic strength in these neurons would have significance for dopamine release and drug-induced changes in different target structures and neural circuits.

5.2. Materials and methods

5.2.1. Injection protocol

TH-GFP or Pitx3-GFP mice (1-3 months) received a single dose of cocaine (15 mg/kg), or the equivalent volume of 0.9% saline, administered via intraperitoneal (IP) injection at a volume of 10 ml/kg. Injections were performed at 10.00 am, and the injected mouse was subsequently transferred to a clean cage for 24 hours before brain slice preparation. I performed all the injections for preliminary experiments, and the majority of the data shown in Fig. 11, but due to Central Biomedical Services (CBS) room restrictions all subsequent injections were carried out by Piotr Czapski.

5.2.2. Electrophysiology

Horizontal or coronal 220 µm brain slices containing the VTA or DRN/vlPAG, respectively, were prepared and maintained as described in chapter 3. Whole-cell patch-clamp electrophysiology was performed as described in chapter 4, using the cesium-based internal solution (composition in mM: $CsCH_3SO_3$ 130**,** NaCl 2.8, HEPES 20, EGTA 0.4, TEA-Cl 5, MgATP 2, Li-GTP 0.5, and 0.1% neurobiotin; pH 7.3, osmolarity 280-290 mosmol/l) and with 100 µM picrotoxin included in the

aCSF. Postsynaptic currents were evoked using a bipolar stimulating electrode (FHC, USA) placed 100-300 µm ventrolateral recorded neuron. Stimulus intensity was controlled using an ISO-flex stimulus isolator (AMPI, Jerusalem, Israel) and adjusted to evoke monosynaptic events, typically using a stimulus intensity of 10-40 V, 500 µs duration. AMPAR:NMDAR ratios were recorded in voltage-clamp at a holding potential of +40 mV as described in chapter 4.

5.2.3. Statistics

Statistical analysis was performed using Prism 5.04 (Graphpad, CA, USA), and values are reported as mean ± standard error of the mean (SEM). Two experimental groups were compared using an unpaired t-test (non-directional), and three or more groups were compared using a one-way ANOVA with Newman-Keuls post-hoc tests. The Pearson's product-moment correlation coefficient was used to analyse correlation between two variables.

5.2.4. Drugs

Cocaine hydrochloride (Sigma Aldrich, St Louis, MO, USA) was stored in a safe and dissolved in 0.9% saline for IP injections.

5.3. Results

Two or three brain slices containing the DRN/vlPAG or VTA were obtained per animal, and one AMPAR:NMDAR ratio was recorded per slice. Both TH-GFP and Pitx3-GFP mice were used for recordings, and no significant difference was found between them in terms of the AMPAR:NMDAR ratio in DRN/vlPAG GFP+ neurons (TH-GFP: 1.23 ± 0.14, n = 7; Pitx3-GFP: 1.24 ± 0.17, n = 6; $t_{11} = 0.009$, p = 0.99), or between identified TH+ and TH- cells (TH+: 1.05 ± 0.15, n = 5; TH-: 0.95 ± 0.38, n = 2; $t_5 = 0.33$, p = 0.75). However, the sample size is too low for accurate statistical comparison between TH+ and TH- GFP+ neurons. A lower proportion of recorded

neurons were successfully labelled and recovered compared to the experiments described in previous chapters, therefore all GFP+ recorded neurons (identified TH+, TH-, VIP+, VIP-, or unidentified) have been included, except where stated, to increase the sample size. This may be due to the tight seal formed during prolonged recordings of this kind, which made it difficult to successfully withdraw the recording electrode without damaging the cell or drawing the cell away with it.

5.3.1. Cocaine-induced potentiation in VTA and DRN/vlPAG dopamine neurons

In the VTA, a single cocaine injection resulted in a significant increase in the AMPAR:NMDAR ratio (0.71 ± 0.09, n = 9; one-way ANOVA: $F_{2,22} = 4.65$, $p < 0.05$) compared to saline-injected (0.48 ± 0.04, n = 6; $p < 0.05$) or naïve animals (0.45 ± 0.05, n = 8; $p < 0.05$), which were not significantly different from each other ($p > 0.05$; Fig. 11a,b). The range of ratios I recorded was similar to those previously reported in mice (e.g., Ungless et al., 2001; Saal et al., 2003; Argilli et al., 2008; Lammel et al., 2011). Given the magnitude of the increase in ratio, and the location of the cells just medial to the MT, these are likely to be mostly NAc lateral shell-projecting neurons, which are mostly frequently targeted in brain slices. Akin to the VTA, in the DRN/vlPAG I found that mice which received a single dose of cocaine exhibited a significantly larger AMPAR:NMDAR ratio (1.82 ± 0.10, n = 8) than naïve mice (1.12 ± 0.11, n = 12; one-way ANOVA: $F_{2,25} = 9.73$, $p < 0.001$; naïve vs cocaine $p < 0.01$; Fig. 11c,d). Unexpectedly, however, I found that mice injected with a single dose of saline also displayed a significantly larger AMPAR:NMDAR ratio (2.00 ± 0.26, n = 8) than naïve mice ($p < 0.01$), which was not significantly different from cocaine-treated mice ($p > 0.05$). This surprising result indicated that an additional factor in both groups of injected mice may have been influencing the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons. Conforming to the method used in Ungless et al., the injection protocol involved transferring injected mice to a clean cage, on their own, for 24 hours before brain slice

preparation (Fig. 11d). This was in order to minimise possible disruption within the cage, due to cocaine-induced hyperactivity in the injected mouse, since the cagemates were to be used in other experimental groups. Therefore the two major differences between injected and non-injected mice were exposure to a novel environment and social isolation for 24 hours before recording. I therefore sought to identify whether either of these factors were contributing to the synaptic potentiation observed in DRN/vlPAG dopamine neurons.

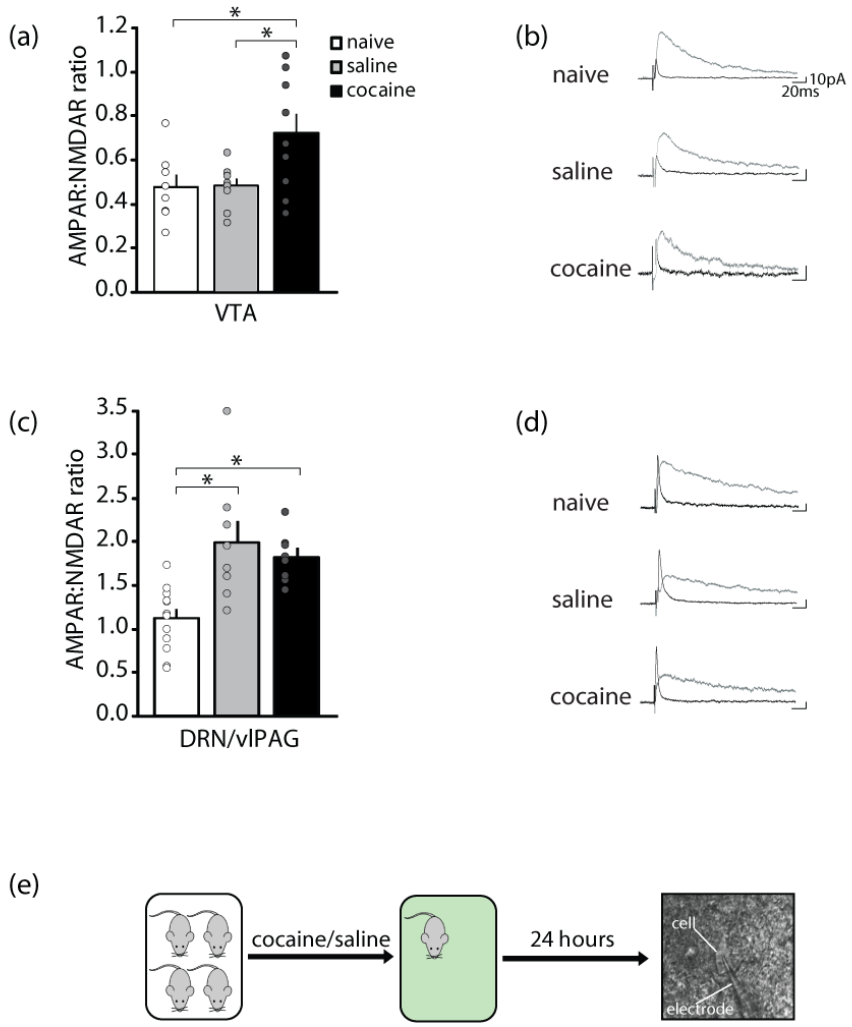


Figure 11. Effect of a single dose of cocaine on the AMPAR:NMDAR ratio. Bar chart (mean + SEM) with superimposed scatter plot, and representative examples of the AMPAR:NMDAR ratio recorded in (a,b) VTA and (c,d) DRN/vlPAG dopamine neurons in naïve mice or following a single dose of saline or cocaine. The mean age of each experimental group was 5.8 weeks (naive VTA), 4.8 weeks (saline VTA), 5.2 weeks (cocaine VTA), 8.2 weeks (naive DRN/vlPAG), 6.2 weeks (saline DRN/vlPAG), and 6.4 weeks (cocaine DRN/vlPAG). (e) The injection procedure used in these experiments. * indicates $p < 0.05$.

5.3.2. Effect of environmental novelty and/or social isolation on DRN/vlPAG synapses

Four protocols were tested, in the absence of a saline or cocaine injection, which involved environmental novelty and/or social isolation for 24 hours prior to brain slice preparation (Fig. 12a). Mice in protocol A were naïve group-housed mice, in protocol B they were transferred to a clean cage alone, in protocol C they were transferred as a group into a clean cage, and in protocol D they were left alone in the original cage. All mice were group-housed in the same cage (containing 2-6 mice) for at least 5 days before these protocols were commenced, and the manipulations in protocols B-D were performed at 10.00 am, 24 hours before brain slice preparation. Additionally, the clean cages were located in the same room, on racks that allowed the mice to see, hear, and smell other mice. AMPAR:NMDAR ratios were recorded in each condition, which revealed a significantly larger ratio (one-way ANOVA: $F_{3,26} = 6.17$, $p < 0.01$) in mice that had been socially isolated with environmental novelty (protocol B: 1.92 ± 0.13, n = 6) or without environmental novelty (protocol D: 2.09 ± 0.21, n = 7), compared to either naïve group-housed mice (protocol A: 1.25 ± 0.13, n = 8; A vs B: $p < 0.05$; A vs D: $p < 0.01$) or mice exposed to environmental novelty without isolation (protocol C: 1.45 ± 0.15, n = 9; C vs B: $p < 0.05$; C vs D: $p < 0.05$; Fig. 12b). Furthermore, the AMPAR:NMDAR ratio recorded in mice socially isolated with environmental novelty was not significantly different from those socially isolated without environmental novelty (B vs D: $p > 0.05$). Similarly, there was no significant difference between the ratio exhibited by naïve mice and those exposed to environmental novelty without isolation (A vs C: $p > 0.05$). This suggested that acute social isolation, but not environmental novelty, can potentiate glutamatergic synapses onto DRN/vlPAG dopamine neurons. Pooling data from multiple groups confirmed there was no difference in the AMPAR:NMDAR ratio from TH-GFP and Pitx3-GFP group-housed mice (TH-GFP: 1.13 ± 0.12, n = 16; Pitx3-GFP: 1.14 ± 0.12, n = 14; $t_{28} = 0.14$, $p = 0.89$) or isolated mice (TH-GFP: 1.96 ± 0.09, n = 12; Pitx3-GFP: 2.09 ± 0.29, n

= 8; t_{18} = 0.48, p = 0.64). There was also no difference in the AMPAR:NMDAR ratio in identified TH+ and TH- neurons from group-housed mice (TH+: 1.29 ± 0.09, n = 19; TH-: 1.10 ± 0.21, n =5; t_{22} = 0.94, p = 0.34) or isolated mice (TH+: 2.03 ± 0.13, n = 19 TH-: 1.78 ± 0.14, n = 2; t_{19} = 0.58, p = 0.57).

Given that there was some variability in the number of animals in each cage (between 2 and 6) prior to social isolation, I considered whether this had any bearing on the AMPAR:NMDAR ratio. There was no significant correlation between the number of mice per cage and the ratio in group-housed mice (Fig. 12c; r^2 = 0.0003, p = 0.94, n = 23). For this analysis, ratios were pooled from mice which were group-housed in protocols A and C, and also mice which had received a single saline injection but remained in the original, group-housed cage (to be reported in 5.3.4.). I also observed the same result when I included only ratios from naive mice in protocol A (r^2 = 0.001, p = 0.92, n = 10). However, I found there was significant positive correlation between the number of mice per cage, and the AMPAR:NMDAR ratio following 24 hours of social isolation (Fig. 12d; r^2 = 0.53, p < 0.01, n = 15). In this case, I pooled ratios from mice which were isolated in their original cage, in a new cage, or given a single dose of saline before isolation. Equally, though, I found a similar positive correlation when I analysed only ratios from mice isolated without any injection (i.e., protocols B and D; r^2 = 0.61, p < 0.05, n = 9) or isolated in their original cage (i.e., protocol D; r^2 = 0.70, p < 0.05, n = 6). Additionally, since there was only one data point at 6 mice per cage, I also analysed the data without this value, which still resulted in significant positive correlation, albeit slightly weaker (r^2 = 0.33, p < 0.05, n = 14), therefore it would be useful to increase the sample for this cage size. However, these results do suggest that social isolation causes a more dramatic increase in the AMPAR:NMDAR ratio when the mouse has been isolated from a larger number of cagemates. Therefore, prior social interaction may be an important factor in determining the synaptic potentiation following social isolation.

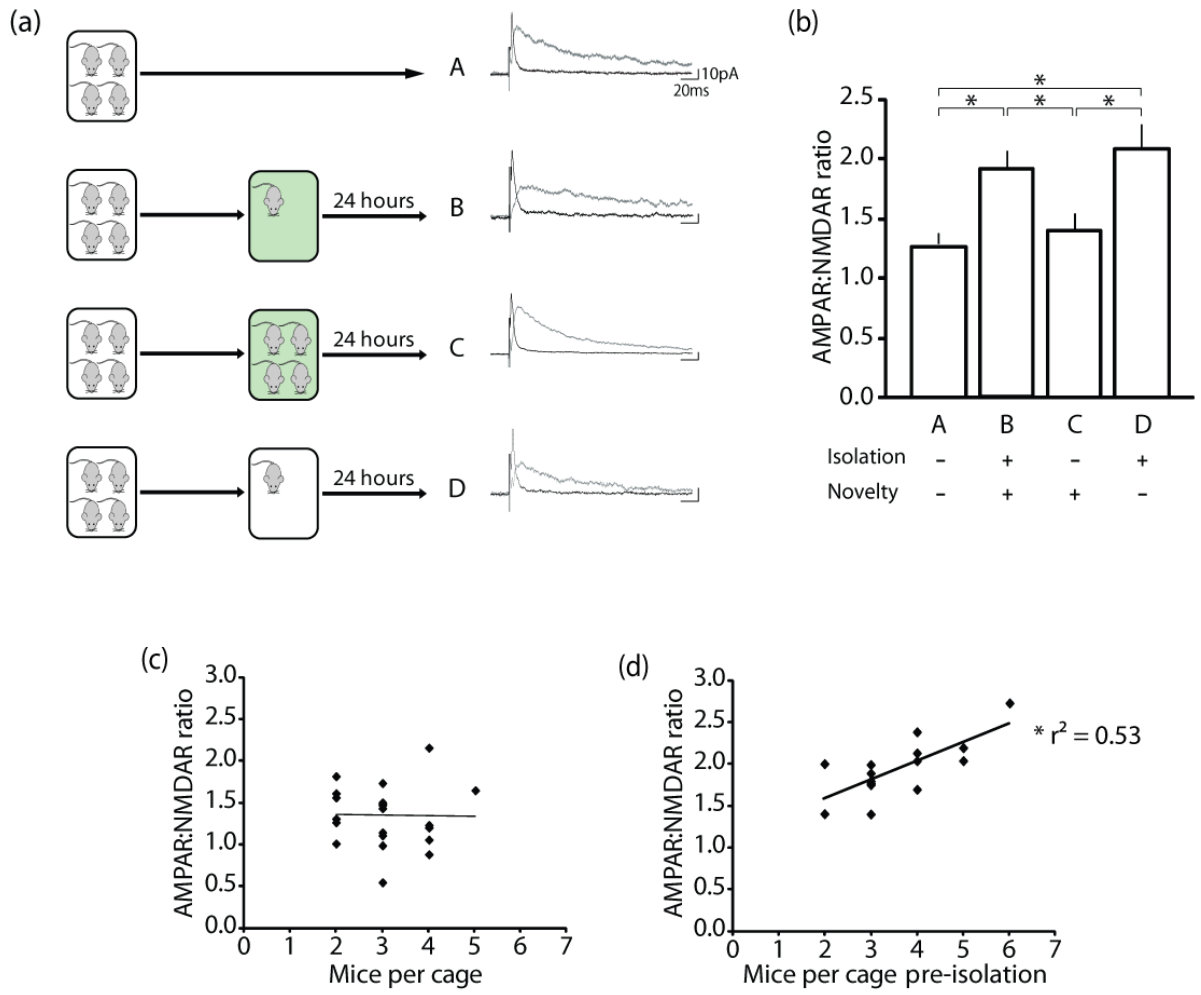


Figure 12. Social isolation, but not environmental novelty, potentiates synapses onto DRN/vlPAG dopamine neurons. (a) Injection protocol with representative AMPAR:NMDAR ratios, and (b) bar chart showing the mean (+ SEM) AMPAR:NMDAR ratio recorded in each condition A-D. The mean age for each experimental group was 7.7 weeks (A), 9.3 weeks (B), 6.6 weeks (C), and 7.8 weeks (D). Scatter graph showing relationship between the number of mice per cage and the AMPAR:NMDAR ratio in (c) group-housed and (d) socially isolated mice. This revealed significant positive correlation in the socially isolated ($r^2 = 0.53$, $p < 0.01$), but not the group-housed mice ($r^2 = 0.0003$, $p = 0.94$). * indicates $p < 0.05$.

5.3.3. Effect of social isolation on VTA synapses

The effect of acute social isolation on glutamatergic synapses onto VTA dopamine neurons has not been directly examined in previous studies. However, indirect evidence suggests that social isolation does not potentiate synapses in the VTA, because isolated saline-injected mice showed no difference in their AMPAR:NMDAR ratio compared to naïve, group-housed mice (present observations and Ungless et al., 2001). Nevertheless, I wanted to test directly whether social isolation potentiated glutamatergic synapses onto VTA dopamine neurons under the same conditions as I had used for examining this in the DRN/vlPAG.

Mice were socially isolated in their original cage (i.e., protocol D in Fig. 12a) for 24 hours prior to brain slice preparation, and AMPAR:NMDAR ratios recorded for comparison with naïve, group-housed mice (i.e., protocol A). VTA dopamine neurons showed no significant difference in the AMPAR:NMDAR ratio recorded in socially isolated mice (0.53 ± 0.04, n = 12) compared to naïve, group-housed mice (0.47 ± 0.05, n = 8; t_{18} = 0.73, p = 0.47; Fig. 13a,b). This suggests that acute social isolation does not potentiate glutamatergic synapses onto dopamine neurons in the VTA. These recordings were mostly performed in the region just medial to the MT, which has been shown to house a considerable proportion of stress-responsive NAc lateral shell-projecting neurons (Saal et al., 2003; Lammel et al., 2011). This, therefore, indicates that social isolation is unlikely to be an acute aversive or stressful stimulus, as there was no potentiation observed in this population. However, the VTA contains a large number of dopamine neurons and, given that there appears to be considerable projection-specificity in the midbrain dopamine population, it is not impossible that certain smaller subpopulations within the VTA, which do respond to social isolation, were not sampled in these experiments.

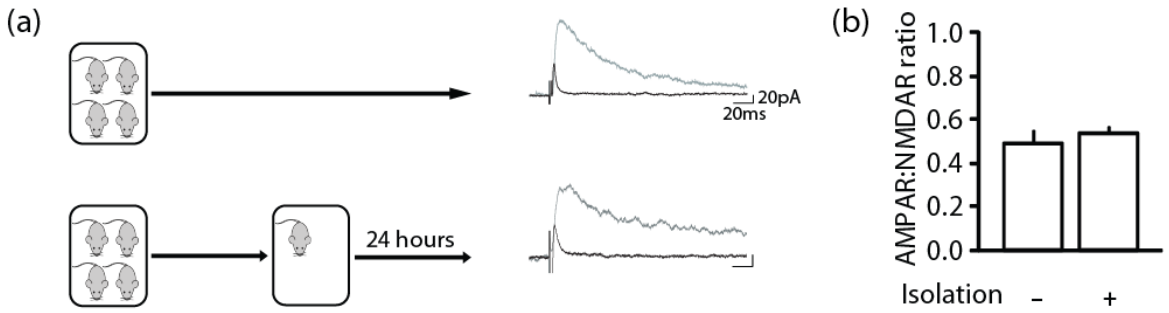


Figure 13. Social isolation does not potentiate synapses onto VTA dopamine neurons. (a) Injection protocol with representative examples and (b) bar chart showing the mean (+ SEM) AMPAR:NMDAR ratio in group-housed and socially isolated mice. The mean age for each experimental group was 5.8 weeks (naive) and 4.6 weeks (isolation).

5.3.4. Effect of cocaine, without social isolation, on DRN/vlPAG synapses

These results suggested that social isolation can increase the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons after just 24 hours. However, as both saline- and cocaine-injected mice were isolated in the initial experiment (Fig. 11), the effect of cocaine on these synapses had yet to be determined. To investigate this, mice were given single saline or cocaine injections, but returned to their original cage, with their original cagemates, for the 24 hours prior to brain slice preparation. Under this protocol, I found that saline-injected mice did not display a significantly different AMPAR:NMDAR ratio compared to naïve mice (saline: 1.39 ± 0.20, n = 8; naïve: 1.25 ± 0.14, n = 11; $p > 0.05$; Fig. 14a,b). However, cocaine-injected mice exhibited a significantly larger ratio (1.96 ± 0.22, n = 8; one-way ANOVA: $F_{2,24} = 4.38$, $p < 0.05$) than either naïve ($p < 0.05$) or saline-injected mice ($p < 0.05$). Therefore, cocaine, in the absence of social isolation was able to potentiate glutamatergic synapses onto DRN/vlPAG dopamine neurons. It is also noteworthy that the size of the AMPAR:NMDAR ratio following a single dose of cocaine given without isolation was no different to that observed following a single dose of cocaine with isolation (Fig. 11c,d; $t_{14} = 0.59$, $p = 0.57$), which suggests that social isolation and cocaine do not have an additive effect at these synapses. Furthermore, the ratio following 24 hours of social isolation (Fig. 12a,b) was no different from a single dose of cocaine without isolation ($t_{13} = 0.41$, $p = 0.69$) suggesting that both these stimuli can potentiate these synapses to a similar extent. Examining the association between the AMPAR:NMDAR ratio and the number of mice per cage, pre-injection, did not reveal any significant correlation for the saline-treated ($r^2 = 0.22$, $p = 0.29$; Fig. 14c) or cocaine-treated mice ($r^2 = 0.11$, $p = 0.42$; Fig. 14d). However, there is insufficient spread of data points for accurate analysis of correlation in the saline-treated group.

