Evidence for a 5-HT_6 receptor-mediated control of midbrain 5-HT neurons

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Julia T Brouard, University College Oxford, Trinity Term 2016

Abstract

The dorsal raphe nucleus (DRN) contains the majority of 5-hydroxytryptamine (5-HT)-producing neurons, whose firing is influenced by homeostatic feedback pathways. Dysfunction of 5-HT neurons is implicated in the pathophysiology of diseases such as depression. Thus, pathways regulating 5-HT neuron activity represent potential therapeutic targets. Negative feedback pathways influencing 5-HT neurons include 5-HT_{1A} autoreceptors located in the DRN, and feedback from 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors on postsynaptic neurons in forebrain regions. One pathway, mediated by 5-HT_4 receptors on medial prefrontal cortex (mPFC) neurons, positively regulates 5-HT neuron activity. Preliminary findings in the Sharp Laboratory suggested that 5-HT_6 receptors may also positively regulate 5-HT neuron activity. Using *in vivo* electrophysiological recordings in combination with drug tools, this thesis investigated the 5-HT_6 receptor control of 5-HT neurons, a proportion of which were identified as 5-HT-containing using a juxtacellular labelling technique.

Electrophysiological recordings in anaesthetised rats found that 5-HT_6 receptor agonists WAY-181187 and WAY-208466 increased 5-HT neuron firing to 58% and 47% above pre-drug firing, respectively. By contrast 5-HT_6 receptor antagonist SB-399886 reduced 5-HT neuron firing to 56% below pre-drug firing. Four other 5-HT_6 ligands, agonist ST-1936, and antagonists AE-58054, SB-258585, and SB-271046, had no significant effect on 5-HT neuron firing. Simultaneous electroencephalogram (EEG) recordings found that WAY-181187 and WAY-208466 reduced frontal cortex slow wave (SW) oscillation power to 62% and 60% below pre-drug values, respectively. It was considered that the frontal cortex may be involved in the effect of these agonists on 5-HT neuron firing. Subsequent experiments investigated the influence of 5-HT_6 receptors over mPFC neural activity.

WAY-181187 reduced mPFC SW oscillation power maximally to 54% below pre-drug values. This occurred in a putatively 5-HT_6 receptor-dependent manner since the effect was blocked by SB-399885 and AE-58054. WAY-181187 also modulated the firing of mPFC pyramidal neurons, including those projecting to the DRN. Specifically, one population of neurons was excited by WAY-181187, with a maximum increase in firing of 161% above pre-drug firing, whereas another population was inhibited by WAY-181187, with a maximum decrease to 73% below pre-drug firing.

In a final set of experiments neurotoxic mPFC lesion attenuated the excitatory effect of WAY-181187 on 5-HT neuron firing. Thus the effect of WAY-181187 at 2mg/kg was significantly reduced in lesioned rats compared to sham controls.

Experiments in this thesis provide evidence to support a role of 5-HT_6 receptor-mediated excitatory control of DRN 5-HT neurons via an mPFC-dependent mechanism. This control may act with previously reported feedback mechanisms to balance inhibitory and excitatory input to 5-HT neurons. Finally, 5-HT_6 receptor-mediated feedback may be a useful target for modulating the 5-HT-system, such as in depression therapy.

Publications arising from this thesis

Parts of this work have been published and presented as follows:

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2) Effect of 5-HT_6 receptor ligands on neural network oscillations and the firing of pyramidal neurons in the rat prefrontal cortex *in vivo* (2015) Brouard, J. T., Frisch Herrik, K., Helboe, L., De Jong, I.E.M., & Sharp, T.
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Home office Licences

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Abbreviations

5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
COVISI	coefficient of variation of the interspike interval
DOI	2,5-dimethoxy-4-iodoamphetamine
DRN	dorsal raphe nucleus
EEG	electroencephalogram
ERK1/2	extracellular signal-related kinase ½
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LFP	local field potential
LHb	lateral habenula
LSD	lysergic acid diethylamide
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
REMs	rapid eye movement sleep
RT-PCR	reverse transcription polymerase chain reaction
SN	substantia nigra
SSRI	selective serotonin reuptake inhibitor
SW oscillations	slow wave oscillations
VGluT	vesicular glutamate transporter
VTA	ventral tegmental area

Chapter 1

General Introduction

1.1 Scope of this thesis

5-HT is a central neurotransmitter that modulates numerous physiological functions, ranging from the regulation of mood and cognition to appetite and the sleep-wake cycle. 5-HT-producing neurons reside in a cluster of midbrain nuclei, with those in the dorsal raphe nucleus (DRN) providing the main source of 5-HT projections to the forebrain region (Törk, 1990). Once released from nerve terminals 5-HT can act on up to 14 receptors which fall into seven families (5-HT_1 to 5-HT_7) and are found throughout the CNS of humans and other species including rodents (for review see Barnes and Sharp, 1999). Evidence suggests 5-HT neurons are under strict homeostatic feedback control from pre-synaptic 5-HT receptors, as well as neurons in forebrain regions expressing various 5-HT receptors (for review see Sharp et al., 2007).

The 5-HT_6 receptor is a Gs-coupled metabotropic receptor and unique amongst 5-HT receptors in being almost exclusively expressed in the brain (Hirst et al. 2003). It localises to post-synaptic neurons in many brain regions and is particularly abundant in the cortex, hippocampus and striatum (Ward et al., 1995; Gerard et al., 1996; Gerard et al., 1997; Helboe et al., 2015). Microdialysis studies have suggested the 5-HT_6 receptor exerts control over the release of various transmitters including dopamine, noradrenaline and acetylcholine (Lacroix et al., 2004; Hirst et al., 2006; Schecter et al., 2008; Valentini et al., 2011). Furthermore, rodent behavioural studies have provided evidence for a role of 5-HT_6 receptors in learning, memory and cognition (Hirst et al., 2006; Arnt et al., 2010; Burnham et al., 2010; de Bruin et al;

2011; Gravius et al., 2011; Woods et al. , 2012; Nikiforuk et al., 2013), and recent clinical data has demonstrated the effectiveness of 5-HT_6 receptor antagonists as augmentation therapy for Alzheimer's disease (Maher-Edwards et al., 2011; Wilkinson et al., 2014). Work in rodents has also shown 5-HT_6 receptor ligands to be antidepressant and anxiolytic (Svenningsson et al., 2007; Wesolowska et al., 2007; Hirano et al., 2009; Nikiforuk et al., 2011; Carr et al., 2011; Jastrzębska-Więsek et al., 2015), and these drugs also exert an influence on sleep and waking parameters (Morairty et al. 2008; Monti and Jantos, 2011; Ly et al., 2013; Monti et al., 2013).

A growing body of evidence suggests 5-HT_6 receptor-expressing neurons may have an influence over the activity of 5-HT neurons. This thesis aims to investigate 5-HT_6 receptor control of 5-HT neurons using a combination of electrophysiological, immunohistochemical and lesion techniques.

1.2 Background

5-HT was first isolated in the periphery, from platelets and enterochromaffin cells in the gut. It was originally conceived as two compounds, named serotonin or enteramine, due to its potent vasoconstrictor properties and effects on gut motility, respectively (Rapport, Green and Page, 1948; for review Gothert, 2013). However, in 1952 these two indoleamine compounds were recognised to be the same, and so renamed 5-hydroxytryptamine (5-HT) (Erspamer and Asero, 1952). Shortly after 5-HT was extracted from brain tissue (Twarog and Page, 1953), Woolley and Shaw began research into the hallucinogenic effects of 5-HT analogues and 5-HT partial agonists such as lysergic acid diethylamide. This led them to hypothesise that 5-HT in the CNS may contribute to various mental health disorders (Woolley and Shaw, 1954). While the proposed link between 5-HT and mental health has since developed

in complexity, this seminal theory sparked decades of research into the nature of 5-HT modulation of emotion, cognition, learning and memory, sexual behaviour, and more.

1.3 The central 5-HT system

The central 5-HT system modulates a range of physiological functions through its actions in cortical and subcortical regions. It can act as a signal causing neuronal differentiation and migration during development, while in the developed brain it has a role in higher functions. Furthermore, changes in 5-HT function have been linked to various CNS disorders including depression, anxiety, Alzheimer's disease, schizophrenia, and Parkinson's disease.

Today several therapeutics targeting the central 5-HT system are in clinical use. This includes the use of serotonin reuptake inhibitors (SSRIs) to treat depression and anxiety, vortioxetine, a recently developed multimodal antidepressant which targets 5-HT_3 and 5-HT_7 receptors among others (Sanchez et al., 2015), and the anxiolytic 5-HT_{1A} receptor agonist buspirone (Feighner, 1987). Other drug therapies include atypical antipsychotics such as risperidone and aripiprazole which exhibit high affinities for 5-HT_{2A} and 5-HT_7 receptors alongside a number of other 5-HT and transmitter receptors (Miyamoto et al., 2005). Finally, in recent years several 5-HT_6 receptor antagonists have entered clinical trial for Alzheimer's disease, with some drugs showing efficacy particularly when combined with acetylcholine esterase inhibitors (Maher-Edwards et al., 2010; Wilkinson et al., 2014). Despite this, many of the networks involved in the 5-HT modulation of CNS functions remain unknown, and understanding these circuits is likely to be essential for future drug development.

1.3.1 The raphe nuclei

5-HT-containing cell bodies reside in clusters which extend from the rostral midbrain to the caudal medulla, as was first identified in rats by Dahlstrom and Fuxe using a histofluorescent technique (Dahlstrom and Fuxe, 1964). This technique involved application of formaldehyde to monoamine-containing cell bodies, causing the monoamine transmitters to be converted into molecules which can be visualised when exposed to UV light (Dahlstrom and Fuxe, 1964). Originally named cell bodies B1-B9, the 5-HT-containing nuclei are now more commonly referred to as the raphe nuclei, derived from the Latin for 'seam', which denotes their location along the midline of the brain (Dahlstrom and Fuxe, 1964; Figure 1.1). The classification of the raphe nuclei broadly overlaps with the B1-B9 cell body divisions (Figure 1.1). 5-HT-containing cell bodies in humans are also found in the brain stem and are considered to have a similar arrangement as those in rats (Takahashi et al., 1986; Hornung and Törk, 1990; Baker et al., 1990).

Studies in rats suggest that during early development 5-HT neurons in the raphe nuclei develop polarity with regard to the direction of their axonal projections, and this polarity is maintained in the adult (Lidov et al., 1982; Aitken and Törk, 1988). Thus, neurons in the rostral raphe nuclei project to the forebrain, whereas neurons in the caudal raphe nuclei project to the spinal cord (Lidov et al., 1982; Aitken and Törk, 1988; Törk, 1990). The rostral group of raphe nuclei is made up of the caudal linear nucleus (B8), the dorsal raphe nucleus (DRN; B6-B7) and median raphe nucleus (B5 and B8) (Törk, 1990). Of these, the majority of 5-HT projections to the forebrain originate from the dorsal and medial raphe nuclei. The caudal group of raphe nuclei include the raphe magnus (B3), raphe pallidus (B1), and raphe obscurus nuclei (B2

and B4), as well as other 5-HT neurons residing in the medullary reticular formation (B3) (Törk, 1990). 5-HT projections originating from these nuclei make up the majority of descending 5-HT projections to the spinal cord (Törk, 1990).

1.4 The dorsal raphe nucleus

The DRN contains the greatest number of 5-HT neurons compared to other raphe nuclei, with approximately 11,000-15,000 5-HT neurons in the rat, which is estimated as 50% of the total 5-HT neuron population in the brain (Steinbusch et al., 1981; Descarries et al., 1982; Baker et al., 1990; Jacobs et al., 1992). The DRN can be subdivided into dorsomedial (midline), ventromedial and lateral regions (Steinbusch et al., 1981; Figure 1.2), and evidence suggests the majority of 5-HT neurons in rats reside in ventromedial areas of the DRN (Steinbusch et al., 1981). By contrast, GABAergic neurons are thought to reside in the lateral wings of the nucleus (Allers and Sharp, 2003).

1.4.1 Forebrain projection sites of DRN 5-HT neurons

In primates as well as rats, DRN 5-HT projections to the forebrain send axons via the medial forebrain bundle, and projection sites have been mapped using a variety of methods including autoradiography with tritiated 5-HT and 5-HT immunohistochemistry (Descarries et al., 1975; Azmitia and Segal, 1978; Steinbusch et al., 1981; Steinbusch and Nieuwenhuys, 1981; Berger et al., 1988; Törk, 1990). These experiments have provided evidence to suggest that almost every region of the forebrain receives a 5-HT innervation including cortical regions, most notably the frontal cortex, regions of the striatum including the caudate putamen and nucleus accumbens, and areas of the limbic system including the hippocampus, thalamus,

hypothalamus, habenula and amygdala (Figure 1.1, Descarries et al., 1975; Azmitia and Segal, 1978; Steinbusch et al., 1981; Steinbusch and Nieuwenhuys, 1981; Berger et al., 1988; Törk, 1990).

Early studies of 5-HT neuron terminals using autoradiography in rats suggested that most 5-HT neurons did not form classical synapses at their projection sites (Descarries et al.,1975). However, later experiments using immunohistochemistry and anti-5-HT antibodies suggest that as many as 80% of 5-HT fibres in the rat cortex do form synapses (Papadopoulos et al., 1987). It is now thought that two types of ascending 5-HT projection fibres exist: fibres arising from the DRN, which are thin and varicose, mediate volume transmission and make few synaptic connections; and median raphe fibres which are non-varicose with large boutons that form classical synapses (Törk, 1990).

1.4.2 Inputs to DRN 5-HT neurons

Evidence suggests DRN 5-HT neurons receive an extensive input from forebrain regions including the hypothalamus, periaqueductal grey, substantia nigra (SN), ventral tegmental area (VTA), and prominent inputs from the medial prefrontal cortex (mPFC) and lateral habenula (LHb) (Aghajanian and Wang, 1977). In addition, DRN 5-HT neurons receive input from the DRN itself, for example from recurrent 5-HT axon collaterals (Wang and Aghajanian, 1977; Wang and Aghajanian, 1978), as well as from local GABAergic interneurons. These inputs serve to regulate 5-HT neuron firing and release, and are thought to utilise various transmitters.

Input from GABAergic and glutamatergic neurons are important mechanisms for regulating 5-HT neuron activity, as will be discussed. However, DRN 5-HT neurons also receive input from neuromodulatory transmitters, in particular dopamine and noradrenaline. For example, evidence from retrograde tracing and electron microscopy studies support the idea that noradrenergic terminals from the locus coeruleus input onto DRN 5-HT neurons in rats and cats (Aghajanian and Wang, 1977; Sakai et al., 1977; Baraban and Aghajanian, 1981). Consequently, α_1-adenoceptor agonists were found to inhibit 5-HT neuron firing, consistent with the presence of these receptors on 5-HT neurons (Gallager et al., 1976; Aghajanian, 1985; Gartside et al., 1997). In addition, studies in rats suggest that dopaminergic neurons arising from the VTA and SN provide input to DRN neurons (Kalen et al., 1988; Peyron et al., 1995). Thus, systemic administration of non-selective $D_{1/2}$ receptor agonist apomorphine in anaesthetised rats resulted in increased 5-HT release in the DRN and striatum, and increased 5-HT neuron firing (Martin-Ruiz et al., 2001). Moreover, these effects were preventable with pre-treatment with both D_1 antagonist SCH-23390 and D_2 antagonist raclopride (Martin-Ruiz et al., 2001).

Local regulation of DRN 5-HT neurons by GABAergic interneurons

Much evidence supports the idea that GABA interneurons are a key source of inhibitory input to DRN 5-HT neurons. For example, several studies have shown the presence of either glutamic acid decarboxylase (GAD) immuno-positive neurons in the DRN or [^{3}H]-GABA labelling in the DRN neurons in rats (Belin et al., 1979; Wang and Nakai, 1993; Varga et al., 2001; Allers and Sharp; 2003). Moreover, the presence of $GABA_A$ and $GABA_B$ receptors on DRN 5-HT neurons provides further support for a role of GABA interneurons in DRN 5-HT neuron regulation (Innis and Aghajanian,1987). Finally, *in vitro* 5-HT neuron inhibitory post-synaptic currents have

been reported to be blocked by $GABA_A$ antagonist bicuculline (Liu et al., 2000), and $GABA_B$ receptor agonist baclofen has been found to inhibit DRN 5-HT neuron firing *in vitro* (Liu et al., 2000; Judge et al., 2004).

mPFC regulation of DRN 5-HT neurons by glutamatergic inputs

The mPFC is thought to be a significant contributor of glutamatergic inputs to the DRN. Evidence from retrograde tracing studies have demonstrated extensive glutamatergic bilateral inputs from the mPFC to the DRN (Aghajanian and Wang, 1977; Hajos et al., 1998; Peyron et al., 1998; Vertes, 2004). These appear to arise predominantly in deeper mPFC layers, specifically layer V, and target the lateral regions of the DRN, where GABA neurons are mainly thought to reside (Hajos et al., 1998; Peyron et al., 1998; Varga et al., 2001; Allers and Sharp, 2003). Thus, evidence suggests that a large number of mPFC glutamatergic terminals synapse onto DRN GABA neurons (Varga et al., 2001; Jankowski and Sesack, 2004). However, more recent evidence from experiments using virally-mediated tracing of monosynaptic inputs to DRN 5-HT neurons also demonstrated the notable presence of direct mPFC inputs to DRN 5-HT neurons (Ogawa et al., 2014; Dorocic et al., 2014).

Electrophysiological studies have further provided insight into the function of mPFC inputs to DRN 5-HT neurons. In particular, several studies in anaesthetised rats have reported that electrical stimulation of the mPFC results in a long latency inhibition in the majority of recorded putative 5-HT neurons (Hajos et al., 1998; Celada et al., 2001; Varga et al., 2001; Varga et al., 2003). Moreover, this effect was preceded by a short latency excitation in non-5-HT neurons in the DRN, which were probably GABAergic (Varga et al., 2001). However, in a smaller population of DRN 5-HT

neurons direct excitation was observed following mPFC electrical stimulation, which may be the result of stimulation of mPFC glutamatergic neurons that form monosynaptic connections with 5-HT neurons (Hajos et al., 1998; Celada et al., 2001). A number of mPFC inputs to DRN neurons are thought to be controlled by 5-HT receptors which act as a homeostatic feedback mechanism to govern 5-HT neurons, as will be discussed later.

1.5 Characteristics of DRN neurons

1.5.1 Chemical characteristics of DRN neurons

Across species approximately 40-50% of neurons in the DRN are thought to be 5-HT- containing (Descarries et al., 1982). However, evidence suggests 5-HT neurons may co-express other transmitters. For example, co-expression of vGluT3 is reported in some 5-HT neurons, suggesting they may co-release glutamate (Liu et al., 2014). Moreover, as many as 80% of medial DRN 5-HT neurons may express nitric oxide synthase, suggesting they co-release nitric oxide (Vasudeva et al., 2011). The remaining DRN neuron population is predominantly made up of GABAergic neurons, although small populations of dopaminergic and cholinergic neurons have been reported (Schweimer et al., 2011; Dougalis et al., 2012; Matthews et al., 2016; Wang et al., 1998; de Oliveira et al., 2015).

Interestingly, recent evidence suggests some 5-HT neurons may co-express GAD and have slower firing rates than 5-HT only expressing neurons in rat DRN slice recordings (Shikanai et al., 2012). This suggests that a number of GABAergic ‘interneurons’ previously identified on the basis of their GAD-expression may also be 5-HT-containing. However, other work has shown that these neurons make up a

small proportion of DRN 5-HT neurons, since only 0.1-0.3% of DRN neurons stained positive for both 5-HT and GAD (Stamp and Semba, 1995).

Mechanisms regulating 5-HT neuron firing can be studied using *in vivo* electrophysiological recordings of DRN 5-HT neurons. In general, little is known about the electrophysiological properties of the smaller populations of DRN cells. However, *in vivo* electrophysiological properties of DRN 5-HT have been widely reported, there are also a few reports of DRN GABA neurons. Differences exist in the firing properties of DRN 5-HT and GABA neurons and this can aid the identification of neurons during electrophysiological recordings.

1.5.2 Electrophysiological characteristics of 5-HT neurons

Intracellular and extracellular *in vivo* recordings of DRN 5-HT neurons in anaesthetised rats have established that a large proportion of 5-HT-producing neurons exhibit a spontaneous, slow (< 2 Hz) and regular firing pattern, as well as broad bi- or tri-phasic action potentials (Aghajainian and Haigler 1974; Wang and Aghajanian 1977; Aghajanian et al., 1978; Aghajanian and Vandermaelen 1982; Sawyer et al., 1985). These cells are typically referred to as 'clock-like' 5-HT neurons. Several studies have characterised the responses of clock-like 5-HT neurons to certain pharmacological agents. For example, administration of 5-HT_{1A} agonists such as LSD or 8-OH-DPAT caused a strong inhibition of firing in these neurons (Aghajanian et al., 1968; Aghajanian and Vandermanelen 1982; Li et al., 2001). Also α_1-adrenoceptor antagonists, such as prazosin, inhibited the firing of 5-HT neurons (Aghajanian, 1985; Gartside et al., 1997). Importantly, DRN neurons exhibiting these electrophysiological and pharmacological properties have been

labelled *in vivo* in rats and found to be 5-HT-immuno-positive (Allers and Sharp, 2003; Schweimer et al., 2010; Schweimer et al., 2011). Thus, these characteristics serve as key identifiers of clock-like 5-HT neurons *in vivo*.

Another reasonably well-studied population of 5-HT neurons in the DRN exhibit burst firing (Hajos et al., 1995; Hajos and Sharp, 1996; Hajos et al., 2007). *In vivo* recordings from anaesthetised rats showed that these neurons exhibited a broad spike width, a slow firing rate and brief high frequency bursts (doublets or triplets), with an otherwise regular firing pattern (Hajos et al., 1995; Hajos and Sharp, 1996). Importantly, experiments combining *in vivo* recordings with juxtacellular labelling provided evidence for the 5-HT-identity of these neurons (Hajos et al., 2007; Schweimer et al., 2010).

Finally, there is evidence of a fast firing population of 5-HT neurons, which have been identified through juxtacellular labelling (Kocsis et al., 2006). *In vivo* recording of these neurons showed they exhibit firing rates > 6 Hz and a narrower spike width than clock-like and bursting neurons (Allers and Sharp, 2003; Kocsis et al., 2006). However, like other 5-HT neuron types, they appeared to be inhibited by the 5-HT_{1A} receptor agonist 8-OH-DPAT, suggesting the presence of 5-HT_{1A} autoreceptors (Allers and Sharp, 2003).

1.5.3 Electrophysiological characteristics of DRN GABA neurons

The majority of non-5-HT neurons in the DRN are thought to be GABAergic neurons. These were first identified as a population of non-5-HT neurons which were active *in*

vivo in rats following 5-HT neurotoxic lesion (Aghajanian et al., 1978). These neurons exhibited different firing characteristics to 5-HT neurons, including a fast and irregular firing pattern, and narrow spike width *in vivo* (Aghajanian et al., 1978; Sawyer et al., 1985; Varga et al., 2001). A later study which combined *in vivo* electrophysiological recordings with juxtacellular labelling confirmed the expression of GAD in DRN neurons with these characteristics (Allers and Sharp, 2003). Furthermore, unlike the 5-HT neurons which were most dense in medial portions of the DRN, GAD-expressing neurons predominantly localised to the lateral wings of the nucleus (Allers and Sharp 2003). Thus, overall current evidence suggests GABAergic neurons exhibit distinct electrophysiological properties compared to classical 5-HT neurons.

1.5.4 Juxtacellular labelling technique

The aforementioned electrophysiological properties can be used to help identify the type of neuron recorded in the DRN. However, the emerging gold standard for determining the neurochemical identity of a recorded neuron *in vivo* is a technique known as juxtacellular labelling, which allows for post-mortem immunohistological identification of the single recorded neuron.

This juxtacellular labelling technique allows individual neurons to be labelled with a marker, e.g. neurobiotin, while the recording electrode is kept juxtaposed to the target neuron. Evidence suggests this technique is highly successful in selectively labelling only the recorded neuron (Pinault, 1996). Moreover, juxtacellular labelling overcomes the difficulties arising from intracellular injection of markers into the recorded neurons, since the process of impaling the neuron to inject the marker can damage the cell, leading to its degradation (Pinault, 1996). By contrast, juxtacellular labelling involves passing positive current pulses of increasing amplitude through the

recording electrode that is placed extracellularly. This process expels the neurobiotin from the electrode and simultaneously causes the recorded neuron to fire in synchrony with the current pulses, a phenomenon known as entrainment. When a neuron is entrained it typically takes up neurobiotin from the extracellular space, allowing the neuron to be later identified using immunohistological techniques.

The mechanism by which neurobiotin is taken up into the neuron remains unknown. One possibility is that the current pulses cause small lesions in the neuronal membrane, allowing the marker to enter (Pinault, 1996). Alternatively, the marker may be taken up through voltage-gated channels which are activated by the current injections (Pinault, 1996; King et al., 1989). Regardless of the mechanism, juxtacellular labelling has been established as successful in labelling several neuron types *in vivo*, including DRN 5-HT neurons and GABAergic neurons (Allers and Sharp, 2003; Hajos et al., 2007; Schweimer et al. 2011), VTA dopamine neurons (Schweimer et al., 2014), cortical glutamatergic pyramidal neurons and several interneuron types (Zhang and Deschenes, 1997; Tierney et al., 2004; and Tseng et al., 2006).

1.6 Feedback mechanisms controlling 5-HT neuron activity

Much evidence suggests that the firing of DRN 5-HT neurons is under strict homeostatic control by feedback pathways located both within and outside the DRN (Sharp et al., 2007). The role of autoreceptors located on the soma, dendrites and terminals of 5-HT neurons in the inhibition of 5-HT neuron firing was the first homeostatic feedback mechanism to be delineated. However, more recent evidence suggests the presence of post-synaptic homoeostatic feedback mechanisms. Thus, 5-HT neurons projecting to certain forebrain regions receive reciprocal inputs from 5-

HT receptor-expressing neurons in these regions. In particular, negative feedback pathways arise from 5-HT_{1A} and 5-HT_{2A} receptor-expressing neurons in the mPFC which project to the DRN and act to inhibit 5-HT neuron firing (Figure 1.3). In addition, positive feedback is putatively elicited by 5-HT_4 receptors expressed on DRN-projecting mPFC pyramidal neurons, and act to increase 5-HT neuron firing (Sharp et al., 2007; Figure 1.3). As will be discussed, these feedback mechanisms are of clinical relevance since they could be pharmacological targets for modulation of 5-HT-neurotransmission. Moreover, some evidence suggests these pathways may contribute to the pathogenesis of neuropsychiatric diseases, including depression and anxiety.

1.6.1 5-HT_{1A} autoreceptor feedback control of 5-HT neurons

5-HT neurons are subject to control mediated by 5-HT_{1A} autoreceptors. In this scenario, 5-HT_{1A} autoreceptors may be activated by 5-HT released from recurrent 5-HT axon collaterals, which predominantly appear to exhibit volume transmission (Wang and Aghajanian, 1977; Wang and Aghajanian, 1978; Descarries et al., 1982). Alternatively, somatodentric release of 5-HT from DRN 5-HT neurons has also been reported in a number of species, including rats (Hery et al., 1986; Becquet et al., 1990; Adell et al., 1993). Early experiments investigating the inhibitory control mediated by 5-HT_{1A} autoreceptors over 5-HT neurons noted that intra-DRN application of certain 5-HT receptor agonists in the anaesthetised rat inhibited 5-HT neuron firing (Aghajanian et al., 1972). Since this time, the use of selective ligands for 5-HT_{1A} receptors has provided evidence that feedback inhibition of 5-HT neurons, at the level of the soma and dendrites, is mediated by 5-HT_{1A} autoreceptors (Figure 1.3). Thus, administration of 5-HT_{1A} agonists such as ipsapirone, LY-165163 and 8-OH-DPAT to anaesthetised rats inhibited DRN 5-HT neuron firing (Sprouse and

Aghjanian 1986; Blier and de Montigny, 1987; Sprouse and Aghajanian, 1987; Lum and Piercey, 1988). Moreover, these effects were blocked by pre-treatment with non-selective 5-HT_{1A} propranolol and spiperone, as well as selective 5-HT_{1A} antagonists WAY-100635 (Sprouse and Aghjanian 1986; Lum and Piercey, 1988; Corradetti et al., 1996).

5-HT_{1A} autoreceptor control over 5-HT neurons has also been demonstrated at the level of 5-HT release. Specifically, microdialysis studies found that systemic administration of selective 5-HT_{1A} agonists including 8-OH-DPAT and ipsapirone to rats reduced extracellular 5-HT in a variety of forebrain regions and the DRN itself (Sharp et al., 1989; Hjorth and Sharp, 1991; Casanovas and Artigas, 1996). These effects were likely to have been 5-HT_{1A}-mediated since they were blocked by pre-treatment with non-selective 5-HT_{1A} antagonists such as pindolol (Sharp et al., 1989). The effects of 5-HT_{1A} autoreceptors on 5-HT neurons are thought to be mediated in the DRN itself, since immunohistochemistry provided evidence for the presence of the receptor on the soma and dendrites of 5-HT-immunoreactive neurons in the DRN (Sotelo et al., 1990; Kia et al., 1996; Riad et al., 2000).

Subsequent studies have suggested that abnormal 5-HT_{1A} autoreceptor expression or function may contribute to pathogenesis of neuropsychiatric diseases. For example, 5-HT_{1A} knockout mice show increased levels of anxiety during adulthood (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Moreover, evidence suggests this phenotype is associated with increased 5-HT neuron firing, increased 5-HT turnover, and increased extracellular 5-HT in the frontal cortex and DRN (Richer et al., 2002; Guilloux et al., 2011). Thus, this work appears to suggest that abnormal function of receptors which are involved with feedback regulation of 5-HT

neurons may result in pathological behaviours, since it can alter the activity of 5-HT neurons.

Regulatory feedback pathways also provide an attractive pharmacological target for modulating 5-HT neuron activity for therapeutic benefits. For example, it is suggested that a delay in onset of therapeutic effect with SSRIs is a consequence of the time it takes for inhibitory 5-HT_{1A} autoreceptors to desensitise; as a result chronic treatment with SSRIs results in reduced potency of 5-HT_{1A} agonists to inhibit 5-HT neuron firing (Chaput et al., 1986; Chaput et al., 1991; Blier and Montigny, 1994; Blier and Bouchard, 1994; Czachura and Rasmussen, 2000). Moreover, 5-HT_{1A} receptor antagonists, and pindolol in particular, have been shown to be efficacious as adjuvants to SSRIs in depression therapy, probably via a mechanism involving blockade of 5-HT_{1A} autoreceptors and prevention of their effects on reducing 5-HT neuron activity (Ballesteros and Callado, 2004; Portella et al., 2011). The growing number of homoeostatic feedback pathways governing 5-HT neuron control which have been discovered could provide more strategies for targeting 5-HT neuron activity in a clinical setting.

1.6.2 5-HT_{1B} autoreceptor feedback control of 5-HT neurons

5-HT_{1B} autoreceptors are thought to exert an inhibitory influence over the release of 5-HT at projection sites (Figure 1.3). Thus, following on from earlier *in vitro* studies (e.g. Göthert et al., 1987), microdialysis studies in anaesthetised rats have demonstrated that systemic administration of 5-HT_{1B} agonists CP-93129 and RU-24969 decreased extracellular 5-HT levels in forebrain regions including the hippocampus and striatum; in the case of CP-93129, at least, these effects were

prevented by pre-treatment with 5-HT_{1B} antagonist GR-127935 (Sharp et al., 1989; Hjorth and Tao 1991; Martin et al., 1992; Knobleman et al., 2000).

5-HT_{1B} receptors appear to be expressed on 5-HT receptors themselves, as well as post-synaptically in various forebrain regions in rodents and primates (Bruinvels et al., 1994; Boschert et al., 1994; Doucet et al., 1995; Barnes and Sharp, 1999). Moreover, localisation of 5-HT_{1B} receptors to 5-HT terminals in forebrain regions, such as striatum and hippocampus, may suggest that 5-HT_{1B} autoreceptors inhibit 5-HT neurons at the site of 5-HT release (Sharp et al., 2007). In further support of this idea and also following earlier *in vitro* studies, 5-HT_{1B} agonists were found to be capable of inhibiting 5-HT release when administered at the site of 5-HT terminals in the forebrain such as the hippocampus (Hjorth and Tao, 1991; Hjorth and Sharp, 1993; Auerbach and Hjorth, 1995; Bosker et al., 1995).