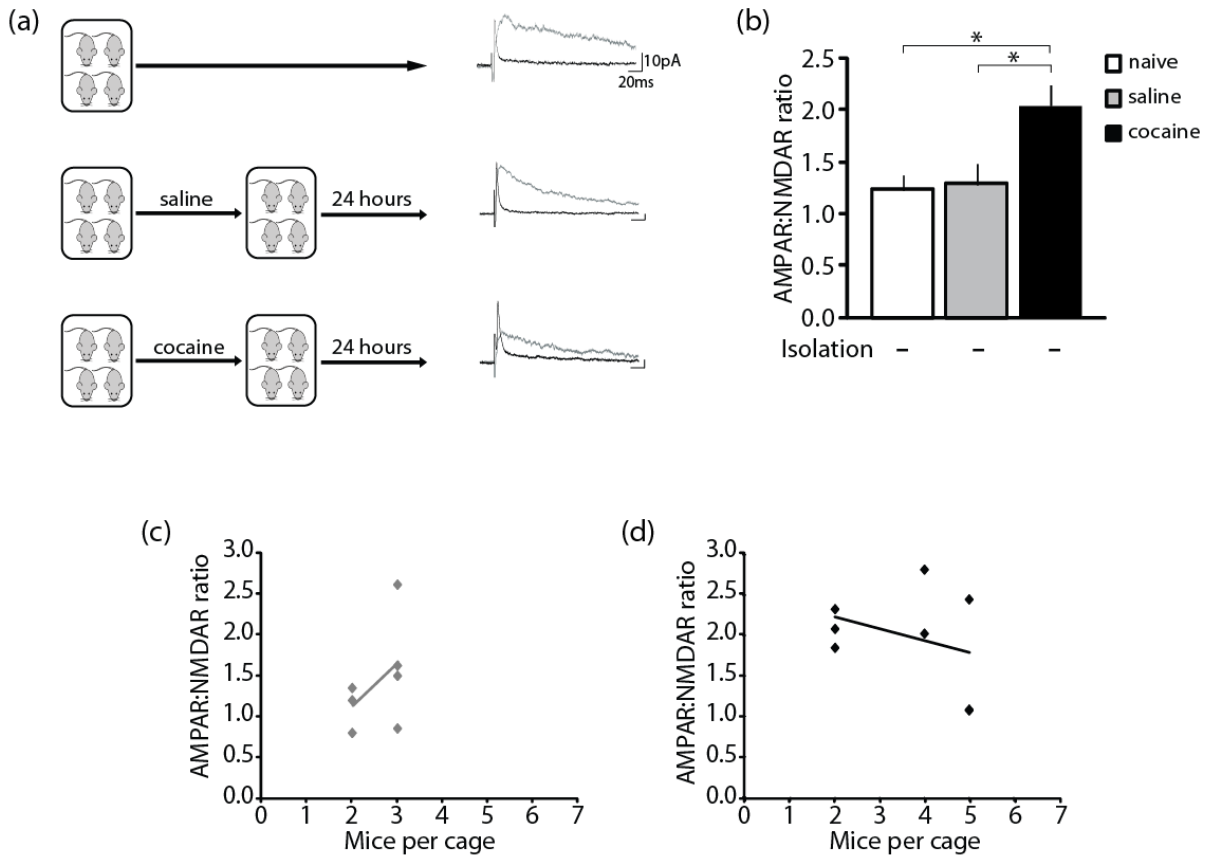


Figure 14. Cocaine, in the absence of social isolation, potentiates synapses onto DRN/vlPAG dopamine neurons. (a) Injection protocol with representative examples, and (b) bar chart showing mean (+ SEM) AMPAR:NMDAR ratio in naïve mice, or following a single dose of saline or cocaine without social isolation. The mean age for each experimental group was 7.7 (naive), 7.0 weeks (saline), and 8.1 weeks (cocaine). Scatter graphs showing the relationship between the number of mice per cage and the AMPAR:NMDAR ratio found no correlation in either (c) saline- or (d) cocaine-treated mice. * indicates $p < 0.05$.

5.3.5. AMPAR:NMDAR ratio in identified VIP+ and VIP- dopamine neurons.

The VIP projection from the DRN/vlPAG has been shown to exclusively target the dorsolateral (dl)BNST (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1998). Co-expression of this neuropeptide may therefore delineate the dopaminergic projection to this structure, whereas VIP- neurons are likely to project to several different targets, including the central amygdala and ventral striatum. Given the projection-specificity of cocaine- and aversion-induced plasticity in the VTA reported by Lammel et al. (2011), it would be interesting to know whether plasticity in the DRN/vlPAG shows a similar organisation. However, I generally found that there was a bias towards recording from the large, multipolar, ventrally-located neurons within the DRN/vlPAG (which are mostly VIP-) for two reasons. Firstly, due to the ventrolateral positioning of the stimulating electrode, recording from the small periaqueductal GFP+ neurons would have required placing the electrode on top of many of these ventral GFP+ neurons, and secondly, it was usually easier to evoke adequate-sized currents in the large, multipolar neurons compared to the small periaqueductal neurons. As mentioned above, a lower proportion of neurons were successfully recovered using immunohistochemistry for post-hoc identification, and so I did not obtain sufficient numbers of identified VIP+ and VIP- neurons in each experimental condition for accurate comparison.

Nevertheless, for several groups I did record from a sufficient number of VIP- neurons to conclude that they showed the same significant changes described for the whole sample. Specifically, VIP- neurons in the DRN/vlPAG showed a significantly larger ratio (one-way ANOVA: $F_{3,14} = 9.46$, $p < 0.01$) in mice that were socially isolated with (protocol B: 2.1 ± 0.10, n = 4) or without (protocol D: 2.3 ± 0.29, n = 4) environmental novelty, compared to naïve mice (protocol A: 1.12 ± 0.16, n = 5; A vs B: $p < 0.05$; A vs D: $p < 0.01$) or those exposed to a novel environment without isolation (protocol C: 1.17 ± 0.21, n = 5; C vs B: $p < 0.05$; C vs D: $p < 0.01$; Fig. 15a).

Additionally, similar to the results from sampling the whole population, there was no difference in the AMPAR:NMDAR ratio of VIP- neurons from naïve mice compared to those exposed to a novel environment without isolation (A vs C: $p > 0.05$), or from mice socially isolated with or without environmental novelty (B vs D: $p > 0.05$). I also recorded from a sufficient number of identified VIP- neurons to confirm the effect of a single dose of cocaine without social isolation (Fig. 15b). The AMPAR:NMDAR ratio in VIP- neurons from saline-injected mice (1.30 ± 0.14, n = 5) was not significantly different from that of naïve mice (1.12 ± 0.16, n = 5; $p > 0.05$), but ratios recorded in cocaine-injected mice (2.05 ± 0.24, n = 6) were significantly larger (one-way ANOVA: $F_{2,14} = 4.28$, $p < 0.05$) than those from either saline-injected ($p < 0.05$) or naïve ($p < 0.05$) mice.

When pooling data from multiple groups, I found that VIP+ neurons showed a trend towards larger AMPAR:NMDAR ratios following social isolation (1.53 ± 0.07, n = 3) compared to group-housed mice (1.17 ± 0.19, n = 6; Fig. 15c). However, this difference was not as pronounced as in VIP- neurons, and did not reach statistical significance ($t_7 = 1.30$, $p = 0.24$). This may be due to the small sample size, and therefore more data is required to confirm the effect of social isolation on the VIP+ population.

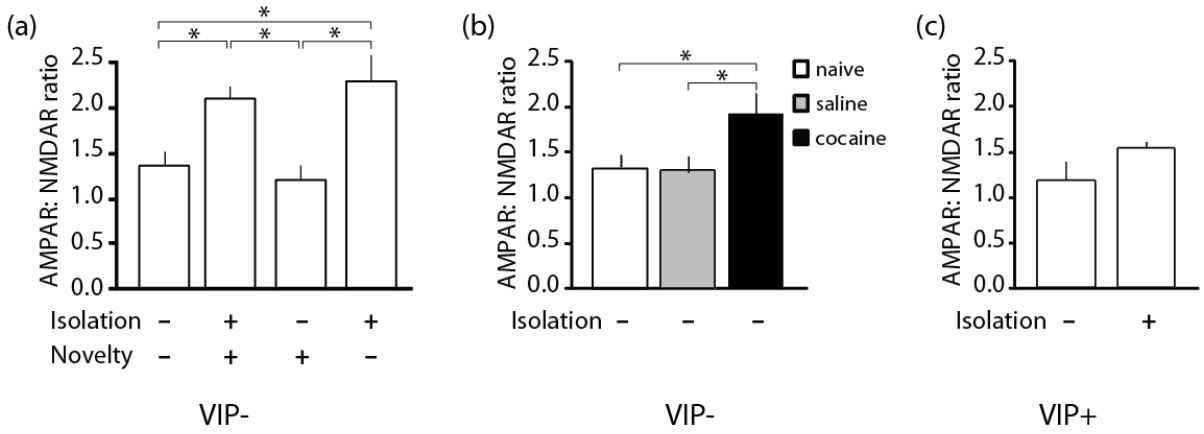


Figure 15. Effect of social isolation, environmental novelty, and cocaine on identified VIP+ and VIP- dopamine neurons. Bar charts showing the mean (+ SEM) AMPAR:NMDAR ratio in identified VIP- neurons following (a) social isolation and/or environmental novelty, and (b) a single dose of saline or cocaine without social isolation. (c) Bar chart showing the mean (+ SEM) AMPAR:NMDAR ratio in identified VIP+ neurons from group-housed and socially isolated mice, pooled from multiple experimental groups. * indicates $p < 0.05$.

5.4. Conclusion

Utilising the AMPAR:NMDAR ratio to assay synaptic strength, I have demonstrated here that acute social isolation and/or a single dose of cocaine can potentiate glutamatergic synapses onto DRN/vlPAG dopamine neurons. Importantly, this adds to the list of manipulations which can potentiate excitatory synapses onto dopamine neurons. The effect of social isolation appears to be exclusive to this population as it was not observed in dopamine neurons of the VTA. However, given the large number of cells within the VTA, and their multiple projection targets, it may be that the whole population was not uniformly sampled in these recordings. Therefore this result does not exclude the possibility that there are certain subpopulations within the VTA that can also respond to social isolation. Nevertheless, these findings support the hypothesis that the DRN/vlPAG dopamine neurons are a distinct functional subpopulation, and intriguingly propose another motivationally-relevant cue that may be processed by the dopamine system. This result also has relevance to neuropsychiatric disorders for which social isolation is frequently used as an animal model, for example depression and schizophrenia (Kim and Kirkpatrick, 1996; Jones et al., 2011).

This potentiation appears to be specific to social isolation, and is not associated with environmental novelty, as animals moved to a new cage with the same conspecifics did not exhibit a larger AMPAR:NMDAR ratio compared to those which remained in their original cage. This is significant, as it has been demonstrated that exposure to a different cage for just 1 hour can increase the AMPAR:NMDAR ratio in medium spiny neurons of the NAc shell, measured after 24 hours (Rothwell et al., 2011). This was attributed to the novelty of the new cage, and disappeared following repeated habituation to the same environment (Rothwell et al., 2011). However, the influence of acute social isolation was not considered in this study. It should also be noted that ‘environmental novelty’ in Rothwell et al. referred to a different type of cage in a

different room, to which the animal had not been previously exposed. However, in my experiments the 'novel environment' refers to a clean cage which is essentially identical to their original cage, and located in the same room near other conspecifics. It is possible that a more robust novel stimulus may induce potentiation in certain populations of dopamine neurons, but this has not been investigated. The findings of Rothwell et al. and the present results do, however, add weight to the idea that more subtle changes in an animal's environmental conditions (which might not be considered strongly rewarding or aversive) may induce synaptic adaptations in the mesolimbic dopamine system and its projection targets. This may have important consequences for dopamine release in downstream projection sites, and may be associated with both short- and long-term changes in behaviour.

I also found that there was a highly significant positive correlation between the number of mice in the cage before isolation, and the AMPAR:NMDAR ratio following 24 hours of social isolation. This suggests that the strength of the potentiation is directly related to the loss of previous social interaction, and is not an artefact relating to another feature of the social isolation procedure. Hence, a mouse which is isolated from just one other cagemate will exhibit a smaller increase in the AMPAR:NMDAR ratio compared to a mouse isolated from a larger number of cagemates. Therefore the degree of loss in social contact appears to be a strong predictor of isolation-induced synaptic potentiation.

The DRN/vlPAG dopamine neurons have several projection targets, but co-expression of VIP may selectively mark the dlBNST projection. It would, therefore, be of interest to determine whether this projection is modified by cocaine and/or social isolation. Due to the bias in recording from large ventral neurons, the majority of the sample is likely to consist of VIP- neurons. Furthermore, recovering recorded, labelled cells with immunohistochemistry was more challenging with these longer-duration recordings, and so few successfully identified VIP+

neurons were obtained. I was, however, able to record a sufficient number of identified VIP- neurons to conclude that they show the same potentiation as described for the whole population. Identified VIP+ neurons also showed the same trend for an increase in the AMPAR:NMDAR ratio following social isolation, but this was not statistically significant, and the sample size was too small to draw a clear conclusion. An essential part of future work would be to increase the sample size of VIP+ neurons in naïve and socially isolated mice in order to determine whether social isolation can produce synaptic adaptations in this population.

Chapter 6. Characterisation of plasticity in DRN/vlPAG dopamine neurons

6.1. Introduction

Many different types of long-term potentiation (LTP) and long-term depression (LTD) have been described, differing in their mechanism of induction and expression (Malenka and Bear, 2004). Several of these have been observed in dopamine neurons of the VTA (Bonci and Malenka, 1999; Jones et al., 2000; Thomas et al., 2000; Bellone and Lüscher, 2005; Liu et al., 2005; Luu and Malenka, 2008; Mameli and Lüscher, 2011). One of the best described forms is NMDAR-dependent LTP, which can be mimicked by high frequency stimulation in brain slices (Bonci and Malenka, 1999). The potentiation observed in the VTA following an acute dose of cocaine closely resembles that of NMDAR-dependent LTP (Ungless et al., 2001). Cocaine-evoked plasticity is reliant on NMDAR activation during induction, and a stimulation protocol used to evoke NMDAR-dependent LTP in dopamine neurons is no longer effective following a dose of cocaine, suggesting an occlusion of further strengthening at these synapses (Ungless et al., 2001; Adams and Sweatt, 2002; Argilli et al., 2008; Luu and Malenka, 2008).

A change in the AMPAR:NMDAR ratio, such as that observed following a single dose of cocaine, could reflect an increase in AMPAR transmission, a reduction in NMDAR transmission, or a concomitant change in both receptors. This usually results from postsynaptic modifications at the synapse, but could also be due to presynaptic changes in the probability of glutamate release, although this is likely to impact equally on the synaptic response of both types of receptor (Malenka and Nicoll, 1999).

6.1.1. Cocaine-induced plasticity: presynaptic expression

In order to assay a change in the relative probability of presynaptic neurotransmitter release (P_r), paired-pulse stimulation in brain slices is frequently employed, and changes in the PPR are usually attributed to changes in presynaptic function (Zucker, 1989; Dobrunz and Stevens, 1997; Zucker and Regehr, 2002). Consistent with the view that cocaine induces a postsynaptic, but not presynaptic, change, there is no difference in the PPR following a single dose of cocaine compared to saline, suggesting that the probability of glutamate release is unaltered (Ungless et al., 2001).

6.1.2. Cocaine-induced plasticity: postsynaptic expression

The cocaine-induced increase in the AMPAR:NMDAR ratio in VTA dopamine neurons was originally attributed to a strengthening of AMPAR transmission, as bath application of exogenous AMPA, but not NMDA, resulted in an increase in receptor current following cocaine (Ungless et al., 2001). Additional evidence for an upregulation of AMPARs was provided through recording miniature (m)EPSCs in VTA dopamine neurons, which increased in both the amplitude and frequency following a single cocaine, but not saline injection (Ungless et al., 2001). Measurement of mEPSCs, which arise from spontaneous exocytosis of presynaptic vesicles of glutamate, is a standard method used to determine the locus of a synaptic change. A change in mEPSC amplitude is generally attributed to a postsynaptic change in receptor number and/or function, whereas a change in frequency could reflect a change in the P_r, or a change in the receptor number due to the uncovering of 'silent' synapses (Isaac et al., 1995; Malenka and Nicoll, 1999). An increase in frequency could also result from an increase in the amplitude of events, as the detection threshold is often very close to the mean, so an increase in amplitude would improve detection of smaller events.

6.1.2.1.Switch in AMPAR subunit composition

It has subsequently been demonstrated that the increase in AMPAR current is associated with a change in subunit composition of the postsynaptic AMPARs, from GluR2-containing to high-conductance GluR2-lacking receptors (Bellone and Lüscher, 2006). AMPARs are heterotetramer assemblies of four types of subunit, GluR1-4, with the presence of the GluR2 subunit dictating the cation-permeability of the AMPAR (Keinanen et al., 1990; Hollmann et al., 1991; Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994; Mano and Teichberg, 1998; Rosenmund et al., 1998; Mayer, 2005; Isaac et al., 2007). GluR2-containing receptors are permeable mostly only to Na^+ and K^+ ions, but the absence of this subunit creates an AMPAR that is permeable to both Ca^{2+} and Zn^{2+} , and also exhibits higher single channel conductance (Jonas and Burnashev, 1995; Kamboj et al., 1995; Liu and Zukin, 2007). GluR2-lacking receptors are susceptible to voltage-dependent block by intracellular polyamines (e.g., spermine), which inhibit the AMPAR current at positive potentials, resulting in a characteristic inwardly-rectifying I-V curve (Verdoorn et al., 1991; Bowie and Mayer, 1995; Jonas and Burnashev, 1995; Kamboj et al., 1995; Koh et al., 1995; Washburn et al., 1997; Pellegrini-Giampietro, 2003). This rectification can be quantified in brain slices by recording the AMPAR current at positive and negative potentials, and calculating the rectification index (RI: typically the peak amplitude at -70 mV relative to the peak amplitude at +40 mV; e.g., Mameli et al., 2007; Mameli et al., 2009).

The incorporation of GluR2-lacking AMPARs was first demonstrated in very young mice which showed a significant increase in the AMPAR RI, and a sensitivity to polyamines following a single dose of cocaine but not saline (Bellone and Lüscher, 2006). This has subsequently been confirmed in older mice (Mameli et al., 2009) and rats (Argilli et al., 2008; Good and Lupica, 2010). Furthermore, the redistribution of the GluR2 subunit from synaptic regions to intracellular compartments has been revealed through the use of electron microscopy (Mameli

et al., 2007; Brown et al., 2010), and the increased single channel conductance of the AMPARs demonstrated through nonstationary fluctuation analysis (Mameli et al., 2007).

The incorporation of AMPARs with a specific subunit composition is reminiscent of LTP in other brain regions such as the hippocampus, in which GluR2-containing AMPARs are transiently replaced by GluR2-lacking AMPARs following LTP induction (Shi et al., 1999; Hayashi et al., 2000; Liu and Cull-Candy, 2000; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004; Plant et al., 2006). Some studies, however, have failed to detect this change in AMPAR rectification following cocaine (e.g., Dong et al., 2004; Faleiro et al., 2004), which may be due to technical differences in experimental conditions (Kauer and Malenka, 2007). However, there is also controversy over whether a switch in subunit composition of AMPARs is required for NMDAR-dependent LTP in other regions (Adesnik and Nicoll, 2007; Kopec et al., 2007).

6.1.2.2. NMDAR transmission

One limitation to the hypothesis that cocaine-evoked potentiation involves a partial exchange of GluR2-containing for rectifying GluR2-lacking AMPARs, is that given that these AMPARs pass minimal current at positive potentials, this would not account for the increase observed in the AMPAR:NMDAR ratio measured at +40 mV (even in the presence of spermine; Mameli et al., 2007; Mameli et al., 2009; Good and Lupica, 2010; Lammel et al., 2011; Mameli et al., 2011). However, a solution to this conundrum was recently provided through the demonstration that cocaine-evoked plasticity is also associated with a reduction in NMDAR transmission (Mameli et al., 2011). This was shown by activating single synapses using 2-photon laser photolysis of caged glutamate, which revealed a reduction in NMDAR current at the same synapses that showed strong-rectification of the AMPAR current, suggesting synapse-specific modification by cocaine (Mameli et al., 2011). This change in NMDAR transmission had not been previously identified

using conventional methods of bath application of exogenous NMDA (Ungless et al., 2001; Borgland et al., 2004), probably due to its non-specific action on both synaptic and extrasynaptic NMDARs, which precluded detection of a selective change in the synaptic population of receptors (Kullmann et al., 1999; Mameli et al., 2011).

6.1.3. Plasticity induced by other addictive drugs

A switch in the subunit composition of AMPARs, and a reduction in NMDAR current, is thought to be integral to the expression of cocaine-induced synaptic plasticity in VTA dopamine neurons. However, in spite of the observation that many different classes of addictive drug can induce this increase in the AMPAR:NMDAR ratio (Saal et al., 2003), there are both similarities and differences in their mechanism of induction and expression. For example, benzodiazepines have been shown to increase the RI of the AMPAR current (Tan et al., 2010), but increase the frequency of mEPSCs rather than the amplitude (Heikkinen et al., 2009), while nicotine-induced potentiation is associated with a change in the probability of glutamate release, as shown by a reduction in the PPR (Gao et al., 2010). Additionally, potentiation induced by self-administration of cocaine, or a natural reward, is associated with an increase in the frequency, but not amplitude of mEPSCs (Chen et al., 2008). Furthermore, stress-induced potentiation of excitatory synapses in the VTA is NMDAR-dependent but, unlike cocaine-evoked potentiation, it can be blocked by a glucocorticoid receptor antagonist, but not by a D_1-receptor antagonist (Saal et al., 2003; Dong et al., 2004), suggestive of a different induction mechanism.

6.1.4. Expression of plasticity in DRN/vlPAG dopamine neurons

Given that cocaine-induced plasticity in VTA dopamine neurons has been well characterised, I considered whether the potentiation observed in DRN/vlPAG dopamine neurons would exhibit a similar mechanism of expression. As discussed, there are a variety of electrophysiological

protocols which are frequently employed to assay presynaptic and postsynaptic changes at synapses. I have utilized three of these, namely the PPR, RI, and mEPSCs, in order to examine AMPAR transmission in DRN/vlPAG dopamine neurons. I have recorded these measures in mice which have been group-housed, socially isolated, or received a single injection of saline or cocaine (without isolation) 24 hours before brain slice preparation. In this way, I have compared the characteristics of both cocaine-induced and social isolation-induced potentiation at these synapses.

6.2. Materials and methods

6.2.1. Injection and social isolation protocol

TH-GFP or Pitx3-GFP mice (1-4 months) received a single dose of cocaine (15 mg/kg), or the equivalent volume of 0.9% saline, administered via intraperitoneal (IP) injection at a volume of 10 ml/kg. Injections were performed at 10.00 am, and the injected mouse was subsequently either transferred to a clean cage alone for 24 hours, or returned to the original cage, before brain slice preparation. For social isolation, all cagemates were removed at 10.00 am to leave a single mouse alone in its original cage for 24 hours before brain slice preparation. All injections were carried out by Piotr Czapski.

6.2.2. Electrophysiology

Coronal or horizontal 220 µm brain slices containing the DRN/vlPAG or VTA, respectively, were prepared and maintained as described in chapter 3. Whole-cell patch-clamp recordings were performed as described in chapter 4, using the cesium-based internal (composition in mM: $CsCH_3SO_3$ 130**,** NaCl 2.8, HEPES 20, EGTA 0.4, TEA-Cl 5, MgATP 2, Li-GTP 0.5, and 0.1% neurobiotin; pH 7.3, osmolarity 280-290 mosmol/l), and with 100 µM picrotoxin in the aCSF throughout the experiments. EPSCs were evoked using a bipolar stimulating electrode (FHC,

USA) placed 100-300 µm ventrolateral recorded neuron. Stimulus intensity was controlled using an ISO-flex stimulus isolator (AMPI, Jerusalem, Israel) and adjusted to evoke monosynaptic events, typically using a stimulus intensity of 10-40 V, 500 µs duration. To measure the PPR, two afferent stimuli were delivered with an inter-stimulus interval of 50 ms while holding the cell at -70 mV in voltage-clamp. The PPR was calculated by dividing the average peak amplitude of the EPSC generated by the second stimulus by the average peak amplitude generated by the first. When recording rectification indices (RIs) 0.1 mM spermine was included in the internal solution. To calculate the RI, 50 µM AP5 was added to the aCSF, and the AMPAR current recorded while holding the neuron at -70 mV, 0 mV, and +40 mV. The RI was calculated by dividing the average peak of the AMPAR current recorded at -70 mV by the average peak recorded at +40 mV. AMPAR:NMDAR ratios were recorded at +40 mV as described in chapter 4. The decay kinetics of the AMPAR-mediated and NMDAR-mediated current recorded at +40 mV were determined by fitting the decay phase of the current with an exponential curve using Spike 2 (Cambridge Electronic Design, UK). The AMPAR current was fitted with a single exponential function and the NMDAR-mediated current was fitted with a double exponential function. Miniature (m)EPSCs were recorded for at least 3 min while holding the neuron at -70 mV, in the presence of 1 µM tetradotoxin citrate (TTX). MiniAnalysis 6.0.7 (Synaptosoft, Fort Lee, NJ) was used to detect and analyse mEPSCs with the threshold set at 7 pA, and events subsequently verified by eye to confirm fast rise (<3 ms) and exponential decay kinetics.