1.6.3 Postsynaptic 5-HT_{1A} receptor feedback

Data suggests postsynaptic 5-HT_{1A} receptors may exert an inhibitory feedback on 5-HT neurons (Figure 1.3). Experiments reported that mPFC lesion or frontal cortex transection in rats prevented the inhibition of 5-HT neuron firing elicited by systemic administration of 8-OH-DPAT (Ceci et al., 1994; Hajos et al., 1999). By contrast, in lesioned rats an inhibitory effect of intra-DRN injection of 8-OH-DPAT on 5-HT neuron firing was still seen (Hajos et al., 1999). Thus, these findings indicate that post-synaptic 5-HT_{1A} receptors, located on neurons in the mPFC (Pompeiano et al., 1992), elicit a feedback inhibition of 5-HT neurons.

More recently, evidence suggests that this postsynaptic 5-HT_{1A} receptor feedback arising from the mPFC may involve GABAergic interneurons, since systemic administration of $GABA_A$ receptor antagonist picrotoxin with 5-HT_{1A} receptor antagonist WAY-100635 caused a partial blockade of the inhibitory effects of mPFC stimulation on 5-HT neuron firing. Moreover, intra-mPFC injection of 8-OH-DPAT inhibited 5-HT neuron firing in anaesthetised rats (Celada et al., 2001). In addition, evidence suggests that 5-HT_{1A} activation increased the firing rat of mPFC neurons (Borsini et al., 1995), putatively pyramidal neurons. Taken together, it has been proposed that mPFC 5-HT_{1A} receptors are likely to mediate inhibition of 5-HT neurons via GABAergic interneurons in them PFC and DRN (Figure 1.3).

1.6.4 Postsynaptic 5-HT_{2A} receptor feedback

Further inhibitory postsynaptic feedback control of DRN 5-HT neurons is thought to be mediated by 5-HT_{2A} receptors (Figure 1.3). Initial reports found that systemic administration of non-selective 5-HT_2 receptor agonists, including DOI, decreased 5-HT neuron firing and reduced extracellular 5-HT levels in the frontal cortex of anaesthetised rats (Wright et al. 1990; Garratt et al., 1991). Evidence for 5-HT_{2A} receptor involvement in this effect was later demonstrated, since administration of selective 5-HT_{2A} antagonist MDL-100907 was capable of reversing these effects of DOI (Martin-Ruiz et al., 2001; Boothman et al., 2003).

Evidence suggests that the mPFC may be a neural substrate for 5-HT_{2A}-mediated feedback control of 5-HT neurons. For example, 5-HT_{2A} agonist administration has been found to increase Fos immunoreactivity in the mPFC, and lesion of the mPFC abolished the effect of 5-HT_{2A} agonists on 5-HT neuron firing (Sharp et al., 2007). In addition, systemic administration of DOI in rats has been found to induce Fos

immunoreactivity in the DRN which colocalised with GAD_{67}-immunopositive cells (Boothman and Sharp, 2005). This effect was prevented by pre-treatment with 5-HT_{2A} antagonist MDL-100907, but not selective 5-HT_{2C} antagonist SB-206553 (Boothman and Sharp, 2005). Collectively these data provide evidence that postsynaptic 5-HT_{2A} receptors localised on pyramidal mPFC neurons exert an inhibitory influence on 5-HT neurons, probably via GABAergic interneurons in the DRN (Figure 1.3; Jakab and Goldman-Rakic, 1998; Sharp et al., 2007).

In addition to the above findings, there is evidence to suggest a local feedback control of 5-HT neurons in the DRN by 5-HT_{2A} receptors. Specifically, bath application of 5-HT onto rat DRN slices caused an inhibition of 5-HT neuron firing, which was blocked by MDL-100907 (Figure 1.3; Liu et al., 2000). In support of this, 5-HT_{2A} receptor mRNA has been detected in the raphe nuclei of rats (Pompeiano et al., 1994).

1.6.5 Postsynaptic 5-HT_{2C} receptor feedback

Evidence suggests that post-synaptic 5-HT_{2C} receptors may also mediate inhibitory feedback of 5-HT neurons, putatively via DRN GABAergic interneurons (Figure 1.3). Electrophysiological studies have demonstrated that the selective 5-HT_{2C} agonists WAY-161503 and Ro 60-0175 inhibited DRN 5-HT neuron firing in anaesthetised rats, effects which were prevented by pre-treatment with selective 5-HT_{2C} antagonist SB-242084 (Boothman et al., 2006; Quérée et al., 2009). Moreover, the effect of WAY-161503 was partially reversed by $GABA_A$ antagonist picrotoxin (Boothman et al., 2006). In addition to this, WAY-161503 increased Fos expression in DRN GAD-positive cells, which also expressed 5-HT_{2C} receptors (Sarrats et al., 2005; Boothman et al., 2006). Taken together, these findings provide evidence in support of a local

inhibitory feedback mechanism mediated by 5-HT_{2C} receptors on GABAergic interneurons over DRN 5-HT neuron firing.

It has been further suggested that 5-HT_{2C} receptors residing outside the DRN also mediate inhibitory feedback of DRN 5-HT neurons (Figure 1.3, Sharp et al., 2007). Specifically, the LHb is dense in 5-HT_{2C} receptors (Pompeiano et al., 1994; Clemett et al, 2000), and it has been reported that administration of 5-HT_{2C} agonist WAY-161503 elicited increased c-Fos expression in LHb neurons (Sharp et al., 2007). Thus, these data suggest that 5-HT_{2C} receptors in the LHb exert an inhibitory control over DRN 5-HT neurons. This pathway may well involve GABAergic interneurons, since projections from LHb to the DRN are predominantly glutamatergic (Kalen et al., 1985; Behzadi et al., 1990; Brinschwitz et al., 2010; Figure 1.3).

1.6.6 Postsynaptic 5-HT_4 receptor feedback

In contrast to the inhibitory nature of feedback pathways discussed so far, evidence suggests 5-HT_4 receptors may mediate positive feedback control of 5-HT neurons (Figure 1.3). In particular, evidence of 5-HT_4 receptor control over DRN 5-HT neurons was apparent in studies by Lucas et al., which found that systemic administration of the 5-HT_4 agonist cisapride to anaesthetised rats increased DRN 5-HT neuron firing, an effect reversed by the 5-HT_4 antagonist GR-125487 (Lucas and Debonnel, 2002). Moreover, administration of GR-125487 alone decreased 5-HT neuron firing, suggesting that 5-HT_4-mediated control of 5-HT neurons was tonically active (Lucas and Debonnel, 2002). This work is supported by findings that systemic administration of selective 5-HT_4 agonist renzapride increased hippocampal 5-HT levels in rats, whereas GR-125487 had the opposite effect (Ge and Barnes, 1996). Finally, virally-mediated overexpression of 5-HT_4 receptors in the mPFC resulted in increased firing

of DRN 5-HT neurons (Lucas et al., 2005). This finding supports the idea that the neural substrate for 5-HT_4 feedback control of 5-HT neurons may be the mPFC, where 5-HT_4 receptors are thought to be highly expressed on pyramidal neurons (Vilaro et al., 1996; Vilaro et al., 2005). Thus, it is hypothesised that 5-HT_4 receptors in the mPFC exert a tonic excitatory influence on DRN 5-HT neurons, putatively via a monosynaptic glutamatergic projection (Figure 1.3, Lucas et al., 2005; Sharp et al., 2007).

Interestingly, 5-HT_4 agonists appear to elicit fast onset antidepressant effects in rats at a behavioural level, with authors speculating that these effects may in part be attributable to the facilitatory effect of 5-HT_4 agonists on 5-HT neurotransmission (Lucas et al., 2007; Lucas et al., 2010). These drugs could offer an advantage over antidepressants such as SSRIs which have a delay in onset of therapeutic effect. Thus, this work highlights the therapeutic potential of ligands targeting feedback pathways governing 5-HT neuron activity.

1.6.7 Evidence of further homeostatic feedback mechanisms

There is evidence for additional homeostatic feedback mechanisms governing 5-HT neuron activity. In particular, 5-HT_7 receptors may exert an inhibitory control over 5-HT neurons (Figure 1.3, Sharp et al., 2007). For example, the 5-HT_7 antagonist SB-269970-A prevented 5-HT efflux from guinea-pig DRN slices evoked by electrical stimulation (Roberts et al., 2004). This effect was blocked by application of $GABA_A$ antagonist bicuculine, suggesting that the mechanism of 5-HT_7 feedback may involve DRN GABAergic interneurons (Roberts et al., 2004). In support of this idea, 5-HT_7 receptors are expressed on non-5-HT, putative GABA neurons within the DRN of guinea pigs and rats (To et al, 1995; Gustafson et al., 1996).

5-HT_{5A} receptor control over DRN 5-HT neurons has been postulated, since the selective 5-HT_{5A} antagonist SB-699551 increased extracellular 5-HT levels in guinea pig frontal cortex (Thomas et al., 2006; Sharp et al., 2007). Given that 5-HT_{5A} receptors are thought to be present on DRN 5-HT neurons, one suggestion is that these receptors act as inhibitory autoreceptors (Thomas et al., 2006; Duncan et al., 2000; Oliver et al., 2000).

Finally, evidence suggests that 5-HT_3 receptors may also exert feedback control over DRN 5-HT neuron activity (Figure 1.3). In particular, application of the 5-HT_3 receptor agonist 2-methyl-5-HT enhanced electrically evoked [^{3}H]5-HT release in frontal cortex, hippocampus and hypothalamus slices in the guinea pig and rat, effects which were reversed by selective 5-HT_3 receptor antagonists (Blier et al., 1993; Bagdy et al., 1998). Thus, this suggests an excitatory control of 5-HT neurons exerted by 5-HT_3 receptors, although the neural substrate of this effect is currently unknown.

1.6.8 Evidence for 5-HT_6 receptor-mediated feedback control of 5-HT neurons

Preliminary findings from the Sharp laboratory suggest that 5-HT_6 agonists and antagonists differentially modulate the firing of putative DRN 5-HT neurons in anaesthetised rats (Figure 1.4; K. Burnham and P.Quérée, unpublished data). Specifically, intravenous administration of the selective 5-HT_6 antagonist SB399885 decreased neuronal firing, an effect reversed by the selective 5-HT_6 agonist WAY181187. These findings raise the interesting possibility that 5-HT_6 receptors are involved in the control of 5-HT neurons.

In addition to this preliminary evidence, indirect evidence also supports a putative interaction between 5-HT_6 receptors and 5-HT neurons. In particular, neuroanatomical evidence from rats suggests that 5-HT_6 receptors localise to regions known to input to the DRN, most notably the mPFC (Ward et al., 1995; Gerard et al., 1996; Gerard et al., 1997; Helboe et al., 2015). Moreover, 5-HT_6 mRNA has also been localised to the DRN itself (Gerard et al., 1996; Helboe et al., 2015). In addition, intra-DRN injection of the selective 5-HT_6 agonist WAY-208466 reduced rapid eye movement (REM) sleep in rats (Monti et al., 2013). These findings suggest the presence of a physiologically relevant population of 5-HT_6 receptors residing within DRN, which could impact on 5-HT neuron activity. Finally, 5-HT_6 ligands appear to modulate behaviour in rats, which could be the result of altered 5-HT neurotransmission. Specifically, 5-HT_6 agonists including WAY-181187 and WAY-208466 elicited antidepressant effects in rats (Svenningson et al., 2007; Carr et al., 2011; Nikiforuk et al., 2011; Jastrzębska-Więsek et al., 2015). Surprisingly, 5-HT_6 antagonists also elicited antidepressant effects in rats in similar paradigms (Wesolowska et al., 2007; Hirano et al., 2009).

Given the evidence above, it was hypothesised that 5-HT_6 receptors may exert an influence over DRN 5-HT neurons (Figure 1.5). However, further investigation of the preliminary findings from this lab was needed to confirm this. A question which needed to be resolved first was whether these effects were mediated through the 5-HT_6 receptor, or were the result of ligand activity at non-5-HT_6 targets. Thus, experiments in this thesis aimed to confirm and extend these preliminary findings by studying the effects of a number of selective 5-HT_6 agonists and antagonists on 5-HT neuron firing. Moreover, experiments aimed to use *in vivo* juxtacellular labelling

techniques in an attempt to confirm the 5-HT-identity of the recorded DRN neurons, something missing from the preliminary study. Finally, experiments in this thesis progressed to investigate the neural site responsible for the effects of 5-HT_6 ligands on 5-HT neurons.

1.7 5-HT_6 receptors

Currently there are 14 identified 5-HT receptors in the CNS of humans and other species, including rodents, which are divided into seven families (5-HT_{1-7}; Table 1.1; Barnes and Sharp, 1999). The classification of 5-HT receptors into families was made on the basis of amino acid sequence, structure and signal transduction pathways, as well as pharmacological profiles.

Among the most recently discovered 5-HT receptors is the 5-HT_6 receptor, which was first identified by Monsma and colleagues in 1993, who sequenced a 1311 base-pair metabotropic receptor from a rat cDNA library (Monsma et al., 1993). This receptor was subsequently cloned and stably expressed in various cell lines, yielding a 437 amino acid receptor with 7 transmembrane regions (Monsma et al., 1993; Ruat et al., 1993). The 5-HT_6 receptor coupled to the Gs-protein *in vitro* and initiated intracellular cAMP accumulation, an effect which could be blocked by certain tricyclic antidepressants and antipsychotics including clozapine (Monsma et al., 1993; Ruat et al., 1993; Barnes and Sharp, 1999).

The 5-HT_6 receptor was subsequently identified in humans, and located to chromosomal position 1p35-p36 (Kohen et al., 1996). The 440 amino acid length human 5-HT_6 receptor was 89% identical to that of the rat, with similar antipsychotic

binding profiles, and 20-40% amino acid identify with other human G-protein coupled 5-HT receptors (Kohen et al., 1996; Barnes and Sharp, 1999).

Mouse 5-HT_6 receptors have also been sequenced. Although they have a high degree of amino acid homology (>80%) with rat and human 5-HT_6 receptors, their CNS expression level is ten-fold lower (Hirst et al., 2003; Setola and Roth, 2003). Furthermore, the pharmacological profile of the mouse 5-HT_6 receptor differs significantly from that of the rat and human receptors (Hirst et al., 2003; Setola and Roth, 2003). In particular, the affinity of the mouse 5-HT_6 receptor for various 5-HT_6 receptor ligands is low compared to that of the rat and human receptors (Hirst et al., 2003). This is likely to be the result of two amino acid residues in the 6th membrane-spanning region of the receptor, which are conserved in rat and human receptors, but different in the mouse (Hirst et al., 2003; Setola and Roth, 2003). Together these findings illustrate important differences between the mouse and human (and rat) receptors, suggesting that rat models of 5-HT_6 receptor function may be better than mice in terms of translational relevance.

1.7.1 5-HT_6 receptor distribution in the CNS

5-HT_6 receptors are almost exclusively expressed in the CNS (Monsma et al., 1993; Ruat et al., 1993). In the rat, 5-HT_6 receptor mRNA expression is high in the striatum, hippocampus, and the olfactory tubercle, moderate in the cortex, hypothalamus, habenula, amygdala, and low but detectable in the ventral tegmental area and raphe nuclei (Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995; Gerard et al., 1996; Helboe et al., 2015). In general, levels of 5-HT_6 mRNA in the rat are in agreement with 5-HT_6 protein expression as shown by immunohistochemistry and

autoradiography studies using 5-HT_6 radioligand [$^{(125)}$I]SB-258585 (Gerard et al., 1997; Roberts et al., 2002).

Study of 5-HT_6 receptor mRNA and protein levels in mice using RT-PCR and [$^{(125)}$I]SB-258585 binding has shown considerably lower 5-HT_6 receptor mRNA and radioligand binding levels in the mouse brain compared to rat and human brains (Hirst et al., 2003). Moreover, unlike rat and human 5-HT_6 receptors, which are highly expressed in the striatum, there was little 5-HT_6 receptor mRNA or radioligand binding in the mouse striatum (Hirst et al., 2003).

Radioligand binding studies using [$^{(125)}$I]-SB-258585 have shown that 5-HT_6 receptor expression in the human brain largely matches its distribution in the rat (East et al., 2002; Marazziti et al., 2012; Marazziti et al., 2013). In particular, high levels of binding have been detected in striatal regions, with lower binding levels in the mPFC and hippocampus (East et al., 2002; Marazziti et al., 2012; Marazziti et al., 2013). These findings have been further corroborated in positron emission tomography imaging studies using the 5-HT_6 ligand ^{11}C-GSK215083 (Parker et al., 2012).

1.7.2 5-HT_6 receptor cellular localisation

A recent, extensive *in situ* hybridisation study in the rat provided insight into the cellular localisation of 5-HT_6 receptors in the rat brain (Helboe et al., 2015). It was found that 5-HT_6 receptor mRNA was located in the soma of post-synaptic, non-5-HT neurons, and not 5-HT neurons themselves (Helboe et al., 2016). Moreover, 5-HT lesions has been shown to have no effect on 5-HT_6 receptor mRNA levels in the rat anterior raphe nuclei, providing further support for the finding that 5-HT neurons do

not express 5-HT_6 receptors (Gerard et al., 1996). Furthermore, no colocalisation was seen between 5-HT_6 mRNA dopaminergic neurons in the VTA or SN, or cholinergic neurons in the basal forebrain (Helboe et al., 2015).

In the cortex, 5-HT_6 receptor mRNA co-localised with vesicular glutamate transporter 1 (vGluT1)-positive neurons and subsets of $GAD_{65/67}$ positive neurons, particularly those expressing 5-HT_3 receptors (Helboe et al., 2015). This expression pattern was found throughout layers 2-6 of the parietal and prelimbic cortices (Helboe et al., 2015). Therefore, evidence suggests that 5-HT_6 receptors localise to glutamatergic principal neurons and GABAergic interneurons in the cortex (Helboe et al., 2015), which may indicate a role of the receptor in balancing excitation and inhibition in these neural networks (Helboe et al., 2015). This theory is further supported by the finding that 5-HT_6 receptor mRNA expression in the hippocampus also co-localised to glutamatergic and GABAergic neurons (Helboe et al., 2015).

In striatal tissue 5-HT_6 receptor mRNA was most dense and expressed on both D_1 and D_2 receptor-expressing medium spiny neurons (Helboe et al., 2015). Interestingly, recent work using virus-mediated overexpression of 5-HT_6 receptors in either direct pathway (D_1 receptor-expressing) medium spiny neurons or indirect pathway (D_2 receptor-expressing) medium spiny neurons in the dorsal medial striatum has supported the differential role of these receptors in facilitation and impairment of instrumental learning, respectively (Eskenazi et al., 2015). Thus, it was suggested that 5-HT_6 receptors here may play a role in balancing the behavioural consequences of direct and indirect pathway activation (Eskenazi et al., 2015).

Few studies have examined the cellular localisation of 5-HT_6 receptors in the human brain. However, there is some support for the idea that the cellular localisation of 5-HT_6 receptors in the human and rat brain is similar. In particular, pyramidal neurons within the human mPFC and hippocampus were shown to express 5-HT_6 receptors as observed in the rat (Lorke et al., 2006; Marazziti et al., 2013). However, unlike the rat, the 5-HT_6 receptor appeared to be expressed by superficial layer glial cells in the human mPFC (Lorke et al., 2006; Marazziti et al., 2013).

1.7.3 5-HT_6 receptor ligands

Experiments in this thesis sought to investigate the influence of 5-HT_6 receptors on 5-HT neurons. Thus, essential tools for this study were selective 5-HT_6 receptor ligands, which included agonists WAY-181187, WAY-208466 and ST-1936, and antagonists SB-399885, SB-271946, SB-258585 and AE-58054. These ligands were selected because they exhibited high 5-HT_6 binding affinity and selectivity for 5-HT_6 receptors over other metabotropic receptors, including other 5-HT receptors. Specifically, WAY-181187 and WAY-208466 exhibit the highest 5-HT_6 affinities of the agonists tested (Ki= 2.2 and 4.8 nM, respectively) and greater than 60-fold selectivity for 5-HT_6 receptors (Schechter et al., 2008). The third agonist used in this thesis, ST-1936, exhibited a lower affinity for 5-HT_6 receptors (Ki= 28.8 nM) and only a ten-fold selectivity for the receptor (Riccioni et al., 2011). Finally, antagonists SB-399885, SB-271046, SB-258585, and AE-58054 all exhibited a high 5-HT_6 receptor affinity (Ki= 1.3, 0.71, 2.3, 0.83 nM, respectively), with selectivity for 5-HT_6 receptors ranging from 50-fold, in the case of AE-58054, to 100-fold, in the case of SB-399885 and SB-271046, compared to other 5-HT receptors (Bromidge et al.,1999; Hirst et al., 2000; Hirst et al., 2006; Arnt et al., 2010).

1.7.4 5-HT_6 ligand signal transduction pathways

The 5-HT_6 receptor positively couples to adenylyl cyclase through interactions with the Gs proteins via its short intracellular 3rd loop, as shown by co-immunoprecipitation studies (Monsma et al., 1993; Ruat et al., 1993; Sebben et al. 1994; Kang et al., 2005). Consequently, selective 5-HT_6 ligands have often been identified on the basis of their action in 5-HT_6 receptor-linked cAMP accumulation assays. Thus, WAY-181187, WAY-208466 and ST-1936 stimulate cAMP accumulation *in vitro* (Schechter et al., 2008; Riccioni et al., 2011). By contrast, all antagonists used in this thesis blocked cAMP accumulation or adenlylyl cyclase activity elicited by 5-HT activation of 5-HT_6 receptors *in vitro,* with the exception of SB-258585 for which data is not available (Routledge et al., 2000; Romero et al., 2006; Hirst et al., 2006).

The 5-HT_6 receptor agonists WAY-181187, WAY-208466, and ST-1936 have also been shown to activate ERK1/2 (extracellular signal-regulated kinase 1/2) signalling in recombinant tissue *in vitro* and *in vivo,* effects which were blocked by 5-HT_6 receptor antagonist SB-258585 in the cases of WAY-181187 and ST-1936 (Riccioni et al., 2011; Pereira et al., 2015). Agonist-induced increases in PKA activity (via adenylyl cyclase signalling) may be responsible for initiating this ERK1/2 activation, since PKA has previously been shown to activate the ERK1/2 cascade (Vossler et al., 1997). Surprisingly, systemic administration of the 5-HT_6 receptor antagonist SB-271046 has been found to elicit ERK1/2 signalling in the hippocampus of rats (Marcos et al., 2010). One proposed explanation is that 5-HT_6 receptor ligands may be capable of eliciting ERK1/2 signalling via receptor c-terminus interactions with Fyn tyrosine kinase, which subsequently initiates ERK1/2 signalling (Yun et al., 2007). Moreover, while it may seem paradoxical that a 5-HT_6 receptor antagonist elicited signalling at the 5-HT_6 receptor, this can be explained by the fact that the

agonist/antagonist classification of 5-HT_6 ligands was based on cAMP accumulation assays rather than ERK1/2 signalling assays (Marcos et al., 2010).

Finally, mTOR pathway activation has been demonstrated by both WAY-181187 and WAY-208466 in rat mPFC tissue, effects which were blocked by SB-258585 (Meffre et al., 2012). In agreement with these findings, SB-399885 was found to suppress seizure-induced mTOR activity in the rat brain (Wang et al., 2015).

1.7.5 Neurochemical and behavioural effects of 5-HT_6 receptor agonists

The 5-HT_6 receptor agonists WAY-181187, WAY-208466 and ST-1936 have been tested in a number of behavioural and neurochemical paradigms in rats, including those which could indicate an interaction between 5-HT_6 receptors and 5-HT neuron activity. For example, evidence suggests that WAY-181187 and WAY-208466 elicit antidepressant and anxiolytic effects in rats using forced swim and defensive burying tests, respectively (Carr et al., 2011). Furthermore, ST-1936 exhibited antidepressant effects in a rat model of depression generated by exposure to inescapable stress (Scheggi et al., 2011). These effects were reversed by pre-treatment with SB-271046, implicating a 5-HT_6-mediated mechanism here (Scheggi et al., 2011). These antidepressant and anxiolytic effects could putatively link to changes in 5-HT neurotransmission.

In support of an effect of 5-HT_6 receptor agonist action on 5-HT neurotransmission, WAY-181187 administration in rats was reported to reduce extracellular 5-HT in the frontal cortex *in vivo*, an effect blocked by SB-271046 (Schechter et al., 2008). While this finding may be at odds with preliminary data from this lab, which suggested

WAY-181187 increased 5-HT neuron firing, there are a number of factors which could account for this. For example, the DRN 5-HT neuron population may have been incompletely sampled in this preliminary study, or changes in 5-HT neuron firing may not be translated into changes in 5-HT release. Changes in extracellular 5-HT have not been reported with WAY-208466 administration in rats. However, in contrast to WAY-181187 effects, administration of ST-1936 to rats had no effect on extracellular 5-HT levels in the prefrontal cortex and nucleus accumbens of rats (Valentini et al., 2011).

Direct evidence that WAY-208466 modulates DRN neuronal activity comes from the finding that intra-DRN injection of WAY-208466 decreased slow wave sleep and REM sleep in rats (Monti et al., 2013). These effects could be mediated via actions on 5-HT neurons, since 5-HT is thought to be a key modulator of sleep and waking (for review see Monti et al., 2010).

Finally, evidence suggests that some of the 5-HT_6 agonists used in this thesis elicit pro-cognitive effects which might be indicative of an interaction with 5-HT neurons. Specifically, WAY-181187 improved executive function in rats during attentional set shifting tasks (Burnham et al., 2010). Furthermore, WAY-181187 administration was also found to increase Fos-immunoreactivity in the mPFC, a region thought to be important in mediation of executive function (Burnham et al., 2010; Miller, 2000). Since the latter effects of WAY-181187 were prevented by pre-treatment with SB-399885, these effects at least appear to be 5-HT_6 receptor-mediated (Burnham et al., 2010). Taken together, evidence from this study may indicate an influence of WAY-181187 over mPFC circuitry. In support of this, 5-HT_6 receptors are found on mPFC pyramidal neurons and interneurons (Helboe et al., 2015). Finally, it is possible that

the effects of WAY-181187 in the mPFC could interact with the 5-HT system, since 5-HT is also implicated in cognitive function (Meneses, 1999; Steinbusch et al., 1981; O'Hearn et al., 1984; Vertes, 1991).

1.7.6 Neurochemical and behavioural effects of 5-HT_6 receptor antagonists

Paradoxically, like 5-HT_6 receptor agonists, a number of 5-HT_6 antagonists, demonstrate antidepressant effects in rats. In particular, SB-399885 and SB-271046 both elicit antidepressant effects in the forced swim test (Hirano et al., 2009). Moreover, evidence suggests SB-399885 elicited anxiolytic effects in rats in an elevated plus maze (Wesolowska and Nikiforuk, 2007). It is currently unclear how both 5-HT_6 receptor agonists and antagonists exert antidepressant effects in rats in the same behavioural paradigms. However, a few possibilities have been speculated; these include suggestions that some antagonists may be unreported inverse agonists, that there may be a tone on 5-HT_6 receptors *in vivo* which varies with regional distribution, or the 5-HT_6 receptor could have some constitutive activity (Benhamu et al., 2014).

Furthermore, SB-399885, SB-271046 and SB-258585 prevent memory deficits induced by the muscarinic antagonist scolopamine in novel object recognition tasks, Morris water maze, and in conditioned fear response paradigms (Hirst et al., 2006; de Bruin et al., 2011; Gravius et al., 2011; Woods et al., 2012). In addition, SB-271046 and AE-58054 reversed deficits induced by NMDA receptor antagonists MK-801 and phencyclidine in conditioned aversive context and novel object recognition tasks in rats (Arnt et al., 2010; Woods et al., 2012). Together, these findings support the theory that 5-HT_6 receptor antagonists mediate effects on cognition in rats via interactions with glutamatergic and cholinergic transmission. Moreover, this may be

the case in humans since AE-58054 has advanced into clinical trials as augmentative therapy with donepezil in Alzheimer's disease patients, and is showing procognitive effects here (Wilkinson et al., 2014). It is possible that these 5-HT_6 receptor antagonists act to improve cognition via distinct mechanisms to 5-HT_6 agonist WAY-181187.

Finally, 5-HT_6 receptor antagonists are thought to mediate effects on feeding behaviour in rats. For example, both administration of 5-HT_6 receptor antagonists RO-046790 and BVT-5182 and intracerebroventricular administration of 5-HT_6 receptor antisense oligonucleotides resulted in reduced weight gain and food intake (Woolley et al., 2001; Svartengren et al., 2003; Svartengren et al., 2004). In general it is thought that 5-HT_6 receptor antagonists mediate these effects via an increase in satiety (Heal et al., 2008). In support of this, a recent study demonstrated that 5-HT_6 receptor antagonist SB-742457 administration to rats reduced glucose intake, with microstructural analysis of licking indicating that the drug elicited a satiety-promoting effect as evidenced by reductions in the number of bouts of licking (Higgs et al., 2016).

1.8 Aim of this thesis

Preliminary experiments in this lab found that the 5-HT_6 receptor antagonist SB-399885 decreased 5-HT neuron firing, an effect which was reversed by the 5-HT_6 receptor agonist WAY-181187. These preliminary data support the hypothesis that 5-HT_6 receptors influence DRN 5-HT neurons akin to the previously reported 5-HT receptor-mediated feedback mechanisms which appear to govern 5-HT neuron activity (Figure 1.5). However, the nature of 5-HT_6 receptor control over 5-HT neurons remained to be fully characterised. Therefore, this thesis aimed to test the

effect of a greater number of 5-HT_6 agonists and antagonists on the firing of DRN in 5-HT neurons, to establish whether the effects of 5-HT_6 receptor ligands were likely to involve the 5-HT_6 receptor, or whether they were the result of activity at non-5-HT_6 targets. Beyond this, experiments aimed to investigate where the 5-HT_6 receptors responsible for these effects may be located and connected to the DRN.

To these ends, experiments in Chapter 3 examined the effect of the 5-HT_6 receptor agonists WAY-181187, WAY-208466 and ST-1936, and 5-HT_6 receptor antagonists SB-399885, SB-271046, SB-258585 and AE-58054 on the firing of DRN 5-HT neurons in the anaesthetised rat. Moreover, these experiments attempted to juxtacellular label recorded 5-HT neurons in order to confirm their 5-HT-identity. Experiments in Chapter 4 aimed to investigate the neural substrate involved in 5-HT_6 receptor agonist-induced responses observed in Chapter 3, and specifically examined the effect of selective 5-HT_6 receptor agonist administration on mPFC neural activity. These experiments were further progressed in a final set of experiments in Chapter 5, which aimed to determine whether the effect of a 5-HT_6 receptor agonist on DRN 5-HT neuron firing occurred in an mPFC-dependent manner. To test this, the effect of a 5-HT_6 receptor agonist administration on 5-HT neuron firing was investigated in rats with neurotoxic lesions of the mPFC.

In summary, experiments described in this thesis aimed to provide direct electrophysiological evidence for 5-HT_6 receptor control over 5-HT neurons *in vivo* and to elucidate the neural substrates involved.

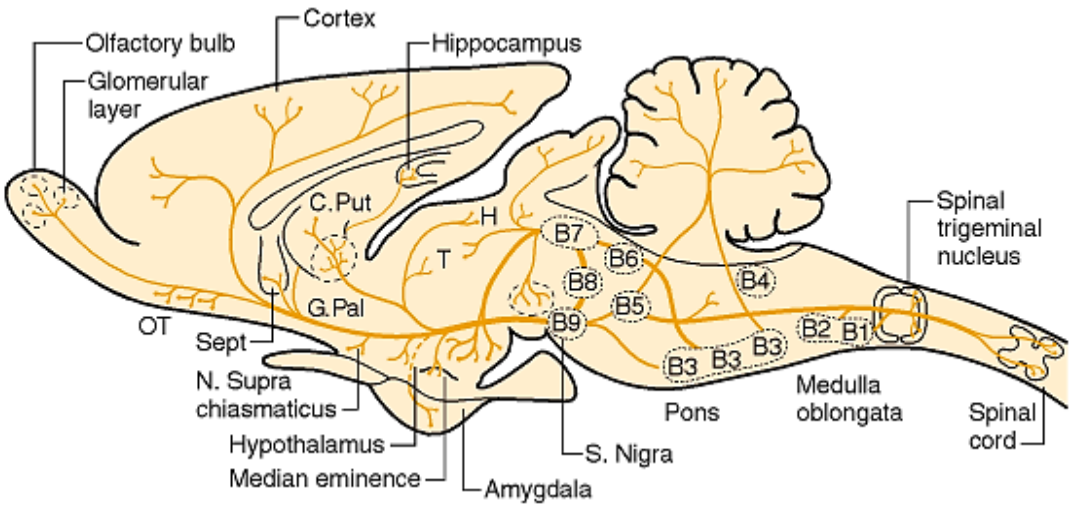
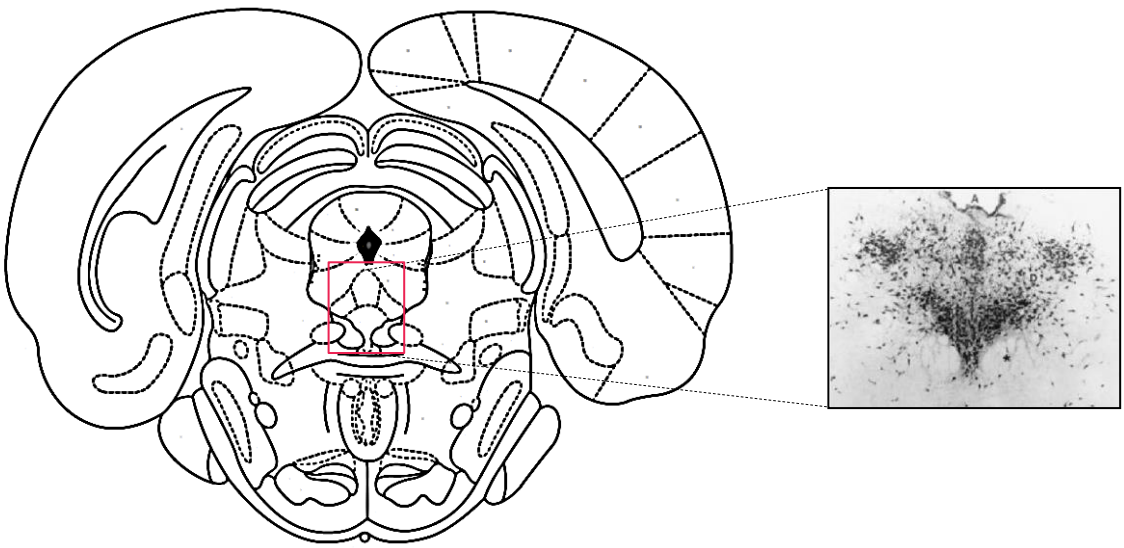


Figure 1.1

The raphe nuclei and their projection sites in the rat brain. A diagram illustrating a sagittal view of the rat brain with the location of raphe nuclei within the midbrain and brainstem: B1, raphe pallidus; B2, raphe obscurus; B3, raphe magnus; B4, raphe obscurus (dorsolateral part); B5, median raphe (caudal part); B6 dorsal raphe (caudal part); B7, dorsal raphe (rostral part); B8, median raphe (rostral part); B9, nucleus pontis orallis. Illustrated in orange are the major projections of 5-HT neurons in the raphe nuclei. Abbreviations: C.Put, caudate putamen; G.Pal, globus pallidus; T, thalamus; H, hypothalamus; S.Nigra, substantia nigra; Sept, septum; OT, olfactory tubercule; N.Supra chiasmaticus, suprachiastmatic nucleus. Figure from Frazer and Hesler, 1999.



Bregma -7.6 mm

Figure 1.2

Location of the DRN and 5-HT neurons in the rat brain. This diagram shows a coronal section (left) of a rat brain illustrating the location of the DRN, which is outlined in red, underneath the lateral ventricle (from Paxinos and Watson, 2007). The photograph (right) corresponds to the area marked by a rectangle on the diagram and shows 5-HT-immunoreactivity within the DRN (from Molliver, 1987).

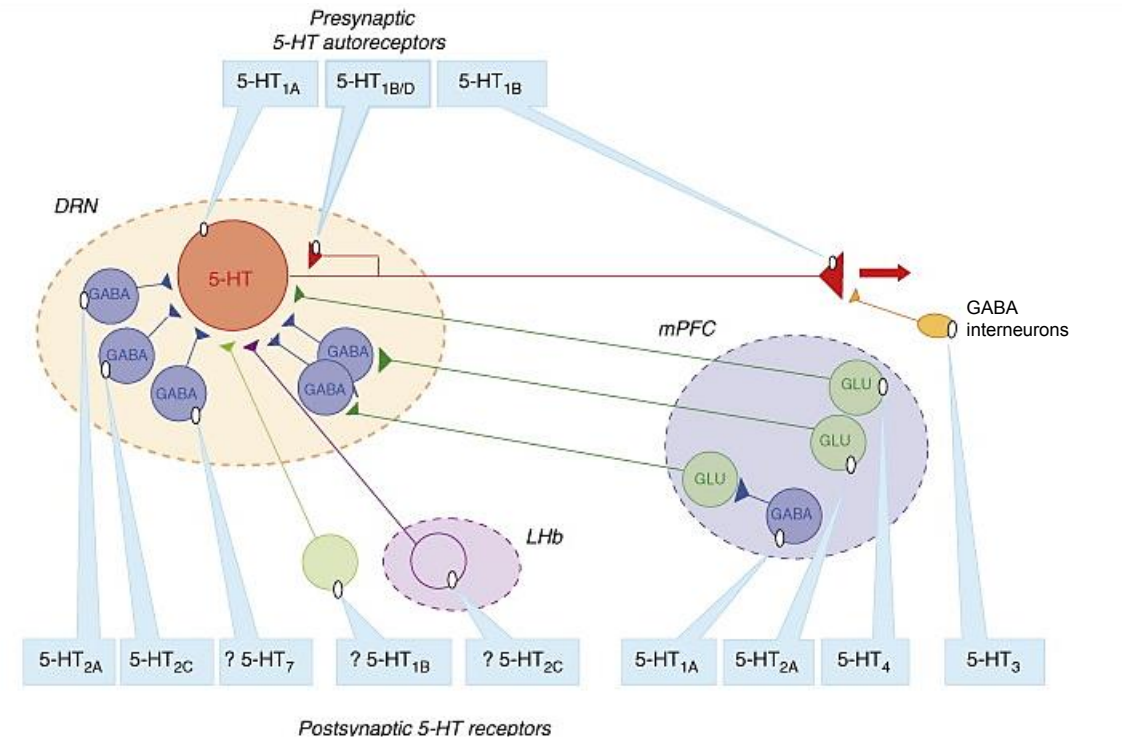


Figure 1.3

Homeostatic feedback mechanisms governing DRN 5-HT neuron activity. This diagram illustrates the pre- and post-synaptic feedback mechanisms which are thought to control DRN 5-HT activity. Figure from Sharp et al., 2007

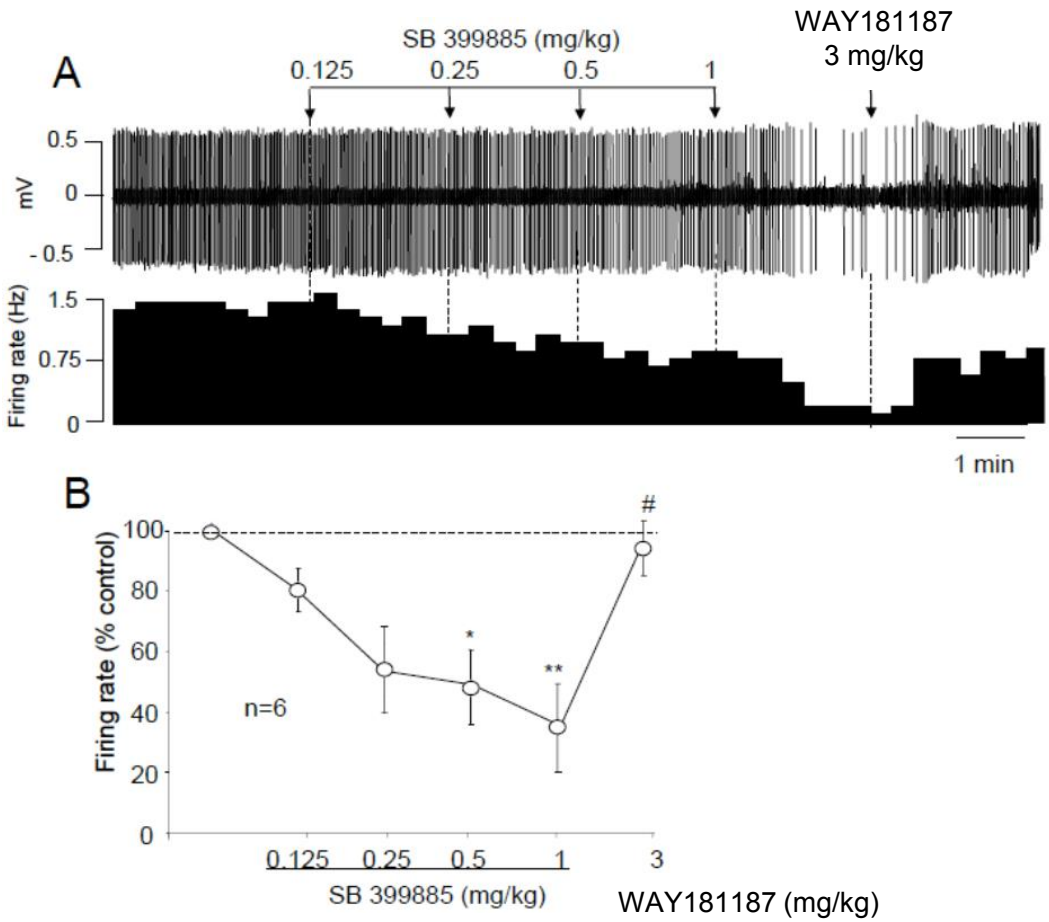


Figure 1.4
Effect of 5-HT_6 antagonist SB399885 and agonist WAY181187 on the firing of putative 5-HT neurons in choral hydrate anaesthetised rats. A) Representative trace from a single 5-HT neuron in the DRN. Following 3 min of baseline rate recording, accumulating doses of SB399885 were given at 2 min intervals (0.125, 0.25, 0.5, 1.0 mg/kg i.v.), causing a decrease in firing rate; this was reversed by WAY181187 administration (3mg/kg i.v.). B) Group data from 5-HT neurons following 5-HT_6 ligand administration. Data are expressed as mean±sem (n=6 neurons). Figure from K. Burnham and P. Quérée, unpublished data.

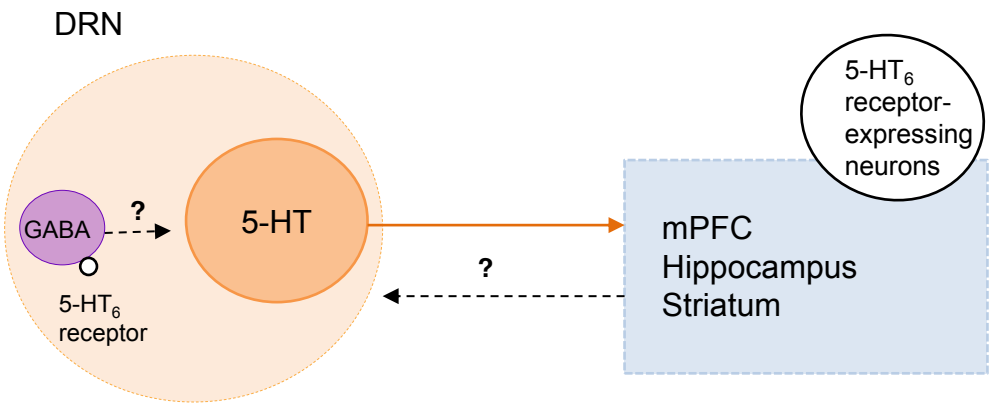


Figure 1.5
5-HT_6 receptor-mediated control of 5-HT neurons. It was hypothesised that 5-HT_6 receptors may influence DRN 5-HT neurons in a homeostatic feedback mechanism. The location of 5-HT_6 receptors involved in this control were unknown but may have included 5-HT_6 receptors expressed on post-synaptic neurons in the forebrain (mPFC, hippocampus or striatum) projecting back to 5-HT neurons, or 5-HT_6 receptors expressed by DRN GABAergic interneurons projecting to 5-HT neurons. Possible mechanisms are indicated by black dashed arrows.

Family	Subtype	Structure	Intracellular Signalling	Central distribution	Cellular localisation	CNS function	Ligands
5-HT_1	5-HT_{1A}	7-transmembrane domain receptor	Gαi coupled, ↓adenylyl cyclase activity, ↓ cAMP	Hippocampus, lateral septum, cortex, raphe nuclei	Postsynaptic, Autoreceptor	Reduces neuronal excitability via membrane hyperpolarisation	8-OH-DPAT (agonist) WAY-100635 (antagonist)
	5-HT_{1B}	as above	as above	Basal ganglia, frontal cortex, hippocampus, raphe nuclei	Postsynaptic, Autoreceptor	as above	Sumatriptan (agonist) 5-CT (antagonist)
	5-HT_{1D}	as above	as above	Basal ganglia, hippocampus, cortex, locus coeruleus, raphe nuclei	Autoreceptor	Reduces 5-HT neuron activity and 5-HT release	Zolmitriptan (agonist) BRL-15572 (antagonist)
	5-HT_{1E}	as above	as above	Frontal cortex, hippocampus, olfactory bulb			Eletriptan (agonist) Methiothepin (antagonist)
	5-HT_{1F}	as above	as above	Cortex, trigeminal ganglion			Naratriptan (agonist) Methiothepin (antagonist)
5-HT_2	5-HT_{2A}	7- transmembrane domain receptor	Gαq coupled, ↑ phospholipase C activity ↑$[Ca^{2+}]_i$	Cortex, nucleus accumbens, hippocampus, dorsal raphe nucleus	Postsynaptic	Increases neuronal excitability via membrane depolarisation	DOI (agonist) MDL-100907 (antagonist)
	5-HT_{2B}	as above	as above	Cerebellum, lateral septum, hypothalamus, amygdala	as above	as above	BW-723C86 (agonist) SB-200646 (antagonist)
	5-HT_{2C}	as above	as above	Cortex, lateral habenula, amygdala, hippocampus, dorsal raphe nucleus	as above	as above	WAY-161503 (agonist) SB-206553 (antagonist)

5-HT_3		Pentameric ligand gated cation channel	N/A	Dosal vagus complex (brainstem), cortex, hippocampus	Presynaptic	Fast neuronal depolarisation resulting in transmitter release	2-methyl-5-HT (agonist) Ondansetron (antagonist)
5-HT_4		7- transmembrane domain receptor	Gαs coupled, ↑ adenylyl cyclase activity ↑ cAMP	Cortex, striatum, hippocampus	Postsynaptic	Increases neuronal excitability via membrane slow depolarisation	Renzapride (agonist) GR-113808 (antagonist)
5-HT_5	5-HT_{5A}	7- transmembrane domain receptor	Gαi coupled, ↓adenylyl cyclase activity ↓ cAMP	Cortex, hippocampus, hypothalamus, thalamus, striatum, dorsal raphe nucleus	Postsynaptic, Autoreceptor	Reduces neuronal excitability via membrane hyperpolarisation	5-CT (agonist) Methiothepin (antagonist)
	5-HT_{5B} (rat)	as above	as above	as above			
5-HT_6		7- transmembrane domain receptor	Gαs coupled, ↑ adenylyl cyclase activity, ↑ cAMP ↑ ERK1/2	Cortex, hippocampus, striatum, olfactory tubercle, dorsal raphe nucleus	Postsynaptic	Increases neuronal excitability via membrane slow depolarisation	WAY-181187 (agonist) SB-399885 (antaganist)
5-HT_7		7- transmembrane domain receptor	Gαs coupled, ↑ adenylyl cyclase activity ↑ cAMP	Thalamus, hypothalamus, hippocampus, cortex, amygdala	Postysnaptic	Increases neuronal excitability via membrane slow depolarisation	LP-44 (agonist) LY-215840 (antagonist)

Table 1.1

5-HT receptors in the rat and human CNS. The following sources were used to compile this table: Barnes and Sharp, 1999; Nelson, 2004; Ciranna, 2006; Thomas, 2006; Pytliak et al., 2011; Hauser et al., 2014.

Chapter 2

Materials and Methods

2.1 Experiment Rationale

Experiments in this thesis combined electrophysiological recordings in the anaesthetised rat with drug tools targeting the 5-HT_6 receptor to determine the direct effect of 5-HT_6 receptor ligands on individual DRN 5-HT neuron firing (Chapters 3 and 5), mPFC network oscillations and individual mPFC pyramidal neuron firing (Chapter 4). This model allowed data to be gathered for dose-response graphs when 5-HT_6 receptor ligands were administered intravenously in accumulating doses. In addition, in the same experiment, 5-HT_6 receptor agonists and antagonists could be interacted to allow for pharmacological characterisation of their effects. Furthermore, *in vivo* recordings allowed DRN and mPFC neuronal populations to be studied with their connections to other brain regions intact. Finally, the use of marker-filled recording electrodes during these experiments enabled the labelling of single cells using a juxtacellular technique (see Chapter 1, Section 1.5.4) and subsequent immunohistological identification of the recorded neuron.

Experiments in Chapter 5 combined the above electrophysiological recording techniques with neurotoxic lesions of the mPFC. This allowed the investigation of the effect of 5-HT_6 receptor agonist WAY-181187 on 5-HT neuron firing in animals with disrupted mPFC-DRN connectivity.

The current chapter provides detailed methods relating to experiments carried out in Chapter 3, 4 and 5 of this thesis, including protocols for mPFC lesions, electrophysiological recordings, and immunohistochemical analysis of juxtacellular labelled neurons. Information relating to the 5-HT ligands used is also provided. A

general overview of the methods used in each chapter is further provided within Chapters 3, 4 and 5 themselves.

2.2 Animals

Male Sprague Dawley rats (230-420 g, Harlan Laboratories, Bicester, U.K.) were group housed at 20°C ± 2°C under 12 hour light-dark cycle (lights on 0800 h) in an enriched environment. Water and food were given ad libitum.

2.3 Surgical Procedures

2.3.1 Neurotoxic lesion of the mPFC

Rats were transported from the housing unit to the procedure room and left to acclimatise prior to surgery. Animals were placed in an induction chamber and anaesthesia was induced with 4% isoflurane (4 l/min oxygen). Following induction, the scalp was shaved, ear canals were infiltrated with local anaesthetic and the rat was placed in a stereotaxic frame after which anaesthesia was maintained with 1.5-2% isoflurane (2 l/min oxygen). Body temperature was maintained at 37°C using a homoeothermic heating blanket (Harvard Apparatus Ltd., Edenbridge, U.K.). The rat's scalp was washed with a sterilising solution and injected with local anaesthetic bupivacaine (2-3 mg/kg s.c., 0.5% Marcain Polyamp Steripack, AstraZeneca, Luton, U.K.). An incision was made in the scalp and small holes were drilled bilaterally over the mPFC according to stereotaxic coordinates in the rat brain atlas (from bregma = i) +3.3 mm anterior-posterior (AP), +0.5 mm medio-lateral (ML), ii) +3.3 mm AP,–0.5 mm ML, iii) +2.6 mm AP,+0.5 mm ML, iv) +2.6 mm AP, –0.5 mm ML, Paxinos and Watson, 2007). At each of the four sites an intracranial injection of neurotoxin quinolinic acid (0.09 M) was administered in 150 nl volumes at two depths within the mPFC (-3.0 mm and -3.5 mm from *dura mata*). After each injection the needle was held in place for 5 minutes to allow the neurotoxin to spread and to prevent it being drawn upwards when the needle was retracted. Sham rats underwent the same

procedure with injections of deionised water rather than neurotoxin. The scalp was then sutured and rats were administered isotonic glucosaline (0.5-1 ml s.c.), after which the isoflurane was turned off. Rats were then moved to a heated recovery cage and administered meloxicam (1 mg/kg s.c., Metacam, Boehringer Ingelheim Vetmedica, Inc, St Joseph, U.S.A.). Recovery from surgery was monitored closely and rats were scored daily for signs of distress. Rats were left to recover and for the lesion to set in for 2-3 weeks before undergoing non-recovery surgery and electrophysiological recordings.

2.3.2 Non-recovery surgery procedure for electrophysiological recording

Rats were transported from the housing unit to the procedure room and left for at least 30 minutes to acclimatise prior to surgery. Following anaesthesia induction using isoflurane (4% isoflurane, 4 l/min oxygen, IsoFlo®, Abbott Laboratories, Maidenhead, U.K.), anaesthesia was maintained with urethane (1.3 mg/kg i.p., ethyl carbamate, Sigma-Aldrich Co. Ltd., Gillingham, U.K.) and supplemental doses of ketamine (30 mg/kg i.p. or i.m., Narketan®, Vetoquinol (UK) Ltd, Buckingham, U.K.) and xylazine (3 mg/kg i.p. or i.m., Rompun®, Bayer Plc., Newbury, U.K.). Ear canals were infiltrated with local anaesthetics, rats were placed in stereotaxic frames (Kopf®, Bilaney Consultants Ltd., Sevenoaks, U.K.), and body temperature was maintained at 37^{O}C. A lateral tail vein was cannulated and used to administer 5-HT$_6$ ligands during electrophysiological recordings or vehicle (isotonic glucose-saline). A craniotomy was performed over the recording site of the DRN (from bregma= - 7.4-7.8 mm AP, and midline) or the mPFC (from bregma= + 3.00-3.20 mm AP, -/+0.5 mm ML) according to coordinates in the rat brain atlas (Paxinos and Watson, 2007). Steel screws were inserted to make contact with *dura mata* for electroencephalogram (EEG) recordings. During DRN electrophysiological recordings (Chapters 3 and 5) the EEG was obtained from over the left frontal cortex (from bregma= +2.7 mm AP, 2.0 mm ML), whereas during mPFC electrophysiological recordings (Chapter 4) the

EEG was obtained from over the left motor cortex (from bregma= -2.6 mm AP, 2.00 mm ML; Paxinos and Watson, 1997). In both setups, reference EEG screws were placed over the ipsilateral cerebellum.

2.4 Electrophysiological recordings

Glass microelectrodes (10-25 MΩ in situ resistance, 1 µm tip diameter) were filled with 1.5% neurobiotin (Vector laboratories, Peterborough, U.K.), and lowered using a microdrive (Inchworm, Burleigh, New York, U.S.A.) to the depth of the DRN (-4.0 mm from *dura mata*) or mPFC (-2.0 mm from *dura mata*). The microelectrode was typically held outside of the target region for 10-15 min so that movement within the tissue caused by the descending microelectrode was reduced before recording began. The microelectrode was then lowered through the target region (1 µm/s) until a neuron of interest was detected or to record local field potentials (LFPs). Single unit and LFP recordings included a pre-drug, baseline period (2- 3 min) followed by recordings made during administration of accumulating doses of 5-HT_6 receptor ligands (or vehicle) at 2 min intervals via the lateral tail vein cannula, with typically 4-5 doses per drug. The drug administration protocols are further outlined within Chapter 3, 4, and 5. Following these recordings, attempts were made to juxtacellular label the recorded neuron. Alternatively, iontophoresis was carried out after LFP recordings. Rats were then left for at least 1 h before transcardial perfusion with phosphate-buffered saline solution followed by 4% paraformaldehyde. Brains were then removed and processed for neurobiotin immunohistochemistry. Rats which did not undergo juxtacellular labelling or iontophoresis were culled by pentobarbitone overdose.

Singe unit, LFP and EEG recordings were acquired onto Spike 2 software (version 8.03; Cambridge Electronic Design, Cambridge, U.K) to be studied during

experiments and later. For this extracellular, single unit and LFP recordings were AC-coupled, then amplified by a headstage (x1000; Cygnus, Pennsylvannia U.S.A, and Neurolog Systems, Digitimer, Welwyn Garden City, U.K.), analogously filtered (between 300 Hz-5 KHz; Neurolog System) as well as filtered at 50 Hz to removed mains electrical noise (HumBug Noise Eliminator, Quest Scientific, Vancouver, Canada), and visualised on Spike 2 software via a Micro1401 analogue-digital converter (Cambridge Electronic Design, Cambridge, U.K.). EEG recordings were band-pass filtered between 0.3−1500 Hz (−3 dB limits; Neurolog System) and amplified (×2000, Neurolog system). The EEG was also filtered at 50 Hz to remove mains noise (HumBug Noise Eliminator), after which signals were passed through the Micro1401 analogue-digital converter (Cambridge Electronic Design) and visualised using Spike 2 software.

2.4.1 DRN stimulation during mPFC recordings

During mPFC single unit electrophysiological recordings, a stimulating electrode was implanted into the DRN so that antidromically activated mPFC pyramidal neurons could be identified (Chapter 4). In these experiments a stimulating electrode was implanted in the DRN. To do this a small hole was drilled in the skull over the site of the DRN (from bregma= - 7.7 mm AP, and midline) and the stimulating electrode was lowered into the DRN (-6.5 mm from the *dura mata*). The stimulating electrode was connected to a current isolator (A360 World Precision Instruments, Florida, U.S.A), which was connected to a Master 8 pulse generator (A.M.P.I, Jerusalem, Israel). The parameters for DRN stimulation were as follows: 0.2 ms square pulses at 0.9 Hz, with an intensity of 0.9-1.2 mA.

2.4.2 Juxtacellular Labelling

Attempts were made to label recorded neurons using a juxtacellular technique so that they could be immunohistologically identified *post mortem* (discussed in Chapter 1 section 1.5.4; Pinault, 1996). This involved passing positive current pulses (200 ms on/off) of 1-10 nA through the microelectrode. This expelled the neurobiotin from the microelectrode into the extracellular space and also caused the neuron to fire in synchrony with the current pulses, a phenomenon known as entrainment (Figure 2.1). In some cases this can lead to the uptake of neurobiotin from the extracellular space, allowing the neuron to be later identified. For successful labelling to occur a neuron was typically entrained for 30 seconds or longer.

2.4.3 Iontophoresis

Iontophoresis was carried out after some LFP recordings to mark the location of the recording electrode. Iontophoresis involved passing large positive current pulses (7 s on/off, 100 nA) through the microelectrode to expel neurobiotin into the extracellular space. As a result, several neurons in the vicinity of the microelectrode were labelled.

2.5 EEG monitoring during electrophysiological recordings

Throughout electrophysiological recordings an EEG was monitored to observe changes in brain state which could indicate a need to top up anaesthetic levels. Monitoring EEG activity also ensured that anaesthetic levels could be kept constant during pre-drug, baseline recording periods which could help to prevent confounding effects of variable anaesthetic levels on neuronal activity. Therefore, animals were maintained in a state of slow wave (SW) oscillatory activity (0.5-1.5 Hz) throughout procedures and pre-drug recording periods. This provided a suitable surgical

anaesthetic depth. Both 5-HT neurons and cortical pyramidal neurons which were recorded during these experiments are reported to be active during SW oscillations (Schweimer et al., 2011; Steriade and Amzica, 1998). SW oscillations were visually identified on EEG traces during electrophysiological recordings, and were later quantified offline (see section 2.7.2).

Supplemental doses of anaesthetic were given as necessary to maintain SW oscillations. However, administration of anaesthetics was avoided at least 30 minutes prior to electrophysiological recordings and during recording periods.

2.6 Immunohistochemical Analysis

Juxtacellular labelled neurons from DRN electrophysiological recordings were stained for 5-HT and neurobiotin immunoreactivity. The 5-HT antibody used in the current experiments was found to specifically label 5-HT neurons. In particular, data collected by other members of the current laboratory found no co-staining with other catecholamine neurons including noradrenergic neurons (J Schweimer, unpublished observations) and dopaminergic neurons (Schweimer et al., 2011). Juxtacellular labelled neurons or iontophoresis from mPFC electrophysiological recordings were only stained for neurobiotin immunoreactivity.

The following protocol describes 5-HT and neurobiotin staining. For neurobiotin only staining (mPFC juxtacellular labelling or iontophoresis) the steps involving incubation with the rabbit anti-5-HT primary antibody (overnight) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (90 minutes) were skipped.

2.6.1 5-HT and neurobiotin staining

Perfused brains were stored overnight in 4% PFA at 4°C, and then transferred into 30% sucrose solution and stored at 4°C. Brains were sectioned (20 µm) using a cryostat at -20°C. Sections were washed in 1X phosphate buffered saline (PBS), followed by 50 mM ammonium chloride to reduce autofluorescence and then PBS containing 0.25% Triton X (PBS-X). Sections were then blocked to prevent non-specific binding for 1 hour at room temperature under continuous agitation with PBS-x with 6% donkey serum, and then incubated overnight at 4°C with an anti-5-HT antibody raised in rabbit (kindly provided by from Professor Harry Steinbusch, University of Maastricht, Netherlands) diluted 1:2500 with PBS-X with 2% donkey serum. Next, sections were washed in PBS-X, and incubated for 90 minutes with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:1000, Invitrogen, UK) and/or Alexa Fluor 594-conjugated streptavidin (1:1000, Invitrogen, UK) in PBS-X with 2% donkey serum. Sections then went through a series of washes (PBS-X, followed by PBS, and finally PB) and onto uncoated slides, cover slipped using Vectashield (Vector Laboratories, Peterborough, U.K.), and were studied using an epifluorescence microscope (DM-5000B, Leica Microsystems, Wetzlar, Germany).

Images produced by through juxtacellular labelling and iontophoresis were studied in Image J, and contrast was enhanced if necessary. Additionally, the outline of features such as the midline in the mPFC, were traced for visibility and orientation purposes.

2.7 mPFC Lesion Validation

Free floating coronal sections of 40 µm were cut through the lesion and sham site at -20°C on a cryostat. Sections were then mounted onto poly-L-lysine coated slides and

left to dry overnight at room temperature. Sections were subjected to hydration in sequential ethanol solutions of diminishing ethanol concentrations and then placed in 0.5% cresyl violet solution for 2 minutes. Subsequently sections were dehydrated in sequential ethanol solutions of increasing concentration. Finally sections were incubated in xylene for a minimum of 2 hours. Sections were then covered slipped and left overnight to dry.

Sections from lesion and sham brains were examined under a light microscope to assess the extent of the lesion with the observer blind to treatment. If rats exhibited unilateral lesions, lesions extending past the mPFC or absence of a lesion they were excluded from the final analysis.

2.8 Data and statistical analysis

2.8.1 Single Unit Analysis

Spike2 (v.8.03) was used for spike-sorting of single unit data to identify spikes arising from one neuron, which included template-matching and principle components cluster analysis. The spike train was also visually inspected to check that all spikes had been detected by the software. Scripts run on Spike2 (v.8.03) software were used to determine spike timing and coherence with EEG or LFP SW oscillations. Data was then imported to Microsoft Excel where the interspike interval, coefficient of variance of the interspike interval (CV_{ISI}), and firing rate could be calculated. Firing rates were then normalised to baseline firing, which was taken as 100%. Data were expressed as mean ± SEM values.

The average spike width for a neuron was calculated in Spike2 (v. 8.03) and measured as the time taken from a 5% increase from baseline to the trough of the waveform, as has been done previously (Allers and Sharp, 2003).

2.8.2 EEG and LFP analysis

LFP and EEG data were analysed offline in Spike 2 (v.8.03). Initially, LFP and EEG traces were visually inspected for interruptions to the trace. To obtain robust measurements of power, approximately 60 seconds of uninterrupted (i.e. no artefacts) trace was required. Once this had been determined, the traces were down-sampled from 5 kHz to 1 kHz. In LFP recordings with single unit contamination a low pass filter was applied to remove these artefacts with minimum attenuation of the LFP.

Spike 2 (v.8.03) was used to generate spectrograms from LFP and EEG data. These spectrograms illustrated the power, i.e. the relative dominance, of a frequency band within a time period. These data were exported into GraphPad Prism 6 (GraphPad Software, Inc., California, U.S.A.) where they were normalised to pre-drug values (taken as 100%) and graphs were produced. Data were expressed as mean ± SEM values.

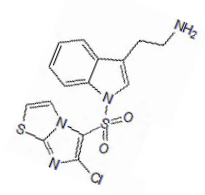
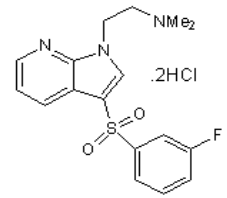
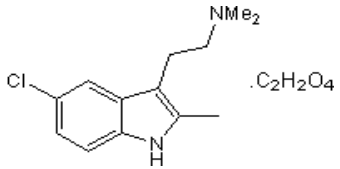
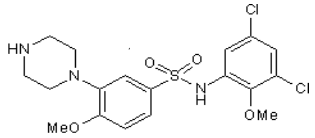
2.8.3 Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 21 software (IBM United Kingdom Limited, Portsmouth, U.K.). The statistical analysis used for each set

of experiments is outlined within the methods sections of Chapters 3, 4, and 5. In all cases, probability values of 0.05 or less were considered statistically significant.

2.9 5-HT ligands

Table 2.1 provides details of the 5-HT ligands used in experiments throughout this thesis. All drug solutions were made up on the day of the experiment from desiccated powders. Solutions were sonicated until dissolved and adjusted to pH 7.4 if necessary.