6.2.3. Statistics

Statistical analysis was performed using Prism 5.04 (Graphpad, CA, USA) and values are reported as mean ± standard error of the mean (SEM). PPRs, RIs, and mean values for mEPSCs were compared using a one-way ANOVA with Newman-Keuls multiple comparison tests, and

cumulative frequency distributions of mEPSCs were compared using a two-way ANOVA, with Newman-Keuls multiple comparison tests.

6.3. Results

As in chapter 5, data collected from all GFP+ neurons in TH-GFP and Pitx3-GFP mice has been pooled, unless otherwise stated. The mean age for each experimental group in the DRN/vlPAG was 8.4 weeks (naive), 6.9 weeks (socially isolated), 7.4 weeks (saline), 9.4 weeks (cocaine), and 6.4 weeks (cocaine with isolation).

6.3.1. Paired-pulse ratio (PPR)

I calculated the PPR from the peak of the AMPAR current response to paired-pulse stimulation at -70 mV, in the presence of picrotoxin. Individual PPRs in the DRN/vlPAG tended towards either slight depression or slight facilitation, and so the mean was generally around 1. I found no significant difference in the PPR between naïve (1.06 ± 0.09, n = 15), socially isolated (1.35 ± 0.21, n = 6), saline-treated (0.908 ± 0.15, n = 11) or cocaine-treated mice (1.07 ± 0.08, n = 13; one-way ANOVA: $F_{13,43} = 1.81$, p = 0.16; Fig. 16). This suggests that the probability of glutamate release is similar in all four groups.

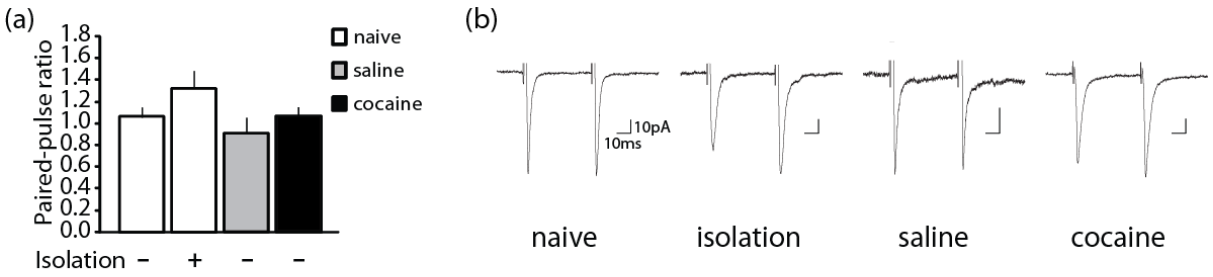


Figure 16. AMPAR PPR in DRN/vlPAG dopamine neurons is unchanged following social isolation or a single dose of cocaine. (a) Bar chart (mean + SEM) and (b) representative examples of the PPR in naïve, socially isolated, saline-, and cocaine-treated mice.

6.3.2. AMPAR currents recorded in the presence of spermine

AMPAR-mediated current was evoked at holding potentials of -70 mV, 0 mV, and +40 mV, in order to calculate the RI. The RI values recorded in DRN/vlPAG GFP+ neurons in naïve mice were generally slightly lower (1.61 ± 0.26, n = 9) than previously reported values for the VTA (∼2; Bellone and Lüscher 2006; Mameli et al., 2009; Tan et al., 2010). This suggests that perhaps the basal subunit composition of the AMPARs in the DRN/vlPAG may differ slightly from the VTA, or this may be a result of the coronal (as opposed to horizontal) slice preparation and the stimulation of different afferent inputs. Following a dose of cocaine in the VTA, the RI has been shown to increase to around 3.0-3.5 in mice (Bellone and Lüscher, 2006; Mameli et al., 2009). I found that either a single dose of cocaine (2.5 ± 0.13, n = 8), or 24 hours of social isolation (2.2 ± 0.19, n = 10) caused a significant increase in the RI (one-way ANOVA: $F_{3,31} = 6.05$, $p < 0.01$), compared to either naïve mice (1.61 ± 0.26, n = 9; naïve vs cocaine: $p < 0.01$; naïve vs isolation: $p < 0.05$) or those which had received a single dose of saline (1.55 ± 0.07, n = 8; saline vs cocaine: $p < 0.01$; saline vs isolation: $p < 0.05$; Fig. 17a,b). This is suggestive of an increased presence of GluR2-lacking receptors, which are inhibited by intracellular polyamines at positive potentials, resulting in positive rectification. Furthermore, there was no significant difference between the RI in naïve and saline-injected mice ($p > 0.05$) or between socially isolated and cocaine-treated mice ($p > 0.05$), suggesting that both social isolation and cocaine induce similar modifications to the AMPAR subunit composition.

Interestingly, although the PPR is routinely used to indicate a presynaptic change at a synapse, under certain conditions it can also indicate a postsynaptic change. In particular, the postsynaptic expression of the GluR2 subunit of the AMPAR can influence the degree of paired-pulse facilitation (Rozov and Burnashev, 1999). This is because intracellular polyamines can partially block GluR2-lacking AMPARs at resting membrane potentials (Bowie and Mayer, 1995).

However, opening of the receptor (i.e. stimulation-evoked synaptic current) can temporarily relieve this blockade, which results in a greater response to subsequent stimulation (Rozov et al., 1998; Rozov and Burnashev, 1999; Armstrong and MacVicar 2001; Bagal et al., 2005). This, therefore, affects short-term plasticity at the synapse, by promoting paired-pulse facilitation at GluR2-lacking but not GluR2-containing synapses (Liu and Zukin, 2007; Savtchouk and Liu, 2011). I therefore analysed PPRs recorded in the presence of spermine, and found a non-significant trend (one-way ANOVA: $F_{3,43} = 2.05$, $p = 0.12$) towards an increase in the ratio following either a single dose of cocaine (1.50 ± 0.21, n = 11), or 24 hours of social isolation (1.38 ± 0.16, n = 13), compared to either saline-injected (1.09 ± 0.10, n = 16) or naïve mice (0.99 ± 0.13, n = 7; Fig. 17c,d). Although this was not statistically significant, the direction of the trend is consistent with what would be expected at synapses where there has been a switch from GluR2-containing to GluR2-lacking receptors (Rozov and Burnashev, 1999). It is likely that utilising a smaller inter-stimulus interval of, for example 10 ms (Savtchouk and Liu, 2011), may be more suitable to reveal this difference, as there would be less time for intracellular polyamines to re-establish their blockade of the receptors. Additionally, the difference may be small and harder to detect than a change in rectification, but increasing the sample size for this protocol would be an important part of future work.

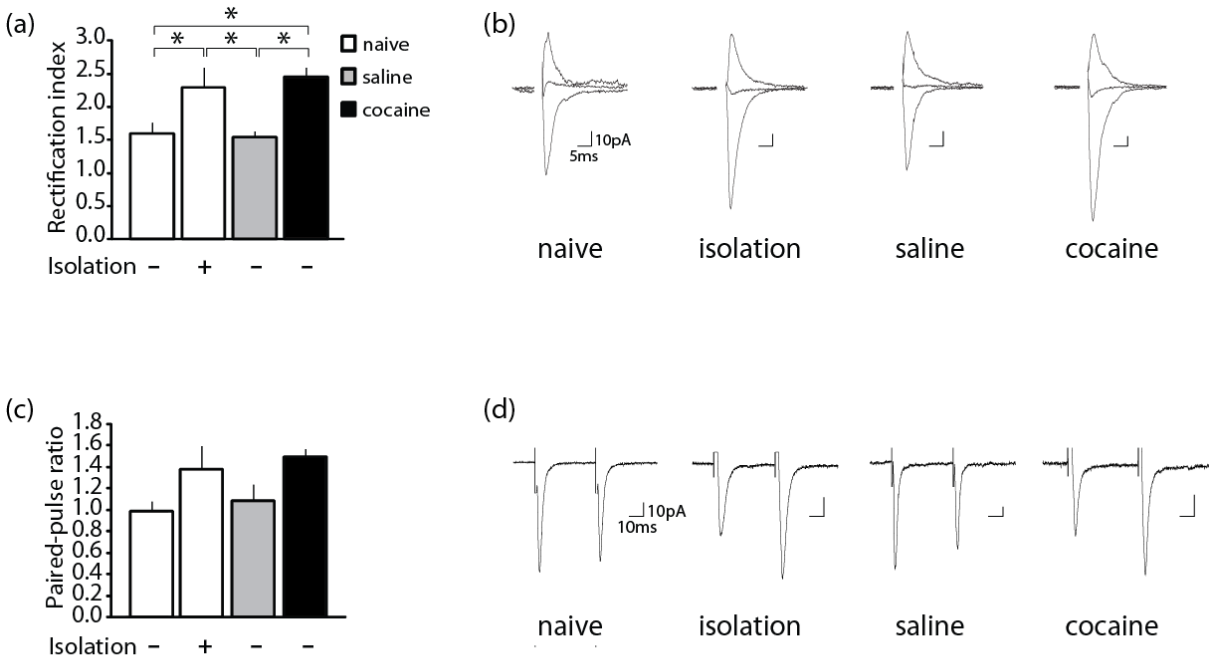


Figure 17. AMPAR rectification index (RI) is increased, and the PPR measured with polyamine blockade shows a trend towards facilitation, following social isolation or a single dose of cocaine. Bar chart (mean + SEM) and representative examples of (a,b) AMPAR RI and (c,d) the PPR in naïve, socially isolated, saline-, and cocaine-treated mice. * indicates $p < 0.05$.

6.3.3. Decay kinetics of evoked AMPAR and NMDAR current

I next examined the decay kinetics of the AMPAR current at +40 mV and -70 mV, and the NMDAR current at +40 mV, by fitting the decay to one or two exponentials for the AMPAR-mediated and NMDAR-mediated current, respectively. There was no significant difference in the weighted time constant (τ_W) of the NMDAR decay (one-way ANOVA: $F_{3,30}$ = 0.52, p = 0.67) between the four groups (naïve: 110.8 ± 16.7 ms, n = 12; isolation: 86.67 ± 15.0 ms, n = 6; saline: 122.9 ± 13.8 ms, n = 8; cocaine: 110.0 ± 27.3 ms, n = 8; Fig. 18a), or the AMPAR decay time constant measured at -70 mV (naïve: 2.53 ± 0.16 ms, n = 9; isolation: 3.08 ± 0.31 ms, n = 10; saline: 3.00 ± 0.32 ms, n = 9; cocaine: 2.92 ± 0.31 ms, n = 8; : $F_{3,32}$ = 0.74, p = 0.54; Fig. 18b). Additionally there was no difference in the decay time constant of the AMPAR current at +40 mV measured either with spermine (naïve: 3.60 ± 0.37 ms, n = 9; isolation: 3.71 ± 0.22ms, n = 9; saline: 4.27 ± 0.50 ms, n = 9; cocaine: 3.34 ± 0.23 ms, n = 8; $F_{3,31}$ = 1.20, p = 0.33; Fig. 18c) or without (naïve: 3.14 ± 0.27 ms, n = 15; isolation: 2.84 ± 0.42 ms, n = 7; saline: 2.65 ± 0.25 ms, n = 8; cocaine: 2.72 ± 0.35ms, n = 8; $F_{3,34}$ = 0.58, p = 0.64; Fig. 18d).

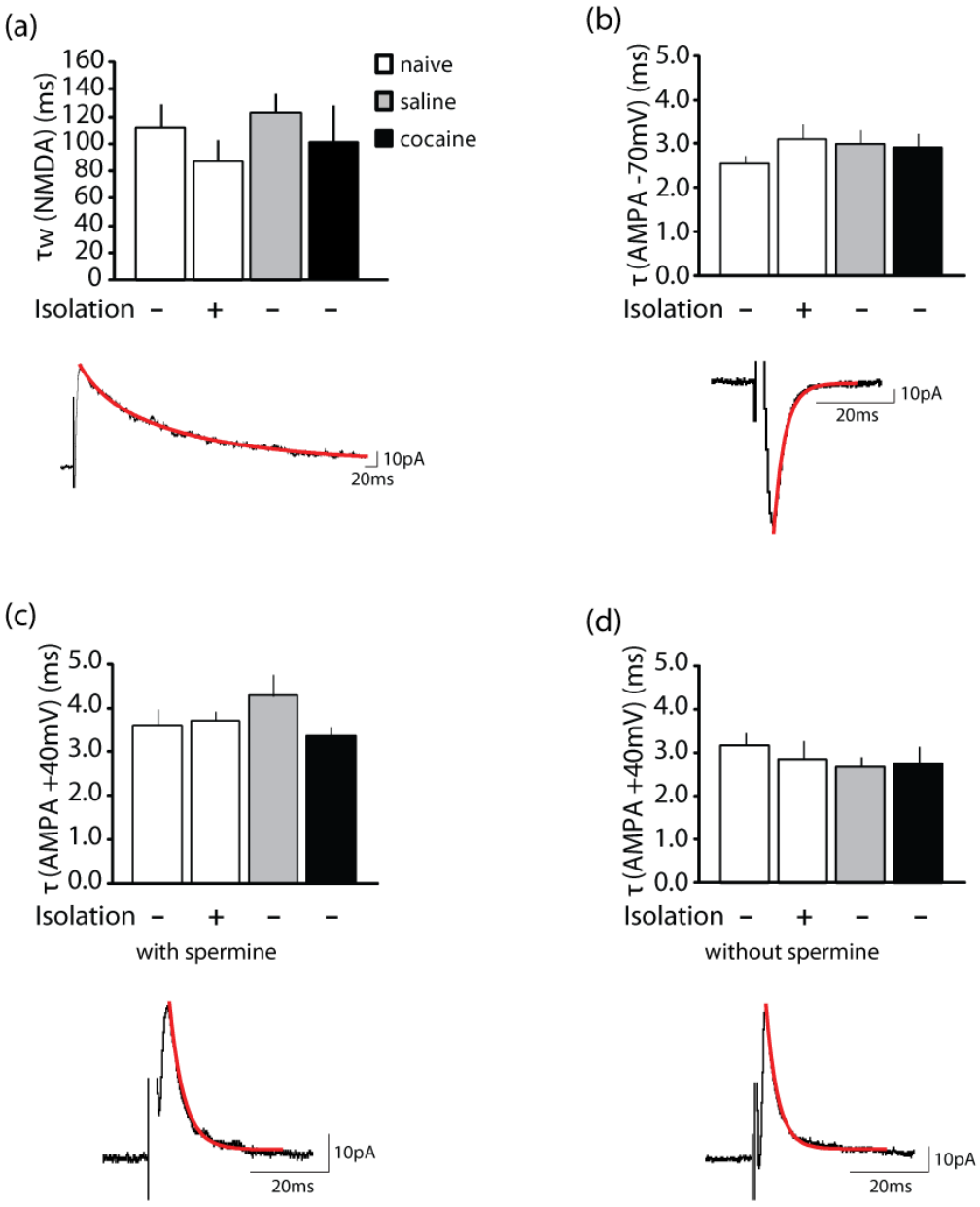


Figure 18. Decay kinetics of NMDAR and AMPAR current in DRN/vlPAG dopamine neurons following social isolation or a single dose of cocaine revealed no major differences. Bar charts (mean + SEM) and example traces with exponential fit (red line) showing the weighted decay time constant (τ_W) of the NMDAR current at +40 mV, (b) the decay time constant (τ) of the AMPAR current at -70 mV, and (c) the AMPAR current at +40 mV with spermine and (d) without spermine.

6.3.4. mEPSC amplitude and frequency

AMPAR-mediated mEPSCs were recorded for typically 3-5 min at -70 mV in the presence of TTX, and events ≥7 pA detected for inclusion in the analysis. For each experimental group the mean amplitude and frequency is reported in bar charts (using an average from each recorded cell), and also the distribution of the events in a cumulative frequency graph. There was considerable variability in the frequency of recorded mEPSCs, from 0.023 Hz to 7.068 Hz, and so using all the recorded events to produce the cumulative frequency distribution would have caused significant bias towards cells with high event frequency. Equally, randomly selecting, for example 50 events per cell, may not have accurately represented the sample of events in these cells. Therefore to evaluate mEPSCs in the most accurate and unbiased manner, I calculated a cumulative frequency distribution for each recorded cell, and averaged these to give the final distribution of events.

6.3.4.1. Effect of cocaine on mEPSCs in the VTA

In the VTA, I observed a trend towards an increase in mEPSC amplitude following a single dose of cocaine (15.29 ± 1.25 pA, n = 13) compared to saline (13.13 ± 0.59 pA, n = 14; Fig. 19a), similar to previous reports (Ungless et al., 2001). This did not reach statistical significance using a t-test on the mean amplitudes (t_{25} = 1.62, p = 0.12; Fig. 19b), but the cumulative distribution of mEPSC amplitude showed a clear shift to the right (Fig. 19c), indicative of a greater proportion of larger amplitude events. A two-way ANOVA revealed a significant main effect of treatment (interaction between treatment and bin amplitude, $F_{22,575}$ = 1.41, p = 0.10; main effect of treatment, $F_{1,575}$ = 12.45, p < 0.0005; main effect of bin amplitude, $F_{22,575}$ = 540.99, p < 0.0001) with a significant difference between 5-15 pA events (p < 0.001). Additionally, I found no significant difference in the frequency of mEPSCs between cocaine-treated (1.15 ± 0.43 Hz, n = 13) and saline-treated (1.09 ± 0.25 Hz) mice (t_{25} = 0.13, p = 0.90; Fig. 19d,e). This contrasts to the

finding in Ungless et al. that both amplitude and frequency increase following a dose of cocaine, but this may have been a result of the high sucrose used for recording these events in this study. These recordings in the VTA, however, are indicative of an increase in the amplitude of AMPAR-mediated events, without a change in presynaptic glutamate release, which is consistent with an increase in AMPAR transmission (Lüscher and Malenka, 2011).

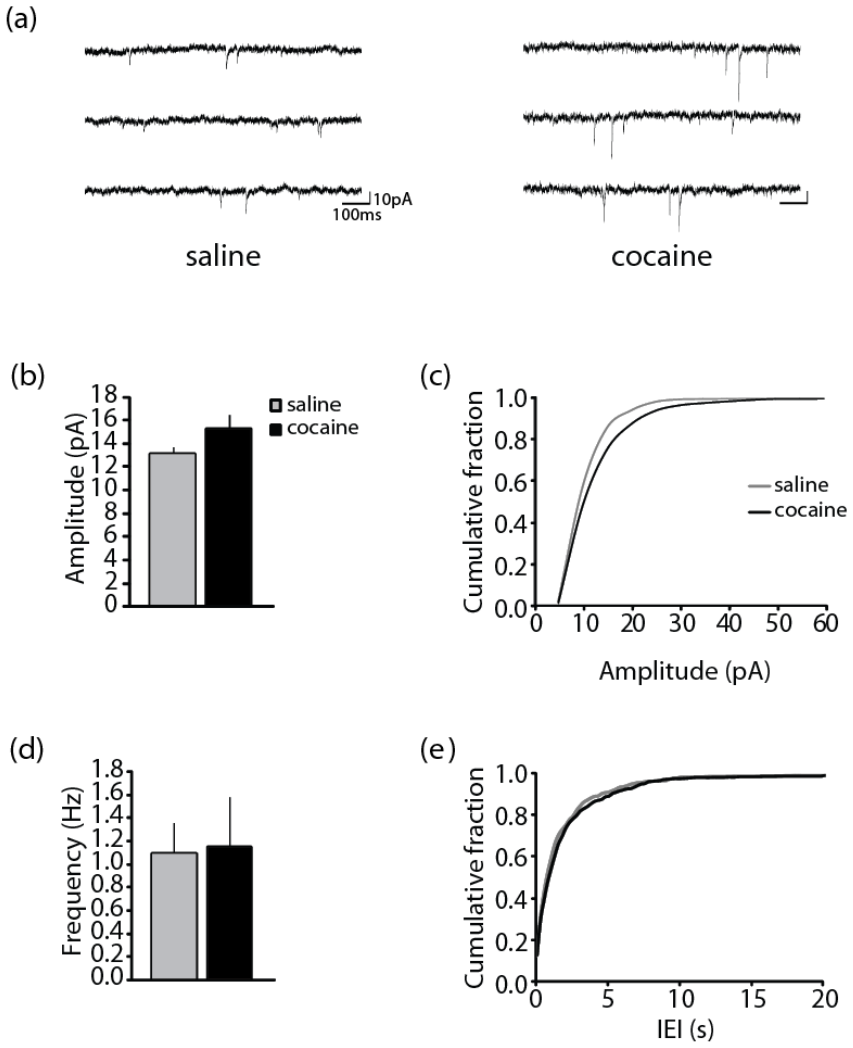


Figure 19. Increase in mEPSC amplitude, but not frequency, in VTA dopamine neurons following a single dose of cocaine. (a) Representative examples of mEPSCs from a saline-treated and a cocaine-treated mouse. (c,d) Bar charts (mean + SEM) and (e,f) cumulative frequency graphs showing mEPSC amplitude, frequency, and inter-event interval (IEI). A two-way ANOVA revealed a significant main effect of treatment on mEPSC amplitude ($p < 0.05$). The mean age for each experimental group was 4.5 weeks (saline) and 4.5 weeks (cocaine).

6.3.4.2. Effect of social isolation or cocaine on mEPSCs in the DRN/vlPAG

In the DRN/vlPAG, I found that there was no significant difference in the mean amplitude of mEPSCs in the four experimental groups (naïve: 19.17 ± 1.11 pA, n = 21; isolated: 22.13 ± 1.60 pA, n = 18; saline: 19.86 ± 0.80 pA, n = 12; cocaine: 21.69 ± 1.06 pA, n = 17; $F_{3,64} = 1.40$, $p = 0.25$; Fig. 20a,b), but again a clear shift towards larger amplitude events was revealed in the cumulative frequency distribution for both mice which had been socially isolated for 24 hours and those which had received a single dose of cocaine (Fig. 20c). A two-way ANOVA found a significant effect of group (interaction between treatment and bin amplitude, $F_{102,2275} = 1.12$, $p = 0.19$; main effect of treatment, $F_{3,2275} = 9.84$, $p < 0.0001$; main effect of bin amplitude, $F_{34,2275} = 739.80$, $p < 0.0001$), with post-hoc tests revealing a significant difference between cocaine-injected and naïve mice at 10-25 pA events ($p < 0.05$) and between cocaine-injected and saline-injected mice at 5-20 pA events ($p < 0.05$). Similarly there was a significant difference between socially isolated and naïve mice at 10-25 pA events ($p < 0.05$) and socially isolated and saline-injected mice at 5-20 pA events ($p < 0.05$). However, there was no significant difference between naïve and saline-injected mice, or between cocaine-injected and socially isolated mice, at any amplitude level. This shift in mEPSC amplitude distribution indicates an increase in AMPAR number or function, which may result from an increase in receptor conductance, as suggested by the RI data.

Interestingly, there was a significant difference in the mean frequency of mEPSCs ($F_{3,64} = 3.02$, $p < 0.05$), which reached statistical significance in post-hoc comparisons between naïve (0.83 ± 0.26 Hz, n = 21) and cocaine-treated mice (2.31 ± 0.56 Hz, n = 17, $p < 0.05$), but not for saline-treated (0.91 ± 0.30 Hz, n = 12, $p > 0.05$), or socially isolated mice (1.17 ± 0.39 Hz, n = 18, $p > 0.05$; Fig. 20d). The cumulative frequency distribution of inter-event intervals (IEIs) showed a clear shift towards smaller IEIs in cocaine-treated mice, with saline-treated and socially isolated

mice displaying an intermediate distribution between naïve and cocaine-treated mice (Fig. 20e). This suggests that a single dose of cocaine or social isolation appear to affect the amplitude of mEPSCs to a similar extent, but only cocaine has a robust effect on event frequency.