Ligand name and target	Structure	Systematic chemical name	Pharmacology	Pharmacokinetics	Vehicle	Manufacturer
WAY-181187		2-(1-(6-chloroimidazo[2,1-b]thiazol-5-ylsulfonyl)-1H-indol-3-yl)ethanamine	5-HT_6 receptor agonist, Ki= 2.2 nM 5-HT_{2C} receptor agonist, Ki= 124 nM Ki ≥ 259 nM for the 5-HT_{2B} receptor, 5-HT_7 receptor, and α_1 adrenoceptor	Moderate blood brain barrier penetration in rats, $t_{t/2}$ (rats, i.v.)= 5.4 h, High systemic clearance (7.4 l/h/kg)	Isotonic glucose-saline (NaCl 0.18% W/V, glucose 4% W/V)	Synthesised by Glaxo Smith Kline (GSK), Harlow, UK or as a base from H. Lundbeck A/S, Copenhagen, Denmark
WAY-208466 dihydrochloride		3-[(-3-Fluorophenyl)sulfonyl]-N,N-dimethyl-1H-pyrrolo[2,3-b]pyridine-1-ethanamine	5-HT_6 receptor agonist, Ki= 4.8 nM 5-HT_{2C} receptor antagonist, Ki= 217 nM Ki ≥ 313 nM for the 5-HT_{2A} receptor, 5-HT_{2B} receptor, and 5-HT_7 receptor.	Not reported	as above	Tocris Bioscience, Bristol, UK
ST-1936 oxalate		5-Chloro-3-[2-(dimethylamino)ethyl]-2-methylindole	5-HT_6 receptor agonist, Ki= 28.8 nM Ki ≥ 245 nM for the 5-HT_{2B} receptor, 5-HT_7 receptor, α_1 adrenoceptor, α_2 adrenoceptor, and α_3 adrenoceptor	Not reported	as above	Tocris Bioscience, Bristol, UK
SB-399885		N-(3,5-dichloro-2-methoxyphenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulfonamide	5-HT_6 receptor antagonist, Ki= 1.3 nM 200-fold selectivity for 5-HT_6 receptors over other 5-HT receptors, ion channels and enzymes (details not reported).	Not reported	as above	Synthesised by GSK, Harlow, UK

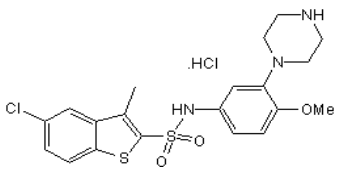
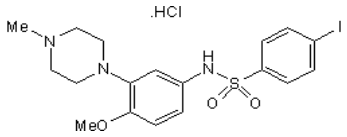
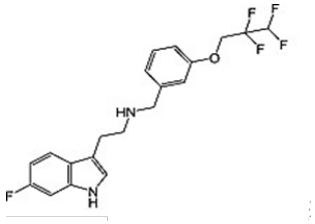
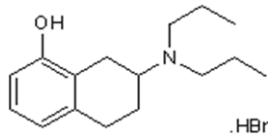
SB-271046 hydrochloride		5-Chloro-N-[4-methoxy-3-(1-piperazinyl)phenyl]-3-methyl-benzo[b]thiophen-2-sulfonamide	5-HT_6 receptor antagonist, Ki= 0.71 nM 200-fold selectivity for 5-HT_6 receptors over other receptors including 5-HT_{1A} receptors, 5-HT_{1B} receptors, 5-HT_{2A} receptors, 5-HT_{2C} receptors, 5-HT_4 receptors, 5-HT_7 receptors, D_2 receptors and D_3 receptors.	Moderate blood brain barrier penetrance in rats, $t_{1/2}$ (rats, i.v.)= 4.8 h, low blood clearance (0.4 l/h/kg)	as above	Tocris Bioscience, Bristol, UK
SB-258585 hydrochloride		4-Iodo-N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]benzene sulfonamide	5-HT_6 receptor antagonist, Ki= 2.3 nM 100-fold selectivity for 5-HT_6 receptors over other receptors including 5-HT_{1A} receptors, 5-HT_{1B} receptors, 5-HT_{2A} receptors, 5-HT_{2C} receptors, 5-HT_4 receptors, 5-HT_7 receptors, D_2 receptors and D_3 receptors.	Not reported	as above	Tocris Bioscience, Bristol, UK
(Lu) AE-58054 hydrochloride		2-(6-Fluoro-1H-indol-3-yl)-N-(3-(2,2,3,3-tetrafluoropropoxy)benzyl)ethanamine	5-HT_6 receptor antagonist, Ki= 0.83 nM 5-HT_{2A} receptor affinity, Ki= 83 nM Ki ≥ 250 nM for the 5-HT_{1A} receptor, 5-HT_{1B} receptor, 5-HT_{2B} receptor, 5-HT_{2C} receptor, and 5-HT_7 receptor.	Not reported	as above	Synthesised by H. Lundbeck A/S/, Copenhagen, Denmark
8-OH-DPAT hydrobromide		(±)-8-Hydroxy-2-(dipropylamino)tetralin	5-HT_{1A} receptor agonist, Ki= 0.48 nM 5-HT_7 antagonist affinity, Ki= 35 nM	High blood brain barrier penetrance in rats, $t_{1/2}$ (rats, i.v.)= 1.5 h, Low systemic clearance (0.06 l/h/kg)	as above	Sigma-Aldrich, Dorset, UK

Table 2.1
The 5-HT_6 receptor ligands and 5-HT_{1A} receptor agonist administered during experiments in this thesis. The following reference were used to compile this table: Shen et al., 1993; Cole et al., 2007; Schechter et al., 2008; Riccioni et al., 2011; Bromidge et al.,1999; Hirst et al., 2000; Hirst et al., 2003; Hirst et al., 2006; Arnt et al., 2010.

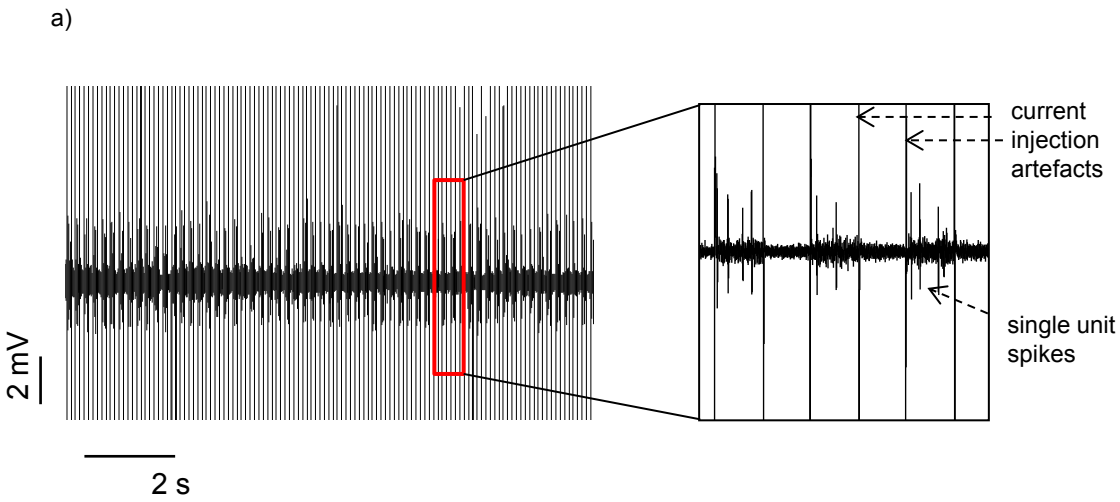


Figure 2.1

Juxtacellular labelling of a representative DRN 5-HT neuron. a) Spike train from a single 5-HT neuron entrained during juxtacellular labelling. The enlarged image indicates the current injection artefacts and single unit spikes. b) An image of the same neuron which was successfully labelled, as identified by neurobiotin staining. Scale bar= 10μm.

Chapter 3

Effect of 5-HT_6 receptor ligands on the firing of DRN 5-HT neurons *in vivo*

3.1 Introduction

As discussed in Chapter 1, preliminary evidence from this lab supported the theory that 5-HT_6 receptors influence 5-HT neurons in the DRN (K. Burnham, P. Quérée, unpublished data). Specifically, in anaesthetised rats systemic administration of the selective 5-HT_6 receptor antagonist SB-399885 dose-dependently decreased the firing of putative DRN 5-HT neurons, an effect reversed by the selective 5-HT_6 agonist WAY-181187. This finding may indicate the involvement of 5-HT_6 receptors in the feedback control of 5-HT neurons. The aim of the experiments described in this chapter was to confirm this phenomenon and then further characterise it using additional 5-HT_6 receptor ligands. The experiments also aimed to provide evidence for the 5-HT identity of recorded neurons using juxtacellular labelling combined with 5-HT immunohistochemistry (Chapter 1).

3.1.1 Feedback control of DRN 5-HT neurons

Much evidence suggests that 5-HT neurons in the DRN are under the control of feedback mechanisms located both within and outside the DRN (Sharp et al., 2007). In particular, electrophysiological and neurochemical data supports the idea that 5-HT neurons are under the inhibitory control of 5-HT_{1A} and 5-HT_{1B} autoreceptors located on 5-HT neurons which inhibit 5-HT neuron firing and 5-HT release *in vivo* (Sprouse and Aghjanian, 1987; Sharp et al., 1989; Hjorth and Tao 1991). In addition, 5-HT_{1A} receptors located postsynaptically in the prefrontal cortex are thought to

mediate the inhibition of 5-HT neurons in the DRN (Ceci et al., 1994; Hajos et al., 1999; Celada et al., 2001). In addition, electrophysiological studies suggest that 5-HT_{2A} and 5-HT_{2C} receptors located postsynaptically in forebrain regions as well as the DRN itself act via GABAergic neurons to inhibit 5-HT neuron firing (Martin-Ruiz et al., 2001; Boothman et al., 2003; Boothman et al., 2006). By contrast, there is evidence for the positive feedback control of 5-HT neurons by 5-HT_4 receptors expressed on glutamatergic neurons projecting from the mPFC to the DRN (Lucas et al., 2005). This mechanism may be tonically active since 5-HT_4 receptor antagonists alone inhibited 5-HT neuron firing (Lucas et al., 2005).

3.1.2 5-HT_6 receptor-mediated feedback control of 5-HT neurons

Preliminary electrophysiological data from this laboratory (discussed above and in Chapter 1) suggest that 5-HT_6 receptors may also influence 5-HT neurons in the DRN, and this is supported by other lines of evidence. In particular, receptor autoradiography and gene expression studies find both 5-HT_6 receptor binding sites and mRNA localised in the DRN (Gerard et al., 1996; Zhang et al., 2011; Helboe et al., 2015), although recent evidence suggests that 5-HT_6 receptors are not expressed by 5-HT neurons themselves (Helboe et al., 2015). Moreover, 5-HT_6 receptors are abundant in the mPFC which is a major source of input to the DRN (Ward et al., 1995; Gerard et al., 1996; Gerard et al., 1997; Helboe et al. 2015). In addition to this, sleep and wake cycle studies in rats show that intra-DRN injection of the 5-HT_6 receptor agonist WAY-208466 increased waking and reduced slow wave sleep (Monti et al., 2013). This effect could be the result of a direct influence of 5-HT_6 receptors on 5-HT neurons which are known modulators of sleep and wake states (for review see Monti, 2010). Finally, 5-HT_6 receptor agonists have been shown to induce behavioural effects in rats that may be 5-HT-mediated. Specifically, agonists

WAY-181187, WAY-208466 and ST-1936 elicited antidepressant effects in rats (Carr et al., 2011; Scheggi et al., 2011). Paradoxically, antagonists SB-399885, SB-271046 and SB-258585 also produced these effects in rats (Hirano et al., 2009; Wesolowska et al., 2007). Despite the above findings, the effect of selective 5-HT_6 receptor ligands on the firing of DRN 5-HT neurons *in vivo* is yet to be fully investigated. Experiments in this chapter report the effect of selective 5-HT_6 receptor agonists and antagonists on the firing of DRN 5-HT neurons.

3.1.3 Pharmacology of 5-HT_6 receptor ligands

Increasing availability of selective 5-HT_6 agonists and antagonists has provided important tools for investigating the interaction between 5-HT_6 receptors and 5-HT neurons. The drugs used in this chapter were the 5-HT_6 receptor agonists WAY-181187, WAY-208466 and ST-1936, and the 5-HT_6 receptor antagonists SB-399885, AE-58054, SB-271046 and SB-258585. Collectively, these ligands exhibited high affinity and selectivity for the 5-HT_6 receptor (Ki values in Chapter 1, Section 1.7.3). In particular, SB-399885, SB-258585 and SB-271046 displayed selectivity for 5-HT_6 receptors over other metabotropic receptors (> 100-fold; Bromidge et al., 1999; Hirst et al., 2000; Hirst et al., 2006; Table 2.1). AE-58054 showed some affinity for the 5-HT_{2A} receptor, although it was otherwise 50-fold selective for the 5-HT_6 receptor (Arnt et al., 2010; Table 2.1). In addition, agonists WAY-181187 and WAY-208466 both exhibited a degree of affinity for the 5-HT_{2C} receptor, although they still retained greater than 60-fold 5-HT_6 receptor selectivity (Schechter et al., 2008; Table 2.1). By contrast, ST-1936 showed some affinity for the 5-HT_{2B} receptor, 5-HT_7 receptor, and α-adrenoceptors, although it was still greater than 10-fold selective for the 5-HT_6 receptor (Riccioni et al., 2011). Despite the current availability of these ligands, few studies have compared multiple 5-HT_6 receptor ligands in the same model to provide

a detailed pharmacological analysis. This chapter aimed to investigate the effect of these 5-HT_6 receptor agonists and antagonists on DRN 5-HT neuron firing.

3.1.4 Characteristics of 5-HT neurons

Previous electrophysiological studies of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptor feedback mechanims have identified 5-HT neurons using electrophysiological and pharmacological criteria (discussed in Chapter 1 and summarised in Table 3.1). However, these criteria may not be sufficient to ensure 5-HT neuron identity. With the advent of *in vivo* juxtacellular labelling methodology (discussed in Chapter 1) the chemical identity of the recorded neuron can be confirmed. Experiments in this chapter aimed to juxtacellular label recorded neurons to establish their 5-HT-identity.

Neurochemical identity	Electrophysiological properties	Pharmacological sensitivity	Other characteristics	References
5-HT Neurons:				
Clock-like	Slow firing (typically <2 Hz) Regular firing pattern (COV_{ISI} < 0.5) Wide spike waveform (≥ 1 ms)	Inhibited by 5-HT_{1A} receptor agonists Inhibited by α1-adreoceptor antagonist	Firing in synchrony with frontal cortex SW oscillations (inactive phase) Firing in synchrony with hippocampal theta oscillations Firing excited by noxious stimulation	Aghajanian et al., 1968 Aghajainian and Haigler 1974, Wang and Aghajanian 1977, Aghajanian et al., 1978, Sawyer et al., 1985 Aghajanian, 1985, Gartside et al., 1997 Li et al., 2001 Kocsis et al., 2006 Schweimer et al., 2010 & 2011
Bursting	Slow firing (typically < 2 Hz) Wide spike waveform (≥ 1 ms) Short burst (2-4 spikes) firing pattern	As above	A proportion fire in synchrony with frontal cortex SW oscillations (inactive phase) Firing inhibited by noxious stimulation	Hajos et al., 1995 & 2007 Hajos and Sharp, 1996 Schweimer et al., 2010 & 2011
Fast firing	Fast firing (typically > 8 Hz) Narrow waveform width (≤ 0.7 ms)	Inhibited by 5-HT_{1A} receptor agonits	Firing in synchrony with hippocampal theta oscillations	Allers and Sharp, 2003 Kocsis et al., 2006
GABA neurons	Fast firing (typically > 10 Hz) Irregular firing pattern (COV_{ISI} > 0.5) Narrow spike waveform (≤ 0.7 ms)	Inhibition by 5-HT_{1A} receptor agonists in some cases		Aghajanian et al., 1978, Sawyer et al., 1985 Allers and Sharp, 2003

Table 3.1
Electrophysiological and pharmacological characteristics of DRN 5-HT and GABA neurons *in vivo.*

3.1.5 Aim of experiments

The aim of the current study was to test the hypothesis that 5-HT_6 receptors influence the firing of DRN 5-HT neurons. To this end, extracellular recordings were made from

single neurons in the DRN of the anaesthetised rat, and the effect of intravenous administration of the 5-HT_6 receptor agonists WAY-181187, WAY-208466 and ST-1639 and 5-HT_6 receptor antagonists SB-399885, SB-271046, SB-258585 and AE-58054 was tested. 5-HT neurons were selected on the basis of their electrophysiological characteristics (defined in section 3.2.4) and then juxtacellular labelling was used in an attempt to confirm their 5-HT identity.

3.2 Materials and Methods

3.2.1. Animals

Male Sprague Dawley rats (230-370g) were group housed at 20°C ± 2°C under 12 hour light-dark cycle (lights on 0800 h) in an enriched environment with water and food given ad libitum. Rats were left to acclimatise for at least 1 week following delivery to the housing facility. On the day of the experiment rats were taken from the housing facility to the experiment room and left to acclimatise for 30 min prior to induction of anaesthesia.

3.2.2 Experiment protocol

In vivo electrophysiological recordings in the DRN were carried out as described in Chapter 2. In brief, anaesthesia was induced with isoflurane and maintained with urethane (1.3 mg/kg i.p.) and supplemental doses of ketamine (30 mg/kg i.m.) and xylazine (3 mg/kg i.m). A craniotomy was performed over the DRN (from bregma= -7.4-7.8 mm AP, and midline) and the recording electrode (filled with neurobiotin) was lowered into the DRN using a Microdrive (-4.0 mm from *dura mata*). Once a neuron was detected the spike amplitude was allowed to increase to around 1 mV and then recorded for 2-3 min to establish a stable baseline. Drug (or vehicle) was then administered in accumulating doses at 2 min intervals via a lateral tail vein cannula, typically with 4-5 doses per drug (or vehicle). A maximum of two 5-HT neurons were recorded per rat; typically the first neuron was recorded during vehicle (isotonic glucose-saline) administration and then a second was recorded during drug administration. If a neuron was lost after no more than one dose of a drug, an alternative neuron was sought. Attempts were then made to juxtacellularly label recorded neurons. This involved passing current of increasing amplitudes through the recording electrode to expel neurobiotin into the extracellular space and at the same

time entrain the recorded neuron to facilitate uptake of neurobiotin. Rats were then perfused with 4% PFA and brains were extracted and processed for 5-HT and neurobiotin immunohistochemistry or rats were culled with pentobarbital overdose. Figure 3.1 illustrates the time course of experiments carried out in this chapter.

Throughout these experiments frontal cortex EEG recordings allowed for monitoring of anaesthetic depth but at the same time provided a measure of oscillatory activity in the cortical neuron networks. Rats were kept in a state of slow wave (SW) oscillations (0.5-1.5 Hz) during baseline recording periods. SW oscillations resemble those occurring during natural sleep and indicate a surgical anaesthesia level (Steriade, 2000). 5-HT neurons are spontaneously active during SW oscillations (Schweimer et al., 2011).

Experiments in this chapter initially examined the effects of 5-HT_6 receptor antagonist SB-399885 and 5-HT_6 receptor agonist WAY-181187 on 5-HT neuron firing in order to confirm the preliminary evidence from this lab. During experiments with SB-399885 administration it was noticed that slow firing neurons showed greater responses to SB-399885 administration. Thus, experiments were restricted to DRN neurons with slower firing rates (approximately ≤ 2 Hz). Experiments in this chapter then developed those of the preliminary data to include testing of WAY-181187 responses after pre-treatment with 5-HT_6 receptor antagonists SB-399885, SB-271046 and SB-258585. These studies were then extended to examine the effects of 5-HT_6 receptor agonists WAY-208466 and ST-1936 and 5-HT_6 antagonists SB-271046, SB-258585 and AE-58054 on 5-HT neurons firing. Table 3.2 provides a summary of the experiments carried out in this chapter. This table also indicates the number of neurons recorded from, and rats used, to generate results in this chapter.

In some experiments more rats are indicated than neurons in the final data set. In some instances this is because the neuron recorded in a rat did not exhibit 5-HT neuron properties. In others, as in the case of SB-399885 experiments, some neurons exhibited a fast firing rate and were not included in the final analysis (see section 3.2.3 Data Analysis).

Treatment	Drugs doses (i.v.)	Number of neurons (rats)
1) Vehicle (isotonic glucose-saline)	0.1 - 0.4 ml	8 (10)
2) WAY-181187	1.0 - 4.0 mg/kg (0.56 - 2.2 ml/kg)	6 (9)
3) SB-271046 followed by WAY-181187	SB-271046: 0.25 - 1.5 mg/kg (0.56- 3.3 ml/kg) WAY-181187: 3.0 mg/kg (1 ml/kg)	7 (9) Same neurons and rats as Experiment 10
4) SB-258585 followed by WAY-181187	SB-258585: 0.25 - 2.0 mg/kg (0.35 – 2.8 ml/kg) WAY-181187: 3.0 mg/kg (1 ml/kg)	7 (7) Same neurons and rats as Experiment 11
5) SB-399885 followed by WAY-181187	SB-399885: 0.125 – 1.0 mg/kg (0.28 – 1.4 ml/kg) WAY-181187: 3.0 mg/kg (1 ml/kg)	8 (13) Same neurons and rats as Experiment 8
6) WAY-208466	Lower doses: 0.5 - 3.0 mg/kg (0.42 – 2.5 ml/kg) Higher doses: 1.25 – 10.0 mg/kg (0.7 – 2.8 ml/kg)	12 (15)
7) ST-1936	Lower doses: 0.25 – 1.0 mg/kg (0.28 – 2.2 ml/kg) Higher doses: 1.25- 10.0 mg/kg (0.7 – 2.8 ml/kg)	9 (9)
8) SB-399885	0.125 – 1.0 mg/kg (0.28 – 1.4 ml/kg)	8 (13)
9) AE-58054	0.25 - 2.0 mg/kg (0.49 – 1.9 ml/kg)	5 (5)
10) SB-271046	0.25 - 1.5 mg/kg (0.56- 3.3 ml/kg)	7 (9)
11) SB-258585	0.25 – 2.0 mg/kg (0.35 – 2.8 ml/kg)	7 (7)
12) WAY-181187 and WAY-208466 on cortical SW oscillations	WAY-181187: 4.0 mg/kg (2.2 ml/kg) WAY-208466: 10.0 mg/kg (2.8 ml/kg)	12 rats (same rats as Experiments 2 & 6)

Table 3.2
Experiments in Chapter 3.

3.2.3. Data analysis

Recordings from individual neurons were acquired online using Spike2 (version 8.03) software. Details regarding the filter and conversion of raw data into Spike2 software are given in Chapter 2. Spike2 was used offline to sort spikes, measure spike widths, and determine firing coherence with cortical EEG recordings. Data were exported into Microsoft Excel to determine firing rates (Hz) and regularity (COV_{ISI}). Firing rates were normalised to pre-drug, baseline periods (taken as 100%). Data are expressed as mean ± SEM values. Neurons included in the final data analysis exhibited a stable baseline firing rate defined as firing during the pre-drug periods which did not fall outside of 2x S.D. of the mean pre-drug firing rate for more than two consecutive 10 second bins.

EEG data were analysed offline in Spike2. Spectrograms were generated to illustrate the power, i.e. relative dominance, of a frequency band within a given time frame. To obtain robust measurements of power, 60 seconds of uninterrupted (i.e. no artefacts) EEG trace was required. Bleeding around the EEG screw sites was found to be a common cause of artefacts which hindered EEG analysis.

Neurons without the characteristics of DRN 5-HT neurons (see section 3.2.4), or which were 5-HT-immunonegative, or outside the DRN, were excluded from the analysis. In early SB-399885 experiments a correlation was seen between firing rate and response to drug. Consequently faster firing neurons (> 2.2 Hz) were excluded from the final analysis of this data set. Finally, during WAY-208466 and AE-58054 one neuron from each data set was lost prior to the final dose could be administered,

and these cells were not included in statistical analysis. The data from remaining neurons was compared to pre-drug and vehicle data use ANOVA analysis, as described below.

3.2.4 Identification of 5-HT neurons

Putative 5-HT neurons were selected according to the following characteristics: a slow firing rate (typically ≤ 2 Hz), a broad waveform width (typically ≥ 1msec) and a regular firing pattern defined by a COV_{ISI} of <0.5 (Allers and Sharp, 2003; Schweimer et al., 2010; Schweimer et al., 2011). Burst firing 5-HT neurons had similar firing rate and waveform characteristics as clock-like 5-HT neurons but had a higher COV_{ISI} (Kocsis et al., 2006; Schweimer et al. 2011).

3.2.5 Statistical analysis

The effect of 5-HT_6 ligands (or vehicle) on the firing rate of 5-HT neurons was tested statistically through comparison with pre-drug values using one-way ANOVA with dose as a repeated measure, followed by Dunnet's post hoc test. The effect of 5-HT_6 ligands on firing rate was also compared to vehicle using two-way ANOVA with dose as a repeated measure, followed by Bonferroni's post hoc test. Pearson's correlation was used to determine the relationship between baseline firing rate and the magnitude of drug response. The effect of antagonist pre-treatment on the effect of agonist was tested statistically using a Student's paired t-test (two-tailed). Also, the effect of drugs on cortical SW oscillation power was compared to pre-drug power using a Student's paired t-test (two-tailed). Probability values of 0.05 or less were considered significant.

3.3. Results

3.3.1. Electrophysiological and pharmacological characteristics of 5-HT neurons

Eighty seven spontaneously active putative 5-HT neurons were recorded. Clock-like 5-HT neurons exhibited a slow, highly regular firing pattern with broad spikes which is characteristic of 5-HT neurons: firing rate= 1.02±0.07 Hz; COV_{ISI}= 0.34±0.1; spike width= 1.24±0.03 ms; n=69/87 (79%). A typical example is shown in Figure 3.2. A proportion of DRN neurons (n= 18/87, 21%) were recorded which fired in short bursts (doublets or triplets) of broad spikes (1.22±0.07 ms), in an otherwise slow (1.07±0.13 Hz) but less regular firing pattern (COV_{ISI}= 0.51±0.0.05). The difference between the COV_{ISI} of clock-like neurons and bursting DRN neurons was statistically significant (unpaired t-test, $p<0.0001$, clock-like neurons n=69, bursting neurons n=18). However, both clock-like and bursting DRN neurons were found to be 5-HT-immunopositive cells (see below), and their baseline firing rates and spike widths did not differ significantly; thus data on the effects of 5-HT_6 receptor ligands on clock-like and bursting neurons were pooled.

In the majority of cases the baseline firing of individually tested putative 5-HT neurons displayed significant coherence with EEG SW oscillations ($p< 0.05$; for both clock-like neurons n= 20/24 (83%) and bursting neurons n= 7/7 (100%)), with firing greatest during the inactive phase of SW oscillations (Figure 3.2). Furthermore, in all neurons tested (n= 6), administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT (10 µg/kg i.v.) caused a complete abolition of firing, as would be anticipated of 5-HT neurons.

Thirty three of the 87 putative 5-HT neurons underwent attempted juxtacellular labelling, and fifteen of these were successfully labelled as evidenced by the presence of neurobiotin in a single DRN neuron. Thus, the labelling success rate for these experiments was approximately 45%. 14 of the labelled neurons were found to be 5-HT immuno-positive (Figures 3.2, 3.3, 3.5, and 3.11). Moreover, the baseline firing properties (spike width, firing rate and COV_{ISI}) of non-juxta-labelled 5-HT neurons (n= 73) did not differ significantly from those of labelled 5-HT neurons (n= 14).

3.3.2 Effect of vehicle on 5-HT neuron firing

Intravenous administration of vehicle (isotonic glucose-saline) in accumulating volumes (0.1, 0.2, 0.3, 0.4 ml) caused a trend for the firing rate of 5-HT neurons to decrease, but this was borderline statistically significant compared to pre-vehicle firing rates (ANOVA $F_{(4,28)}$= 2.643, p=0.055; Figure 3.3 and 3.4). The vehicle data were used as a control group for experiments on the effects of 5-HT_6 receptor ligands. Of the total 15 labelled neurons in this chapter, 2 were in the vehicle-treated group, and both were found to be 5-HT immuno-positive (Figure 3.3).

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3.3.3 Effect of 5-HT_6 receptor agonists on 5-HT neuron firing

Administration of the 5-HT_6 agonist WAY-181187 (1, 2, 3, 4 mg/kg i.v.) caused a dose-dependent increase in firing of 5-HT neurons, with a maximum effect of 58% above pre-drug levels at the highest dose (4 mg/kg) (Figure 3.5 and 3.6). This effect of WAY-181187 was statistically significant compared to pre-drug values at doses of 3 and 4 mg/kg (ANOVA $F_{(4,20)}$ = 0.979, p= 0.01; Dunnet's post hoc test, p<0.01 for 3 and 4 mg/kg). The effect of WAY-181187 was also statistically significant compared

to vehicle controls (ANOVA $F_{(4,60)}$= 6.58, p < 0.001; Bonferroni post hoc test, p<0.01 all doses). Two of the 15 labelled neurons in this chapter were from the WAY-181187-treated group, and both were found to be 5-HT immuno-positive (Figure 3.5). The magnitude of effect of WAY-181187 was not correlated with pre-drug firing rate.

The effect of WAY-181187 (3.0 mg/kg i.v.) on 5-HT neuron firing was robustly blocked by pre-treatment with 5-HT_6 receptor antagonist SB-258585 (2.0 mg/kg i.v.). Thus, WAY-181187 did not cause a statistically significant increase in firing compared to firing after SB-258585 administration alone (SB-2858585 versus SB+WAY, p= 0.64; Student's paired t-test), or pre-drug firing (pre-drug versus SB-258585+WAY, p= 0.39; Student's paired t-test; Figure 3.7). Following pre-treatment with a second 5-HT_6 receptor antagonist, SB-271046, WAY-181187 caused an increase in the firing rate of neurons; however, this was not significant compared to firing after antagonist administration (SB-271046 versus SB+WAY, p= 0.081; Student's paired t-test)) or pre-drug firing (pre-drug versus SB-271046+WAY, p= 0.31; Student's paired test; Figure 3.7). The effect of 5-HT_6 antagonist SB-399885 (1 mg/kg) on WAY-181187-induced (3 mg/kg) increases in 5-HT neuron firing was also tested. SB-399885 caused a decrease in 5-HT neuron firing (see Section 3.3.4 and Figures 3.10 and 3.12), and this effect was significantly reversed by WAY-181187 (SB-399885 versus WAY-181187, p=0.019, Student's paired t-test; Figure 3.7). However, the effect of WAY-181187 on 5-HT neuron firing following SB-399885 pre-treatment was not statistically significant compared to pre-drug, baseline firing (pre-drug versus SB-399885+WAY, p=0.74, Student's paired t-test).

In an initial experiment, administration of the 5-HT_6 receptor agonist WAY-208466 at lower doses (0.5, 1.0, 2.0, 3.0 mg/kg i.v.) had no statistically significant effect on the

firing of 5-HT neurons compared to pre-drug values (ANOVA: $F_{(4,24)}$= 0.847 p= 0.509) and vehicle controls (ANOVA: F(3,64)= 0.241 p= 0.867; Figure 3.8). Surprisingly, a second dose-response experiment which involved WAY-208466 administration at comparable doses to previously and higher doses (1.25, 2.5, 5, 10 mg/kg i.v.) found that WAY-208466 increased 5-HT neuron firing, with a maximum increase of 47% above pre-drug levels at the highest dose of WAY-208466 (Figure 3.5 and 3.8). This effect of WAY-0208466 was not statistically significant compared to pre-drug values (ANOVA: F(4,12)= 2.758, p= 0.078), however it was statistically significant compared to vehicle controls (ANOVA: $F_{(4,50)}$= 2.56, p= 0.05; Bonferroni post hoc test, P<0.01 at all doses; Figure 3.8). Two of the 15 labelled neurons in this chapter were in the WAY-208466 treated group and found to be 5-HT immuno-positive (Figure 3.5).

By comparison, administration of the 5-HT_6 receptor agonist ST-1936 at lower doses (0.25, 0.5, 0.75 and 1.0 mg/kg i.v.) had no statistically significant effect on the firing of 5-HT neurons compared to pre-drug values (ANOVA: $F_{(4,12)}$=1.528, p= 0.256). Similarly, higher doses of ST-1936 (1.25, 2.5, 5.0 and 10.0 mg/kg i.v.) had no statistically significant effect compared to pre-drug firing (ANOVA: $F_{(4,12)}$= 0.556, p= 0.698). Moreover, ST-1936 had no statistically significant effect on 5-HT neuron firing compared to vehicle controls at either the lower (ANOVA: $F_{(4,55)}$=0.246, p=0.092) or higher (ANOVA: $F_{(4,60)}$= 0.235, p=0.918) doses (Figure 3.5 and 3.9). Two of the 15 labelled neurons in this chapter were in the ST-1936-treated group and found to be 5-HT immuno-positive (Figure 3.5).