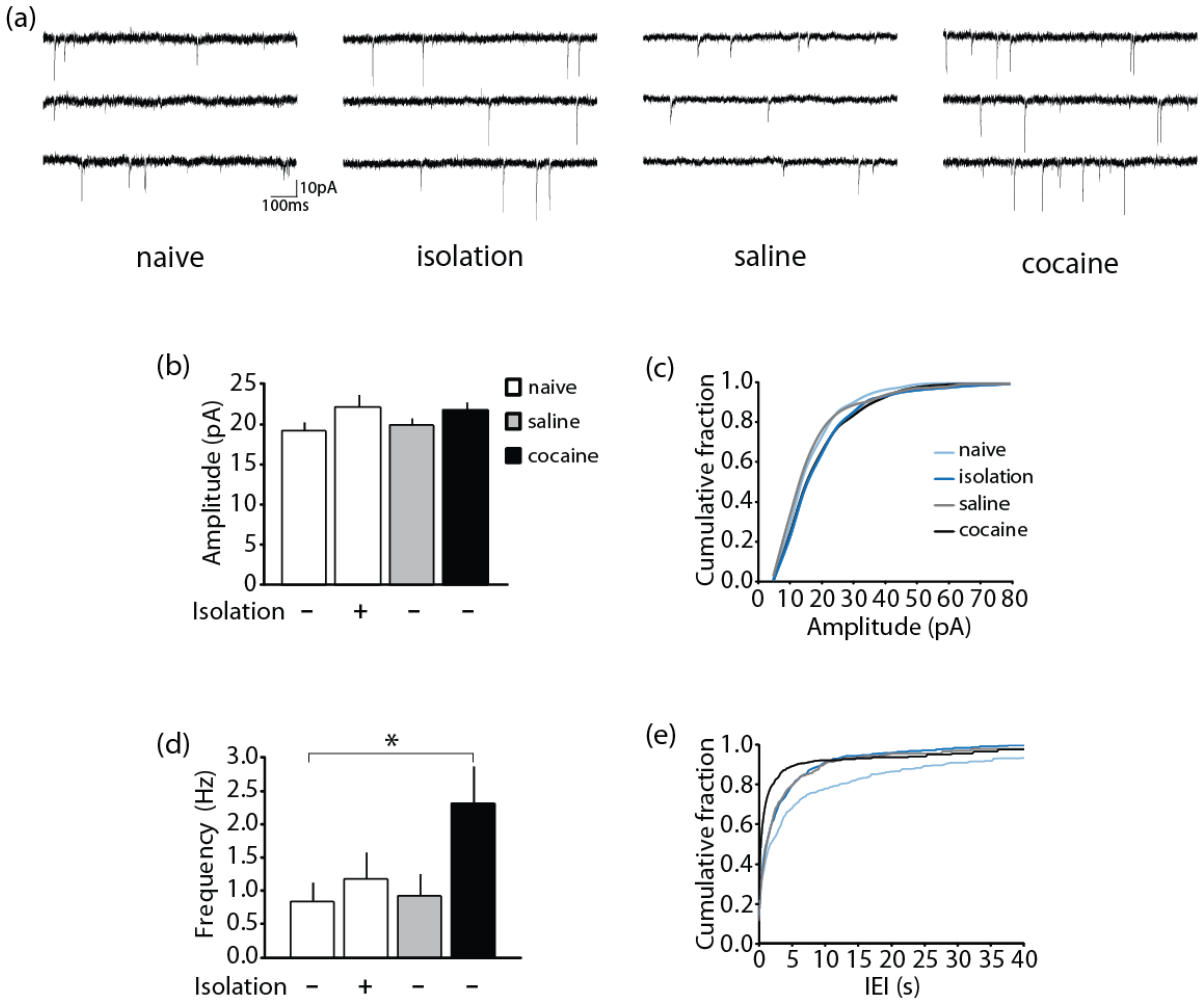


Figure 20. Social isolation or a single dose of cocaine increases mEPSC amplitude in DRN/vlPAG dopamine neurons, but only cocaine increases the frequency. (a) Representative examples of mEPSCs, (b,d) bar charts (mean + SEM), and (c,e) cumulative frequency graphs showing mEPSC amplitude, frequency, and IEI, in naïve, socially isolated, saline-, and cocaine-treated mice. A two-way ANOVA revealed a significant main effect of treatment on mEPSC amplitude ($p < 0.05$). * indicates $p < 0.05$.

6.3.4.3.Cocaine in the presence or absence of social isolation

I also examined mEPSCs in mice which received cocaine with social isolation, and mice which received cocaine without social isolation. I found no difference in the mean mEPSC amplitude (cocaine with isolation: 23.49 ± 2.84 pA, n = 12; cocaine without isolation: 21.69 ± 1.06 pA, n = 17, t_{27} = 0.67, p = 0.51; Fig. 21a) or the cumulative distribution of events (interaction between treatment and bin amplitude, $F_{27,756}$ = 0.18, p = 1.00; main effect of treatment, $F_{1,756}$ = 3.84, p = 0.06; main effect of bin amplitude, $F_{27,756}$ = 211.40, p < 0.0001; Fig. 21b). There was also no difference in mEPSC frequency (cocaine with isolation: 1.86 ± 0.40 Hz, n = 12; cocaine without isolation: 2.32 ± 0.56 Hz, n = 17; t_{26} = 0.59, p = 0.56; Fig. 21c,d) between these two groups. This suggests (consistent with the AMPAR:NMDAR ratios) that there is no additive effect of cocaine and social isolation at these synapses.

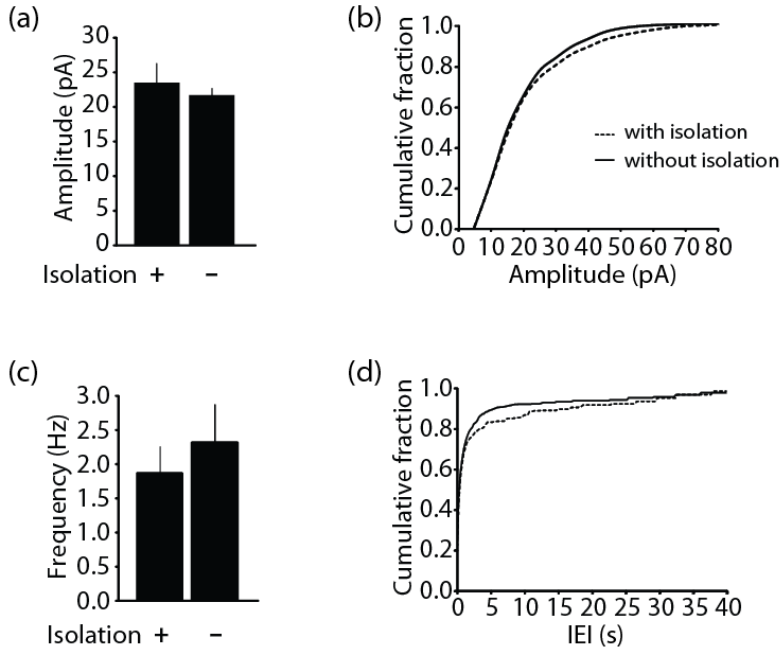


Figure 21. Cocaine with social isolation has the same effect on mEPSC amplitude and frequency as cocaine without social isolation. (a,b) Bar charts (mean + SEM) and (c,d) cumulative frequency graphs showing mEPSC amplitude, frequency, and IEI.

6.3.5. Characteristics of AMPAR current in identified VIP+ and VIP- neurons

Similar to the recordings in chapter 5, I found a general bias towards recording from VIP- neurons, and the longer-duration recordings often precluded post-hoc identification of recorded neurons. However, for many of the protocols I recorded from a sufficient number of identified neurons to confirm the characteristics of VIP- neurons, and in some cases (using pooled data) also VIP+ neurons.

6.3.5.1. PPR

Interestingly, under these recording conditions (cesium-based internal and picrotoxin in the aCSF, compared to potassium gluconate-based internal and the absence of picrotoxin in chapter 4) I found no difference in the PPR between VIP+ and VIP- neurons (VIP+: 1.10 ± 0.17, n = 5; VIP-: 1.11 ± 0.15, n = 7; t_{10} = 0.03, p = 0.98). This suggests that perhaps this reflected differences in $GABA_AR$-mediated modulation of AMPAR transmission or glutamate release. There was also no difference in the PPR in VIP- neurons between naïve (1.11 ± 0.15, n = 7), socially isolated (1.40 ± 0.33, n = 4), saline-treated (0.90 ± 0.16, n = 10) or cocaine-treated mice (1.03 ± 0.16, n = 6; $F_{3,23}$ = 1.06, p = 0.39; Fig. 22a), indicating that there is no change in presynaptic glutamate release in the VIP- subpopulation.

6.3.5.2. AMPAR current in the presence of spermine

The RI in identified VIP- neurons displayed a similar trend to the whole population sample, with an increase following social isolation (2.18 ± 0.15, n = 7) or a single dose of cocaine (2.60 ± 0.15, n = 3), compared to naïve (1.49 ± 0.26, n = 7) or saline-treated mice (1.41 ± 0.12, n = 3; one-way ANOVA: $F_{3,16}$ = 5.31, p < 0.01; Fig. 22b), but this only reached statistical significance for the post-hoc comparison between cocaine-treated mice and naive (p < 0.05) or saline-treated mice (p < 0.05). Furthermore, the same trend towards paired-pulse facilitation was displayed by cocaine-

injected (1.34 ± 0.29, n = 4) and socially isolated mice (1.41 ± 0.32, n = 6) in identified VIP-neurons, when recorded in the presence of spermine, in comparison to naïve (0.92 ± 0.06, n = 4) or saline-treated mice (0.97 ± 0.22, n = 6; Fig. 22c). However, as with the result from all recorded GFP+ cells, this was not overall statistically significant ($F_{3,16}$ = 0.91, p = 0.46). Some of these groups are only made up of a few cells for these protocols, therefore increasing the sample size may provide a more conclusive result.

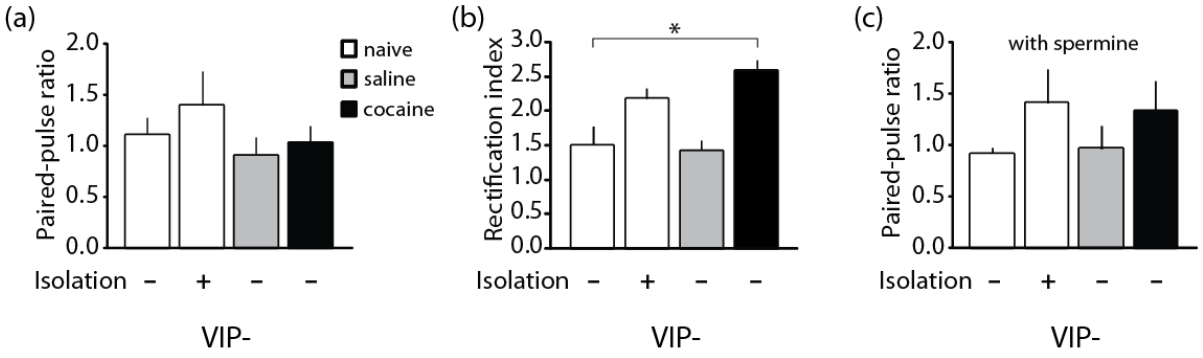


Figure 22. AMPAR current in identified VIP- neurons following social isolation or a single dose of cocaine. Bar charts (mean + SEM) showing (a) PPR, (b) RI, and (c) PPR with polyamine block in naïve, socially isolated, saline-, and cocaine-treated mice. * indicates $p < 0.05$.

6.3.5.3. Decay kinetics of evoked AMPAR and NMDAR current

The τ_W of the NMDAR current decay did not differ significantly in VIP- neurons between the four experimental groups (naïve: 76.54 ± 37.24 ms, n = 4; social isolation: 66.83 ± 12.22 ms, n = 4; saline: 104.92 ± 16.31 ms, n = 5; cocaine: 79.00 ± 18.69 ms, n = 7; $F_{3,16} = 0.52$, p = 0.68; Fig. 23a), similar to results for the whole population. Additionally, identified VIP- neurons showed no significant difference in the AMPAR current decay measured at +40 mV with spermine (naïve: 3.63 ± 0.55 ms, n = 6; social isolation: 3.99 ± 0.24 ms, n = 6; saline: 2.65 ± 0.34 ms, n = 6; cocaine: 2.78 ± 0.40 ms, n = 7; $F_{3,14} = 0.30$, p = 0.82; Fig. 23c) or without (naïve: 3.69 ± 0.52 ms, n = 5; social isolation: 2.31 ± 0.29 ms, n = 4; saline: 3.33 ± 0.74 ms, n = 3; cocaine: 3.55 ± 0.50 ms, n = 3; $F_{3,18} = 1.77$, p = 0.19; Fig. 23d), or measured at -70 mV (naïve: 2.47 ± 0.19 ms, n = 7; social isolation: 3.28 ± 0.38 ms, n = 7; saline: 2.76 ± 0.57 ms, n = 3; cocaine: 2.50ms ± 0.35 ms, n = 3; $F_{3,16.} = 1.39$, p = 0.28; Fig. 23b).

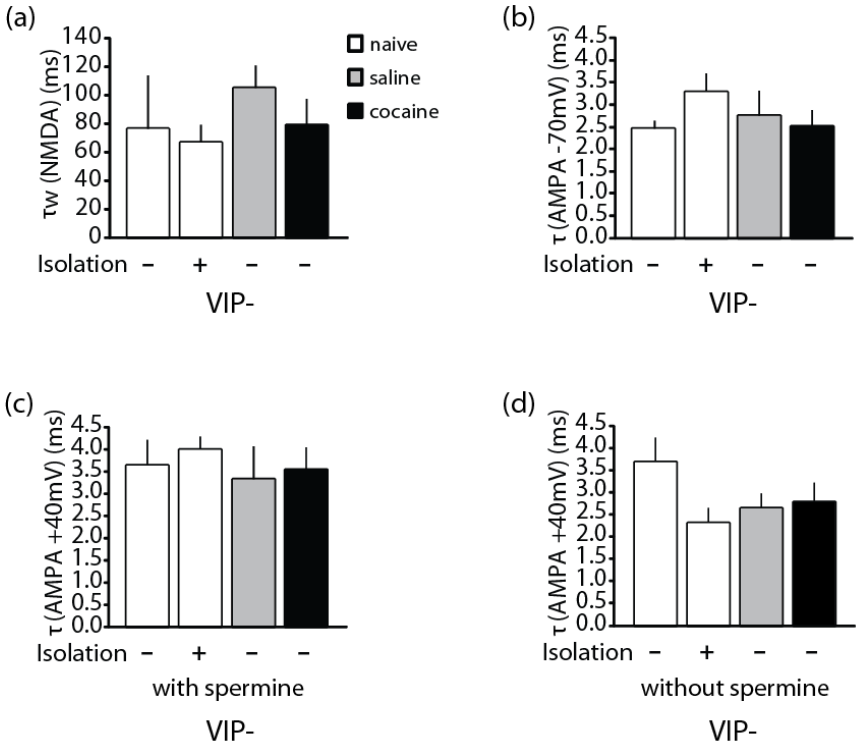


Figure 23. NMDAR and AMPAR current decay in identified VIP- neurons revealed no significant changes following social isolation or a single dose of cocaine. (a) Weighted decay time constant (τ_W) of the NMDAR current at +40 mV, (b) the decay time constant (τ) of the AMPAR current at -70 mV, and (c) the AMPAR current at +40 mV with spermine and (d) without spermine.

6.3.5.4.mEPSC amplitude and frequency

I pooled mEPSCs from all groups of socially isolated and group-housed mice in order to compare VIP+ and VIP- neurons in these two conditions. I found that in socially isolated mice both VIP+ and VIP- neurons showed a strong trend towards larger mEPSC amplitudes (VIP+: 22.99 ± 2.12 pA, n = 12; VIP-: 22.16 ± 0.88 pA, n = 20; Fig. 24a,c) and higher frequency (VIP+: 1.43 ± 0.39 Hz, n = 12; VIP-: 2.07 ± 0.44 Hz, n = 20; Fig 24b,d), in comparison to group-housed mice (amplitude – VIP+: 19.48 ± 1.03 pA, n = 19, t_{29} = 1.66, p = 0.11; VIP-: 18.87 ± 1.39 pA, n = 11, t_{29} = 2.10, p < 0.05; frequency – VIP+: 0.62 ± 0.23Hz, n = 19, t_{29} = 1.94, p = 0.06; VIP-: 1.28 ± 0.46Hz, n = 11, t_{29} = 1.16, p = 0.26), but this was only statistically significant for the amplitude in VIP- neurons. This suggests that social isolation may potentiate the AMPAR-mediated current in both neuronal subtypes, but perhaps to a greater extent in VIP- neurons. Additionally, I did not observe a significant difference in either amplitude or frequency of mEPSCs between VIP+ and VIP- neurons for either the pooled group-housed mice (amplitude: t_{28} = 0.35, p = 0.73; frequency: t_{28} = 1.45, p = 0.16), or solely the naïve mice (amplitude: t_{13} = 0.35 p = 0.73; frequency: t_{13} = 0.83, p = 0.42). Similar to the PPR, it appears that under these conditions, as opposed to those in chapter 4, there are no detectable differences in glutamatergic currents between VIP+ and VIP- neurons.

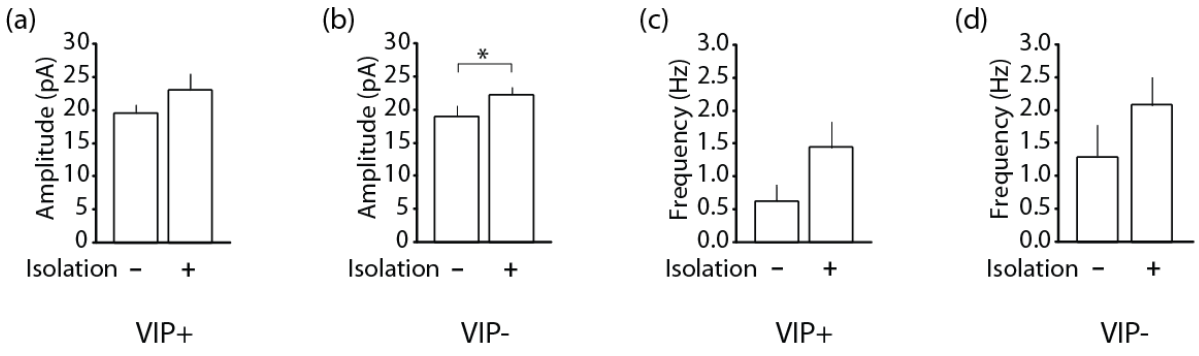


Figure 24. Social isolation increases mEPSC amplitude in VIP- neurons. Bar charts (mean + SEM) showing (a,b) mEPSC amplitude and (c,d) frequency in identified VIP+ and VIP- neurons. * indicates $p < 0.05$.

6.4. Conclusion

I have utilised several electrophysiological protocols to examine different properties of AMPAR and NMDAR transmission in DRN/vlPAG dopamine neurons. These measures can be used to determine the locus of a change in synaptic efficacy, such as that observed in these neurons following a single dose of cocaine or social isolation. I therefore focused mainly on examining these measures of synaptic strength in four groups of mice: naïve, socially isolated, saline-treated, and cocaine-treated.

Overall, these results indicate that cocaine and social isolation induce similar changes at these excitatory synapses, which, importantly, also resemble the changes seen following cocaine in the VTA. Specifically, I observed an increase in the RI of the AMPAR current, without a change in the PPR, suggesting a postsynaptic, rather than presynaptic, modification at the synapse. Additionally, the PPR in the presence of spermine, showed a trend towards paired-pulse facilitation, only in cocaine-treated and socially isolated mice, which supports the notion that there is an increased insertion of GluR2-lacking receptors at these synapses. Although mEPSCs did not yield a clear result, they tended towards larger amplitudes in cocaine-treated and socially isolated mice, which is consistent with what would be expected from this change in AMPAR subunit composition.

It has been noted that a large number of cells are often required to reveal differences in the characteristics of mEPSCs, and the lack of robust differences observed here may be due to the relatively small sample size. Indeed in the VTA, a considerable increase in the AMPAR:NMDAR ratio is observed following cocaine, but this is accompanied by a change of only a few pA in the mEPSC amplitude (Ungless et al., 2011). Therefore mEPSCs may not be the most appropriate or sensitive measure to use when examining this form of synaptic plasticity. Conversely, it could be that the observed increase in the AMPAR:NMDAR ratio is not a result of a substantial change in

AMPAR number or function, but instead may be mediated by a reduction in NMDAR transmission. This is more difficult to assay in brain slices, and may require analysis at the single synapse resolution, as in Mameli et al. (2011), which could be usefully investigated in future studies.

The AMPAR and NMDAR current decay kinetics can be used to demonstrate changes in the receptor subunit composition, as incorporation of certain subunits can confer different properties on the receptor. No significant difference was found between any of the groups examined in terms of the decay time constant of these currents, suggesting that perhaps there is no change in kinetics of the current through these receptors. However, equally, this may not be the most sensitive method for detecting a clear change. Furthermore, the NMDAR current was produced by digitally subtracting the AMPAR current from the mixed response, but analysing the pure recorded current might yield a more reliable result.

The basal AMPAR RI in DRN/vlPAG dopamine neurons was generally lower than previously reported values in the VTA, which could be related to a specific feature of the slice preparation, or may indicate a difference in the basal subunit composition of the AMPARs on these two dopamine neuron subpopulations. There is some evidence for the latter interpretation, for example, in the macaque GluR1 and GluR2 are the most abundantly expressed transcripts in both the VTA and DRN/vlPAG, but there is a higher proportion of GluR1 in the midbrain reticular formation (housing the VTA) compared to the DRN/vlPAG, while the DRN/vlPAG also expresses the GluR3-4 subunits (Beneyto and Meador-Woodruff, 2004). Although this is not specific to the dopamine population, it indicates that perhaps a different composition of AMPARs could underlie the difference in rectification observed in DRN/vlPAG compared to VTA dopamine neurons.

Due to the general bias in recording from VIP- neurons, and the difficulty in recovering high numbers of labelled, recorded neurons, I was not able to determine every feature of AMPAR and NMDAR transmission in both VIP+ and VIP- neurons. However, I was able to confirm that the RI, PPR, and current decay showed the same result for both cocaine and social isolation when only identified VIP- neurons were included in the analysis. Additionally, using pooled mEPSC data, I found that both VIP+ and VIP- neurons appeared to show a trend towards a greater amplitude and frequency of events in socially isolated mice, although perhaps to a lesser extent in VIP+ neurons. Furthermore, under these recording conditions, I did not observe a difference between VIP+ and VIP- neurons in terms of the PPR or mEPSC characteristics, suggesting that perhaps the differences I observed in chapter 4 were mediated by a $GABA_A$-receptor mechanism, or were a result of the different internal solution. It would be interesting to confirm the effect of social isolation and cocaine on VIP+ neurons, as these neurons are likely to be providing a significant dopaminergic (and VIPergic) input to the dlBNST. Furthermore, the type of postsynaptic change that my results suggest is likely to have consequences for the excitability of these dopamine neurons, and the processing of synaptic inputs, which would ultimately impact on the output of this neuronal circuit (Liu and Savtchouk, 2011).

Chapter 7. Is the synaptic potentiation in DRN/vlPAG dopamine neurons anxiety-induced?

7.1. Introduction

7.1.1. Consequences of social isolation

Studies on socially isolated animals have revealed multiple behavioural abnormalities, including hyperreactivity to a novel environment (Sahakian et al., 1977; Gentsch et al., 1981; Gentsch et al., 1982; Domeney and Feldon, 1998; Varty et al., 2000; Del Arco et al., 2004), increased aggression and poor social interaction (Harlow et al., 1965; Valzelli, 1973; Wongwitdecha and Marsden, 1996), enhanced response to psychomotor stimulants (Sahakian et al., 1975), and a range of cognitive deficits (Schrijver and Würbel, 2001; Bianchi et al., 2006). Social isolation is therefore frequently used as an animal model for several neuropsychiatric disorders including schizophrenia and depression (Ehlers et al., 1993; Geyer et al., 1993; Jaffe et al., 1993; Myhrer, 1998; Whitaker-Azmitia et al., 2000; Lapiz et al., 2003; Heim et al., 2004; Fone and Porkess, 2008; Wilkinson et al., 2009; Fabricius et al., 2011; Fischer et al., 2012; Moller et al., 2012). Furthermore, a wealth of evidence implicates the dopamine system in mediating these isolation-induced behavioural changes (Guisado et al., 1980; Bean and Lee, 1991; Jones et al., 1992; Rilke et al., 1995; Hall et al., 1998a; Heidbreder et al., 2000; Peters and O'Donnell, 2005; Djouma et al., 2006; Wallace et al., 2009; Fabricius et al., 2010; Han et al., 2011; Han et al., 2012).