3.3.4 Effect of 5-HT_6 receptor antagonists on 5-HT neuron firing

Administration of the 5-HT_6 receptor antagonist SB-399885 (0.125, 0.25, 0.5, 1.0 mg/kg i.v.) produced a dose-dependent decrease in the firing of 5-HT neurons, with a maximum decrease of 56% below pre-drug values at the highest dose (Figure 3.10 and 3.12). This effect of SB-399885 was statistically significant when compared to pre-drug values at doses 0.25, 0.5, and 1.0 mg/kg (ANOVA: $F_{(4,28,)}$ = 10.359, $p< 0.001$; Dunnet's post hoc test, $p<0.05$ at 0.25 mg/kg, $p< 0.01$ at 0.5 and 1.0 mg/kg). The effect of SB-399885 was also statistically significant compared to vehicle controls at doses 0.5 and 10.0 mg/kg (ANOVA: $F_{(4,70)}$= 2.513, p= 0.049; Bonferroni post hoc test, $p<0.01$ at both doses). There was a statistically significant correlation between the baseline firing rate of 5-HT neurons and their response to SB-399885, with slower firing neurons showing the greatest inhibition ($r=0.70$, $p<0.05$; n=12; Figure 3.13).

In contrast to the effects of SB-399885, the three other selective 5-HT_6 receptor antagonists tested, AE-58054 (0.25, 0.5, 1.0, 2.0 mg/kg i.v.; ANOVA: $F_{(5,20)}$= 0.591, p= 0.707), SB-271046 (0.25, 0.5, 1.0, 1.5 i.v.; ANOVA: $F_{(4,24)}$=0.897), p= 0.481) and SB-258585 (0.25, 0.5,1.0, 2.0 mg/kg i.v.; ANOVA $F_{(4,24)}$= 2.755, p= 0.051) had no statistically significant effects on 5-HT neuron firing rates compared to pre-drug values (Figures 3.10 and 3.11). Moreover, effects of AE-58054 (ANOVA: $F_{(4,55)}$= 0.242, p= 0.913), SB-271046 (ANOVA: $F_{(4,70)}$= 0.095, p= 0.984), and SB-258585 (ANOVA: $F_{(4,65)}$= 0.479, p= 0.751) were not statistically significant compared to vehicle controls (Figures 3.14 - 3.16). The lack of effect of these 5-HT_6 receptor antagonists occurred despite recorded neurons predominantly exhibiting slower firing rates, as had previously been found to correlate with response to SB-399885 (Figure 3.13). Both SB-271046- and SB-258585-treated groups contained 2 of the 15 neurons successfully labelled in this chapter, and all were found to be 5-HT immuno-positive in both (Figure 3.11).

3.3.5 Effect of WAY-181187 and WAY-208466 on cortical SW oscillations

EEG recordings were made using surface electrodes placed over the frontal cortex throughout DRN recordings. During WAY-181187 and WAY-208466 administration, activation of the brain state was evident by decreased SW oscillations, which were not evident during ST1936 administration or during administration of the 5-HT_6 receptor antagonists SB-399885, AE-58054, SB-271056 and SB-258585. These effects were quantified at the highest doses of WAY-181187 (4.0 mg/kg i.v.) and WAY-208466 (10.0 mg/kg i.v.), and it was found that both drugs caused a statistically significant decrease in SW oscillation (0.5-1.5 Hz) power compared to pre-drug values (pre-drug versus WAY-181187, $p<0.0001$; pre-drug versus WAY-208466, $p<0.0001$; Student's paired t-test) and vehicle controls (vehicle versus WAY-181187, $p< 0.0001$; vehicle versus WAY-208466, $p< 0.0001$; unpaired t-test; Figure 3.17 and 3.18). In contrast to the effects of 5-HT_6 receptor agonists on SW oscillation power, it was noted that administration of vehicle caused a significant increase in the power of SW oscillations, with a maximum increase of 45% above pre-vehicle values at 0.2 ml vehicle administration (ANOVA: $F_{(4,64)}= 8.4$, $p< 0.0001$; Dunnett's post hoc $p< 0.001$ at 0.2 ml, $p<0.01$ at 0.3 and 0.4 ml; Figure 3.19).

3.4 Discussion

Experiments in this chapter aimed to test the hypothesis that 5-HT_6 receptors influence the firing of DRN 5-HT neurons. This was investigated using electrophysiological recordings of single 5-HT neurons during administration (i.v.) of selective 5-HT_6 receptor agonists and antagonists in anaesthetised rats. One key finding was that the selective 5-HT_6 receptor agonist WAY-181187 increased the firing rate of 5-HT neurons. In addition, the selective 5-HT_6 receptor antagonist SB-399885 decreased 5-HT neuron firing, an effect reversed by WAY-181187. These observations confirm preliminary findings from this lab (K. Burnham and P. Quérée, unpublished data). In the current experiments a number of DRN neurons responding to the 5-HT_6 ligands were confirmed as 5-HT-containing through juxtacellular labelling. Taken together these data are consistent with the theory that 5-HT_6 receptors exert a positive feedback control over 5-HT neuron firing. A further five 5-HT_6 receptor ligands were tested to add to the pharmacological characterisation of the phenomenon, specifically the agonists WAY-208466 and ST-1936, and antagonists SB-258585, SB-271046 and AE-58054. It was found that WAY-208466, like WAY-181187, increased 5-HT neuron firing. However, ST-1936 did not consistently increase the firing of 5-HT neurons. Furthermore, in contrast to the decrease in 5-HT neuron firing induced by SB-399885, the other antagonists SB-258585, SB-271046 and AE-58054 had no significant effect. Thus, the theory that 5-HT_6 receptors are involved in the feedback control of 5-HT neurons needs further consideration.

3.4.1 Electrophysiological and pharmacological properties of 5-HT neurons

The electrophysiological properties of the DRN neurons recorded here are consistent with those previously described for 5-HT neurons. Specifically, clock-like neurons

fired broad spikes in a slow and regular firing pattern (Allers and Sharp, 2003; Schweimer et al., 2010; Schweimer et al., 2011). Another population of DRN neurons exhibited short, high frequency bursts in an otherwise regular firing pattern, but these neurons have also been shown to be 5-HT-containing (Allers and Sharp, 2003; Kocsis et al., 2006; Schweimer et al., 2010; Schweimer et al., 2011). The current study found that the majority of recorded DRN neurons were clock-like (80%), while a smaller proportion exhibited properties of bursting 5-HT neurons (20%). In general these findings agree with previous reports that found fewer bursting DRN neurons than clock-like neurons (Allers and Sharp, 2003; Schweimer et al., 2010; Schweimer et al., 2011; Hajos et al., 2007). However, there is some variation in these reports. For example, Schweimer et al. (2011) reported that 25% of recorded DRN neurons were bursting, whereas Hajos et al. (2007) reported 40% of recorded neurons to be bursting. Moreover, Schweimer et al. (2010) reported that 68% of recorded DRN neurons were clock-like, while Allers and Sharp (2003) reported 50% of recorded neurons to be clock-like.

The firing of individual DRN neurons exhibited strong coherence with the inactive phase of cortical SW oscillations, as previously described for 5-HT neurons (Schweimer et al., 2011). Also, the 5-HT_{1A} receptor agonist 8-OH-DPAT completely inhibited the firing of the neurons, as was expected of 5-HT neurons from earlier studies (Sprouse and Aghjanian, 1987; Allers and Sharp, 2003). Finally, 15 of the total 87 recorded DRN neurons were successfully juxtacellular labelled and all but one judged to be 5-HT-immunopositive. Moreover, the electrophysiological properties of these 5-HT-immunopositive neurons were indistinguishable from those of non-juxtacellular labelled neurons, thereby strongly suggesting that the vast majority, if not all, DRN neurons included in the final analysis were 5-HT-containing.

3.4.2 Evidence of 5-HT_6 receptor targeting by ligands used in the current study

Several lines of evidence indicate that the 5-HT_6 receptor ligands used here are capable of specifically targeting 5-HT_6 receptors. In particular, the agonists WAY-181187 and WAY-208466 display high 5-HT_6 binding affinity (Ki values of 2.2nM and 2.8nM, respectively) and both ligands show at least 60-fold selectivity for the 5-HT_6 receptor over other metabotropic receptors including various 5-HT receptors (Schechter et al., 2008). Furthermore, both ligands elicited full agonist effects in a cAMP accumulation assay (WAY-181187 E_{max}= 93%, WAY-208466 E_{max} = 100%; Schechter et al., 2008). In comparison, the other 5-HT_6 receptor agonist used ST-1936, displayed lower 5-HT_6 receptor binding affinity (Ki 28.8 nM) and had only 10-fold selectivity for the 5-HT_6 receptor over other receptors; however, ST-1936 exhibited near full agonist properties in cAMP accumulation assays (E_{max} 85-90%, Riccioni et al., 2011).

Previous studies found that WAY-181187 at 3 mg/kg i.p. produced a 5-HT_6 receptor occupancy of 80% 30 min after administration, as estimated on the basis of its bioavailability and 5-HT_6 receptor binding affinity (Cole et al., 2007). Here, similar doses of WAY-181187 were given i.v., suggesting a high degree of 5-HT_6 receptor occupancy with this drug. Moreover, at comparable doses to those used here all three agonists have been reported to induce 5-HT_6 receptor-dependent effects. For example, 0.5-1.6 mg/kg i.v. WAY-181187 and 0.4 mg/kg i.v. ST-1936 modulated the firing of VTA dopamine neurons in the anaesthetised rat, effects which were blocked with the 5-HT_6 receptor antagonist SB-271046 (Borsini et al., 2015). Also, in microdialysis studies 10mg/kg i.p. ST-1936 elevated extracellular dopamine and noradrenaline in the rat frontal cortex, effects that were blocked by the SB-271046 and SB-399885 (Valentini et al., 2011). In addition, 10 mg/kg i.p. WAY208466

reduced rapid eye movement sleep in freely moving rats, an effect which was blocked by SB-399885 (Monti et al., 2013).

In the case of the 5-HT_6 receptor antagonists, each of SB-399885, SB-258585, SB-271046 and AE-58054 show high 5-HT_6 binding affinity (Ki= 9.1, 8.9, 9.0, and 0.83 nM respectively; Bromidge et al., 1999; Hirst et al., 2000; Hirst et al., 2006; Arnt et al., 2010). SB-399885 and SB-271046 exhibit a 200-fold selectivity for the 5-HT_6 receptor over other receptors including various 5-HT receptors (Routledge et al., 2000; Hirst et al., 2006). Similarly, SB-258585 displays a 100-fold selectivity for 5-HT_6 receptors, while AE-58054 shows greater than 30-fold selectivity (Hirst et al., 2000; Arnt et al., 2010). *In vivo* 2 mg/kg i.v. AE-58054 produced a 5-HT_6 receptor occupancy close to 100% (unpublished data from H. Lundbeck A/S). The same occupancy measurements for 3-10 mg/kg i.p. SB-399885 and 10-30 mg/kg i.p. SB-271046 where found to be 62-96% and 56-84%, respectively (Hirano et al., 2009). Although the latter doses of SB-399885 and SB-271046 are higher than those used here (0.125-1.0 mg/kg and 0.25-1.5 mg/kg, respectively), i.v. dosing will cause greater plasma drug concentrations and receptor occupancy that i.p. dosing. Finally, at similar doses to those used here, these 5-HT_6 antagonists reportedly block 5-HT_6 receptor agonist induced effects. For example, 10 mg/kg i.p. SB-399885 blocked the effects of WAY-181187 in rats in a test of executive function (Burnham et al., 2010). In addition, 0.1-0.2 mg/kg i.v. SB-271046 reversed the effects of ST-1936 on VTA dopamine neuron firing (Borsini et al., 2015).

3.4.3 Involvement of 5-HT_6 receptors in the effects of 5-HT_6 ligands on 5-HT neurons

Both the 5-HT_6 receptor agonists WAY-181187 and WAY-208466 increased 5-HT neuron firing, while ST-1936 had no significant effect. WAY-208466 and WAY-181187 exhibit a similarly high 5-HT_6 receptor affinity and selectivity. Both drugs are reported to evoke similar effects *in vivo* in rats, including elevated extracellular GABA levels in the cortex and eliciting antidepressant effects (Schechter et al., 2008; Carr et al., 2011). Blockade of the increase in 5-HT firing induced by WAY-181187 by SB-258585, and to a lesser extent SB-271046, gives weight to the idea that WAY-181187 is acting via a 5-HT_6 dependent-mechanism. Moreover, others have reported the blockade of the antidepressant effect of WAY-208466 by SB-258585 in a rat model (Li et al, 2015).

In contrast to WAY-181187 and WAY-208466, ST-1936 did not increase 5-HT neuron firing. ST-1936 has lower 5-HT_6 receptor affinity and selectivity compared to WAY-181187 and WAY-208466. Moreover, another study has reported divergent effects of ST-1936 and WAY-181187 *in vivo;* specifically ST-1936 increased VTA dopamine neuron firing, whereas WAY-181187 inhibited firing (Borsini et al., 2015). As a potential explanation of this finding, the latter study noted possible differences in the signalling pathways activated by ST-1936 and WAY-181187 (Borsini et al., 2015), as also discussed by others (Riccioni et al., 2011; Schechter et al., 2008). Of particular relevance to current findings, reports suggest that WAY-181187 has a higher potency than ST-1936 in cAMP accumulation assays. In particular, two studies report WAY-181187 elicits cAMP accumulation *in vitro* at the human 5-HT_6 receptor at an Emax > 90% (Cole et al., 2007; Schechter et al., 2008). By contrast, ST-1936 is reported to have an Emax as low as 70% in these assays (Riccioni et al., 2011). These data would suggest ST-1936 has reduced potency in 5-HT_6 receptor

signal transduction, perhaps contributing to the differing effects of ST-1936 and WAY-181187 on 5-HT neuron firing.

The 5-HT_6 receptor antagonist SB-399885 decreased 5-HT neuron firing when administered alone. This finding is of interest since it could suggest tonic activity of a putative 5-HT_6 receptor controlled excitatory feedback mechanism, since previous studies have explained the inhibitory effect of 5-HT_4 antagonists on 5-HT neuron firing as evidence for an excitatory feedback pathway which is tonically active (Lucas et al., 2005). However, the lack of effect of the antagonists SB-271046, SB-258585 and AE-58054 on 5-HT neuron firing raises the question of the role of the 5-HT_6 receptor in SB-399885-induced inhibition of 5-HT neuron firing. One possible explanation is that SB-399885 has inverse agonist properties not possessed by SB-271046, SB-258585 and AE-58054, although currently there is no published evidence of this. In another context, data suggest that SB-399885 has 5-HT_6 receptor-independent effects. Specifically, SB-399885 elicited antidepressant effects in a forced swim test in rats, despite CNS 5-HT depletion (Wesolowska, 2007). Moreover, evidence in support of a 5-HT_6 receptor-independent mechanism of SB-399885 in the current study, is the finding that SB-399885 failed to block WAY-181187 effects on 5-HT neuron firing, whereas these effects were blocked by SB-271046 and SB-258585. Thus, although SB-399885 mediated a decrease in 5-HT neuron firing, these effects may be independent of actions on the 5-HT_6 receptor.

3.4.4 Site of action of 5-HT_6 receptor ligands

Overall, there is evidence to support the hypothesis that the increase in 5-HT neuron firing induced by WAY-181187 and WAY-208466 is mediated by 5-HT_6 receptors.

This is consistent with the idea that 5-HT_6 receptors exert an excitatory feedback control over 5-HT neurons, although this may not be tonically active under the current conditions. This idea is further supported by evidence that 5-HT_6 mRNA is expressed in the DRN (Gerard et al., 1997; Helboe et al., 2015). Furthermore, these receptors appear to be physiologically relevant since intra-DRN application of the 5-HT_6 agonist WAY-208466 reduced sleep in rats (Monti et al., 2013). More recently, studies report a lack of co-localisation between 5-HT_6 mRNA and 5-HT in the DRN, suggesting the receptor may be present on non-5-HT neurons (Helboe et al., 2015). However, 5-HT_6 receptors may also reside on forebrain neurons which input to the DRN. For example, 5-HT_6 receptor-expressing neurons are present in the mPFC which sends extensive inputs to the DRN (discussed in Chapter 1).

3.4.5 Effect of 5-HT_6 receptor agonists on cortical SW oscillations

In the current study, an unexpected finding was that WAY-181187 and WAY-208466 produced SW oscillations (0.5-1.5 Hz) in the frontal cortex, as measured by EEG recordings. In support of these findings, WAY-208466, as well as 5-HT_6 agonist EMD386088, was reported to decrease slow wave sleep in non-anaesthetised rats (Monti et al., 2013 and Ly et al., 2013). Like SW oscillations which occur in the anaesthetised rat, slow wave sleep is characterised by oscillations around 1 Hz (Azmica and Steriade, 1998). Thus, SW oscillations occurring under anaesthesia in the current study can be considered to mimic oscillations which occur during slow wave sleep in non-anaesthetised rats (Azmica and Steriade, 1998).

It was noted that vehicle (isotonic glucose-saline) administration induced a significant increase in SW oscillation power. One explanation for this effect may be changes in

blood volume resulting from the injections. However, against this argument is the observation that the increased SW oscillation power did not exhibit a linear increase with administration volumes. Thus the greatest effect of vehicle on SW oscillation power occurred at an intermediate point in the administration regime, and then remained stable with increasing volumes. Nevertheless, this result highlights the importance of examining the effect of vehicle on EEG activity as well as single neuron firing in electrophysiological recordings. An alternative vehicle with no effect on EEG activity may be favourable in future studies.

EEG recordings are generated from the propagation of small amplitude, long duration post-synaptic currents in the extracellular space (Buzsaki, 2006).The SW oscillations in the frontal cortex result from the synchronous firing of cortical and thalamic neurons, characterised by periods of depolarisation and hyperpolarisation (Contreras and Steriade, 1995, Steriade, 2000). This synchronous activity in cortical neurons results in the entrainment of neuronal activity in subcortical structures, as a result of a synchronous discharge in cortical afferents. This phenomenon occurs in other regions including the basal ganglia and is also reported in the DRN (Schweimer et al., 2011). Thus, DRN 5-HT neurons are found to firing in synchrony with frontal cortex SW oscillations (Schweimer et al., 2011), as also documented in the current experiments. As Schweimer and colleagues describe, the activity of DRN 5-HT neurons is entrained to cortical activity during SW oscillations. Thus, a reduction in these oscillations, as induced by WAY-181187 and WAY-208466 administration, could in turn influence DRN neuronal activity.

EEG recordings have low spatial resolution and consequently represent a measurement of activity within the cortical neural network and not a specific neuronal

population (Buszaki, 2006). One frontal cortex region which could contribute to the EEG recordings is the mPFC, since 5-HT_6 receptors are thought to be highly expressed on pyramidal neurons and interneurons here (Helboe et al., 2015). Importantly, much data suggests the mPFC exhibits considerable bilateral inputs to the DRN (Sesak et al., 1989; Hajos et al., 1998; Peyron et al., 1998). Moreover, data from electrophysiological studies has demonstrated an influence of mPFC neurons in the DRN. For example, electrical stimulation of the mPFC predominantly inhibited DRN 5-HT neuron but also increased firing in some 5-HT neurons (Hajós et al. 1998, Celada et al. 2001, Varga et al. 2001). Thus, the mPFC represents a possible site of action of WAY-181187 and WAY-208466 on EEG activity, and is also a candidate region in the influence of 5-HT_6 ligands on 5-HT neuron firing. Given this, the next chapter aimed to further characterise the effect of 5-HT_6 ligands on mPFC neuronal activity, including on individual DRN projecting cortical neurons.

3.4.6 Conclusion

The current chapter found that 5-HT_6 agonists WAY-181187 and WAY-208466 increased DRN 5-HT neuron firing, while 5-HT_6 agonist SB-399885 decreased firing. The effects of WAY-181187 and WAY-208466 are likely to be 5-HT_6 receptor-mediated, whereas SB-399885 may produce its effects independent of the 5-HT_6 receptor since three other 5-HT_6 receptor antagonists had no effect on 5-HT neuron firing. Interestingly, WAY-181187 and WAY-208466 both decreased frontal cortex SW oscillations. One region which is likely to contribute to the frontal cortex EEG is the mPFC. Since the mPFC expresses a high level of the 5-HT_6 receptors and is a key source of inputs to the DRN, this region may be a site of action of 5-HT_6 receptor ligands in mediating effects on 5-HT neuron firing. Experiments described in the next chapter aimed to further characterise the influence of 5-HT_6 receptor ligands on cortical activity at a network and single neuron level.

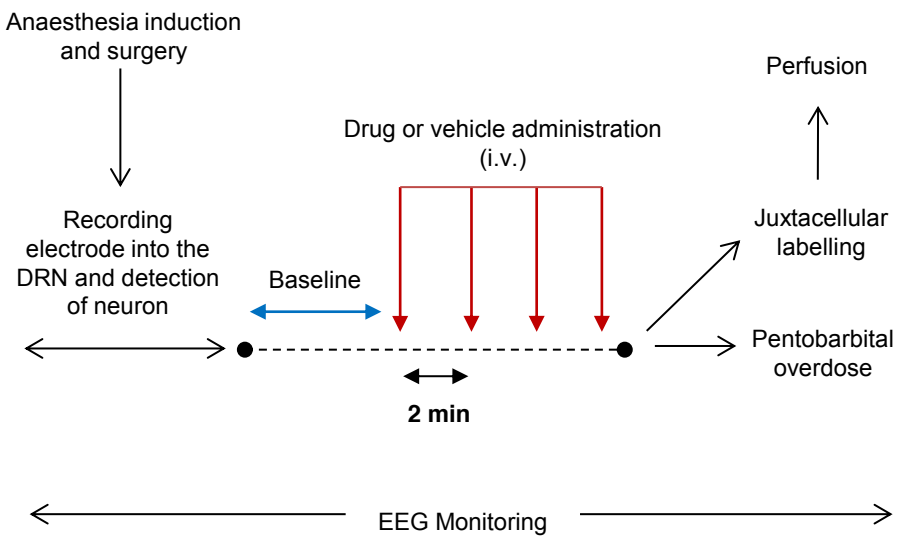


Figure 3.1
General protocol for experiments described in Chapter 3. Rats were anaesthetised and a recording electrode was lowered into the DRN. Neurons were recorded for 2-3 min to establish a stable baseline followed by administration of drugs or vehicle in 2 min intervals. Then, either recorded neurons were juxtacellular labelled followed by perfusion, or otherwise rats were culled with pentobarbital overdose.

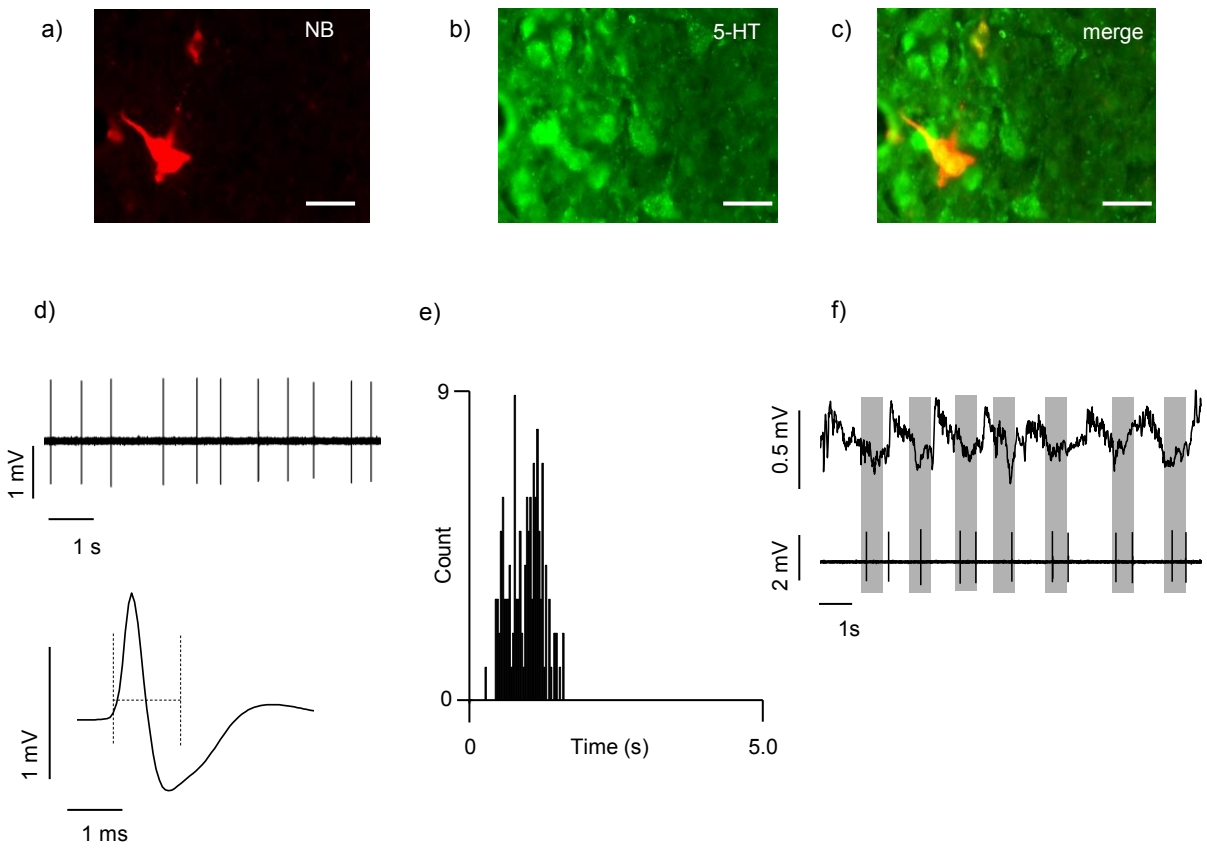


Figure 3.2

Electrophysiological and chemical properties of a representative 5-HT neuron. The neuron was juxtacellular labelled and found to be 5-HT immuno-positive (a, b and c), scale bars= 20µm. d) Spike train (top) exhibiting a slow firing rate with a broad spike width (bottom). e) Inter-spike interval histogram illustrating a regular firing pattern. f) Spike train with matching EEG trace demonstrating firing during the inactive phase of SW oscillations.

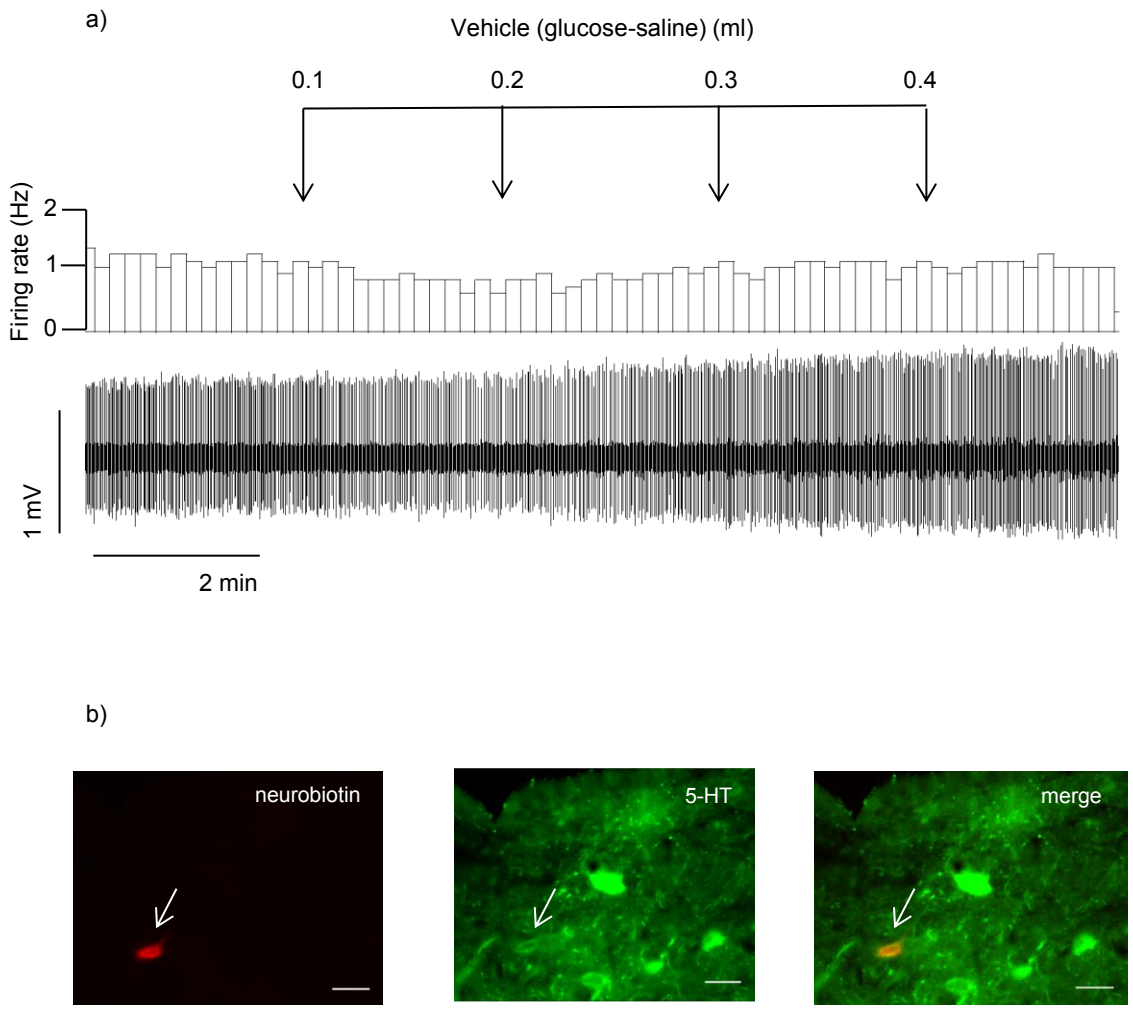


Figure 3.3
Effect of vehicle (gluose-saline) on the firing of a single 5-HT neuron in the rat DRN. a) Rate meter (top) and spike train (bottom) recorded during vehicle administration. b) The same cell was successfully labelled with neurobiotin and identified as 5-HT immuno-positive, scale bar = 20μm.

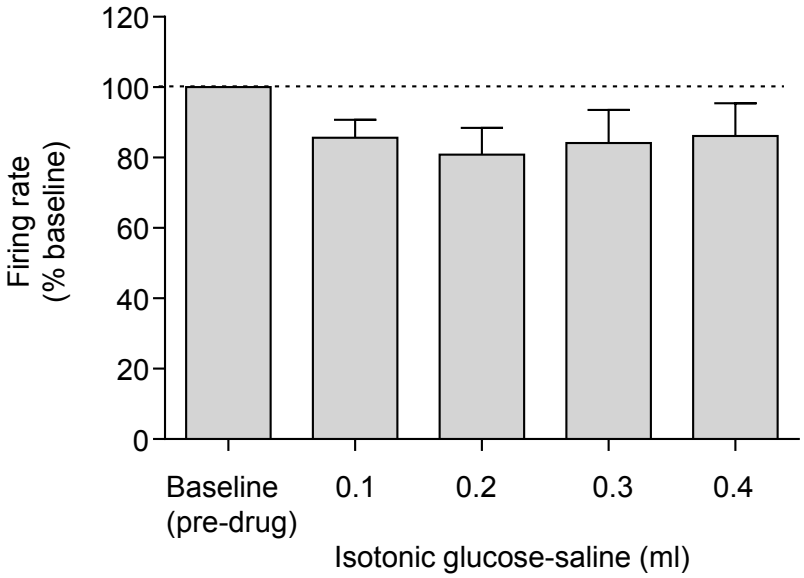


Figure 3.4
Effect of vehicle (glucose-saline) on DRN 5-HT neuron firing across a group of neurons. Vehicle was administered i.v. in accumulating volumes at 2 min intervals. Data expressed as mean ±SEM values, (n=8 neurons).