7.1.1.1. Response to addictive drugs

One interesting observation is that the psychostimulant effects of cocaine, amphetamine, and ethanol are increased by prior social isolation (Jones et al., 1990a,b; Fowler et al., 1993; Ahmed

et al., 1995; Smith et al., 1997; Hall et al., 1998b; Michel and Tirelli, 2002; Lu et al. 2003). Animals exhibit an increased propensity to self-administer cocaine (Matthews et al., 1999; Howes et al., 2000; Kosten et al., 2000; Zhang et al., 2005), suggestive of enhanced impulsivity or motivation for cocaine (Schenk et al., 1987), and more frequently infuse the drug, suggesting that social isolation also reduces its reinforcing properties (Schenk et al., 1986; LeSage et al., 1999; Ding et al., 2005), similar to other addictive drugs (Alexander et al., 1978; Hadaway et al., 1979; Schenk et al., 1986; Phillips et al., 1994; Wongwitdecha and Marsden, 1995; Wongwitdecha and Marsden, 1996). It has also been hypothesised that isolation rearing may interact with dopamine-dependent mechanisms to modify the response to addictive drugs (Jones et al., 1990b; Broseta et al., 2005; Estelles et al., 2005).

7.1.1.2. Behavioural changes

Isolation rearing has been shown to increase anxiety levels measured, for example, using the elevated-plus maze and open field tests (Hatch et al., 1965; Koch and Arnold, 1972; Sahakian et al., 1977; Morinan and Parker, 1985; Parker and Morinan, 1986; Wright et al., 1991; Bickerdike et al., 1993; Fone et al., 1996; Da Silva et al., 1996; Hellemans et al., 2004; Weiss et al., 2004; Chourbaji et al., 2005; Kwak et al., 2009; Conrad et al., 2011), and causes a greater physiological reaction to stress (Baldwin et al., 1995; Ruis et al., 1999). Furthermore, it has been noted that, in contrast to group-housed animals, socially isolated animals spend significantly less time engaging in social behaviours (when subsequently re-introduced to conspecifics), and display higher levels of aggression (Estelles et al., 2004).

Although social stimuli are innately rewarding (Krach et al., 2010), the lack of social interaction is not the only factor that would be important in determining the emotional and physiological state of an isolated animal. For example, social isolation would also involve an increase in metabolic activity to maintain body temperature, a reduction in the complexity of the

surrounding environment, loss of tactile stimulation, and decreased visual, auditory, gustatory and olfactory stimulation (Bates et al., 1985; Kim and Kirkpatrick 1996). Therefore both social and non-social factors are likely to contribute to the neurobiological repercussions of social isolation.

7.1.1.3.Acute social isolation

The majority of these studies typically isolate animals in the neonatal or adolescent period, for around 2-12 weeks, but the effects of a short period of social isolation in adulthood have not been extensively investigated, with only a handful of studies examining the effect of just 24 hours of social isolation. Furthermore, it has been suggested that the effects of isolation in adulthood can be distinct from isolation during childhood or adolescence (King et al., 2009). For example, isolation in adulthood is not thought to affect the self-administration of cocaine (Bozarth et al., 1989), or ethanol (Schenk et al., 1990), and conversely increases social interaction (Lister and Hilakivi 1988; Ferdman et al., 2007). However, along with the duration of isolation, many other methodological variations are likely to affect the outcome of these isolation studies, including animal species, strain, age, and the frequency of handling (Lapiz et al., 2003; Fone and Porkess, 2008). Therefore contradictory results are common in these types of investigations.

The few studies that have investigated short-term social isolation in adulthood, in a similar manner to this investigation, have revealed interesting deficits. For example, Conrad et al. (2011) report a trend for increased anxiety levels (in the open field test and elevated-plus maze), along with blunting of LTP in the dorsolateral (dl)BNST, following just 24 hours of social isolation. Additionally, it has been reported that 24 hours of isolation produces a significant decrease in striatal dopamine D_2 receptor density, indicating increased synaptic dopamine concentration in this region (Rilke et al., 1995). Therefore even a short period of isolation in

adulthood is likely to have consequences for behaviour, synaptic plasticity, and dopaminergic transmission.

7.1.2. Effect of cocaine on social behaviour and anxiety

Cocaine administration exerts complex actions on many aspects of social behaviour. Although mixed results have been reported with regard to the effect of cocaine on aggressive behaviours in both animals (Miczek and O'Donnell, 1978; Miczek, 1979; Hadfield et al., 1982; Emley and Hutchinson, 1983; Filibeck et al., 1988; Long et al., 1996; Estelles et al., 2004) and humans (Miller et al., 1991; McCormick and Smith, 1995; Bukstein, 1996; Gordon et al., 1996; Denison et al., 1997; Moeller et al., 1997; Dhossche, 1999; LeSage et al., 1999), many studies have noted a reduction in social contacts with conspecifics following cocaine administration (Darmani et al., 1990; Rademacher et al., 2002; Estelles et al., 2004; Estelles et al., 2007). Furthermore, isolation-induced reduction in social contact is further impaired by an acute dose of cocaine, leading to almost a complete absence of social investigation (Estelles et al., 2004). Interestingly, both the dopamine and glutamate systems have been implicated in the effect of cocaine on social behaviour (Lluch et al., 2005). Furthermore, there is general agreement that cocaine increases flight and avoidance (i.e., defensive behaviours), which is specifically expressed in the presence of other conspecifics (Blanchard and Blanchard, 1999; Estelles et al., 2004), and can also elicit an anxiogenic response in various behavioural assays of anxiety (Costall et al., 1989; Rogerio and Takahashi, 1992a,b; Yang et al., 1992; Paine et al., 2002). This can be observed following just a single dose of cocaine, or after chronic exposure during withdrawal (Paine et al., 2002; Hayase et al., 2005).

7.1.3. Is synaptic potentiation in DRN/vlPAG dopamine neurons induced by acute anxiety?

Previous work indicates that cocaine administration and social isolation have in common that they induce a reduction in social interaction and an increase in anxiogenic behaviour. This suggests that these factors may be integral to the effects observed here. I therefore hypothesised that the synaptic potentiation in DRN/vlPAG dopamine neurons was induced by acute anxiety, resulting from either social isolation or a single dose of cocaine. However, previous studies have been performed over varying timescales and examined animals of different ages, with different cocaine dose ranges, and behavioural assays. Therefore as a first step to investigate this hypothesis, the open field test was used to assess anxiety-like behaviour, under the same conditions used for electrophysiological recordings. Secondly, I investigated the effect of a single dose of diazepam on the AMPAR:NMDAR ratio at synapses onto DRN/vlPAG dopamine neurons. Diazepam is an addictive drug, which has been shown to potentiate excitatory synapses onto VTA dopamine neurons following a single dose, in a similar way to cocaine (Heikkinen et al., 2009; Tan et al., 2010). However, diazepam is an anxiolytic compound, and therefore elicits a different behavioural profile to cocaine administration. Thirdly, I tested whether the potentiation observed following social isolation could be prevented by two different compounds: diazepam and ketamine. Diazepam and other benzodiazepines are widely prescribed to treat anxiety disorders (Woods et al., 1992; Gallager and Primus, 1993; Carlini, 2003). Ketamine has more recently been suggested to have anxiolytic and antidepressant-like effects (Berman et al., 2000; Zarate et al., 2006; Machado-Vieira et al., 2009; Li et al., 2010; Vollenweider and Kometer, 2010; Murrough, 2011), and is an NMDAR antagonist (Harrison and Simmonds, 1985). The potentiation observed in DRN/vPAG dopamine neurons bears a certain resemblance to cocaine-induced plasticity in the VTA, which is blocked by systemic administration of an NMDAR antagonist (Ungless et al., 2001). It would also, therefore, be

interesting to determine whether NMDAR blockade can prevent social isolation-induced plasticity.

7.2. Materials and methods

7.2.1. Open field test

The open field arena consisted of a wooden chamber 45 cm x 45 cm, with 30 cm walls, under constant illumination in a sound-attenuated room, which was separate from the room in which the mice were housed. Mice were acclimatised to the new surroundings for at least 20 min, in their own cage, before being placed in the centre of the open field arena. Their activity was recorded using a video camera suspended above the arena, interfaced with a computerised tracking system (HVS Image 2100, Hampton, UK). For analysis, the arena was divided into a grid of 36 squares (7.5 cm x 7.5 cm), and the time spent in each square, the total number of entries into new squares, the % time spent moving, and the total distance travelled were calculated using the HVS Image 2100 software. Subsequent analysis was performed in Excel, and the level of anxiety quantified by calculating the proportion of time spent in the periphery (outer 1125 cm^2) of the arena.

7.2.2. Injection protocol

Diazepam (5 mg/kg), ketamine hydrochloride (10 mg/kg), or the equivalent volume of vehicle, was administered via IP injection in a volume of 10 ml/kg. Mice were injected at 10.00 am and either returned to their original cage, with their cagemates, or moved to a clean cage on their own for 24 hours before brain slice preparation.

7.2.3. Electrophysiology

Coronal 220 µm brain slices containing the DRN/vlPAG were prepared, and whole-cell electrophysiology was used to record AMPAR:NMDAR ratios as described in chapter 4. The cesium-based internal solution (composition in mM: $CsCH_3SO_3$ 130**,** NaCl 2.8, HEPES 20, EGTA 0.4, TEA-Cl 5, MgATP 2, Li-GTP 0.5, and 0.1% neurobiotin; pH 7.3, osmolarity 280-290 mosmol/l) was used for all recordings and 100 µM picrotoxin was included in the aCSF.

7.2.4. Drugs

Diazepam (Sigma, UK) was dissolved by sonication in 0.9% saline with 0.3% Tween-80, and ketamine hydrochloride (Fort Dodge Animal Health, US) was dissolved in 0.9% saline.

7.2.5. Statistics

Statistical analysis was performed using Prism 5.04 (Graphpad, CA, USA) and values are reported as mean ± standard error of the mean (SEM). Behaviour in the open field test was analysed using a one-way ANOVA with Newman-Keuls post-hoc tests, and the Pearson's product-moment correlation coefficient. AMPAR:NMDAR ratios from diazepam or ketamine-treated mice were compared using an unpaired t-test (non-directional).

7.3. Results

7.3.1. Open field test

Mice were socially isolated, or received a single injection of saline or cocaine at 10.00 am, and their behaviour was monitored in the open field test between 9.00 – 11.00 am the following day. I collected around half of the data in the naïve and socially isolated groups, and the remainder was collected by Rebecca Davis. The open field test has been extensively used to

study general anxiety-like behaviour, by exploiting the natural aversion of mice to open spaces (Carola et al., 2002). Mice therefore tend to spend more time near the edges of the arena, and less time in the centre. I quantified the level of anxiety by the percentage of time spent in the periphery of the open field over the first 5 min of the trial (Fig. 25a,b). This revealed that mice which were socially isolated (78.13 ± 1.50%, n = 13) or received a single dose of cocaine (80.81 ± 2.12%, n = 8) spent a greater proportion of time in the periphery compared to naïve (73.60 ± 1.55%, n = 12) or saline-treated mice (76.09 ± 1.97%, n = 7), but this was more pronounced for cocaine, and so only the comparison between the naïve and cocaine-treated groups was significant in post-hoc tests ($F_{3,36}$ = 3.08 , $p < 0.05$; naïve vs cocaine $p < 0.05$; Fig. 25b). There was, however, no difference in the total distance travelled over this same time period (naïve: 51.85 ± 5.30 m, n = 12; socially isolated: 51.21 ± 5.90 m, n = 13; saline: 54.07 ± 4.24 m, n = 7; cocaine: 54.31 ± 6.92 m, n = 8; $F_{3,36}$ = 0.07, p = 0.98; Fig. 25c), indicating that there is no change in general locomotor activity. This suggests that a single dose of cocaine and, perhaps to a lesser extent, social isolation can increase general anxiety levels in mice. The mean time spent in the periphery for saline-treated mice was slightly higher than the naïve group, which may have been due to the stress related to the injection and handling. Increasing the sample size for this experiment may reveal a more clear separation between these four groups. However, it has already been noted that the effect of 24 hours of social isolation on anxiety levels is subtle (Conrad et al., 2011).

Interestingly, I noticed a trend for positive correlation between the number of mice in the cage pre-isolation, and the time spent in the periphery after 24 hours of isolation (Fig. 25e). This did not quite reach statistical significance (r^2 = 0.17, p = 0.16), which may be because there was limited spread of the data points, since the majority of mice had been in a cage of 3 before isolation. However, this is similar to the relationship that I had observed between the number of

mice per cage pre-isolation and the AMPAR:NMDAR ratio post-isolation, suggesting that both the AMPAR:NMDAR ratio and anxiety levels are both directly related to the previous social interaction of the animal. Additionally, similar to the AMPAR:NMDAR ratio, I found no correlation between the number of mice per cage and the time spent in the periphery for the naive mice ($r^2 = 0.0005$, $p = 0.94$; Fig 25d). Contrastingly, however, I found negative correlation between the number of mice per cage and the time spent in the periphery following a cocaine injection ($r^2 = 0.61$, $p < 0.05$; Fig 25f). This indicates that perhaps greater social contact reduces the anxiogenic effects of cocaine. However, there is limited spread of the data points in these three groups, and therefore more data is required for accurate statistical analysis.

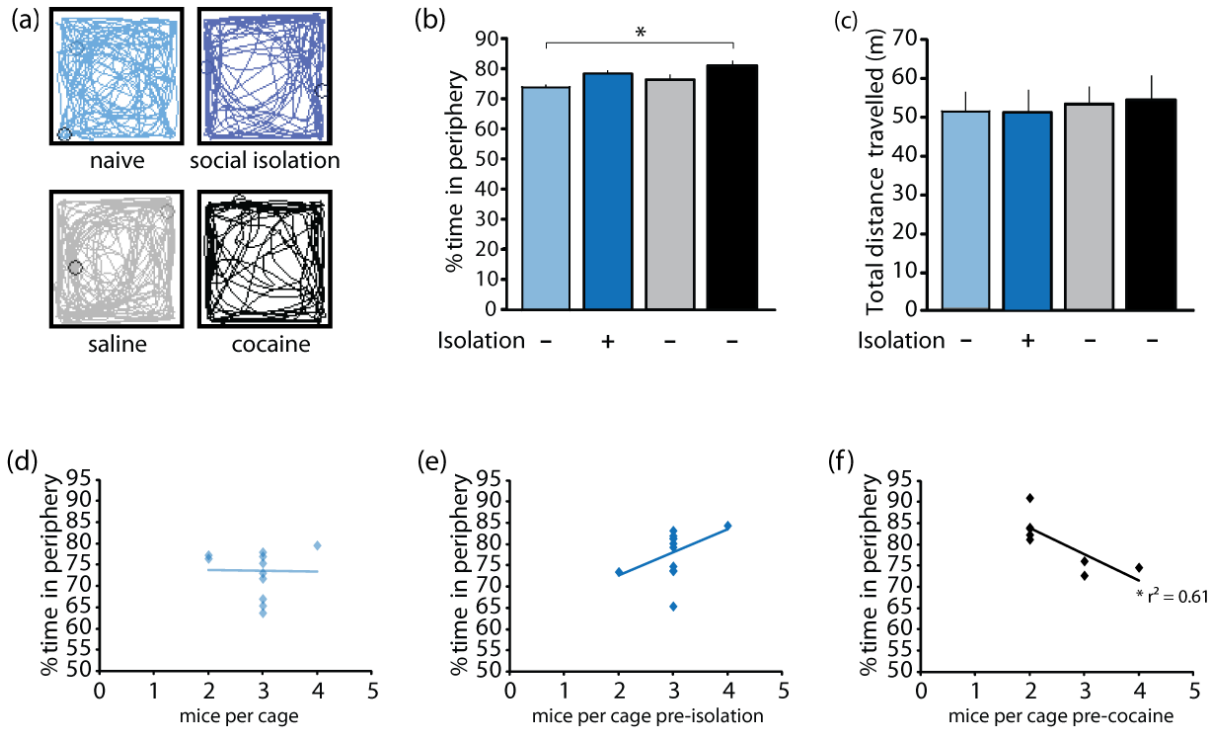


Figure 25. A single dose of cocaine increases anxiety measured in the open field test. (a) Representative path examples and bar charts (mean + SEM) showing (b) % time spent in the periphery of the open field and (c) total distance travelled in naïve, socially isolated, saline-, and cocaine-treated mice. The mean age for each experimental group was 12 weeks (naive), 10.6 weeks (socially isolated), 13.3 weeks (saline), and 11.6 weeks (cocaine). Scatter graphs showing the relationship between the number of mice per cage and the % time spent in the periphery in (d) naïve, (e) socially isolated, and (f) cocaine-treated mice. This revealed significant negative correlation in cocaine-treated mice ($r^2 = 0.61$, $p < 0.05$), but no correlation in naïve or socially isolated mice. * indicates $p < 0.05$.

7.3.2. Effect of a single dose of diazepam

Mice received a single injection of 5 mg/kg diazepam, or vehicle, at 10.00 am and were subsequently returned to their original cage, with their cagemates, before brain slice preparation 24 hours later. This dose of diazepam has been shown to not only induce an anxiolytic effect on behaviour (Chaouloff et al., 1997; Griebel et al., 1998), but also potentiate excitatory synapses onto VTA dopamine neurons after just 24 hours (Heikkinen et al., 2009; Tan et al., 2010). I found that the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons was not significantly different following a single dose of diazepam (1.55 ± 0.09, n = 7) compared to vehicle (1.40 ± 1.61, n = 6, $t_{11} = 0.83$, p = 0.42; Fig. 26). This indicates that, unlike VTA dopamine neurons, excitatory synapses onto DRN/vlPAG dopamine neurons are not potentiated by diazepam.

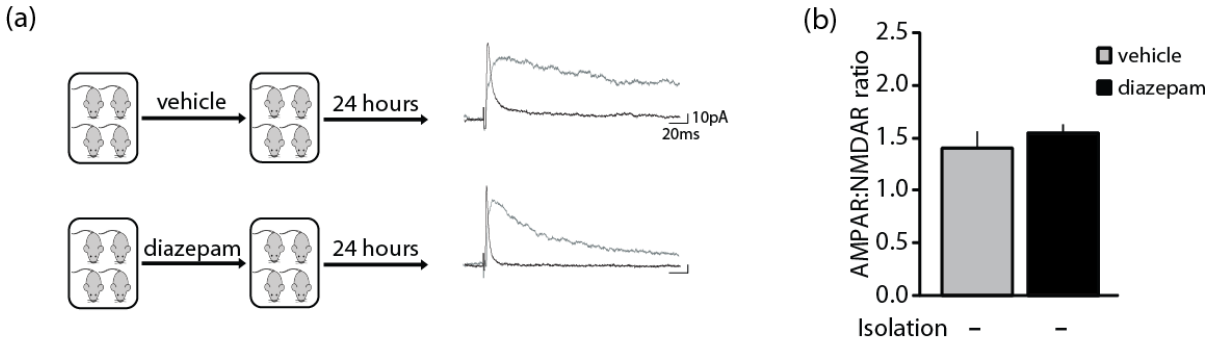


Figure 26. Diazepam does not potentiate synapses onto DRN/vlPAG dopamine neurons. (a) Injection protocol with representative examples and (b) bar chart showing mean (+ SEM) AMPAR:NMDAR ratio in mice given a single dose of diazepam or vehicle. The mean age for each experimental group was 5 weeks (vehicle) and 5.6 weeks (diazepam).

7.3.3. Effect of diazepam or ketamine on social isolation-induced potentiation

In order to investigate whether the synaptic potentiation induced by social isolation could be prevented by alleviating anxiety, I administered diazepam, ketamine, or vehicle before socially isolating the mouse. The effect of an acute dose of diazepam was compared to administration of the equivalent volume of vehicle, whereas the effect of ketamine was compared to the saline-injected and isolated group from an earlier experiment (chapter 5, Fig. 11c). I found that there was no significant difference in the AMPAR:NMDAR ratio in mice isolated with a single injection of diazepam (1.77 ± 0.19, n = 6) compared to vehicle (1.71 ± 0.1, n = 6; t_{10} = 0.24, p = 0.81; Fig. 27a,b), or a single injection of ketamine (1.59 ± 0.31, n = 7) compared to saline (2.00 ± 0.26, n = 8; t_{13} = 1.02, p = 0.33; Fig. 27c,d), although there was a small trend for lower ratios following ketamine with isolation. This suggests that neither of these compounds is effective in reducing the potentiation induced by social isolation. However, this could be due to a number of reasons. For example anxiety levels were not assayed, and therefore these two compounds may or may not have produced a measurable effect on anxiety after isolation for 24 hours. Equally the acute effect of the drug would have worn off after a few hours, but the mouse would have remained isolated for the ensuing 24 hours. Therefore, it is difficult to accurately evaluate the effect of these compounds on the induction of plasticity in these neurons.

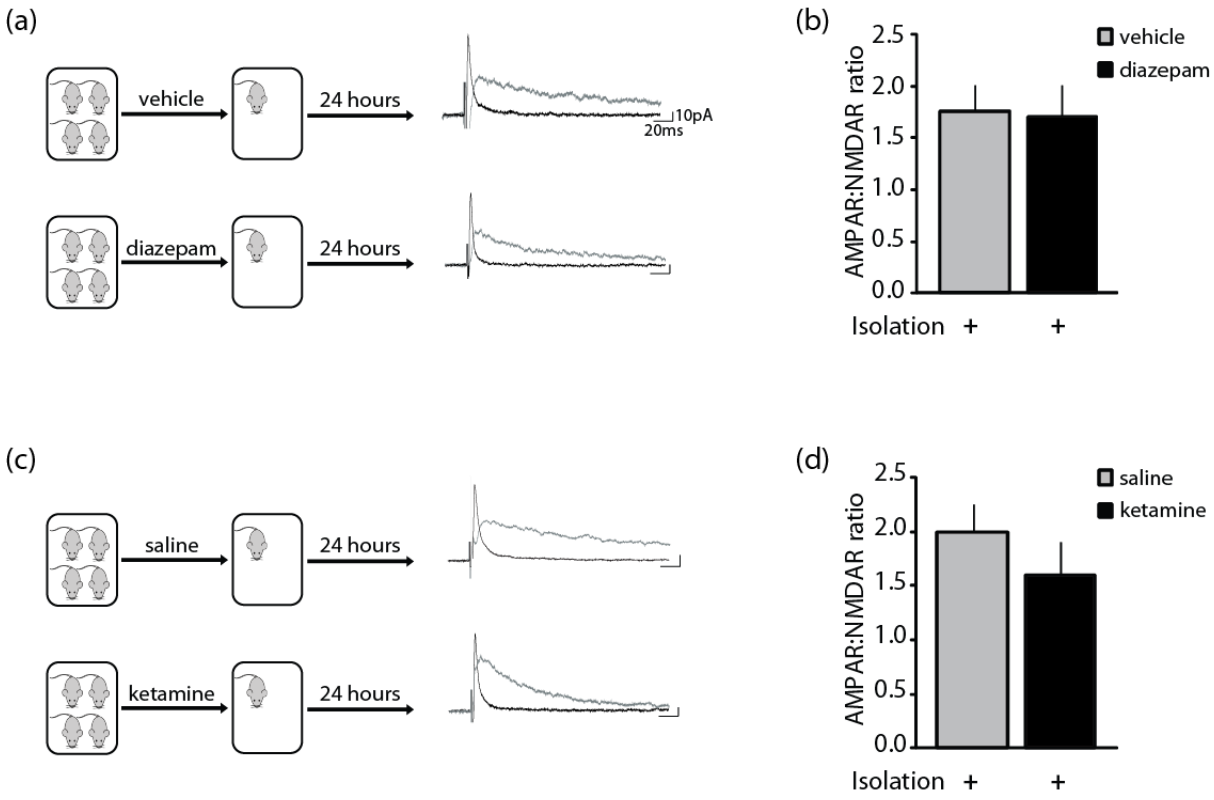


Figure 27. Neither diazepam nor ketamine prevent social isolation-induced potentiation in DRN/vlPAG dopamine neurons. (a,c) Injection protocol with representative examples and (b,d) bar charts showing the mean (+ SEM) AMPAR:NMDAR ratio following a single dose of diazepam or ketamine with social isolation. The mean age for each experimental group was 5.5 weeks (vehicle with isolation), 6.3 weeks (diazepam with isolation), 6.2 weeks (saline with isolation) and 5.8 weeks (ketamine with isolation).

7.4. Conclusion

In this chapter I have tested the hypothesis that cocaine- and social isolation-induced potentiation in DRN/vlPAG dopamine neurons is a result of acute anxiety. I found that there was a trend for increased anxiety measured in the open field test in both these groups of animals, in comparison to group-housed or saline-treated controls. However, this was only statistically significant for cocaine, with a more subtle effect observed following social isolation, in line with previous work (Conrad et al., 2011). Furthermore, an acute dose of diazepam did not increase the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons (unlike in the VTA; Heikkinen et al., 2009), suggesting that it may be the anxiogenic quality of cocaine, rather than its addictive properties, which is important for the induction of plasticity in this region. I also investigated the ability of diazepam or ketamine to prevent the potentiation induced by social isolation. While I noted a trend for smaller AMPAR:NMDAR ratios following isolation with ketamine, neither of these compounds produced a significantly smaller ratio compared to administration of vehicle or saline. However, this result is difficult to interpret because the isolation will still be in play even after the acute effect of the drug has worn off. If the time course for this plasticity was known, these compounds could be tested during a critical period of induction, which may be more appropriate. Additionally, the dependence of plasticity on NMDAR transmission could be evaluated using mice with selective disruption of NMDAR signalling in dopamine neurons (Engblom et al., 2008; Zweifel et al., 2008).

The observation that social isolation only exhibited a trend towards higher anxiety levels in the open field test, and a trend towards correlation of anxiety with prior social interaction, could be due to the relatively small sample size and/or the limited sensitivity of this behavioural assay for detecting a difference in anxiety levels. Indeed, Conrad et al. (2011) note that under more anxiogenic testing conditions the trend they observed for an increase in anxiety-like behaviour

may become more apparent. In contrast, however, it may be that social isolation does not have a robust effect on general anxiety levels, and the synaptic potentiation observed may be instead more directly related to the lack of social contact, rather than an increase in general anxiety. This would also be one explanation for why the anxiolytic compounds had no effect on isolation-induced potentiation. This idea is also supported by the correlation observed between the AMPAR:NMDAR ratio following isolation and the number of mice per cage, suggesting that the extent of prior social interaction is directly associated with isolation-induced synaptic potentiation. However, conversely, cocaine-treated mice showed negative correlation between anxiety-like behaviour and the number of mice per cage, suggesting that perhaps social interaction can alleviate the anxiogenic effects induced by cocaine.

An alternative hypothesis could be that social isolation-induced plasticity is a result of stress. However, whereas chronic social isolation is considered a stressful experience for adult mice (Cabib et al., 2002; Avitsur et al., 2003), acute isolation is suggested to be anxiogenic, without effecting depressive-like behaviour (Kwak et al., 2009) or stress (Hilakivi et al., 1989). Furthermore, if 24 hours of social isolation was acutely stressful for the animals, I would have expected an increase in the AMPAR:NMDAR ratio in the VTA (Saal et al., 2003; Lammel et al., 2011), but this was not the case in this study, or in others (Ungless et al., 2001).

The behavioural and neurochemical alterations arising from long-term isolation rearing have been extensively examined, but acute social isolation has received considerably less attention. Furthermore, even though multiple lines of evidence suggest that modifications to the dopamine system result from social isolation, the precise nature of these changes is not clear (Fone and Porkess, 2008). Using this simple, acute manipulation in adult mice, however, I have observed that social isolation can induce changes to excitatory synapses, at a particular subset of dopamine neurons, which supports the involvement of the dopamine system in the response

to isolation. The two experimental conditions which induce potentiation in DRN/vlPAG dopamine neurons have in common that they are both thought to increase general anxiety and reduce social interaction. While these effects are brought on by the drug in cocaine-treated mice, enforced isolation directly abolishes social contact. Therefore even though the emotional state of the animals is generated in different ways, the resulting behaviour (and synaptic alterations) does have similarities. My results suggest the possibility that this potentiation may be related to acute anxiety, but it would be interesting to see whether a manipulation that increases anxiety, but does not alter social interaction *per se*, would cause synaptic potentiation in DRN/vlPAG dopamine neurons.

Chapter 8. Discussion and future work

8.1. Introduction and summary

Psychostimulant addiction represents a serious medical and social problem for which no effective treatment currently exists (McLellan et al., 2000; Gorelick et al., 2004; Vocci and Ling, 2005; Kalivas, 2007; Karila et al., 2008). Cocaine dependence is particularly common, with an estimated 1.5 million dependent users in the US (Substance Abuse and Mental Health Services Administration, 2011). Chronic exposure to psychostimulants, such as cocaine, provokes profound structural and neurochemical alterations in neural circuitry conserved for motivation and natural rewards (Hyman and Malenka, 2001; Jones and Bonci, 2005; Hyman et al., 2006). This is thought to underlie the enduring drug craving and drug-seeking behaviour apparent even after long periods of withdrawal, which are defining features of addiction (Robinson and Berridge, 1993; Robinson and Berridge, 2000; Hyman and Malenka, 2001; Kelly and Berridge, 2002; Robinson and Berridge, 2003; Kauer, 2004; Vezina, 2004; Kalivas, 2009). A key component of this neural circuit mediating the effects of addictive drugs is the midbrain dopamine system, which consists of dopamine neurons projecting to, and receiving input from, a variety of limbic and cortical structures (Björklund and Dunnett, 2007a). Excitatory synapses onto these dopamine neurons are a critical site of drug-induced neuroadaptation, exhibiting potentiation within just a few hours of a single drug exposure (Ungless et al., 2001; Argilli et al., 2008). This transient LTP-like plasticity is thought to be involved in the early induction of behavioural sensitisation and conditioned place preference (CPP; Kalivas and Alesdatter, 1993; Vezina, 1996; Wolf, 1998; Vezina and Queen, 2000; Carlezon and Nestler, 2002; Harris and Aston-Jones, 2003; Borgland et al., 2004; Harris et al., 2004; Wolf et al., 2004; Dunn et al., 2005; Mead et al., 2005; Borgland et al., 2006; Zweifel et al., 2008), and is also essential for longer-term synaptic changes

in downstream structures following continued drug exposure (Mameli et al., 2009). Additionally, potentiation at these synapses is thought to contribute to cue- and drug-induced reinstatement during withdrawal (Engblom et al., 2008; Mameli et al., 2009), which highlights the importance of this plasticity at different stages in the addiction process.

However, while the dopamine system is traditionally associated with reward, several studies in the last few years have added weight to the hypothesis that dopamine neurons also respond to aversive stimuli (Horvitz, 2000; Di Chiara, 2002; Pezze and Feldon, 2004; Redgrave and Gurney, 2006; Brischoux et al., 2009; Lammel et al., 2011). This supports the emerging evidence that the midbrain dopamine neurons are a heterogeneous population, with distinct molecular, electrophysiological, and functional properties (Neuhoff et al., 2002; Ford et al., 2006; Margolis et al., 2006a,b; Lammel et al., 2008; Margolis et al., 2008; Brischoux et al., 2009; Lammel et al., 2011). Aversion-responsive dopamine neurons had previously been overlooked, as they display 'unconventional' electrophysiological properties (Lammel et al., 2008) and are located more medially within the VTA, so they have rarely been targeted in brain slices or *in vivo* (Brischoux et al., 2009). Another understudied subset of midbrain dopamine neurons is located in the DRN/vlPAG (Lindvall and Björklund, 1974; Hökfelt et al., 1976; Ochi and Shimizu, 1978; Hökfelt et al., 1984; Trulson et al., 1985; Descarries et al., 1986; Oades and Halliday, 1987; Arsenault et al., 1988; Stratford and Wirtshafter, 1990; Charara and Parent, 1998), which has received substantially less attention than dopamine neurons residing in the VTA. This is likely to be a result of their relatively small size and number, and the fact that they are in the minority in this region (Descarries et al., 1986). Therefore although numerous studies have targeted this region, there is only one report of a single recorded dopamine neuron *in vivo* (Schweimer and Ungless, 2010), and no accounts of *ex vivo* electrophysiology. Furthermore, in spite of the observation that these neurons provide the majority of the dopamine input to the central amygdala and

BNST (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006; Shin et al., 2008), and selective lesioning has hinted at their role both in the sleep-wake cycle (Lu et al., 2006) and mediating the effects of opiates (Flores et al., 2004; Flores et al., 2006; Meyer et al., 2009), no clear function has been ascribed to the DRN/vlPAG dopamine neurons.

I have studied this dopaminergic subpopulation using immunohistochemistry and electrophysiology *ex vivo*, in combination with drug administration, environmental manipulations, and behavioural analysis. In **chapters 2-4,** I assessed the suitability of transgenic mice for targeting these dopamine neurons, and characterised their basal neurochemical and electrophysiological properties. They generally expressed the same dopaminergic markers, and exhibited similar properties to 'unconventional' VTA dopamine neurons (Lammel et al., 2008), including their response to depolarisation and hyperpolarisation. Additionally, around half of these dopamine neurons co-expressed the neuropeptide VIP. These VIP+ neurons displayed more irregular firing, lower maximal firing frequencies, and a larger I_h than VIP- neurons. In **chapter 5,** I examined the ability of cocaine to induce potentiation at excitatory synapses onto these neurons, in order to determine if drug-evoked neural modifications were a key feature of this population, similar to the VTA. I found that not only could cocaine potentiate the glutamatergic synapses onto DRN/vlPAG dopamine neurons, but this was also observed following 24 hours of social isolation. This acute environmental manipulation caused a similar magnitude increase in the AMPAR:NMDAR ratio compared to a single dose of cocaine. Further characterisation in **chapter 6** revealed that this plasticity was associated with a change in AMPAR transmission, indicative of a switch from GluR2-containing to GluR2-lacking receptors, which resembles that of cocaine-induced plasticity in the VTA. Contrastingly, however, I found that social isolation did not potentiate glutamatergic synapses onto VTA dopamine neurons. In **chapter 7,** I hypothesised that this potentiation was related to the acute anxiety induced by

either 24 hours of social isolation or a single dose of cocaine. Only cocaine-treated animals showed a statistically significant increase in anxiety measured using the open field test, but the addictive, anxiolytic drug diazepam was not able to potentiate these synapses, as it has been reported to do so in the VTA (Heikkinen et al., 2009; Tan et al., 2010). It is not clear whether general anxiety levels are increased by social isolation, but interestingly the AMPAR:NMDAR ratio measured following social isolation directly correlated with the number of mice housed together in the cage before isolation, suggesting that the loss of social interaction may be a key factor contributing to this potentiation. However, conversely, the number of mice per cage was negatively correlated with the anxiety levels following cocaine, suggesting that perhaps social interaction is able to counteract the anxiogenic effects of this drug.

My findings provide an initial immunohistochemical and electrophysiological description of this understudied population of dopamine neurons, on which future studies can build. This includes not only identifying potential caveats with GFP mouse models used to target this population, but also proposing use of the neuropeptide VIP to delineate the two subsets of dopamine neuron in this region. The observation that both cocaine and social isolation can induce potentiation at glutamatergic synapses implicates the DRN/vlPAG neurons as an important component of the midbrain dopamine population, which can respond to a different type of salient cue. These results are discussed in relation to the functional properties of other midbrain dopamine neurons, the possible implications for downstream targets of this dopamine subpopulation, and the potential clinical relevance of these findings.

8.2. Immunohistochemical and electrophysiological characterisation

8.2.1. Suitability of GFP mouse models

An initial step towards characterising the functional properties of the DRN/vlPAG population involved confirming the suitability of transgenic GFP mice for targeting this population in brain slices. Utilising both TH-GFP and Pitx3-GFP mice, I found that while the majority of GFP+ neurons in the DRN/vlPAG were TH+, the proportion was slightly lower than previously reported in the VTA (Matsushita et al., 2002; Zhao et al., 2004). GFP expression in TH-GFP mice was stronger, more efficient, and more uniformly distributed throughout the TH+ population in this region, compared to Pitx3-GFP mice. In particular, Pitx3-directed GFP expression appeared stronger in the small periaqueductal neurons compared to the larger, multipolar, and more ventrally located neurons, in which it was often weak or absent. The reason for this is unclear, as in the VTA and SNc, there is near complete overlap between GFP and TH in Pitx3-GFP mice (Zhao et al., 2004). However, it has been noted that although Pitx3 is expressed in all midbrain dopamine neurons, there appears to be a differential reliance on this transcription factor in different dopamine subpopulations (van den Munckhof et al., 2003). The majority of SNc and about half of VTA dopamine neurons fail to develop in Pitx3$^{-/-}$ mice, but a subset of VTA dopamine neurons are unaffected, suggesting potential differences in Pitx3 expression and function (Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005). The DRN/vlPAG population may also exhibit differential dependence on Pitx3 for development and maintenance, which could result in the variable Pitx3-directed GFP expression pattern seen here.

Interestingly, GFP+/TH- neurons generally corresponded to the small, periaqueductal neurons, even in TH-GFP mice, suggesting that perhaps these cells are dopaminergic, but that TH is expressed at low levels in these cells, which has been previously suggested (Hökfelt et al., 1976;

Rogers, 1992). One reason for this could be that TH is transported to the terminals of these neurons, as is suggested to occur in some dopaminergic neurons (Frain and Leviel, 1998), rendering somatodendritic TH undetectable using immunohistochemistry. A second reason could be the location of these neurons, just ventral to the aqueduct of Sylvius. It has been noted using glutaraldehyde fixation, that there is particularly intense autofluoresence in this region (Hasue and Shammah-Lagnado, 2002), which may impair immunohistochemical detection of TH. One method of improving the strength of TH staining could be to use colchicine. This prevents axonal transport, which results in accumulation of proteins in the soma. Restricted injection of colchicine into the striatum has been successfully used to confirm the presence of TH+ neurons in this region, which express low levels of TH but are GFP+ in a TH-GFP mouse (Ibáñez-Sandoval et al., 2010). Therefore, in the same way, this method could be used to confirm the presence of TH in the small periaqueductal GFP+ neurons.

The enzyme AADC colocalised with a similar proportion of GFP+ neurons as TH, confirming that these neurons are enzymatically capable of producing dopamine, while the dopamine transporter (DAT) was mostly expressed by the large, ventral GFP+ neurons. The absence of DAT in some of these neurons does not necessarily indicate a non-dopaminergic phenotype, because different subtypes of VTA dopamine neuron have been shown to express variable levels of DAT, or even lack detectable expression (Lammel et al., 2008). Mice expressing GFP under the control of AADC or DAT could act as alternatives for targeting this dopamine population. However, these are likely to be less suitable, as AADC is also expressed by the 5-HT neurons in the DRN/vlPAG, and DAT shows variable expression both in the DRN/vlPAG and the VTA dopamine populations (Lammel et al., 2008).

8.2.2. VIP distinguishes between two subsets of DRN/vlPAG dopamine neuron

Two subsets of dopamine neuron in the DRN/vlPAG have been described, based on their size, morphology, and anatomical location (Hökfelt et al., 1976; Stratford and Wirtshafter, 1990; Charara and Parent, 1998; Flores et al., 2004). I found that expression of the neuropeptide VIP could distinguish between these two subtypes, as it was co-expressed by the small periaqueductal dopamine neurons, but not by the large, multipolar more ventrolaterally located neurons. The functional relevance of these two subpopulations remains to be established. However, most of the functions so far attributed to the DRN/vlPAG dopamine neurons relate to the large, ventral subtype, since these appear to be selectively affected by 6-OHDA lesion (Flores et al., 2004; Flores et al., 2006; Lu et al., 2006). Consistent with this, I noted that DAT was expressed in the large ventral neurons, but not generally in the small periaqueductal neurons, suggesting that this may prevent uptake of the neurotoxin in this dopaminergic subtype. The low, and often absent expression of DAT, resembles that described for VTA dopamine neurons projecting to the NAc core, NAc medial shell, mPFC, and BLA, which are generally found more medially (Lammel et al., 2008).

Apart from 6-OHDA, DRN/vlPAG dopamine neurons have also been shown to display differential vulnerability to the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), with more extensive dopaminergic cell loss noted in the ventral DRN than the dorsal part (Unguez and Schneider, 1988). Furthermore, the ventral DRN has been suggested to house striatal-projecting dopamine neurons (Descarries et al., 1986), whereas the dorsal DRN is thought to contain more neurons projecting to the extended amygdala (Hasue and Shammah-Lagnado, 2002), suggesting that selective vulnerability may also relate to projection target. This is consistent with the more dorsal location of the VIP+ neurons, and their projection to the dlBNST (Petit et al., 1995; Kozicz et al., 1998). Interestingly, even though there have so far been no reports linking VIP to

dopaminergic function, many studies have demonstrated a robust neuroprotective role for VIP following exposure of dopaminergic neurons to neurotoxins including 6-OHDA and MPTP (Offen et al., 2000; Delgado and Ganea, 2003). Consequently, VIP may serve as a neuroprotective agent in these dopamine neurons, limiting their vulnerability to neurodegeneration. Therefore the existence of different subtypes of dopamine neuron within this nucleus may have implications for the vulnerability of these neurons to different toxic agents.

8.2.3. VIP population of the DRN/vlPAG

Sparse co-expression of PHI/VIP with TH+ neurons in the periventricular region has been previously reported (Seroogy et al., 1988a), but the findings here suggest that all VIP+ neurons in the DRN/vlPAG may be dopaminergic. Presumably, therefore, dopamine and VIP are co-released, which may have functional significance for the downstream targets of this neuronal subpopulation. Indeed it has been shown that VIP is able to modulate synaptic transmission (e.g. Ciranna and Cavallaro, 2003; Itri and Colwell, 2003) and intrinsic membrane currents (Wang and Aghajanian, 1990; Haug and Storm, 2000; Sun et al., 2003; Pakhotin et al., 2006), implying that its release from these dopamine neurons may be functionally important in regulating neural activity. The VIP projection from this region appears to be restricted to the dlBNST (Petit et al., 1995; Kozicz et al., 1998), which has also been described as a major target of DRN/vlPAG dopamine neurons (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). This suggests that co-expression of VIP in the DRN/vlPAG may selectively mark the dopaminergic projection to the dlBNST.

Very little is known about the VIP population in the DRN/vlPAG, although its presence has been noted in several species (Sims et al., 1980; Moss and Basbaum, 1983; Eiden et al., 1985; Antonopoulos et al., 1987; El Kafi et al., 1994; Smith et al., 1994; Petit et al., 1995). There is evidence from electron microscopy that these VIP neurons make local excitatory contacts onto

inhibitory interneurons within the PAG to form a local circuit (Paspalas et al., 2000). Furthermore, it has been hypothesised that VIP signalling may also occur via the CSF of the aqueduct of Sylvius, as VIP neurons and processes appear to extend into the aqueduct (Paspalas et al., 2000). While this could be a sectioning artefact, the existence of 'liquor-contact' neurons and fibres, which form a supraependymal system, has been proposed (Leonhardt and Lindemann, 1973). Therefore the influence of VIP (and possibly dopamine) release may occur via different means in this neuronal subpopulation.

My findings suggest that VIP is expressed in a significant proportion of this dopaminergic population, but this does not preclude the possibility that other neuropeptides and/or neuromodulators are also co-expressed. Indeed, multiple neuropeptides have been described in the DRN/vlPAG (Fu et al., 2010), and there is evidence for co-expression of cholecystokinin (CCK) in some dopamine neurons in this region (van der Kooy et al., 1981; Seroogy et al., 1988a; Seroogy and Fallon, 1989) as well as CCK and neurotensin in some VTA dopamine neurons (Hökfelt et al., 1984; Seroogy et al., 1987; Seroogy et al., 1988a,b). However, the functional significance of this co-expression remains to be established.

8.2.4. Electrophysiological characterisation

Utilising TH-GFP and Pitx3-GFP mice to record from the DRN/vlPAG dopamine neurons in brain slices *ex vivo* revealed that they generally exhibit similar electrophysiological properties to VTA dopamine neurons. Specifically, they best resemble 'unconventional' dopamine neurons (i.e. those projecting to the NAc core, NAc medial shell, BLA, and mPFC) but not 'conventional' dopamine neurons (i.e. those projecting to the dorsal striatum or NAc lateral shell; Neuhoff et al., 2002; Ford et al., 2006; Lammel et al., 2008; Lammel et al., 2011). This was based on their average firing rate, action potential width, high frequency firing in response to depolarisation, and small I_h following hyperpolarisation.

8.2.4.1. Identified TH+ and TH- GFP+ neurons

Post-hoc immunohistochemical identification of recorded GFP+ neurons revealed no differences between TH+ and TH- neurons in terms of any electrophysiological measures assayed. This supports the suggestion that GFP+/TH- neurons may be dopaminergic, but with very low TH levels. Moreover, previous work has demonstrated that the electrophysiological properties of these dopamine neurons are distinct from those of putative serotonergic (Dougalis et al., 2012), and putative GABA (Dougalis, unpublished) neurons in the DRN/vlPAG, suggesting that they may be distinguishable based on these properties alone. However, this does not overcome the initial difficulty in locating these dopamine neurons, being the minority in this nucleus.

8.2.4.2. Identified VIP+ and VIP- GFP+ neurons

I compared the electrophysiological characteristics of identified VIP+ and VIP- GFP+ neurons. The slightly higher proportion of GFP+/VIP+ neurons I had observed in Pitx3-GFP mice, compared to TH-GFP mice, translated to recordings, in which just under and just over half of recorded GFP+ neurons were VIP+ in TH-GFP and Pitx3-GFP mice, respectively. This demonstrated that both neuronal subtypes can be targeted in each GFP mouse model, but with a slight bias towards VIP+ neurons in Pitx3-GFP mice. However, due to the pattern of expression of these two subtypes, it is possible (to some degree) to attempt to visually target small periaqueductal neurons or large ventrolateral neurons for a greater likelihood of recording a VIP+ or VIP- neuron, respectively.

Identified VIP+ and VIP- neurons generally exhibited very similar electrophysiological properties, but they did differ in terms of their whole-cell capacitance, action potential firing pattern, maximal firing frequency in response to depolarisation, and I_h amplitude. A difference in the I_h may be due to differences in the expression or function of the hyperpolarization-activated cyclic

nucleotide-gated (HCN) channels, which mediate this current (Santoro and Tibbs, 1999). However, a difference in firing pattern may reflect differences in the intrinsic properties of the neurons (Person and Kudina, 1972; Overton & Clark, 1997; Bennett and Wilson, 1999; Powers and Binder, 2000), for example SK3 channel expression (Wolfart et al., 2001), and/or differences in the temporal and spatial properties of their synaptic input (Calvin and Stevens, 1968; Stevens and Zador, 1998; Gonzalez-Forero et al., 2001). Support for the latter interpretation was provided by the observation that certain features of the glutamatergic and GABAergic input differed between VIP+ and VIP- neurons. Hence a difference in the ratio of excitatory to inhibitory input may contribute to the variation observed in firing regularity. However, the differences in the properties of glutamatergic synapses were not apparent in the presence of picrotoxin and a cesium-based internal solution. This indicates that perhaps differences in the $GABA_AR$-mediated influence over the glutamatergic currents may underlie the differences observed.

Interestingly, a high CV-ISI, such as that observed in VIP+ neurons, is generally associated with a greater probability of burst firing *in vivo* (Brischoux et al., 2009). An important part of future work would be to assess the characteristics of these neurons *in vivo*. However, targeting these dopamine neurons would be challenging since they are the minority in the DRN/vlPAG. An optogenetic approach may overcome this difficulty, by expressing a light-activated channel selectively in dopamine neurons, and using the response to brief illumination to confirm dopaminergic identity (Lima et al., 2009), which has been successfully employed in the VTA (Cohen et al., 2012).