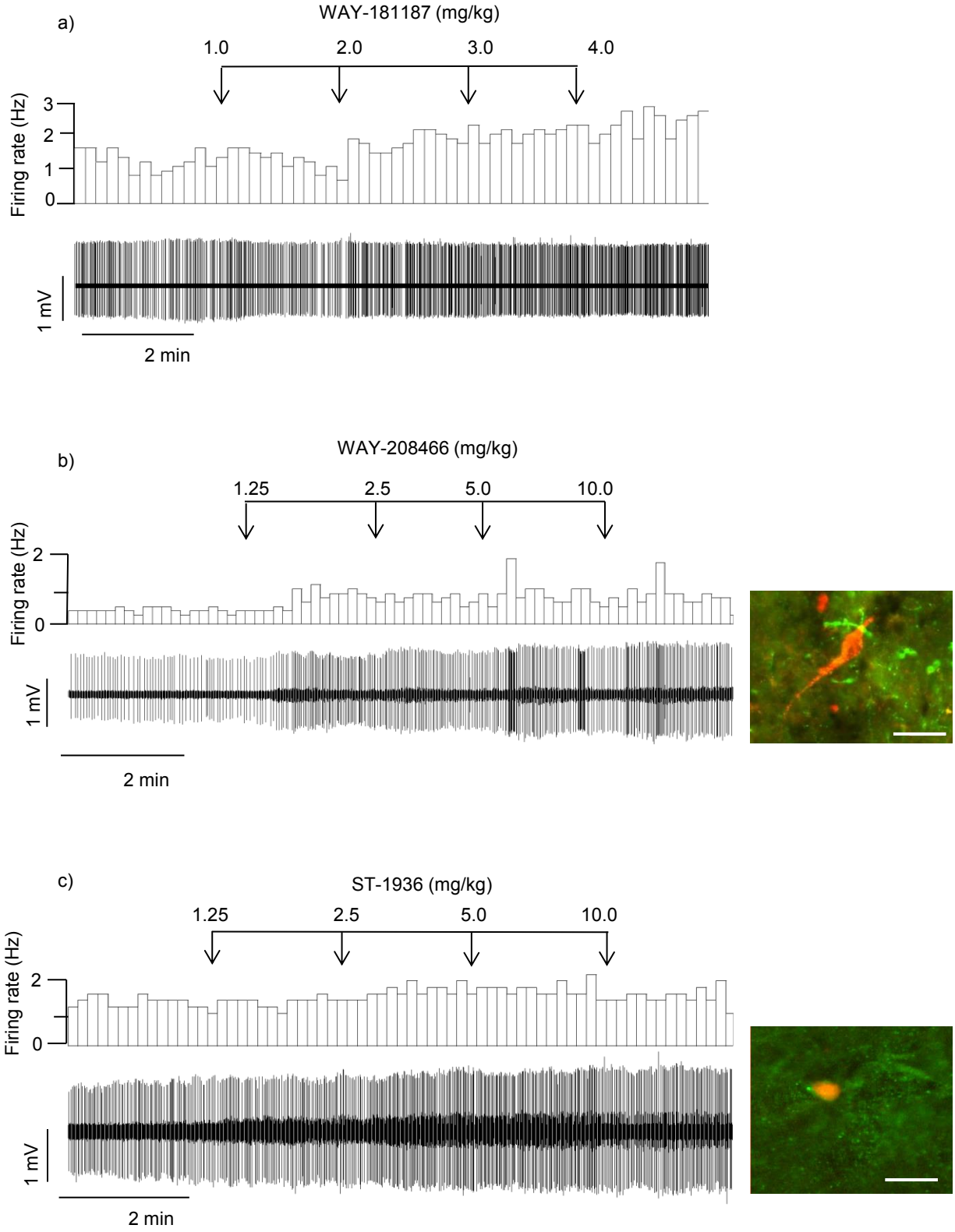


Figure 3.5
Electrophysiological traces illustrating the effect of 5-HT_6 agonists on single 5-HT neurons. Representative rate meters (top traces) and spike trains (bottom traces) from a) WAY-181187; b) WAY-208466; and c) ST-1936. Neurons in b) and c) were successfully labelled and found to be 5-HT immuno-positive; red = neurobiotin, green = 5-HT, scale bar = 20μm.

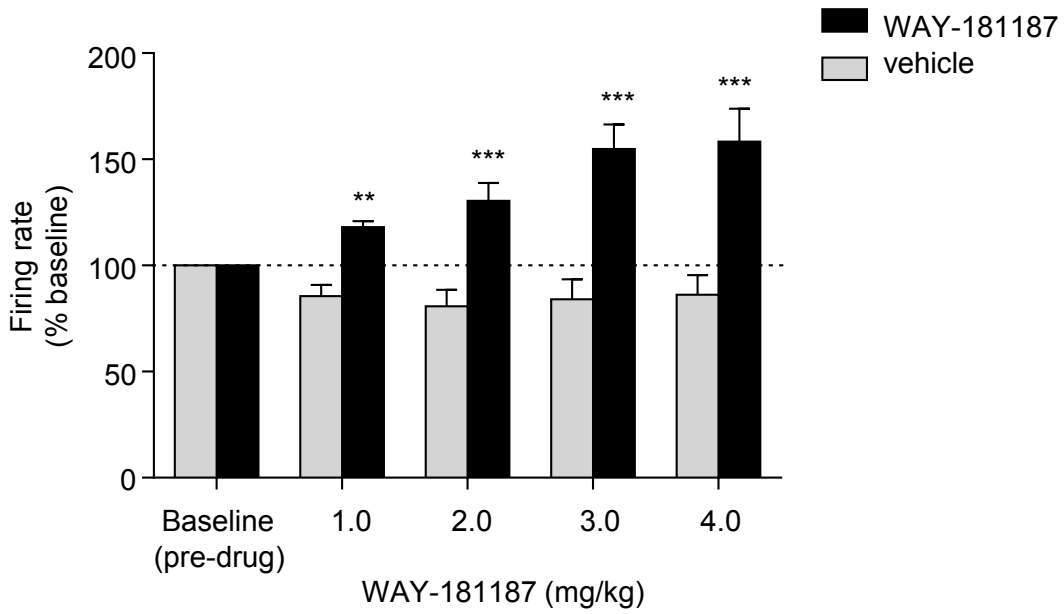


Figure 3.6
Effect of WAY-181187 on DRN 5-HT neuron firing across a group of neurons. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values, (n=6 neurons). **p<0.01, ***p<0.001 versus vehicle (ANOVA with Bonferroni's post hoc test).

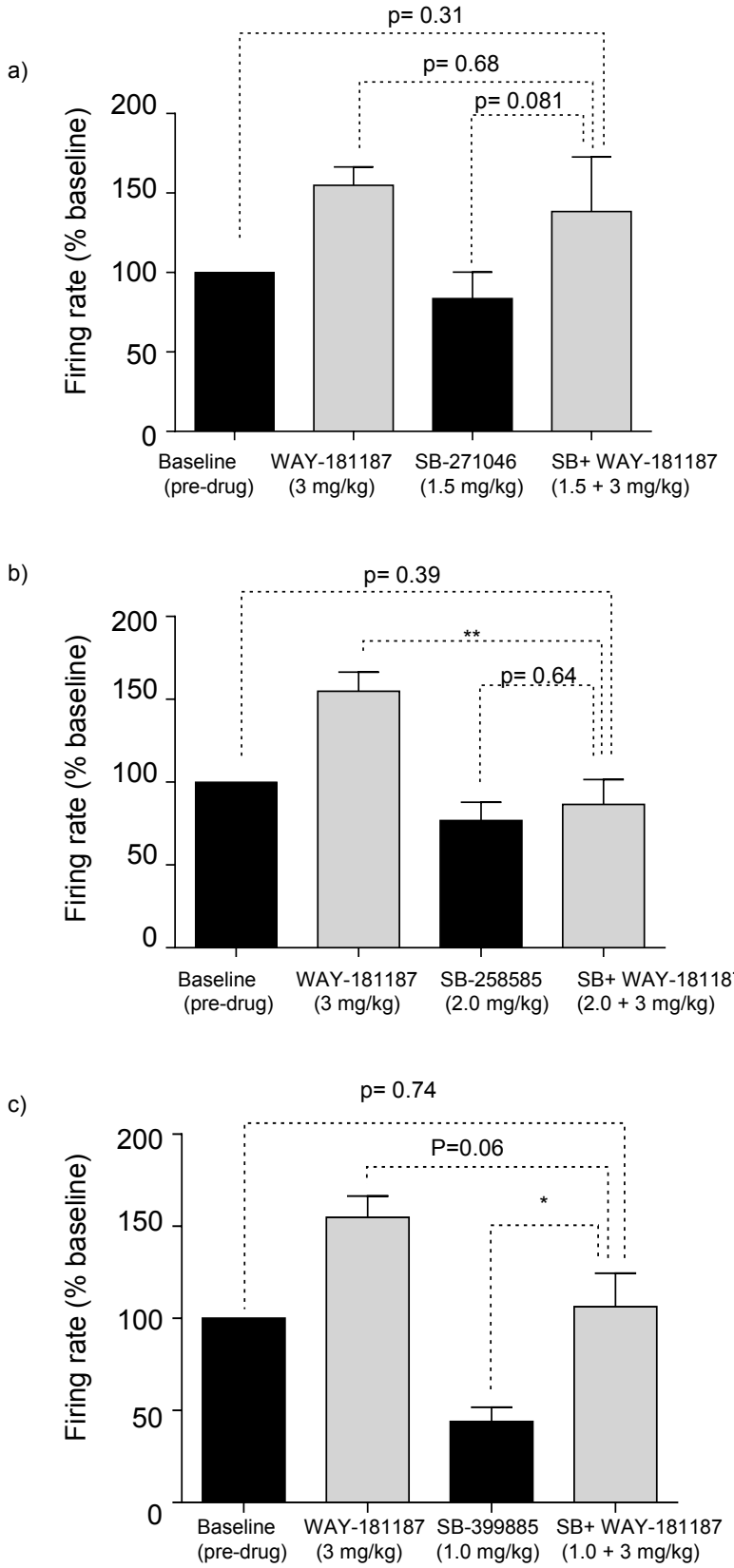


Figure 3.7
Effect of antagonist pre-treatment on WAY-181187-induced increases in DRN 5-HT neuron firing. Group data illustrating the effect of pre-treatment with a) SB-271046 (n=7 neurons); b) SB-258585 (n=7 neurons); and c) SB-399885 (n=8 neurons). Data expressed as mean ± SEM values *p<0.05 **p<0.01 versus group indicated.

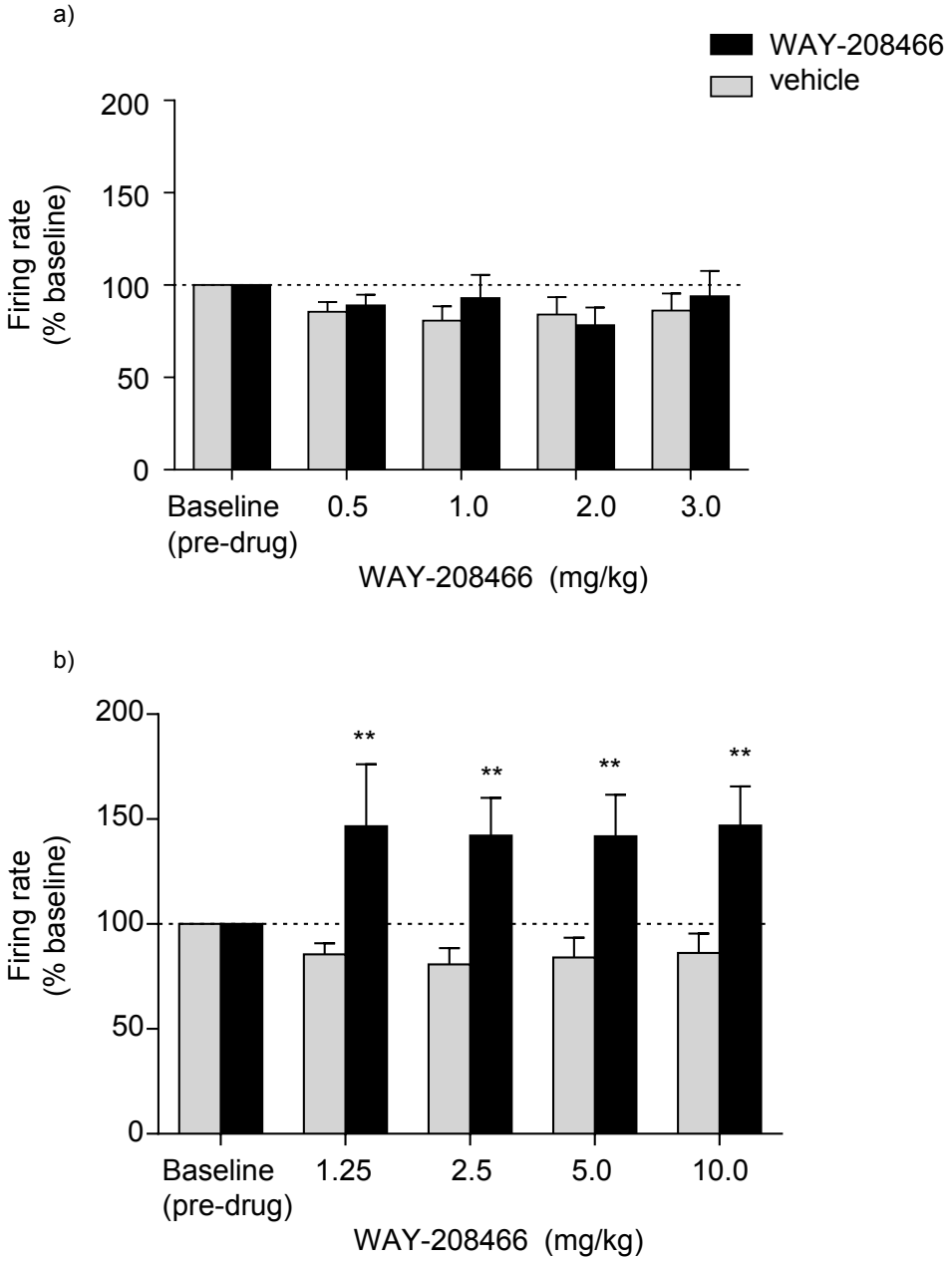


Figure 3.8
Effect of WAY-208466 on DRN 5-HT neuron firing across a group of neurons. WAY-208466 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values. a) lower doses, n=7 neurons and b) higher doses, n=4 neurons. **p<0.01 versus vehicle (ANOVA with Bonferroni's post hoc test).

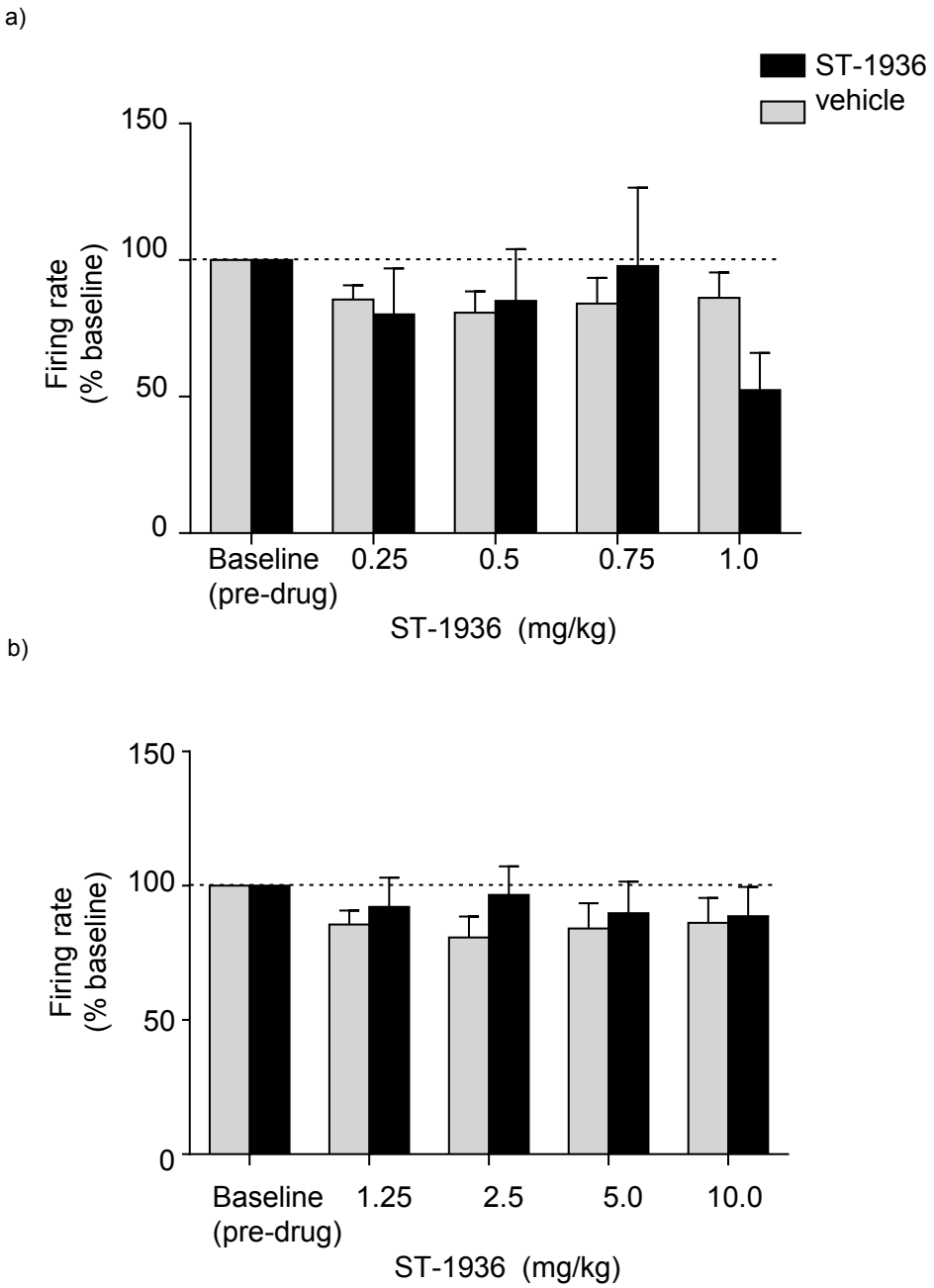


Figure 3.9
Effect of ST-1936 on DRN 5-HT neuron firing across a group of neurons. ST-1936 was administered in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values. a) lower doses, n=4 neurons, and b) higher doses, n=5 neurons.

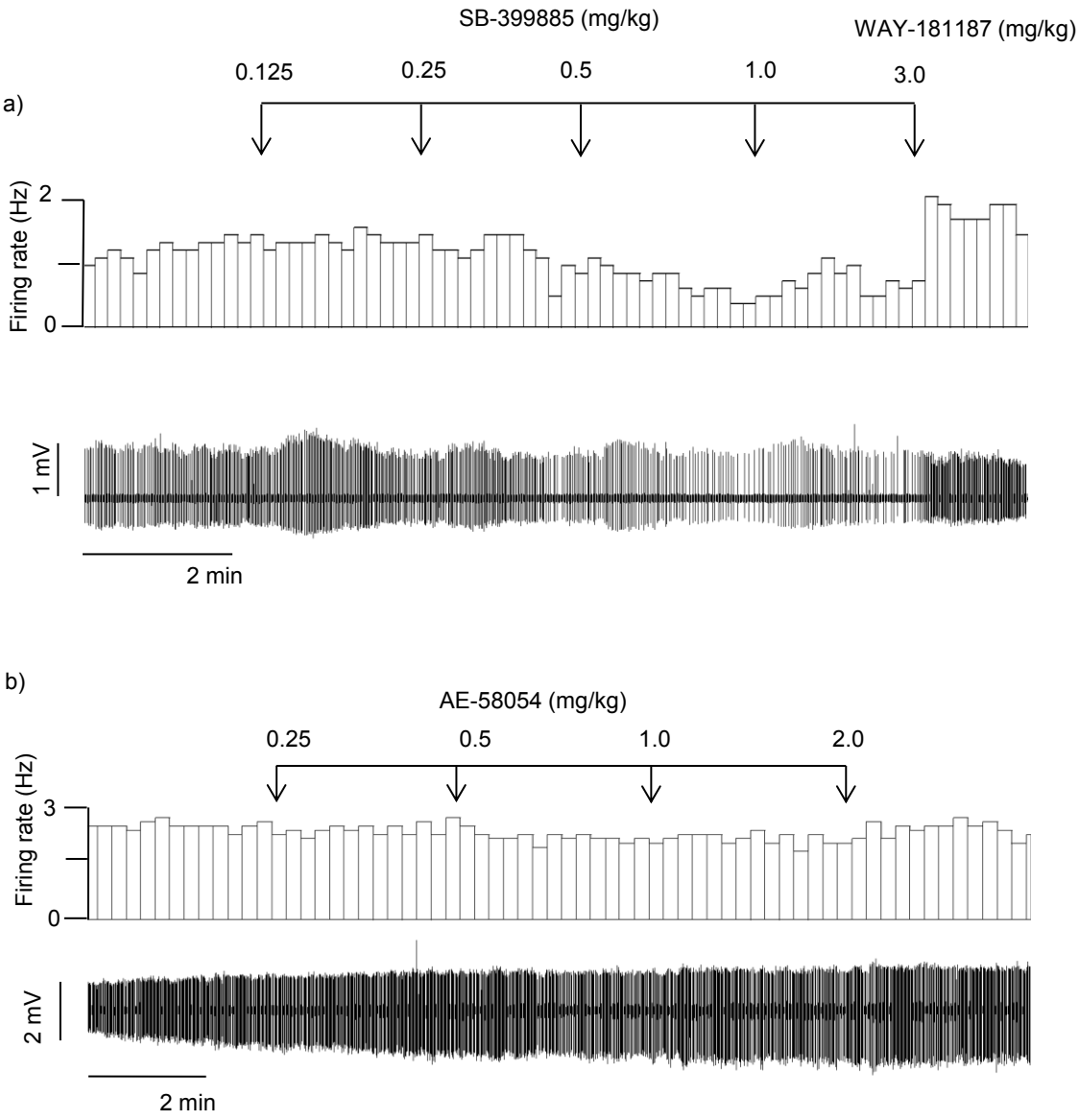


Figure 3.10
Electrophysiological traces illustrating the effect of 5-HT_6 antagonists on single 5-HT neurons. Representative rate meters (top traces) and spike trains (bottom traces) from experiments with a) SB-399885 and b) AE-58054.

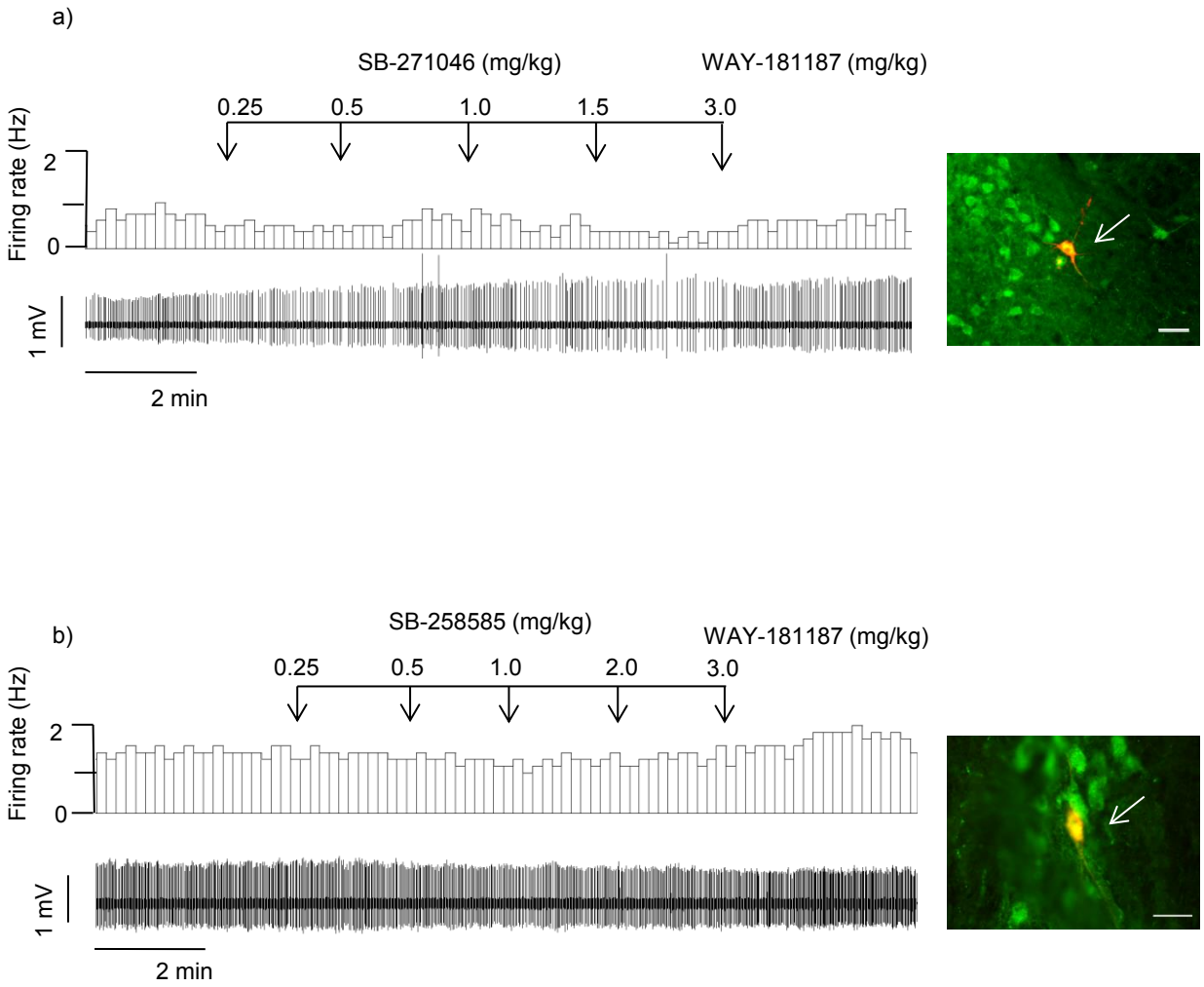


Figure 3.11
Electrophysiological traces illustrating the effect of 5-HT_6 agonists on single 5-HT neurons. Representative rate meters (top traces) and spike trains (bottom traces) from a) SB-271046 and b) SB-258585. The same neurons were 5-HT immuno-positive; red = neurobiotin, green = 5-HT, scale bar = 20μm.

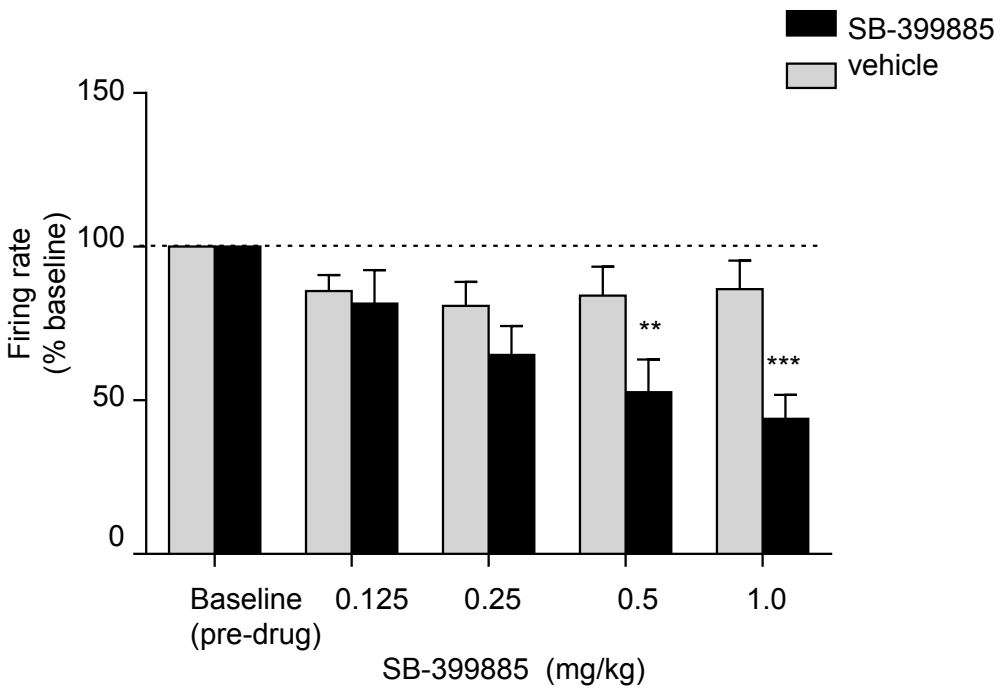


Figure 3.12
Effect of SB-399885 on DRN 5-HT neuron firing across a group of neurons. SB-399885 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values (n= 8 neurons). **p<0.01, ***p<0.001 versus vehicle (ANOVA with Bonferroni's post hoc test).

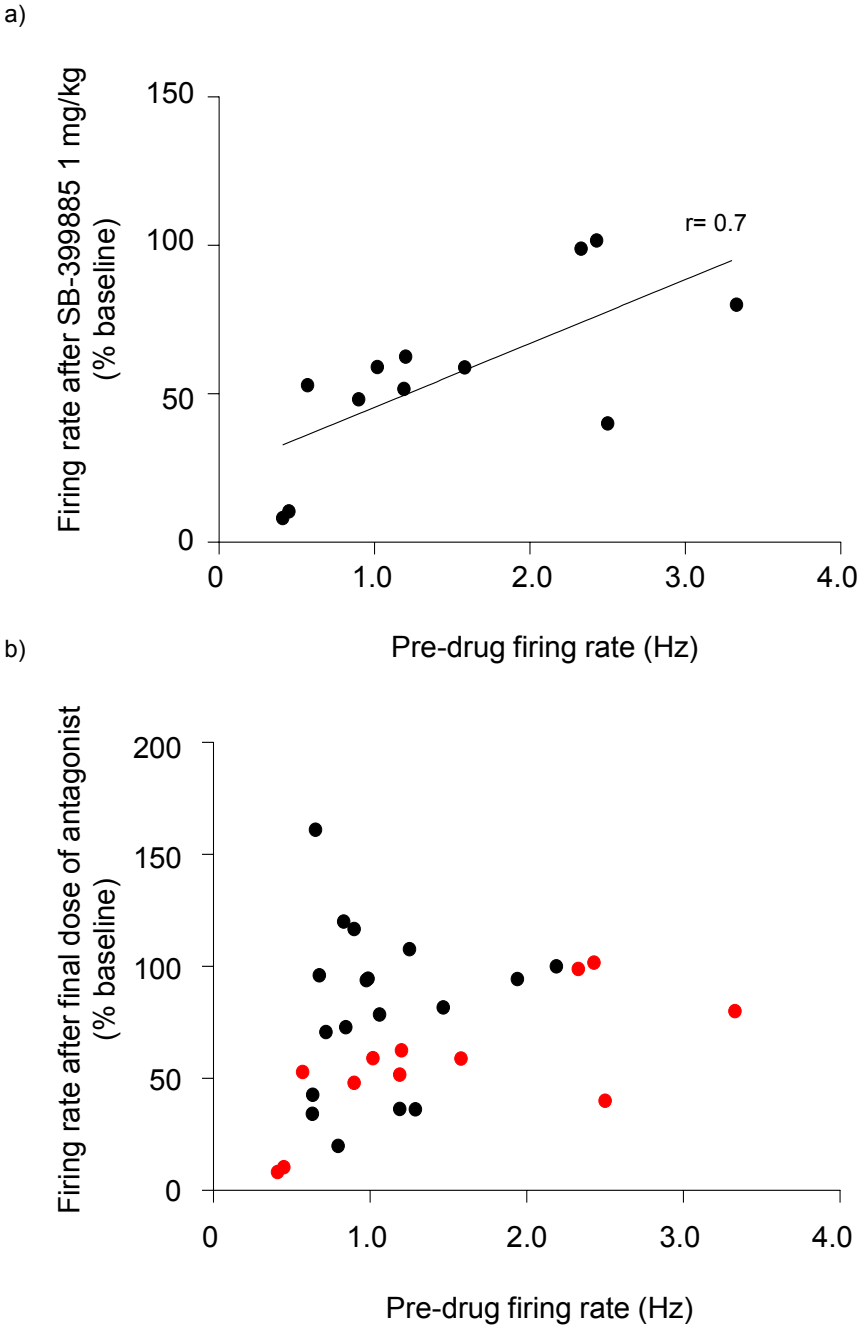


Figure 3.13
The correlation between pre-drug firing rate and response to 5-HT_6 receptor antagonists. a) The response of neurons to the final dose of SB-399885 (1mg/kg) as a percentage of the pre-drug (baseline) firing rate (taken as 100%) compared to the pre-drug firing rate of that neuron (n= 12). A line of best fit has been drawn following a Pearson's correlation (r= 0.7). b) The response of neurons to the final dose of 5-HT_6 receptor antagonists SB-399885 (indicated in red), SB-271046, SB-258585, and AE-58054 as a percentage of the pre-drug (baseline) firing rate compared to the pre-drug firing rate of that neuron (n= 30).