While it has been shown that the I_h can contribute to the firing frequency in a subpopulation of SNc dopamine neurons (with the largest I_h amplitudes; Luthi and McCormick, 1998; Neuhoff et al., 2002), it is not thought to influence the basal firing of VTA dopamine neurons, which

generally exhibit smaller I_h amplitudes (Neuhoff et al., 2002; Appel et al., 2003). It would follow, therefore, that the small I_h observed in DRN/vlPAG dopamine neurons is unlikely to influence the firing properties of these neurons at rest, although this remains to be determined, and may differ between VIP+ and VIP- neurons due to the difference in the average size of their I_h. Another widely used hallmark of dopamine neurons is hyperpolarisation by a D_2-receptor agonist (Lacey et al., 1989), which is important in dopamine-mediated feedback inhibition to regulate neuronal excitability (Beckstead et al., 2004). However, this is only characteristic of a subpopulation of VTA dopamine neurons (Johnson and North, 1992a; Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2008), and therefore an important future experiment to conduct would be to determine the response of the DRN/vlPAG dopamine population to a D_2 agonist.

8.2.5. Immunohistochemical and electrophysiological characterisation: conclusion

DRN/vlPAG dopamine neurons resemble the 'unconventional', generally more medially-located, VTA dopamine neurons (Lammel et al., 2008) in terms of their molecular and electrophysiological properties. Interestingly, a subset of VTA dopamine neurons have been found to co-express the vesicular glutamate transporter, VGLUT2 (Kawano et al., 2006; Yamaguchi et al., 2011), and shown to co-release glutamate in the NAc shell (Hnasko et al., 2010; Stuber et al., 2010; Tecuapetla et al., 2010). These dopamine neurons are generally found in more medial and caudal regions of the VTA, particularly the interfascicular nucleus, rostral linear nucleus, and rostral part of the caudal linear nucleus (Kawano et al., 2006; Yamaguchi et al., 2011). Additionally, it is noted by Kawano et al. that a small proportion of A10dc (DRN/vlPAG) dopamine neurons co-express VGLUT2. It would therefore be intriguing to examine the possibility of co-release of glutamate in this population, as it may have important functional consequences for neural activity in projection targets.

The differences observed between VIP+ and VIP- dopamine neurons, while reasonably subtle, may have implications for the neuronal response to synaptic input, and the pattern of dopamine release in target regions. Given that the VIP+ dopamine neurons may project more exclusively to the dlBNST, this hints at projection-specific properties, which is a feature of VTA dopamine neurons (Lammel et al., 2008). Indeed, differences in maximal firing frequency and I_h amplitude have been associated with VTA dopamine neurons projecting to different targets (Lammel et al., 2008), which is also suggested by these results. Future work could therefore include analysing the neurochemical and electrophysiological characteristics of the DRN/vlPAG dopamine neurons in a projection-specific manner, possibly utilising retrogradely-transported fluorescent beads, as has been done for several major projection targets of the VTA (Lammel et al., 2008). Specifically, the vlBNST, dlBNST, and centromedial and centrolateral subregions of the central amygdala would be intriguing targets to examine.

8.3. Cocaine- and social isolation-induced plasticity

8.3.1. Characterisation of plasticity in the DRN/vlPAG

An acute dose of an addictive drug (Ungless et al., 2001; Saal et al., 2003), an aversive stimulus (Saal et al., 2003; Lammel et al., 2011), or a reward-predicting cue (Stuber et al., 2011) have all been shown to strengthen excitatory synapses onto VTA dopamine neurons. Here, I have demonstrated that an additional type of salient stimulus – social isolation – can potentiate excitatory synapses onto DRN/vlPAG dopamine neurons. Either 24 hours of social isolation or a single dose of cocaine resulted in an increase in the AMPAR:NMDAR ratio measured in DRN/vlPAG dopamine neurons, but social isolation appeared ineffective at potentiating these synapses in the VTA. Further electrophysiological analysis suggested that both social isolation- and cocaine-induced plasticity in the DRN/vlPAG are associated with a postsynaptic change in AMPAR subunit composition, which is also a feature of cocaine-induced potentiation in the VTA

(Bellone and Lüscher, 2006). Specifically, the AMPAR rectification index and AMPAR-mediated mEPSC amplitude were significantly increased following either social isolation or a single dose of cocaine, while the AMPAR PPR (in the presence of polyamine block) showed a trend in the same direction. This indicates that both types of stimuli induce a similar potentiation of AMPAR transmission, characterised by a switch from GluR2-containing to GluR2-lacking receptors. Additionally, however, cocaine (in the presence or absence of social isolation) caused an increase in the frequency of AMPAR-mediated mEPSCs, which was not observed following purely social isolation. Therefore a distinct property of this psychostimulant may induce additional changes at these glutamatergic synapses. Importantly, however, cocaine administered with isolation did not have an additive effect on synaptic strength, in terms of the AMPAR:NMDAR ratio, or mEPSC amplitude and frequency. This again suggests that the mechanism of expression of plasticity resulting from these two stimuli may be similar. It is also reminiscent of the effect of cocaine and an aversive stimulus on the AMPAR:NMDAR ratio in the VTA, which also does not exert an additive effect at these synapses (Dong et al., 2004).

8.3.2. Potentiation following social isolation correlates with prior social contact

The AMPAR:NMDAR ratio following social isolation was positively correlated with the number of mice housed together prior to isolation. However, no relationship was observed between these two variables in naïve or cocaine-treated mice. This suggests that the greater the prior social interaction, the stronger the potentiation following removal of that interaction. In the VTA, cocaine-induced plasticity is proposed to involve a 'switch-like' mechanism at excitatory synapses, whereby the synapses are maximally potentiated following a single injection, and cannot be further potentiated by chronic exposure (Ungless et al., 2001; Borgland et al., 2004; Mameli et al., 2009). However, several studies have associated variability in this synaptic enhancement with measures of cocaine-induced behaviour. For example, in drug-naïve rats the

magnitude of cocaine-induced locomotor activity, following a single dose of cocaine, was positively correlated with the increase in the AMPAR:NMDAR ratio (Borgland et al., 2004). Given that no correlation was observed between locomotor activity and the ratio in saline-treated rats, this suggests that the locomotor activity itself does not drive the increase in synaptic strength (Borgland et al., 2004). Therefore in the same way here, social contact in naïve mice may not itself determine synaptic strength, but the magnitude of the social change imposed by isolation may dictate the strength of the potentiation.

Furthermore, in mice trained to lever press for intravenous cocaine administration, the rectification index of the AMPAR current correlated with the number of active lever presses when tested during withdrawal, suggesting that greater synaptic strength is associated with more intense drug craving (Mameli et al., 2009). Additionally, an intense stress experience induces robust potentiation in a subset of VTA dopamine neurons (Saal et al., 2003; Lammel et al., 2011). However, it is frequently noted that the AMPAR:NMDAR ratio is generally slightly higher in saline-injected compared to naïve mice, which is routinely attributed to the stress of the injection. Hence, this mildly stressful stimulus may have a small effect at these synapses (or only affect a certain subset of synapses) to result in a small, but noteworthy increase. Therefore, the isolation-induced increase in the AMPAR:NMDAR ratio may be dependent on the degree of change in social contact, i.e. separation from a large social group represents a more intense, motivationally salient, stimulus, which results in greater synaptic potentiation. Indeed, it has been suggested that the magnitude of cocaine-induced locomotor activity reflects the degree of motivational significance of the drug (Borgland et al., 2004).

8.3.3. Time course of plasticity

The time course of cocaine-induced plasticity in the VTA has been well-studied, and is thought to be expressed within a few hours (Argilli et al., 2008), and lasts for around a week following a

single exposure (Ungless et al., 2001). It would be interesting to know whether the potentiation in DRN/vlPAG dopamine neurons exhibits the same time course as the VTA, and whether this is similar for both cocaine- and social isolation-induced plasticity. It is conceivable that these two stimuli may be distinct in terms of the time required to induce the potentiation, since the effect of social isolation may not progress as quickly as the rapid actions of cocaine within the brain. Furthermore, it has been shown that seven daily injections of cocaine increased the AMPAR:NMDAR ratio in the VTA to the same extent as a single dose, and still only remained for around a week following the final exposure (Borgland et al., 2004). This would be harder to test with social isolation, as it could involve either leaving the mouse in social isolation for a week, or repeatedly returning the animal to its cagemates for a fixed period of time between daily isolations.

Interestingly, even though addictive drugs, aversive stimuli, and rewarding-predicting cues are the only identified stimuli which can potentiate excitatory synapses onto VTA dopamine neurons, other salient stimuli have been shown to induce plasticity in other parts of the reward circuitry. One notable example is the potentiation observed in NAc medium spiny neurons 24 hours after exposure to a novel environment (Rothwell et al., 2011). This supports the notion that a more subtle, but salient, environmental change can potentiate excitatory synapses within 24 hours. Interestingly, physiological responses and plasticity induced by mild forms of stress (e.g. environmental novelty or physical restraint), but not intense forms of stress (e.g. forced swim or social defeat), have been shown to habituate with repeated exposure (Covington and Miczek, 2005; Grissom and Bhatnagar, 2009; Kreibich et al., 2009; Rothwell et al., 2011). Therefore, in the same way, social isolation-induced potentiation may reverse with repeated exposure, as long as the animals are intermittently returned to their cagemates. However, continued isolation may represent a more intense form of stress, which may not exhibit

habituation. Indeed, multiple lines of evidence indicate that chronic social isolation induces significant neurochemical changes in the brain (e.g. Heidbreder et al., 2000, Lapiz et al., 2003; Fone and Porkess, 2008; Koike et al., 2009; Fabricius et al., 2010). Therefore even if the plasticity observed here is only transient, it may promote longer-term changes in downstream regions of the neural circuit, in a similar way to cocaine-induced plasticity in the VTA (Mameli et al., 2009). Intriguingly, both acute and chronic social isolation have been shown to alter LTP in the dlBNST (Conrad et al., 2011), which is one target of this population (discussed in 8.4.2).

Reversal of cocaine-induced potentiation in the VTA requires mGluR-LTD, which resets the synapses back to their basal GluR2-containing state (Bellone and Lüscher, 2006; Mameli et al., 2007). Given that potentiation in DRN/vlPAG dopamine neurons also appears to involve a switch in AMPAR subunit composition, it would be interesting to test whether mGluR-LTD is necessary and/or sufficient to reverse the potentiation observed following cocaine or social isolation in the DRN/vlPAG.

8.3.4. What is the underlying cause of this potentiation?

One key similarity between social isolation and acute cocaine is that they are both proposed to be anxiogenic. Consequently, one interpretation of my results is that excitatory synapses onto DRN/vlPAG dopamine neurons are strengthened by acute anxiety. To test this hypothesis I assayed behaviour in the open field test and also examined whether an acute dose of diazepam could increase the AMPAR:NMDAR ratio at these synapses. Anxiety-like behaviour (quantified by the time spent in periphery of the open field) was significantly increased 24 hours after a single dose of cocaine, with a (non-significant) increase also observed following 24 hours of social isolation. Additionally, a single dose of the addictive, but anxiolytic, drug diazepam did not increase the AMPAR:NMDAR ratio in DRN/vlPAG dopamine neurons, suggesting that it may not be the addictive quality of cocaine which is important for inducing potentiation in DRN/vlPAG

dopamine neurons, as it is in the VTA. I also tested the ability of diazepam, or the NMDAR antagonist ketamine, to prevent the potentiation following social isolation. Diazepam administration with social isolation had no effect on the AMPAR:NMDAR ratio compared to social isolation with vehicle, and ketamine administered with social isolation caused a modest reduction in the AMPAR:NMDAR ratio, although this was not statistically significant.

8.3.4.1. Is the potentiation purely anxiety-induced?

There was a significant difference across the four experimental groups in the time spent in the periphery of the open field, which revealed a difference between naïve and cocaine-treated mice in post-hoc tests following a one-way ANOVA. A trend for increased anxiety levels was also observed in socially isolated mice, but this was not significantly different from naïve, saline- or cocaine-treated mice. However, this was consistent with the non-significant trend for increased anxiety found following 24 hours of social isolation in a previous study (Conrad et al., 2011), suggesting that this period of isolation may only have a mild effect on general anxiety. Increasing the sample size of the four groups may reveal whether or not social isolation does have a clear effect on anxiety levels. Alternatively, using a more sensitive behavioural assay, such as the elevated plus-maze (Lister, 1987), may clarify this result. Indeed, different behavioural tests are likely to reflect different underlying features of anxiety, and so may be modelling different components of this emotional state (File, 1992; Belzung and Le Pape, 1994; Ramos et al., 1997; Flaherty et al., 1998; Cheeta et al., 2001). It may, therefore, be useful to employ multiple behavioural assays to evaluate the behavioural state of the animal. In order to strengthen the relationship between anxiety levels and synaptic potentiation it would also be useful to assay anxiety-like behaviour prior to recording from these dopamine neurons, in group-housed, socially isolated, saline-, and cocaine-treated mice, in order to determine whether anxiety levels correlate with synaptic strength. However, one caveat of this approach

could be that the behavioural assay itself has an acute stress-like effect, which may produce synaptic alterations in these neurons.

Another possible approach to testing the hypothesis that this potentiation is anxiety-induced could be to administer an acute dose of an anxiogenic pharmacological agent. A corticotrophin-releasing factor receptor 1 (CRFR1) agonist, for example, has been shown to increase anxiety levels measured in the open field or elevated-plus maze (Sutton et al., 1982; Koob et al., 1985; Moreau et al., 1997; Momose et al., 1999; Valdez et al., 2002; Bakshi et al., 2002; Zorrilla et al., 2002). A single dose of corticosterone has also been shown to increase anxiety-like behaviour, although this is only apparent 12 days, and not 24 hours, after exposure, suggesting a delayed anxiogenic effect (Mitra and Sapolsky, 2008). However, similar to the effect of acute social isolation, there is a modest effect of corticosterone after just 24 hours on anxiety in the elevated zero-maze (Conrad et al., 2011), suggesting perhaps, that subtle responses are beginning to emerge after 24 hours, but are not fully apparent. Other well established anxiogenic pharmacological agents that could be tested are yohimbine (Handley and Mithani, 1984; Pellow et al., 1985; Baldwin et al., 1989; Johnston and File, 1989; Cole et al., 1995; Redfern and Williams, 1995) and meta-chlorophenylpiperazine (mCPP; Mueller et al., 1985; Kennett et al., 1989; Benjamin, 1990; Kahn et al., 1990; Rodgers et al., 1992).

Equally, it would also be interesting to examine whether acute social isolation increases circulating levels of corticosterone, or CRF, which may indicate an acute stress response. Indeed other environmental manipulations, e.g. exposure to a novel environment, have been shown to increase levels of this stress hormone (Badiani et al., 1998; Piazza et al., 1991). However, this would not confirm whether this is a cause or consequence of the synaptic potentiation observed, or simply an additional side-effect.

8.3.4.2. The importance of prior social interaction

Regardless of whether social isolation causes an increase in anxiety or not, it clearly abolishes social interaction. It is intriguing, therefore, that an acute dose of cocaine is reported to reduce social interaction (Darmani et al., 1990; Rademacher et al., 2002; Estelles et al., 2004; Estelles et al., 2007), which presents a second similarity between these two stimuli. Synaptic strength following social isolation appears to be directly correlated with the extent of social contact prior to isolation. However, it remains to be determined whether this potentiation results from: (1) anxiety directly resulting from the loss of social interaction, or (2) loss of social interaction alone (i.e. independent of anxiety). However, any manipulation which increases anxiety is likely to also reduce social interaction (e.g., Dunn and File, 1987; Mele et al., 1987; Kennett et al., 1989; Elkabir et al., 1990), therefore teasing apart the relative importance of (and relationship between) anxiety and social interaction, is a considerable challenge.

I also found there was a trend for a positive correlation between the number of mice per cage prior to isolation, and the time spent in the periphery of the open field post-isolation, hinting that possibly higher anxiety levels result from a greater loss of social contact (i.e. in support of hypothesis 1). However, this correlation was not significant, probably because the majority of mice were isolated from a cage of three, with only one mouse isolated from a cage of two, and one from a cage of four. This limited sample range is not sufficient to reveal the relationship between prior social interaction and the anxiety response to social isolation. It would, therefore, be important to establish whether anxiety levels are directly related to the loss of social contact by increasing the sample size of isolated mice from different sized social groups. Given that I also observed strong positive correlation between the number of mice per cage prior to isolation, and the AMPAR:NMDAR ratio post-isolation, a positive relationship between cage size and anxiety would suggest that this potentiation may be related to the anxiety induced by the

loss of social contact (although it would not be sufficient to demonstrate a direct causal link between the two). However, if increasing loss of social contact does not predict higher anxiety levels, it may be that another feature resulting from the loss of social interaction is important in inducing this potentiation (hypothesis 2).

It was also observed that cocaine-treated mice housed with a larger number of cagemates exhibited lower anxiety levels in the open field test, suggesting that social interaction may be able to counteract the anxiogenic effect of cocaine. This suggests that anxiety and social interaction are intimately connected, and under certain conditions, an increase in social interaction may be able to reduce anxiety. Indeed, social interaction is often used as a behavioural test for anxiety, and increased interaction is interpreted as reduced anxiety levels. However, the AMPAR:NMDAR ratio following a single dose of cocaine did not correlate with the number of cagemates, suggesting that perhaps cocaine-induced potentiation is independent of social interaction.

Whereas there are a few potential experiments which could be used to test whether an anxiogenic stimulus (regardless of a change in social interaction) can induce potentiation (described in 8.3.4.1), assessing the reverse is much harder, because any manipulation which alters social housing conditions is likely to have an impact on anxiety. One possible approach would be to examine the social hierarchy within a group of mice. Interestingly it has been shown, using the tube-test for social dominance in mice, that social rank correlates with synaptic strength in the mPFC (Wang et al., 2011). Furthermore, increasing or decreasing this synaptic efficacy causes an upwards or downwards movement, respectively, in the social rank of the mouse (Wang et al., 2011). This study also speculates that downstream targets of the mPFC, including the VTA, DRN, hypothalamus, and amygdala, are likely to be important in setting this social dominance hierarchy, and mediating different features of its behavioural expression

(Wang et al., 2011). Therefore the association observed here, between synaptic strength in DRN/vlPAG dopamine neurons and loss of social interaction, hints at the involvement of this system in social behaviour.

8.3.4.3. Can social isolation-induced potentiation be prevented?

Neither the anxiolytic drug diazepam, nor the NMDAR antagonist ketamine, prevented the social isolation-induced increase in the AMPAR:NMDAR ratio. This could be evidence in support of hypothesis 2, that the potentiation is independent of anxiety levels. However, the ineffectiveness of diazepam could be due to the acute effect of the drug compared to the longer period of social isolation. It has also been noted that single-housed rats are less sensitive to the anxiolytic effects of diazepam compared to pair-housed rats (Gardner and Guy, 1984), which may also have contributed to its lack of effectiveness. Another way to examine this could be to assay anxiety-like behaviour after social isolation with administration of diazepam, to ascertain whether this acute dose has any effect on anxiety levels after 24 hours. If it does reduce anxiety levels (but the potentiation is still evident) then this would suggest that the plasticity is independent of anxiety.

Furthermore, the result obtained with ketamine administration is also open to interpretation, as (although a trend towards smaller ratios was observed) the negative result does not necessarily indicate that the plasticity is NMDAR-independent. It could simply be that the time frame within which the ketamine is acting does not correspond to the time-scale over which isolation-induced changes are taking place. Although it is not an established anxiolytic like diazepam (O'Brien, 2005), ketamine has been reported to have therapeutic potential in the treatment of anxiety-related disorders (Vollenweider and Kometer, 2010). Another method to assess whether this plasticity is NMDAR-dependent would be to abolish NMDAR signalling, by selective deletion of the obligatory NR1 subunit in dopamine neurons. This has been shown to prevent cocaine-

induced plasticity in the VTA (Engblom et al., 2008; Zweifel et al., 2008), and so may reveal the necessity of NMDAR transmission for the induction of plasticity in the DRN/vlPAG.

8.3.5. Alternative hypotheses and considerations

8.3.5.1. Stress

An alternative hypothesis is that this potentiation is stress-induced. However, several lines of evidence caution against this interpretation. Firstly, excitatory synapses onto VTA dopamine neurons are potentiated by an acutely stressful experience (Saal et al., 2003; Lammel et al., 2011), and therefore I would have expected to observe an increase in the AMPAR:NMDAR ratio in the VTA following social isolation. However, this was not the case in this study, or following social isolation with a saline injection in Ungless et al. (2001). Secondly, it has been suggested that acute social isolation is anxiogenic, but not stressful *per se* (Morinan and Leonard, 1980) or associated with a depression-like phenotype (Kwak et al., 2009). However, distinguishing between an 'anxiogenic' and a 'stressful' stimulus is also difficult, as there may be considerable inter-animal variability in the emotional result of such a manipulation. Interestingly, even though natural rewards, addictive drugs, and stress all increase circulating levels of glucocorticoids, and increase dopamine release in the NAc (Kalivas and Duffy, 1995; Piazza et al., 1996; Piazza and Le Moal, 1997), only stress-induced, but not cocaine-induced, potentiation in the VTA is glucocorticoid-dependent (Saal et al., 2003). It would therefore be interesting to examine whether cocaine- and/or social isolation-induced plasticity in the DRN/vlPAG is dependent on glucocorticoid receptor activation.

These findings are consistent with the idea that neuroadaptations can be triggered by mild forms of stress (Grissom and Bhatnagar, 2009; Rothwell et al., 2011). Even the relatively mild injection stress has been suggested to contribute to synaptic changes in the dopamine system

(Barrot et al., 2000; Argilli et al., 2008). Therefore, aside from the functional implications of social isolation-induced synaptic plasticity, these results also highlight the importance of controlling environmental factors in studies of this kind.

8.3.5.2.Sleep deprivation

The DRN/vlPAG dopamine neurons have been proposed to promote arousal in the sleep-wake cycle, and their c-Fos expression is correlated with wakefulness (Lu et al., 2006). Therefore another hypothesis is that this potentiation is related to the sleep deprivation induced by either acute social isolation or a single dose of cocaine.

However, sleep deprivation and anxiety are closely related, therefore separating the contribution of these two factors is difficult. Chronic sleep deprivation in humans has been reported to lead to pathological anxiety (Peeke et al., 1980; Dinges et al., 1980; Roy-Byrne et al., 1986; Sagaspe et al., 2005), while the majority of people suffering from anxiety disorders are also sleep deprived (Wyatt et al., 1971). Moreover, animal models of sleep deprivation suggest a strong association with anxiety. However, although many of these studies report that sleep deprivation is anxiogenic (Pokk and Zharkovsky, 1995; Silva et al., 2004; Kumar and Garg, 2009; Kumar and Singh, 2009; Xu et al., 2010), others suggest that it is anxiolytic (Van Hulzen and Coenen, 1981; Pokk et al., 1996; Pokk and Zharkovsky, 1997; Pokk and Zharkovsky, 1998; Suchecki et al., 2002). This implies that the type of sleep deprivation protocol used, and the anxiety-like measure employed, is likely to heavily impact on the interpretation of the results. To investigate the relative contribution of sleep deprivation to potentiation at these synapses, a minimally-stressful protocol could be used, in group-housed mice, to see if sleep deprivation without a loss of social interaction can induce potentiation.

Interestingly, it has recently been shown that socially isolated mice display impaired recovery from sleep deprivation compared to paired mice (Ramesh et al., 2012). Furthermore, social interaction has been shown to not only reduce fear-conditioned responses (Harlow and Harlow, 1965; Davitz and Mason, 1955; Hake and Laws, 1967; Kikusui et al., 2006; Kiyokawa et al., 2007), but also to reduce the REM sleep fragmentation induced by conditioned fear, compared to socially isolated animals (DaSilva et al., 2011). Therefore social isolation may also induce neuroadaptations in the neural circuitry controlling sleep.