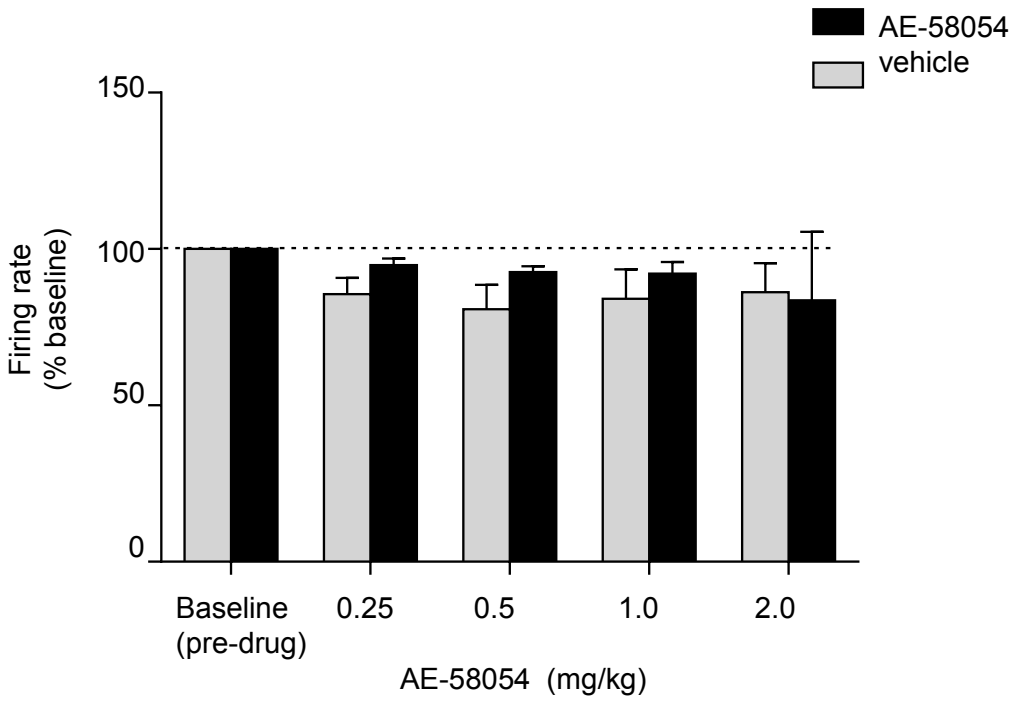


Figure 3.14
Effect of AE-58054 on DRN 5-HT neuron firing across a group of neurons. AE-58054 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM (n=4 neurons).

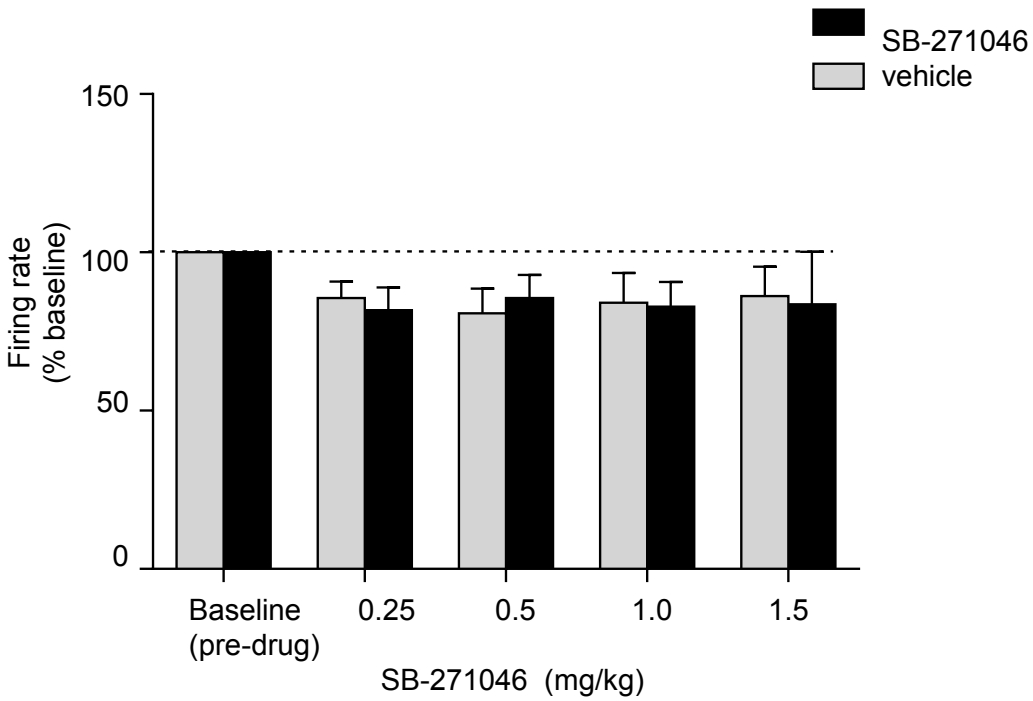


Figure 3.15
Effect of SB-271046 on DRN 5-HT neuron firing across a group of neurons. SB-271046 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM (n=7 neurons).

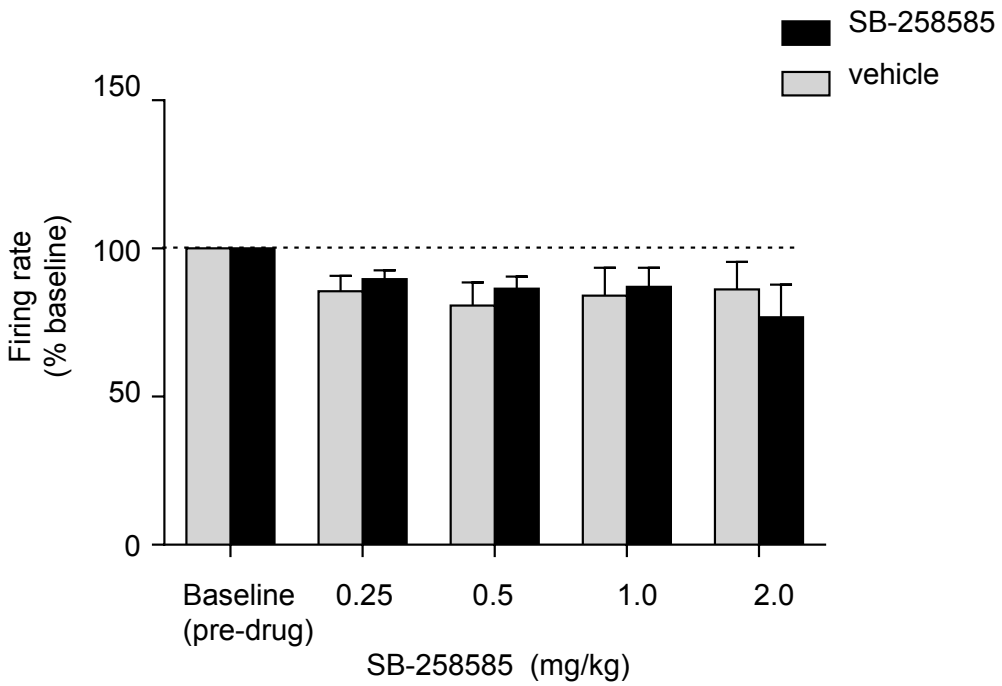


Figure 3.16
Effect of SB-258585 on DRN 5-HT neuron firing across a group of neurons. SB-258585 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values (n=7 neurons).

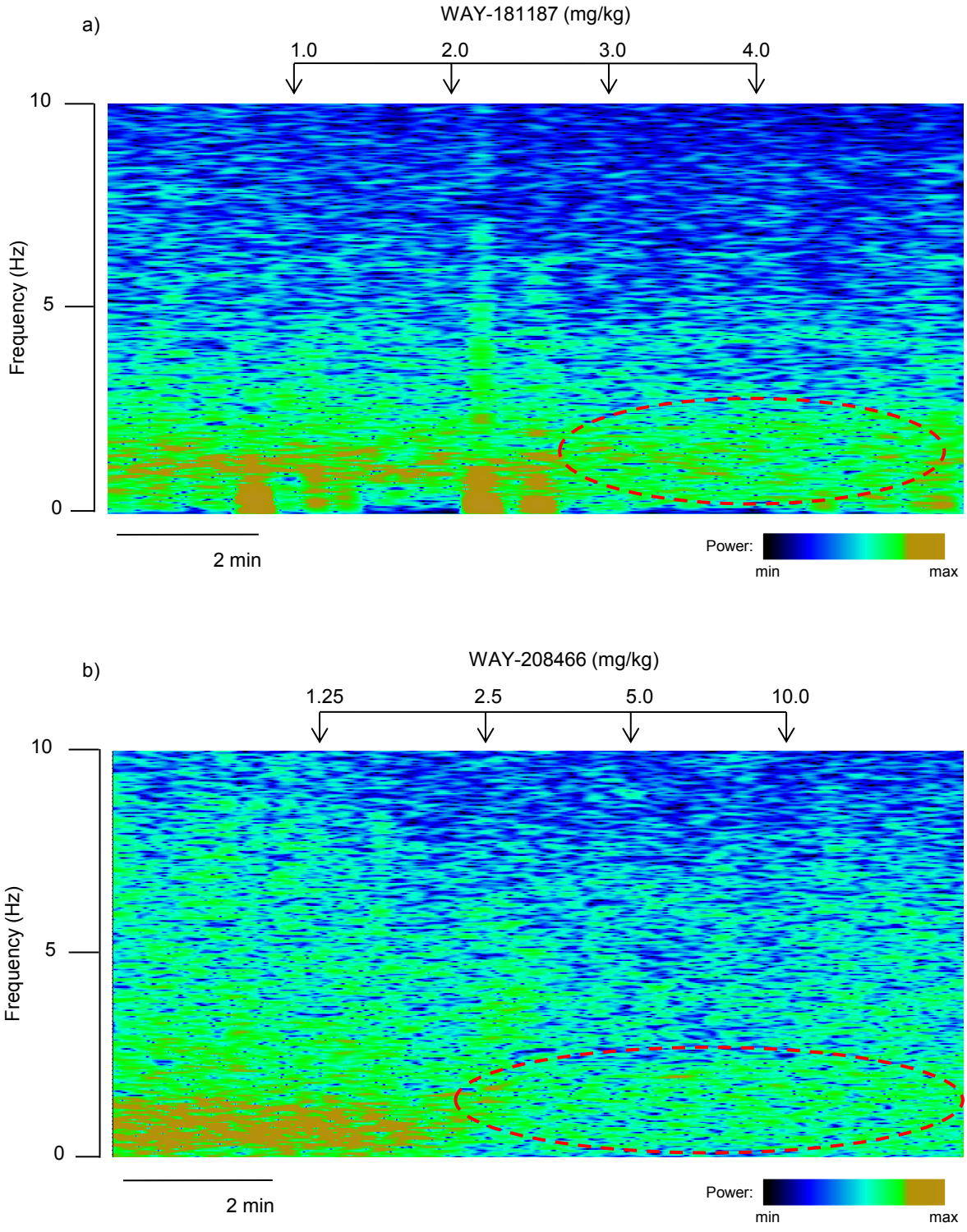


Figure 3.17
Effect of WAY-181187 and WAY-208466 on cortical SW oscillations. Representative spectrograms from single EEG recordings during a) WAY-181187 and b) WAY-208466 administration. Key denotes power with high power orange, moderate power green, lower power blue. Note the high SW oscillation power between 0.5 and 1.5 Hz which is lost following WAY-181187 and WAY-208466 administration (circled in red).

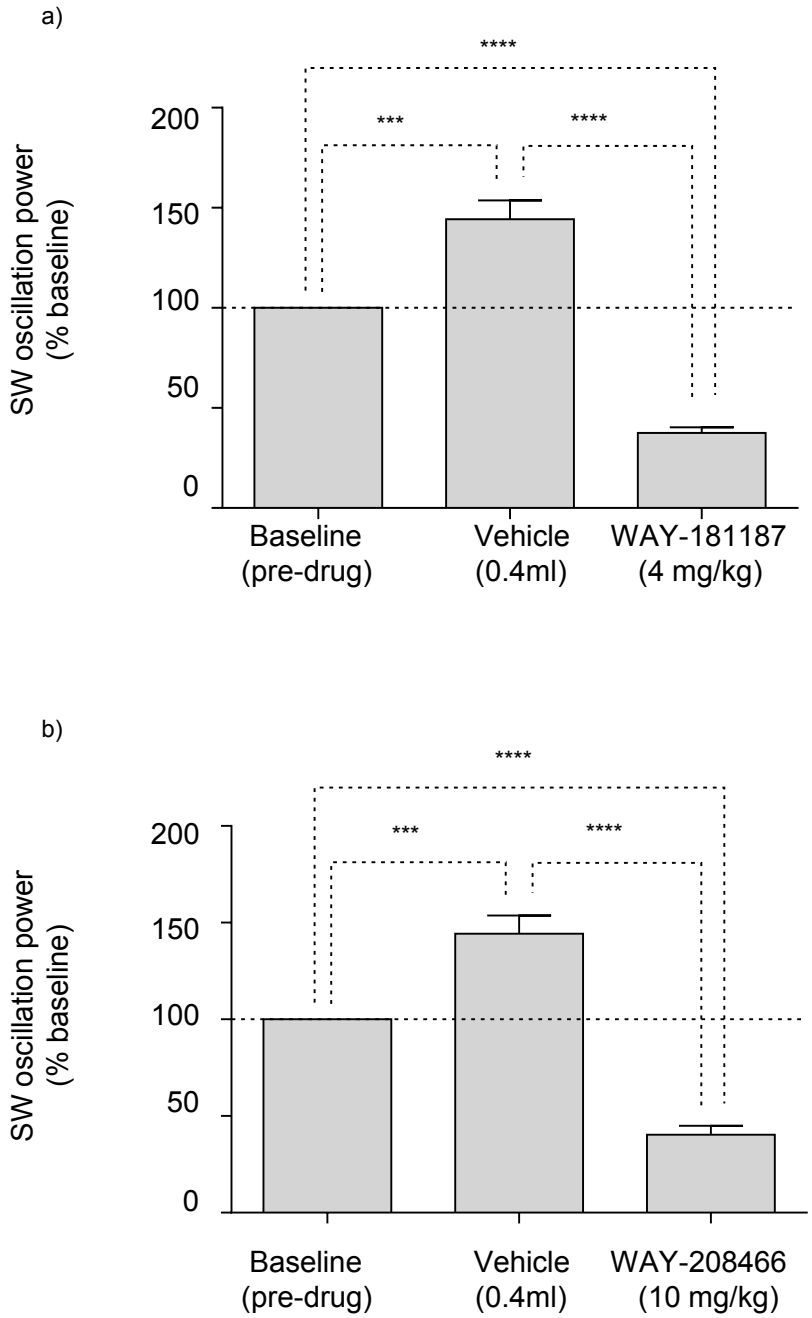


Figure 3.18
Effect of WAY-181187 and WAY-208466 on cortical SW oscillations across groups of rats. a) Effect of WAY-181187 on SW oscillation power (n=5); b) effect of WAY-208466 on SW oscillation power (n=3). ***p< 0.001 ****p<0.0001 verses vehicle (n=8). Data expressed as mean ±SEM values.

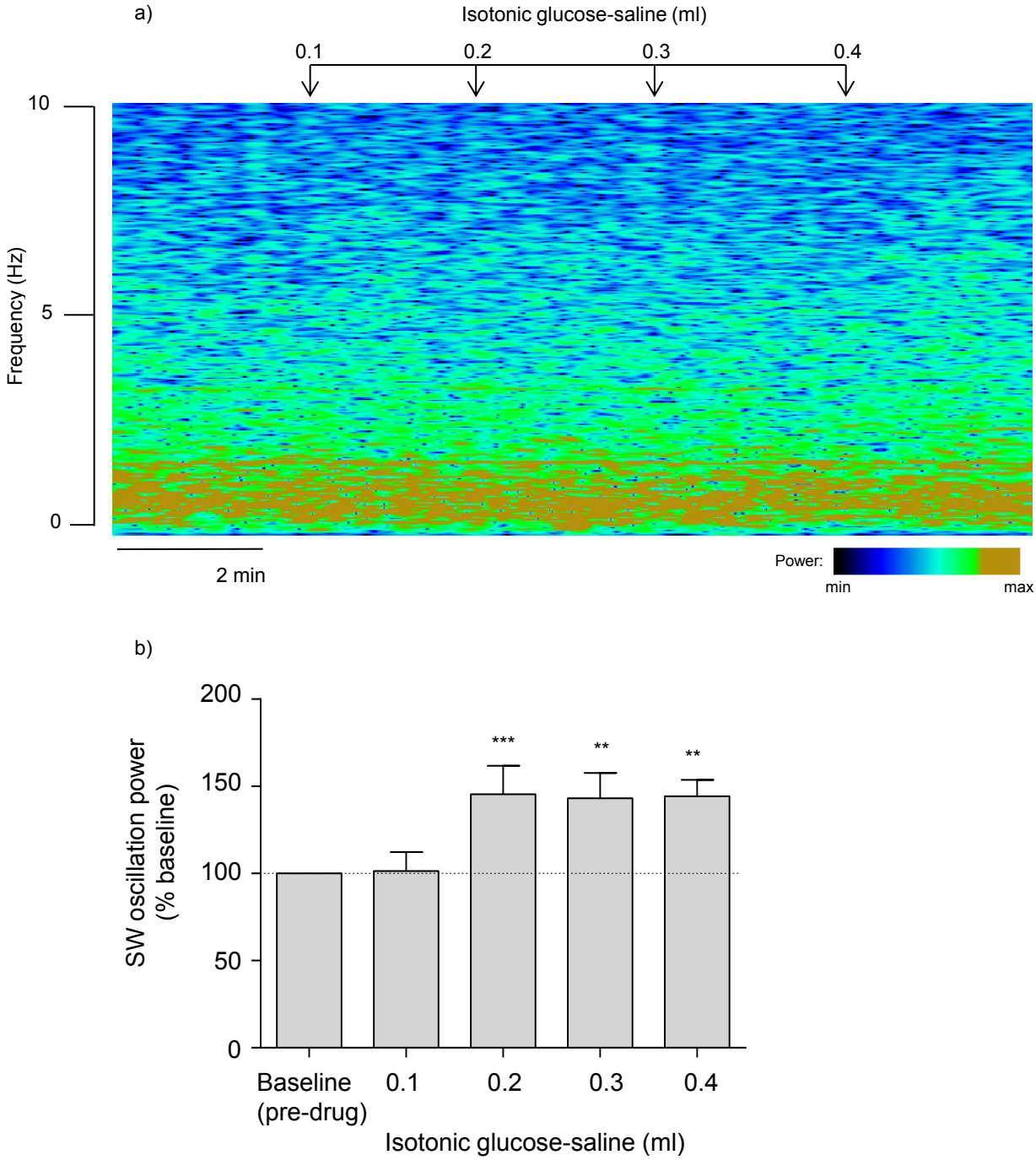


Figure 3.19
Effect of vehicle administration on cortical SW oscillations. a) Representative spectrogram from a single EEG recording during vehicle administration. Key denotes power with high power orange, moderate power green, lower power blue. b) Effect of vehicle on SW oscillation power (n=8). **p< 0.01 ***p<0.001 verses pre-vehicle. Data expressed as mean ±SEM values.

Chapter 4

Effect of 5-HT_6 receptor ligands on mPFC neural network activity and pyramidal neuron firing *in vivo*

4.1 Introduction

Experiments in the previous chapter provided evidence to suggest that 5-HT_6 receptor ligands exert an excitatory influence over DRN 5-HT neurons. Specifically, the 5-HT_6 receptor agonists WAY-181187 and WAY208466 increased 5-HT neuron firing in the DRN of anaesthetised rats, probably via a 5-HT_6 receptor-mediated mechanism. Therefore, it was considered that these findings may be evidence of a 5-HT_6-mediated feedback pathway governing 5-HT neuron activity. The neural circuits mediating these effects of 5-HT_6 agonists on 5-HT neuron firing is unknown. However, evidence from the previous chapter that WAY-181187 and WAY-208466 induced a reduction in frontal cortex SW oscillations suggested that the frontal cortex may be involved. In particular, the mPFC is a region within the frontal cortex which contains high levels of 5-HT_6 receptors and is well known to influence 5-HT neuron activity.

The current chapter aimed to address the following points. Firstly, it needed to be established whether the effect of 5-HT_6 agonists on the activity of the frontal cortex was mediated via the 5-HT_6 receptor. Secondly, since 5-HT_6 receptors are expressed on pyramidal neurons in the mPFC (Helboe et al., 2015), experiments investigated whether individual mPFC pyramidal neurons were sensitive to 5-HT_6 agonist administration. Finally, experiments determined whether mPFC neurons projecting to the DRN were sensitive to 5-HT_6 agonist administration. To these ends, the effect of

administration of the 5-HT_6 receptor agonist WAY-181187 and 5-HT_6 receptor antagonists SB-399885 and AE-58054 on mPFC slow wave oscillations and the firing of individual putative pyramidal neurons, including those projecting directly to the DRN, was tested in anaesthetised rats.

4.1.1 Evidence of mPFC-DRN connectivity

Data from neuronal tracing studies have suggested that glutamatergic neurons in the mPFC project in a bilateral pathway to the DRN (Sesack et al., 1989, Hajos et al. 1998, and Peyron et al., 1998; Lee et al., 2003; Vertes, 2004). In the DRN, mPFC inputs appeared to predominantly terminate on GABAergic neurons (Hajos et al., 1998; Peyron et al., 1998; Varga et al., 2001; Allers and Sharp, 2003; Jankowski and Sesack, 2004). However, recent evidence from studies using virally-mediated tracing of monosynaptic inputs to DRN 5-HT neurons have supported the idea that a significant number of mPFC terminals also input directly to 5-HT neurons (Ogawa et al., 2014; Pollak Dorocic et al., 2014).

Electrical stimulation of the mPFC has been shown to principally inhibit the firing of DRN 5-HT neurons (Hajós et al. 1998; Celada et al. 2001). This effect is likely to be mediated indirectly via the targeting of DRN GABA neurons, since electrical stimulation of the mPFC was found to increase the firing of non-5-HT, putative GABA neurons in the DRN (Varga et al. 2001). In contrast, electrical stimulation of the mPFC was also found to increase the firing of a smaller population of 5-HT neurons, which could be the result of direct glutamatergic mPFC input (Hajos et al., 1998; Celada et al., 2001). Taken together, these anatomical and electrophysiological studies support the idea that mPFC neurons send inputs to both GABAergic and 5-HT neurons in the DRN.

4.1.2 Evidence of mPFC pathways in feedback control of 5-HT neurons

As discussed above, mPFC neurons are thought to input to the DRN where they elicit inhibitory and excitatory effects on 5-HT neuron firing (Hajós et al. 1998; Celada et al. 2001; Varga et al., 2001). Furthermore, evidence suggests that some of these mPFC inputs to DRN 5-HT neurons are controlled by specific 5-HT receptor subtypes, suggesting that the mPFC is an important substrate for homoeostatic feedback control of 5-HT neurons. In particular, electrophysiological studies combined with cortical lesions suggest that 5-HT_{1A} receptors located on mPFC pyramidal neurons inhibit DRN 5-HT neuron firing (Hajos et al., 1999; Celada et al., 2001). Moreover, findings from other studies indicate that 5-HT_{2A} receptors localised on mPFC pyramidal neurons also inhibit 5-HT neuron firing in the DRN, probably via connections with DRN GABAergic interneurons (Martin-Ruiz et al., 2001; Boothman et al., 2003; Boothman and Sharp, 2005). By contrast, other studies find that 5-HT_4 receptors putatively on mPFC pyramidal neurons excite 5-HT neuron firing (Lucas and Debonnel, 2002, Lucas et al., 2005).

Experiments in Chapter 3 provided evidence for a 5-HT_6 receptor-mediated excitatory control of 5-HT neuron firing, and it was speculated that this effect might also be mediated through an interaction with the mPFC. Experiments in the current chapter aimed to investigate further the interaction between 5-HT_6 receptors and the mPFC.

4.1.3 Localisation of 5-HT_6 receptors in the mPFC

A number of studies report the presence of 5-HT_6 receptor mRNA and protein in the mPFC (Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995; Gerard et al., 1996;

Gerard et al., 1997; Roberts et al., 2002; Helboe et al., 2015). Moreover, recent studies have provided evidence that 5-HT_6 receptors co-localise with vGlut1-expressing mPFC neurons (Helboe et al., 2015). Thus, it was concluded that more than 70% of mPFC pyramidal neurons may express 5-HT_6 receptors (Helboe et al., 2015). In support of these findings it has previously been reported that the 5-HT_6 receptor agonist WAY-181187 increased Fos-immunoreactivity in GAD-negative mPFC neurons in rats, effects which were reversed by 5-HT_6 receptor antagonist SB-399885 (Burnham et al., 2010). This work also suggested that 5-HT_6 receptors co-localise to mPFC pyramidal neurons, and provides evidence that these neurons may be activated by a 5-HT_6 receptor agonist.

Finally, there are findings to support the hypothesis that a small proportion (~13%) of GABAergic interneurons in the mPFC express 5-HT_6 receptors (Helboe et al., 2015). Specifically, it was found that 5-HT_6 receptor mRNA co-localised with GAD-expressing mPFC neurons, particularly those also expressing 5-HT_3 receptor mRNA (Helboe et al., 2015). Interestingly, Burnham et al. reported small numbers of Fos/GAD co-labelled neurons in the mPFC following administration of WAY-181187, which supports the finding that a small proportion of mPFC GABAergic interneurons express 5-HT_6 receptors (Burnham et al., 2010).

4.1.4 *In vivo* electrophysiological recording in the mPFC

Local field potential recordings

Experiments in the current chapter made recordings of local field potentials (LFP) in the mPFC, which are analogous to the EEG recordings described in Chapter 3 (Buszaki, 2006). However, LFPs are recorded by the implanted recording electrode

rather than on the brain surface so can record neurons more deeply located in the cortex than an EEG. Moreover, since the recording electrode has a small diameter it detects neurons in the vicinity of the electrode, although the exact number of neurons contributing to the LFP recording is not known (Buszaki, 2006).

In vivo recording of pyramidal neurons in the mPFC

The principal neuron types in the mPFC are glutamatergic pyramidal neurons and multiple types of GABAergic interneurons including those co-expressing parvalbumin (PV), calbindin, and cholecystokinin (Tseng et al., 2006; Hartwich et al., 2009; Massi et al., 2012). The *in vivo* firing properties of putative pyramidal neurons and interneurons in the mPFC based on studies combining electrophysiological recordings of mPFC neurons with juxtacellular labelling, are summarised in Table 4.1. Overall, pyramidal and interneurons in the mPFC appear to exhibit similar firing properties, however, there are distinctions between the two. For example, it has been suggested that compared to pyramidal neurons mPFC interneurons exhibit faster firing rates and narrower spike widths, in a study which identified labelled interneurons as PV-positive and pyramidal neurons as PV-negative (Tseng et al., 2006). However, since many mPFC interneurons are PV-negative, this may have led to the erroneous identification of PV-negative neurons as pyramidal (Tseng et al., 2006). In support of this, another study which juxtacellular-labelled mPFC neurons and later identified them based on morphology, suggested that interneurons and pyramidal neurons exhibited similar firing rates (Tierney et al., 2004). However, the latter study suggested that pyramidal neurons had broader spike widths than interneurons which may help identification (Tierney et al., 2004). Together, these reports suggest that identification of mPFC neurons on the basis of their *in vivo* firing

properties alone may not be sufficient for discriminating between interneurons and pyramidal neurons (Table 4.1).

Juxtacellular labelling of the recorded neurons can allow for post-mortem identification (Chapter 1, section 1.5.4). Compared to interneurons, pyramidal neurons exhibit a distinct morphology including a triangular shaped soma, an apical dendrite extending into superficial layers of the mPFC, and short basal dendrites (Tierney et al., 2004; Tseng et al., 2006; Spruston, 2008; Table 4.1). These morphological characteristics could be used to identify mPFC pyramidal neurons following juxtacellular labelling. Therefore, experiments in this chapter aimed to juxtacellularly label recorded neurons to confirm their pyramidal morphology.

Finally, the antidromic response of mPFC neurons to DRN stimulation can identify pyramidal neurons with inputs to the DRN. In these neurons, DRN stimulation causes antidromic propagation of action potentials from the DRN to the soma in the mPFC. The characteristics of antidromic spikes include a constant latency, high fidelity, and collision with spontaneously occurring spikes (Celada et al., 2001, Hajos et al., 2003, and Puig et al., 2003). These properties (defined further in section 4.2.7) were used in the current study to identify DRN-projecting pyramidal neurons.

Neurochemical identity	Electrophysiological properties	Other characteristics	Morphological features	References
Pyramidal neurons (glutamatergic)	Slow firing rate (up to 14 Hz, typically 1-2 Hz) Broad spikes waveform width (typically > 0.7 ms) Complex burst firing (2-7 spikes, ~10 ms apart)	Firing increases with transition to active state of mPFC SW oscillations.	Triangular shaped soma, typically residing in deeper mPFC layer (III-V1) Distinct apical dendrite extending to shallower mPFC layers. Short basal dendrites	Tierney et al., 2004; Tseng et al., 2006; Hartwich et al., 2009; Massi et al., 2012
Interneurons (GABAergic)	Fast firing (up to 38 Hz, typically 4-6 Hz, PV-expressing) Narrow spike waveform width (typically < 0.65 ms, PV-expressing)	Firing with active states of mPFC SW oscillations (PV-expressing, axo- axonic interneurons) Firing with transition between inactive to active states of mPFC SW oscillations (PV-expressing, basket interneurons). Increased firing with tail pinch (PV-expressing axo-axonic interneurons)	Majority aspiny stellate morphology (e.g. basket and axo axonic interneurons) Smaller populations have bitufted morphology and spiny stellate morphology	As above

Table 4.1
Electrophysiological and morphological characteristics of mPFC neurons recorded using single unit recordings combined with juxtacellular labelling *in vivo*.

4.1.5 Aim of experiments

The aim of experiments in this chapter was to investigate the influence of 5-HT_6 receptor ligands over mPFC neural activity. Initial experiments determined whether the 5-HT_6 receptor agonist WAY-181187 elicited 5-HT_6-mediated effects on neural network oscillations in the mPFC, as determined by blockade with the 5-HT_6 receptor antagonists SB-399885 and AE-58054. Subsequent experiments aimed to investigate the effect of WAY-181187 on the firing of mPFC pyramidal neurons, including those projecting to the DRN. Extracellular recordings were made of LFPs

and putative pyramidal neurons in the mPFC of anaesthetised rats, and the effect of administration of the 5-HT_6 receptor agonist WAY-181187, and 5-HT_6 receptor antagonists SB-399885 and AE-58054 was tested. Putative pyramidal neurons were selected on the basis of their electrophysiological characteristics (defined in section 4.2.6), and then juxtacellular labelling was used to confirm their pyramidal morphology.

4.2 Materials and Methods

4.2.1. Animals

Experiments were carried out on male Sprague Dawley rats (250-420g) which were group housed at 20^{o}C $\pm$ 2^{O}C under 12 hour light-dark cycle (lights on 0800 h) in an enriched environment with water and food given ad libitum. Rats were left to acclimatise for at least 1 week following delivery to the housing facility. On the day of the experiment rats were taken from the housing facility to the experiment room and left to acclimatise for 30 min prior to induction of anaesthesia.

4.2.2 Experimental Design

Initially experiments investigated the effect of the 5-HT$_6$ agonist WAY-181187 on mPFC SW oscillations and the firing of single putative pyramidal neurons. Subsequent experiments investigated the effect of pre-treatment with the 5-HT$_6$ antagonists SB-399885 and AE-58054 on WAY-181187-induced changes on mPFC SW oscillations. Table 4.2 below provides a summary of the experiments carried out.

Experiment	Doses (i.v.)	Number of neurons/rats
1) Vehicle (isotonic glucose-saline) during LFP recordings	0.1 - 0.5 ml	8 rats
2) WAY-181187 during LFP recordings	0.1 - 9.0 mg/kg (0.14 – 4.3 ml/kg)	10 rats
3) SB-399885 followed by WAY- 181187 during LFP recordings	SB-399885: 1.0 mg/kg (1.4 ml/kg) WAY-181187: 0.1 - 9.0 mg/kg (0.14 – 4.3 ml/kg)	10 rats
4) AE-58054 followed by WAY-181187 during LFP recordings	AE-58054: 2.0 mg/kg (1.9 ml/kg) WAY-181187: 0.1 – 9.0 mg/kg (0.14 – 4.3 ml/kg)	7 rats
5) Vehicle during single neuron recordings	0.1 – 0.5 ml	6 neurons (6 rats)
6) WAY-181187 during single neuron recordings	0.1 - 9.0 mg/kg (0.14 – 4.3 ml/kg)	19 neurons (22 rats)

Table 4.2
Experiments carried out in Chapter 4.

4.2.3. LFP recordings

Details of the *in vivo* electrophysiological recording methods are described in Chapter 2. In brief, animals were anaesthetised with urethane (supplemented with ketamine and xylazine) and a craniotomy was performed over the mPFC (from bregma= + 3.00-3.20 mm AP, -/+0.5 mm ML). A recording electrode (filled with neurobiotin) was then lowered into the mPFC using a microdrive (-3.5 mm from *dura mata*).

Some LFP recordings were made simultaneously with single unit recordings but the majority were performed independently. Baseline LFP recordings were made during

a pre-drug period and recordings continued during drug (or vehicle) administration via a lateral tail vein cannula. In some experiments antagonists were administered (i.v.) after the pre-drug period and 4-5 min before WAY-181187, which was administered in accumulating doses at 2 min intervals in 5 doses. Typically two LFP recordings were made per rat: the first studied the effect of vehicle administration and the second studied the effect of agonist/antagonist administration. After LFP recordings, the electrode usually contents were expelled by iontophoresis to confirm electrode placement in the mPFC. Rats were then perfused either with 4% PFA and brains were extracted and processed for neurobiotin immunohistochemistry, or culled by pentobarbital overdose. Figure 4.1 illustrates the time course of these experiments.