It has also been proposed that drug addiction is associated with dysfunction in regions of the brain that regulate sleep (Brower and Perron, 2009), and several studies have demonstrated an association between sleep disturbances in childhood (particularly insomnia) and early-onset drug abuse (Wong et al., 2009). REM sleep-deprived rats display hypersensitivity of post-synaptic dopamine receptors (Tufik et al., 1978) and subsensitivity of pre-synaptic DATs (Tufik et al., 1987). Furthermore, D_2 receptors appear to be directly involved in many of the behavioural effects induced by REM sleep deprivation (Nunes Junior et al., 1994; Andersen et al., 2003) since D_2 blockade suppresses REM sleep (Lima et al., 2008). This is an interesting observation, since a link has been proposed between reduced D_2-receptor expression and vulnerability to drug abuse (Dalley et al., 2007). Furthermore, repeated amphetamine administration leads to considerable disruption of the sleep-wake cycle, marked by a clear reduction in REM sleep and an increased latency to REM and non-REM sleep (Andersen et al., 2008). It has also been proposed that sleep disturbance is a universal risk factor for relapse in addiction to all psychoactive drugs (Brower and Perron, 2009), and it is a symptom reported in all substance-related withdrawal syndromes (American Psychiatric Association [DSM-IV], 2000). Both cocaine and social isolation, therefore, have an important impact on sleep regulation.

8.4. Importance of plasticity for downstream targets: central amygdala and BNST

An enhancement of excitatory synaptic strength in dopamine neurons increases the likelihood of burst firing, and subsequent dopamine release, which is crucial in dopamine-dependent plasticity observed in projection regions (Montague et al., 2004; Wickens et al., 2007; Kreitzer and Malenka, 2008; Surmeier et al., 2009; Maia and Frank, 2011). This is thought to be a particularly important mechanism by which addictive drugs promote learned associations with drug-related stimuli, thereby promoting drug-seeking behaviour (Hyman et al., 2006). In the same way that chronic cocaine exposure leads to persistent alterations of neural function in the reward circuitry that ultimately leads to addiction, it is thought that chronic anxiety-like stressors may result in maladaptive neural changes, which could underlie the anxiety component of chronic neuropsychiatric disorders (Vyas et al., 2002; Rainnie et al., 2004; de Kloet et al., 2005; Shekhar et al., 2005; McEwen, 2007). Therefore it is tempting to speculate that just as cocaine-induced potentiation in the VTA is required for subsequent induction of plasticity in the NAc (Mameli et al., 2009), acute anxiety-induced potentiation at synapses onto dopamine neurons of the DRN/vlPAG may be essential for synaptic changes in downstream structures. Interestingly, the central amygdala and BNST have been suggested to receive a major dopaminergic input from the DRN/vlPAG neurons (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006; Shin et al., 2008).

The amygdala is a complex, highly interconnected structure, which has a well-established role in regulating emotional behaviour, including the response to stress, fear and anxiety (Phillips and LeDoux, 1992; Davis et al., 1994; Pitkanen et al., 1997; LeDoux, 2000; Davis and Whalen, 2001; Everitt et al., 2003; Millan, 2003; Alvarez et al., 2008; Engin and Treit, 2008; Pérez de la Mora et al., 2008; Ehrlich et al., 2009; Herry et al., 2010). Learning to recognise and respond to negative cues is evolutionary conserved to facilitate survival (Cho and Fudge, 2010). There is also strong

evidence implicating the amygdala in social behaviours, as lesioning in nonhuman primates causes profound deficits in social interaction (Kling, 1968; Dicks et al., 1969; Kling et al., 1970; Kling and Cornell, 1971; Kling and Steklis, 1976).

The central amygdala and BNST (part of the extended amygdala) are both heavily implicated in mediating anxiety-like behaviour (Inoue et al., 1994; Walker et al., 2003; Kalin et al., 2004) and their dopaminergic input has been suggested to play an important role in modulating the response to aversive stimuli (Asan 1997; Guarraci and Kapp, 1999; Inglis and Moghaddam, 1999; Pezze and Feldon, 2004; Engin and Treit, 2008; de la Mora et al., 2010). Dopamine release in the amygdala has also been shown to facilitate LTP (Bissiere et al., 2003; Kash et al., 2008; Tye et al., 2010), which may underlie long-lasting changes in behaviour, and is likely to be responsible for maladaptive behaviours which manifest in neuropsychiatric disorders.

8.4.1. Central amygdala (CeA)

The CeA has been heavily implicated in attention and motivation in response to both rewarding and aversive events (Holland and Gallagher, 1999; Baxter and Murray, 2002; Merali et al., 2003; Balleine and Killcross, 2006). Along with other subnuclei of the amygdala, neurons of the CeA have been proposed to signal motivational salience, and their activity is correlated with behavioural measures of arousal (Nishijo et al., 1988; Belova et al., 2007; Shabel and Janak, 2009). The CeA serves as the major output of the amygdala, and participates in the control of physiological and behavioural defensive responses to both conditioned and unconditioned aversive stimuli (Kapp et al., 1979; Hitchcock and Davis, 1986; Beaulieu et al., 1987; Iwata et al., 1987; LeDoux et al., 1988; Roozendaal et al., 1990; Davis, 1992; Davis et al., 1994; Campeau and Davis, 1995; Walker and Davis, 1997; LeDoux, 2000; Kalin et al., 2004; Gozzi et al., 2010).

There is a strong dopaminergic input to the CeA (Fuxe, 1965a,b; Asan, 1993; Freedman and Cassell, 1994; Eliava et al., 2003; Fuxe et al., 2003), which is considered to play an important role in the modulation of fear and anxiety (Pezze and Feldon, 2004; Engin and Treit, 2008; de la Mora et al., 2010). Dopamine is reportedly released in the amygdala under stressful conditions (Abercrombie et al., 1989; Coco et al., 1992; Young and Rees, 1998; Inglis and Moghaddam, 1999; Yokoyama et al., 2005), and while there is a high density of both D_1 and D_2 dopamine receptors in this region (Boyson et al., 1986; Dawson et al., 1986; Scibilia et al., 1992; Maltais et al., 2000; Fuxe et al., 2003), D_1 receptors have been implicated in promoting anxiogenic behaviours (Lamont and Kokkinidis, 1998; Guarraci et al., 1999a,b; Nader and LeDoux, 1999; de la Mora et al., 2005). The role of D_2-receptors appears more complex, with contradictory findings likely to be a result of antagonist infusion into different subregions of the amygdala, and the type of behavioural assay employed (Nader and LeDoux, 1999; Greba et al., 2001; de Oliveira et al., 2011; Guarraci et al., 2000; Perez de la Mora et al., 2012). Nevertheless, there is strong evidence of a modulatory role for dopamine within the CeA.

8.4.2. BNST

The BNST plays an established role in mediate stress- and anxiety-like responses to diffuse or unpredictable stimuli (Davis, 1998; Walker et al., 2003). Anxiogenic compounds or experiences increase neuronal activation within the BNST (Singewald et al., 2003; Straube et al., 2007), stimulation of the BNST elicits an anxiety-like physiological response (Casada and Dafny, 1991; Kalin et al., 2005), and inactivation of the BNST blocks many anxiogenic behaviours (Davis et al., 1997; Fendt et al., 2003; Hammack et al., 2004; Sullivan et al., 2004; Waddell et al., 2006). The BNST has been particularly associated with mediating long-term ‘tonic’ anxiety-like behavioural responses to sustained, diffuse and/or unpredictable threats (Walker et al., 2003; Waddell et al., 2006; Walker and Davis, 2008; Walker et al., 2009; Davis et al., 2010), as opposed to the CeA

which is thought to be more important in the rapid, acute response to anxiety-like stressors (Walker et al., 2003; Kalin et al., 2005; Davis et al., 2010). Consistent with this, chronic stress promotes maladaptive plasticity and remodelling within the BNST, which is associated with pathological anxiety-like behaviour (Schulkin et al., 1998; Vyas et al., 2002; Vyas et al., 2003; Pego et al., 2008). Therefore, stress-induced alterations in neuronal function and synaptic plasticity within this region are thought to underlie anxiety disorders in humans (Straube et al., 2007). The BNST also contains a high density of D_1- and D_2-receptors, and receives a strong dopaminergic input, which is thought to contribute to BNST-mediated behaviours and synaptic plasticity (Wamsley et al., 1989; Phelix et al., 1992; Epping-Jordan et al., 1998; Hurd et al., 2001; Eiler et al., 2003; Kash et al., 2008).

It is of particular interest to note that one of the very few studies to have investigated 24 hours of social isolation found that this acute manipulation resulted in blunting of NMDAR-dependent LTP in the dlBNST (Conrad et al., 2011). This was also observed following chronic social isolation (6 weeks) or chronic corticosterone administration, both of which (unlike acute social isolation) significantly increased anxiety measured in the open field test and elevated-zero maze (Conrad et al., 2011). This supports the notion that acute social isolation can induce alterations in synaptic strength after just 24 hours, and implicates the DRN/vlPAG dopamine neurons as a key participant in this neural response. It was also hypothesized by Conrad et al. (similar to the speculation discussed here) that these acute changes may have significance for long-term neuroadaptations. They suggest that the chronic isolation-induced state of enhanced anxiety, driven in part by synaptic changes in the dlBNST, was only beginning to develop after 24 hours (Conrad et al., 2011). Interestingly, chronic social isolation is thought to be associated with multiple changes to dopamine neurotransmission, including that within the amygdala (Guisado et al., 1980; Bardo and Hammer, 1991; Jones et al., 1992; Phillips et al., 1994; Smith et al., 1997;

Fulford and Marsden, 1998; Hall et al., 1998a; Heidbreder et al., 2000; Howes et al., 2000; Del Arco et al., 2004; Peters and O'Donnell, 2005; Djouma et al., 2006). However, the precise role of dopamine and the amygdala in mediating the effects of social isolation remains unknown.

8.4.3. Significance of projection target

Increasingly, evidence is accumulating that the projection target (Lammel et al., 2011) and the source of afferent input (Good and Lupica, 2010) are crucial determinants of the functional output of a dopamine neuron. The demonstration that only a particular subset of synapses are potentiated by cocaine, or an aversive event, dependent on their projection target (Lammel et al., 2011), suggests that these salient stimuli influence dopaminergic activity in restricted neural circuits. Therefore, although this presents a greater technical challenge, determining the connections of a neuron prior to recording allows changes to be interpreted in terms of distinct neural circuits, rather than generalised neurotransmitter release. Therefore, one key future experiment would be to assess cocaine- and social isolation-induced plasticity in a projection-specific manner. This could be achieved by employing the same retrograde labelling technique as Lammel et al. (2008, 2011), which involves injection of fluorescently-tagged retrobeads into specific target sites, followed by recording from identified dopaminergic neurons. Specifically, the dlBNST and central amygdala would be intriguing projection targets to examine, given their proposed roles in anxiety-like behaviour. While this would be an important aspect of future work, its absence does not detract from the importance of these findings. More, these results provide a useful guide as to the role of this understudied subset of dopamine neurons, proposing a new type of salient stimulus processed by dopamine neurons, and possibly including a considerable number of mesoamygdaloid neurons, which are sparsely distributed, and therefore rarely recorded from, in the VTA.

Indirectly, the use of VIP expression in the DRN/vlPAG dopamine neurons could be used as an indicator of projection target, as these neurons appear to project selectively to the dlBNST (Petit et al., 1995; Kozicz et al., 1998). However, for long duration recordings involving evoked synaptic currents, I found that not only were less neurons successfully recovered with immunohistochemistry, but there was a bias towards recording from the larger, more ventrally-located VIP- neurons. This was mainly due to the relative position of the stimulating electrode, and the difficulty in evoking currents in the small, periaqueductal VIP+ neurons. Therefore, while I was able to confirm the majority of my electrophysiological findings in identified VIP- neurons, I did not have a sufficient number of identified VIP+ neurons to also confirm the synaptic response of this subpopulation. When pooling data from multiple groups, VIP+ neurons appeared to show a trend towards higher AMPAR:NMDAR ratios following social isolation, suggesting that perhaps this acute manipulation does affect the dlBNST-projecting dopamine neurons. However, the sample size was not sufficient for a conclusive result, therefore future work would involve collecting more data from the VIP+ population to determine the effect of social isolation or cocaine on this subpopulation.

One approach to testing a causal role for the DRN/vlPAG dopaminergic projection to the amygdala in anxiety-like and social behaviour would be to exploit the spatial and temporal precision offered by optogenetics (Boyden et al., 2005; Tye and Deisseroth, 2012), in order to selectively control a specific dopaminergic projection. Optogenetic stimulation of VTA dopamine neurons has already demonstrated their requirement in reward-seeking behaviour (Tsai et al., 2009), while studies in the amygdala have demonstrated the capacity to modify anxiety-like behaviour by optogenetically controlling a specific set of synapses (Tye et al., 2011). Specifically, mice expressing channelrhodopsin-2 (ChR2; a light-activated cation channel for neural activation; Boyden et al., 2005) or halorhodopsin (eNpHR3.0; a light-activated chloride pump for

neural inhibition; Gradinaru et al., 2010), selectively in DRN/vlPAG dopamine neurons, could be examined in behavioural assays for general anxiety or social interaction. This could be combined with selective optogenetic control over the dopaminergic terminals within a specific target structure, for example the central amygdala or dlBNST, in order to reveal the role of these dopaminergic projections in specific aspects of behaviour. Additionally, combining optogenetic activation of the dlBNST projection, with infusion of vasoactive intestinal polypeptide receptor (VPAC) antagonists into this region, could indicate the requirement for co-release of VIP. Alternatively, VIP-Cre mice (Song et al., 2012) could be used to selectively control the VIP+ population. This could be combined with fast scan cyclic voltammetry to determine dopamine release, and VPAC or dopamine receptor antagonists to uncover the necessity of each neurotransmitter for a particular function.

8.4.4. Functional role of VIP

Although VIP expression within the central nervous system is limited, one major group of VIP neurons reside within the suprachiasmatic nucleus (SCN; Welsh et al., 1995; Romijn et al., 1997; Guillaumond et al., 2007). VIP release from these neurons, acting through the VPAC2 receptor, is thought to be critical in promoting circadian rhythmicity and behaviour, and maintaining synchrony between intrinsically rhythmic SCN neurons (Shen et al., 2000; Harmar et al., 2002; Colwell et al., 2003; Aton et al., 2005; Brown et al., 2006). However, interestingly, the VIP population within the DRN/vlPAG has also been implicated in the regulation of sleep and wakefulness, possibly exerting their effects via local inhibition of the serotonergic system (Bourgin et al., 1997; Simon-Arceo et al., 2003; Ahnaou et al., 2006). Given that it has also been suggested that the dopaminergic neurons in this region participate in regulating the sleep-wake cycle (Lu et al., 2006), it is likely that both dopamine and VIP release from these neurons is important in this function. VIP has also been implicated in pain sensitivity and analgesia (Sullivan

and Pert, 1981; Mácsai et al., 1998), which is another function attributed to the DRN/vlPAG dopamine neurons (Flores et al., 2004; Meyer et al., 2009), again suggesting a functional significance for the co-expression of these neurotransmitters.

Interestingly, VIP has also been implicated in anxiety and social behaviour. Blockade of VIP signalling during a critical period of embryogenesis disrupts development and causes a permanent change in brain neurochemistry (Gozes et al., 1989; Gressens et al., 1993; Hill et al., 1994; Wu et al., 1997; Glazner et al., 1999; Hill et al., 1999; Servoss et al., 2001; Hill et al., 2007a,b). This results in impaired learning, increased anxiety-like behaviour, and deficits in social behaviour in adulthood, which is proposed to bear similarities to autism-like social deficits (Hill et al., 2007a,b). It has also been shown that disruption of VIP signalling is associated with cognitive, and memory and learning deficits (Cottrell et al., 1984; Flood et al., 1990; Glowa et al., 1992, Gozes et al., 1993; Takashima et al., 1993a,b; Itoh et al., 1994; Chaudhury et al., 2008), which are functions routinely ascribed to the dopaminergic system. Reduced plasma concentrations of VIP have been found in patients with anxiety and depressive disorders, as well as reduced levels in the cerebrospinal fluid of patients with depression, which suggests a possible role for this neuropeptide in the neurobiological mechanisms of affective disorders (Gjerris et al., 1984; Rafaelsen and Gjerris, 1985; Gjerris, 1988; Saiz Ruiz et al., 1992). Furthermore, duplications in the VIP receptor gene, VIPR2, have been associated with an increased risk for schizophrenia (Vacic et al., 2011). Given that this has predominantly been viewed as a disorder of dopamine dysfunction, this represents another potential functional link between these two neurotransmitter systems. Consistent with this, it has been noted that the orphan nuclear receptor, Nurr1, which is essential for the formation of midbrain dopamine neurons during development (Zetterstrom et al., 1997), can directly regulate VIP transcription,

and activate its expression (Luo et al., 2007). These studies point towards a functional association between VIP and dopamine, which would be intriguing to examine further.

8.5. Clinical relevance of these findings

The results presented here implicate the DRN/vlPAG dopamine neurons in the response to social isolation, and possibly anxiogenic stimuli, in contrast to the rewarding stimuli processing by the VTA. The DRN/vlPAG provides a substantial dopaminergic input to the central amygdala and BNST (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006; Shin et al., 2008), and therefore synaptic changes in this population may have functional implications for dopamine release and neuroadaptations, particularly in these structures. The findings reported here are of relevance not only to anxiety-related disorders, but also to those with a social dysfunction component, such as autism, which have all been associated with the amygdala in humans. Dopamine has also been linked to many neuropsychiatric disorders with anxiety-like and social impairments, including schizophrenia (Swerdlow and Koob, 1987; Davis et al., 1991; Lodge and Grace, 2011), depression (Dailly et al., 2004; Nestler and Carlezon, 2006), ADHD (Swanson et al., 1998; Swanson et al., 2000; Tye et al., 2010), and autism (Lake et al., 1977; Ernst et al., 1997; Baron-Cohen, et al., 2000; Robinson et al., 2001; Gadow et al., 2008; Nakasato et al., 2008). Additionally, chronic social isolation is frequently used as an animal model of schizophrenia and depression, since many of the behavioural manifestations bear similarities to those characterising these disorders (Ehlers et al., 1993; Geyer et al., 1993; Jaffe et al., 1993; Myhrer, 1998; Lapiz et al., 2003; Heim et al., 2004; Fone and Porkess, 2008; Wilkinson et al., 2009; Fischer et al., 2012; Moller et al., 2012).

8.5.1. Anxiety disorders

Anxiety disorders span a range of diagnoses including panic disorder, agoraphobia, post-traumatic stress disorder, social anxiety disorder/social phobia, generalized anxiety disorder,

and obsessive-compulsive disorder (American Psychiatric Association [DSM-IV], 2000). Anxiety disorders have the highest prevalence of all psychiatric diseases, both in the US (Kessler et al., 2005) and in many European countries (Alonso and Lepine, 2007), and show high co-morbidity with other neuropsychiatric diseases including addiction and major depression (de Graaf et al., 2002; Ressler and Mayberg, 2007). Furthermore, these disorders are associated with a huge socioeconomic burden (François et al., 2010), with the direct cost of anxiety disorders estimated at $63.1 billion in the US in 1998 (Greenberg et al., 1999). Current treatments, however, are limited in their efficacy and consistency. For example, selective serotonin reuptake inhibitors are associated with undesirable side-effects including headaches, nausea and insomnia, while benzodiazepines carry the risk of dependence and cognitive impairments (Woods et al., 1992; Stein, 2004). This highlights the need for more effective therapeutic interventions, which first requires a comprehensive understanding of the neural circuits underlying anxiety.

Aside from the wealth of animal evidence which implicates the amygdala in anxiety-like behaviour, hypersensitivity in the amygdala has been associated with depression and related disorders in humans (Sheline et al., 2001; Siegle et al., 2002). This is proposed to result from misinterpretation of, and an exaggerated response to, negative cues (Wenzlaff et al., 1988; Oaksford and Stenning, 1992; Cho and Fudge, 2010). Furthermore it has been shown in humans that increased dopamine release potentiates amygdala function (Hariri et al., 2002; Tessitore et al., 2002), suggesting that variability in dopamine signalling may underlie the individual variability in amygdala function and emotional behaviours. Consistent with this, it was demonstrated that amygdala dopamine storage capacity is correlated with amygdala activation in response to an aversive stimulus (Kienast et al., 2008). It has therefore been hypothesised that differences in amygdala dopamine contribute to individual differences in trait anxiety (Kienast et al., 2008).

8.5.2. Autism and social interaction

Autism Spectrum Disorders (ASDs) are characterised by abnormal social behaviour, in addition to deficits in communication, repetitive behaviours, and narrowly focused interests (Howlin and Moore, 1997; Schultz, 2005; White and Roberson-Nay, 2009; Landrigan, 2010). Furthermore, up to 74% of autistic persons have generalized anxiety disorder (Fombonne, 1992; Kim et al., 2000; Gillott et al., 2001; Hill and Furniss, 2006). Despite tremendous research efforts aimed at identifying the cause of autism, no singular cause has emerged, and available medications are 'off-label' therapies that are typically prescribed for other conditions (Happe et al., 2006; Filipek et al., 2006; Oswald and Sonenklar, 2007).

In humans, the amygdala has been linked to a number of processes directly relevant to the core symptoms of autism, including social anxiety (Amaral, 2003; Etkin et al., 2004; Killgore and Yurgelun-Todd, 2005; Phan et al., 2006), the recognition of emotional facial expressions (Morris et al., 1996; Schultz 2005; Ashwin et al., 2007), the size of our social networks (Bickart et al., 2011) and even the concept of personal space (Kennedy et al., 2009). Amygdala lesions in animals produce some autism-like behaviours (Hetzler and Griffin, 1981), while in patients this results in errors in social judgement (Adolphs et al., 1994; Young et al., 1996). Additionally, fMRI has revealed altered amygdala volume (Abell et al., 1999; Sparks et al., 2002; Schumann et al., 2004) and reduced activation (Baron-Cohen et al., 1999) in individuals with autism, while signs of neuropathology have been observed in the amygdala of post-mortem autistic brains (Bauman and Kemper, 1985; Abell et al., 1999; Aylward et al., 1999; Howard et al., 2000; Pierce et al., 2001; Schumann and Amaral, 2006). This has led to the hypothesis that dysfunction of the amygdala may be partly responsible for the deficits in social behaviour seen in autism (Baron-Cohen et al., 2000).

Interestingly, hyperactivation of the dopamine system has been hypothesized to play a role in autism (Nakasato et al., 2008), based on evidence that risperidone, which blocks postsynaptic dopamine receptors, is effective in treating many symptoms and stereotyped behaviours of individuals with autism (McCracken et al., 2002; McDougle et al., 2005). In addition, of relevance to the findings here, it is also interesting to note that increased VIP levels have been reported in newborns with autism (Nelson et al., 2001), implicating this neuropeptide in the pathophysiology of this neurodevelopmental disorder.

8.6. Conclusion

The results presented here are relevant to several neuropsychiatric disease states, which are currently limited in their understanding and available treatments. Given that the DRN/vlPAG dopamine population is tightly integrated into the neural circuit which mediates many of the maladaptive behaviours associated with these disorders, it is likely to play a crucial regulatory role in the expression of these responses. Finally, and most importantly, the amygdala (Sander et al., 2003) and the dopamine system (Pani and Gessa, 1997) display remarkable conservation throughout evolution, suggesting that findings in animals will be relevant to humans, and thus have considerable translational potential. These results are therefore of interest to disease states modelled by social isolation, and disorders of anxiety, as well as to the rapidly evolving field of social neuroscience (Eisenberger and Cole, 2012).

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Acknowledgements

I would like to thank my supervisor Mark Ungless for his guidance, support, encouragement, and numerous motivational pep talks over the last few years.

I am very grateful to Matthew Bishop for his unwavering patience while teaching me electrophysiology, and to Frederic Brischoux for assisting me with perfusions and immunohistochemistry (and teaching me the odd bit of French). I am also grateful to Piotr Czapski for performing the IP injections, Rebecca Davis for the behavioural work, and to all members of the Neurophysiology group – past and present – for their valuable discussions, support, and friendship.

I will be forever indebted to my wonderful family, particularly my parents Val & Paul and my sister Helen, for their limitless patience, support, and encouragement; and also to my long-suffering boyfriend Conall, for cooking me countless meals and being a constant source of motivation.