4.2.4 Single unit recordings of pyramidal neurons

Rats were anaesthetised with urethane (supplemented with ketamine and xylazine) and a craniotomy was performed. A stimulating electrode was stereotaxically implanted into the DRN (from bregma= - 7.4-7.8 mm AP, and midline, typically -6.0 mm from *dura mata*), and then a recording electrode (filled with neuroiotin) was lowered into the mPFC (bregma= + 3.00-3.20 mm AP, -/+0.5 mm ML, -2.0 mm from the *dura mata*).

During recordings, a neuron was detected in the mPFC and the spike amplitude was allowed to increase to around 1mV. The DRN was then stimulated and the response of the neuron was monitored typically for 1-2 min. DRN stimulation was turned off and neuron was then recorded for 2-3 min to establish a stable baseline. Typically, rats exhibited SW oscillations (0.5-1.5 Hz) during the baseline recording periods, as evident on either LFP or EEG traces. mPFC pyramidal neurons were spontaneously

active during SW oscillations (see also Steriade and Amzica, 1998). WAY-181187 (or vehicle) was then administered in accumulating doses at 2 min intervals via a lateral tail vein cannula, with 5 doses per drug. A maximum of two pyramidal neurons were recorded per rat: typically the first neuron was recorded during vehicle administration, and then a second was tested for its response to WAY-181187. Attempts were then made to juxtacellular label the recorded neuron (Chapter 2 section 2.3.2). Rats were then perfused with 4% PFA and brains were extracted and processed for neurobiotin immunohistochemistry. The location of the stimulating electrode was also confirmed post-mortem. If no juxtacellular labelling was carried out, rats were culled with pentobarbital overdose and brains were extracted to confirm the placement of the simulating electrode. Figure 4.1 illustrates the time course of these experiments.

4.2.5 Data analysis

LFP recordings were acquired using Spike2 (v.8) software online and then analysed offline. Spectrograms were generated from LFP recordings to illustrate the power, i.e. the relative dominance, of a frequency band within a time period. To obtain robust measurements of power, at least 60 seconds of uninterrupted (i.e. no artefacts) LFP trace was required. LFP data were then normalised to pre-drug values (taken as 100%). Data are expressed as mean ± SEM values.

Single unit recordings were acquired online using Spike2 (version 8.03) software. Details regarding the filtering and conversion of raw data into Spike2 software are given in Chapter 2. Spike2 was used offline to sort spikes, measure spike width, and determine firing coherence with LFP recordings. Data were exported into Microsoft Excel to determine firing rates (Hz) and regularity (COV_{ISI}). Firing rates were normalised to pre-drug, baseline firing (taken as 100%). Neurons included in the

final data analysis exhibited a stable baseline firing rate defined as firing during the pre-drug period (approximately 2 mins) which did not fall outside of 2x S.D. of the mean pre-drug firing rate, for more than two consecutive 10 second bins.

mPFC neurons were classified as being excited or inhibited by WAY-181187 administration. Excitatory responses were defined as firing rates of 2x S.D. greater than the pre-drug mean firing rate for the majority of WAY-181187 doses. Conversely, inhibitory responses were defined as firing rates of 2x S.D. less than the pre-drug mean firing rate for the majority of WAY-181187 doses.

4.2.6 Identification of mPFC pyramidal neurons

Putative pyramidal neurons were selected according to the following characteristics: a firing pattern consisting of complex bursts (2-7 spikes), firing in synchrony with the active phase of mPFC SW oscillations measured through LFP recordings, an antidromic response to DRN stimulation, and pyramidal morphology when successfully labelled. Neurons included in the final analysis included at least 2 of these characteristics.

4.2.7 Recording of antidromically identified mPFC neurons

Antidromic responses to DRN stimulation were characterised as spikes occurring in response to almost every simulation with a consistent latency of approximately 10 msec as previously reported (Celada et al., 2001, Hajos et al., 2003 and Puig et al., 2003). Antidromic spikes also exhibited collision with a spontaneously occurring spike. Here, a spontaneous spike is propagated from the soma at the same time as

an antidromic spike is propagated from the nerve terminals by DRN stimulation. When these spikes meet on the axon, neither spike is further propagated since the portion of axon ahead is in a refractory period. In this instance the antidromic spike is not detected post-stimulation.

4.2.8 Statistical analysis

The effect of vehicle or WAY-181187 on SW oscillation power was compared to pre-drug values and tested statistically using a one-way ANOVA with dose as a repeated measure, followed by a Dunnet's post hoc test. The effect of WAY-181187 on SW oscillation power was also compared to vehicle controls using a two-way ANOVA with dose as a repeated measure, followed by Bonferroni's post hoc test. The effect of WAY-181187 alone on SW oscillation power was compared to the effect of WAY-181187 with antagonist pre-treatment and tested statistically using a two-way ANOVA followed by Bonferroni's post hoc test.

The effect of WAY-181187 on the SW oscillation frequency was tested statistically in comparison to pre-drug values using a one-way ANOVA with dose as a repeated measure, followed by Dunnet's post hoc test. The effect of antagonist pre-treatment on SW oscillation power and frequency was tested statistically compared to pre-drug values or vehicle controls using a Student's paired t-test (two-tailed) or an unpaired t-test (two-tailed), respectively.

The effect of vehicle and WAY-181187 on pyramidal neuron firing rate was compared with pre-drug values and tested statistically using a Student's paired t-tests (two-tailed), correcting for multiple comparisons using the Sidak-Bonferroni method. The

effect of WAY-181187 on pyramidal neuron firing was compared to vehicle controls and tested statistically using Student's unpaired t-test (two-tailed), correcting for multiple comparisons using the Sidak-Bonferroni method. ANOVA analysis was not used for these comparisons since group sizes varied between drug doses.

Probability values of 0.05 or less were considered significant.

4.3 Results

4.3.1 Localisation of the recording electrode to the mPFC

Post-mortem analysis confirmed the location of the recording electrodes in the mPFC in all cases tested (Figure 4.2, n= 11).

4.3.2 Effect of vehicle on SW oscillations

SW oscillations were measured in the mPFC by LFP recordings. Administration of vehicle (isotonic glucose-saline) in accumulating volumes (0.1, 0.2, 0.3, 0.4 ml i.v.) had no statistically significant effect on SW oscillations (0.5-1.5 Hz) compared to pre-vehicle values (ANOVA: $F_{(5,\ 35)}$= 1.824, p= 0.134; Figure 4.3 and 4.4). These data were used as the vehicle control group for experiments on the effects of 5-HT_6 WAY-181187 on mPFC SW oscillations.

4.3.3 Effect of WAY-181187 on SW oscillations

WAY-181187 (0.1, 0.3, 1.0, 3.0, 9.0 mg/kg i.v.) administration caused a decrease in the power of SW oscillations recorded in the mPFC (Figure 4.5), and this effect was statistically significant compared to pre-drug values (ANOVA: $F_{(5,45)}$= 5.291, p=0.001; Dunnet's post hoc p< 0.05 at 0.3 and 9.0 mg/kg). The effect of WAY-181187 was also statistically significant when compared to vehicle controls (ANOVA: $F_{(5,96)}$=4.120, p=0.002; Bonferroni's post hoc: p<0.05 at 1.0 and 3.0 mg/kg, p<0.0001 at 0.3 and 9.0 mg/kg; Figure 4.6). Furthermore, WAY-181187 caused a statistically significant shift in the frequency of SW oscillations compared to pre-drug values (ANOVA $F_{(5,45)}$, p=0.022; Dunnet's post hoc p<0.05 at 9 mg/kg; Figure 4.7).

4.3.4 Effect of SB-399885 on WAY-181187-induced decrease in SW oscillations

As shown in Figure 4.8, administration of SB-399885 (1 mg/kg i.v.) alone had no significant effect on the power of SW oscillations in the mPFC compared to pre-drug values (p=0.235; Student's paired t-test) as well as the vehicle control group (p= 0.578; Student's unpaired t-test; Figure 4.8). However, pre-treatment with SB-399885 (1 mg/kg i.v.) attenuated the decrease in SW oscillation power induced by WAY181187 (ANOVA: $F_{(5,108)}$= 4.309, p= 0.0013, Bonferroni's post hoc p< 0.01 at 0.3 mg/kg WAY-181187; Figure 4.9 and 4.10). Furthermore, SB-399885 reduced the magnitude of the shift in SW oscillation frequency induced by WAY-181187 when compared to pre-drug values. Thus, in the presence of SB-399885, WAY-181187 (9 mg/kg) no longer caused a significant increase in SW frequency compared to pre-drug values (p=0.40; Student's Paired t-test; Figure 4.10).

4.3.5 Effect of AE-58054 on WAY-181187-induced decrease in SW oscillations

Administration of AE-58054 (2 mg/kg, i.v.) alone had no statistically significant effect on mPFC SW oscillation power compared to pre-drug values (p= 0.274; Student's paired t-test) and vehicle controls (p=0.157; Student's unpaired t-test; Figure 4.11).

However, as with SB-399885, pre-treatment with AE-58054 (2 mg/kg i.v.) blocked the WAY-181187-induced decrease in SW oscillation power. This effect was statistically significant over a range of WAY-181187 doses (ANOVA: F(5,90)=6.773, p<0.0001, Bonferroni's post hoc p<0.0001 at 0.3 mg/kg, p<0.05 at 1.0 and 3.0 mg/kg, and p<0.001 at 9.0 mg/kg; Figure 4.12 and 4.13). Pre-treatment with AE-58054 also blocked the increase in SW oscillation frequency induced by WAY-181187. Thus, in the presence of AE-58054, WAY-181187 (9 mg/kg) no longer caused a significant

increase in SW frequency compared to pre-drug values (p=0.53; Student's Paired t-test; Figure 4.13).

4.3.6 Electrophysiological recording of mPFC pyramidal neurons

Nineteen spontaneously active putative pyramidal neurons in the mPFC were recorded. Collectively, these neurons exhibited a slow firing rate (1.04±0.19 Hz) and broad spike width (1.1±0.07 ms), properties which are thought to be characteristic of mPFC pyramidal neurons. Moreover, all of the neurons exhibited an irregular firing pattern with periods of complex burst firing, resulting in a high COV_{ISI} (1.12±0.05; Figure 4.14). Finally, the neurons displayed significant coherence with SW oscillation in the mPFC ($p< 0.05$, n=14), with firing occurring during the active phase of SW oscillations (Figure 4.14).

Of the 19 recorded pyramidal neurons, 10 exhibited antidromic responses to DRN stimulation. Antidromic spikes had a short latency (13±1 ms), and high fidelity, consistent with previous findings (Figure 4.15; Celada et al., 2001; Hajos et al., 2003; Puig et al., 2003). In addition, when tested for collision, the antidromic spike was extinguished by a spontaneously occurring spike (n=7). All neurons exhibiting antidromic responses to DRN stimulations were spontaneously active and demonstrated firing properties that were not significantly different from other putative pyramidal cells (see below). In all the cases tested, post-mortem histological analysis confirmed the localisation of the stimulating electrode in the DRN (n= 9; Figure 4.16).

Juxtacellular labelling was attempted for 7 of the 19 recorded neurons, and 6 were successfully labelled. Thus the success rate for labelling mPFC neurons was 85%.

All six labelled neurons were identified as pyramidal on the basis of their morphological properties: a distinct apical dendrite, a triangular shaped soma and basal dendrites (examples in Figures 4.14, 4.17 and 4.21). The baseline firing properties of identified pyramidal neurons (i.e. those juxtacellular-labelled or exhibiting antidromic responses to DRN stimulation, n=12) were not different from those identified on the basis of firing characteristics alone (n=7; Table 4.3).

	Identified (number of cells)	Non-identified (number of cells)	Identified versus non-identified (Student's unpaired t-test)
Spike width (ms)	1.14 ± 0.08 (12)	1.12 ± 0.12 (7)	p=0.950
Firing rate (Hz)	1.21 ± 0.29 (12)	0.66 ± 0.15 (7)	p=0.194
COV_{ISI}	1.14 ± 0.08 (12)	1.10 ± 0.08 (7)	p=0.751

Table 4.3
Comparison of the firing properties of identified versus non-identified pyramidal neurons. Data are mean ± SEM values.

4.3.7 Effect of vehicle on the firing of mPFC pyramidal neurons

Administration of accumulating volumes of vehicle (isotonic glucosaline; 0.1, 0.2, 0.3, 0.4 ml i.v.) had no statistically significant effect on the firing rate of mPFC pyramidal neurons compared to pre-vehicle values ($p>0.1$; Student's paired t-test; Figure 4.17 and 4.18). In the vehicle group one neuron was successfully labelled and found to be a pyramidal neuron. The data for the vehicle group were used as a control for experiments investigating the effect of WAY-181187.

4.3.8 Effect of WAY-181187 on the firing of mPFC pyramidal neurons

Administration of WAY-181187 (0.1, 0.3, 1.0, 3.0, 9.0 mg/kg i.v.) induced mixed responses from individual pyramidal neurons. On final analysis neurons could be divided into two populations: those excited (n=11) by WAY-181187 and those

inhibited (n=8). Thus, in one group of neurons, accumulating doses of WAY-181187 (0.1, 0.3, 1.0, 3.0, 9.0 mg/kg i.v.) increased firing rate and the effect was statistically significant compared to pre-drug values (p= 0.023, 0.3 mg/kg; p= 0.003, 1.0 mg/kg; p=0.013, 3.0 mg/kg; Student's paired t-test). This effect of WAY-181187 was also statistically significant compared to vehicle controls (p= 0.001, 0.3 mg/kg; p= 0.042, 1.0 mg/kg; Student's unpaired t-test; Figure 4.19 and 4.20). Furthermore, five of the neurons in this group were labelled and exhibited pyramidal morphology.

In another group of pyramidal neurons, accumulating doses of WAY-181187 (0.1, 0.3, 1.0, 3.0, 9.0 mg/kg i.v.) decreased the firing rate. This effect was statistically significant when compared to both pre-drug values (p=0.005, 0.1 mg/kg; p=0.0004, 0.3 mg/kg; p=0.031, 1.0 mg/kg; p=0.016, 3.0 mg/kg; p< 0.0001, 9.0 mg/kg; Student's paired t-test) and vehicle controls (p=0.035, 0.1 mg/kg; p=0.007, 0.3 mg/kg; p=0.003, 9.0 mg/kg; Student's unpaired t-test; Figure 4.21 and 4.22). Furthermore, one neuron in the group was successfully labelled and exhibited pyramidal morphology (Figure 4.21).

4.3.9 Effect of WAY-181187 on the firing of antidromically activated mPFC neurons

mPFC neurons antidromically activated from the DRN fell into both the group of neurons excited by WAY-181187 (n=5; Figure 4.20) and the group inhibited by WAY-181187 (n=5; Figure 4.22). Figure 4.21 shows a representative spike train from an antidromically activated neuron which was inhibited by WAY-181187 and subsequently juxtacellular labelled.

4.4 Discussion

Experiments in this chapter aimed to test the hypothesis that 5-HT_6 receptors exert an influence over neuronal activity in the mPFC, including mPFC neurons projecting to the DRN. This was investigated using electrophysiological recordings of LFPs and single neurons during administration of 5-HT_6 receptor agonist WAY-181187 and 5-HT_6 receptor antagonists SB-399885 and AE-58054. Initial experiments demonstrated that WAY-181187 reduced SW oscillations in the mPFC, an effect which was fully blocked by AE-58054, and partially blocked by SB-399885. Neither antagonist affected mPFC SW oscillations when administered alone. Investigation of the activity of single pyramidal neurons found that WAY-181187 modulated the firing of neurons: one population was excited, the other inhibited. A number of pyramidal neurons were successfully juxtacellular labelled and found to be pyramidal based on their morphology. Finally, and importantly, it was found that DRN-projecting pyramidal neurons, which were identified by antidromic responses to DRN stimulation, also responded to WAY-181187 administration. Thus, half of the antidromically activated cells were excited by WAY-181187 administration, while the other half were inhibited by WAY-181187. Taken together, the findings in this chapter support the hypothesis that 5-HT_6 receptors exert an influence over mPFC neurons, including those that directly impact the activity of DRN neurons. Therefore, this may suggest that mPFC pyramidal neurons could be involved in a 5-HT_6-mediated feedback pathway controlling 5-HT neuron firing.

4.4.1 Electrophysiological and morphological properties of pyramidal neurons

The electrophysiological properties of the recorded mPFC neurons are consistent with those previously described for pyramidal neurons (Tierney et al., 2004, Tseng et al., 2006, Hartwich et al., 2009). Specifically, neurons exhibited slow firing rates,

broad spike widths, and fired in high frequency bursts. Moreover, the firing of recorded neurons exhibited strong coherence with the active phase of mPFC SW oscillations, as has previously been described for pyramidal neurons (Massi et al., 2012). Approximately 45% of recorded neurons displayed antidromic responses to DRN electrical stimulation, a feature of pyramidal neurons that project to the DRN (Celada et al., 2001, Hajos et al., 2003, Puig et al., 2003). Finally, 6 of the total 19 recorded neurons were successfully juxtacellular labelled and exhibited distinct pyramidal neuron morphology. Moreover, the electrophysiological properties of labelled or antidromically-activated pyramidal neurons were indistinguishable from neurons identified on their baseline firing characteristics alone, thereby strongly supporting the pyramidal identity of the vast majority, if not all, of the neurons included in the final analysis.

4.4.2 Effect of WAY-181187 on mPFC neural activity

In the current chapter WAY-181187 reduced mPFC SW oscillations as recorded by LFP measurements. This supports findings in Chapter 3 that WAY-181187 administration reduced frontal cortex EEG SW oscillations and extends this work, since the LFP recordings are likely derived from the mPFC itself. It was also found that WAY-181187 induced a decrease in SW oscillation power in a 5-HT_6 receptor-dependent manner. Thus, this effect of WAY-181187 was blocked by AE-58054 at a dose of the antagonist known to produce close to 100% 5-HT_6 occupancy and partially blocked by SB-399885. The partial blockade by SB-399885 might reflect less than 100% 5-HT_6 receptor occupancy at the dose used.

As well as SW oscillation power, WAY-181187 caused a shift in the frequency of SW oscillations. A previous study found a comparable shift in mPFC SW oscillation

frequency induced by DRN stimulation (Puig et al., 2010). Since Chapter 3 provided evidence for WAY-181187 increasing 5-HT neuron firing, if this increased firing was translated to changes in 5-HT release, it could be the basis of the shift in SW oscillation frequency.

In the single unit recordings, administration of WAY-181187 increased the firing of one population of pyramidal neurons, whereas it decreased the firing of another. Since these effects of WAY-181187 were not tested in combination with 5-HT_6 antagonists, direct evidence of a role for 5-HT_6 receptors is lacking. However, the WAY-181187-induced changes in SW oscillations were blocked by AE-58054 and SB-399885 at the same doses of WAY-181187 tested on individual neurons. Moreover, at comparable doses as used here, WAY-181187-induced increases in 5-HT neuron firing were blocked by 5-HT_6 receptor antagonists SB-258585 and SB-271046 (Chapter 3). Together this supports the 5-HT_6 receptor targeting of WAY-181187 to cause changes in the firing of individual pyramidal neurons in the mPFC.

4.4.3 Site of action of 5-HT_6 receptor ligands

There is strong support for the influence of local 5-HT_6 receptors in the effect of WAY-181187 on mPFC neural activity. In particular, 5-HT_6 receptors are reported to be expressed by 75% of mPFC pyramidal neurons, and 15% of mPFC interneurons (Helboe et al., 2015). It has been suggested that 5-HT_6 receptors act to balance excitation and inhibition within cortical circuits as evident by expression on both pyramidal neurons and interneurons (Helboe et al., 2015). Thus, this expression pattern could underlie the mixed excitatory/inhibitory effects elicited by WAY-181187 on mPFC pyramidal neuron firing. Specifically, activation of 5-HT_6 receptors localised to mPFC pyramidal neurons themselves could cause increased firing of these

neurons. By contrast, other pyramidal neurons may receive input from 5-HT_6 receptor-expressing interneurons in the mPFC (Spruston, 2008) and activation of these 5-HT_6 receptors could increase inhibitory drive onto connected pyramidal neurons.

Further support for mPFC 5-HT_6 receptors as the site of action of WAY-181187 comes from behavioural studies in rats. Specifically, intra-PFC administration of WAY-181187 has been found to evoke cognitive changes, an effect reversed by the 5-HT_6 receptor antagonist SB-271046 (Loiseau et al., 2008). This effect suggests the presence of 5-HT_6 receptors in the mPFC which influence local neuronal activity. In addition, several 5-HT_6 receptor ligands, including WAY-181187 and AE-58054, have been shown to elicit pro-cognitive effects in rats in tasks such as attentional set shifting which are thought to rely on mPFC circuity (Burnham et al., 2010; Arnt et al., 2010). Together this evidence supports an effect of local 5-HT_6 receptors in the modulation of mPFC neural activity.

5-HT_6 receptors are also located outside the mPFC. For example, the hippocampus has a high level of 5-HT_6 receptor expression on glutamatergic pyramidal neurons (Helboe et al., 2015), and such neurons have been shown to input to the mPFC (Jay and Witter, 1991; Jay et al., 1992). Moreover, intra-hippocampal injection of the 5-HT_6 receptor agonist WAY-208466 and the 5-HT_6 receptor antagonist SB-258585 has been reported to differentially modulate mPFC dopamine and noradrenaline release (Liu et al., 2015), which could conceivably alter neural activity in the mPFC. Alternatively, non-5-HT neurons in the DRN are found to express 5-HT_6 receptors (Gerard et al., 1996; Helboe et al., 2015), and intra-DRN injection of WAY-208466 has been shown to influence the sleep-wake cycle in rats (Monti et al., 2013). Finally

5-HT_6 receptors are abundant in the striatum, particularly the caudate putamen and nucleus accumbens (Helboe et al., 2015). In these regions 5-HT_6 receptors are thought to be expressed on D_1 and D_2 receptor-expressing GAD-positive medium spiny neurons and calretinin-positive interneurons (Helboe et al., 2015). Thus, 5-HT_6 receptors in striatal circuits, such as in the nucleus accumbens, could exert an indirect influence over mPFC neuronal activity via a striatal-thalamus-cortex pathway (Cummings, 1993; Salgado and Kaplitt, 2015).

4.4.4 Influence of 5-HT_6 receptors in mPFC-DRN connectivity

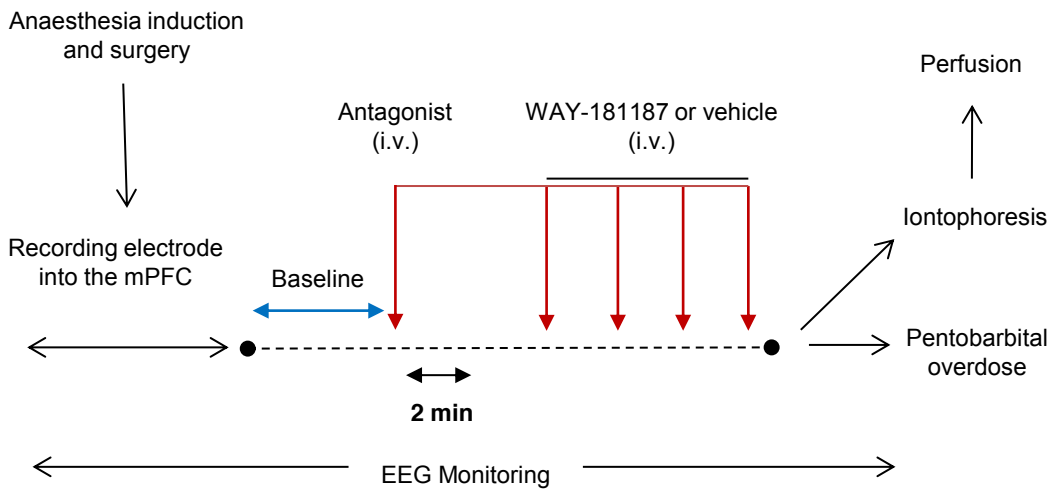
Administration of WAY-181187 modulated the firing of mPFC pyramidal neurons, as demonstrated by antidromic activation to project to the DRN, which may suggest the effect of WAY-181187 on the firing of DRN-projecting pyramidal neurons contributes to the excitation of DRN 5-HT neuron firing reported in Chapter 3. In support of this there is strong evidence linking other 5-HT receptors to the control of mPFC-DRN connectivity. For example, electrophysiological studies combined with cortical lesions suggest that 5-HT_{1A} receptors located on mPFC pyramidal neurons inhibit DRN 5-HT neuron firing (Hajos et al., 1999; Celada et al., 2001). Thus, in rats with intact mPFC-DRN connections, pharmacological inhibition of mPFC pyramidal neurons by intra-mPFC injection of the 5-HT_{1A} agonist 8-OH-DPAT reduced DRN 5-HT neuron firing (Celada et al., 2001). Another example is the 5-HT_4 receptor-mediated feedback mechanism thought to originate in the mPFC. In studies of this pathway, virally-mediated overexpression of 5-HT_4 receptors in mPFC neurons resulted in increased firing of 5-HT neurons (Lucas et al., 2005). Thus, together with evidence that 5-HT_6 receptors are expressed across mPFC layers (Helboe et al., 2015), including deeper layers where most DRN-projecting mPFC neurons reside (Hajos et al., 1998; Peyron

et al., 1998), these studies support the idea that 5-HT_6 receptor activation by WAY-181187 may modulate mPFC-DRN projections.

4.4.5 Conclusion

Experiments in this chapter found that the 5-HT_6 receptor agonist WAY-181187 decreased the power of mPFC SW oscillations, an effect which was antagonised by the 5-HT_6 receptor antagonists AE-58054 and SB-399885. This supports the role of 5-HT_6 receptors in WAY-181187-induced changes on mPFC neural activity. WAY-181187 also strongly modulated the firing of pyramidal neurons in the mPFC, including those projecting to the DRN and able to impact on the firing of DRN 5-HT neurons. The next chapter aimed to use lesions of the mPFC to further test the hypothesis that DRN-projecting mPFC pyramidal neurons mediate the effect of 5-HT_6 receptor agonist administration on 5-HT neuron firing, as described in Chapter 3.

a) LFP recordings



b) Pyramidal neuron recordings

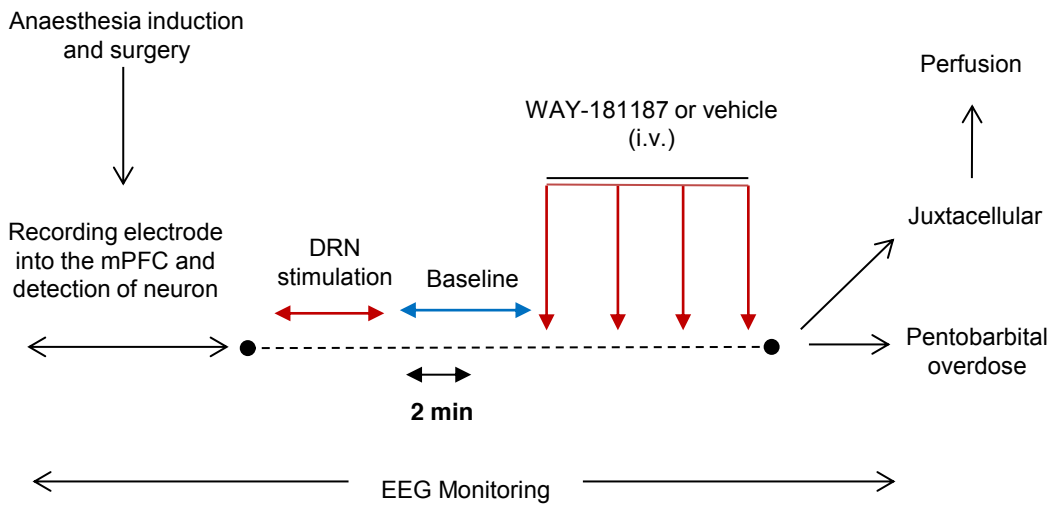


Figure 4.1
General protocol for experiments described in Chapter 4. Rats were anaesthetised and a recording electrode was lowered into the mPFC, and a stimulating electrode was lowered into the DRN. a) LFPs recordings: a stable baseline was established (2-3 min), followed by administration of a 5-HT_6 antagonist in some experiments (4-5 min interval), followed by WAY-181187 administration (2min intervals). b) Pyramidal neuron recordings: DRN stimulation was carried out (2-3 min) followed by recordings to establish a stable baseline (2-3 min), followed by WAY-181187 or vehicle administration (2-3 min intervals). Neurons were then juxtacellular labelled or iontophoresis was carried out, followed by perfusion, or otherwise rats were culled with pentobarbital overdose.

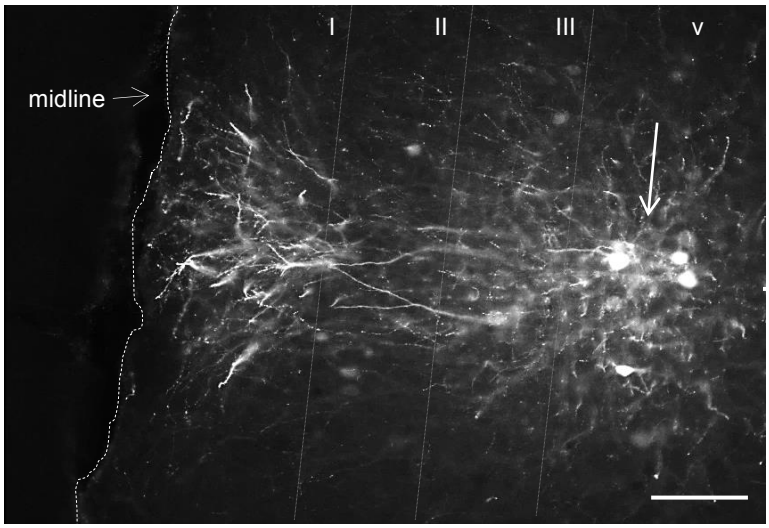


Figure 4.2
Localisation of the recording site in the mPFC. Representative microscopic image from an experiment showing neurobiotin staining as a result of iontophoresis within the mPFC. The arrow in layer 5 indicates approximately where the recording electrode would have been located. Scale bar = 40μm.

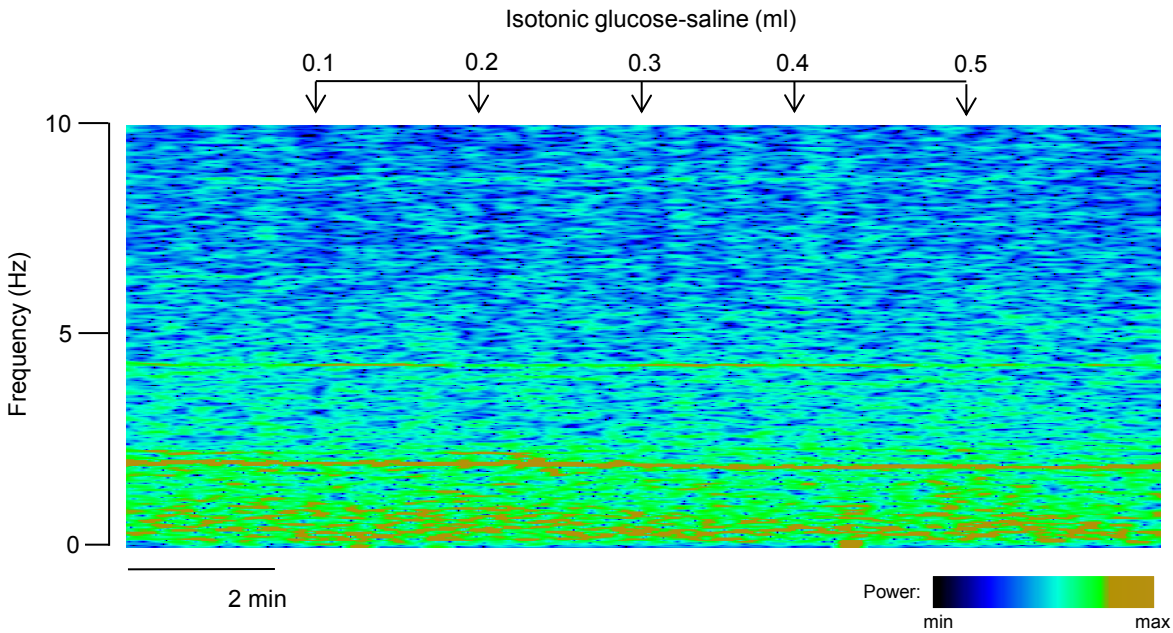


Figure 4.3
Lack of effect of vehicle administration on SW oscillations in the mPFC. A representative LFP spectrogram from a single rat during vehicle (isotonic glucose-saline) administration. Key denotes power with high power orange, moderate power green, lower power blue. Note the higher power SW oscillation power between 0.5 and 1.5 Hz. The line of high power running through the trace around 2 Hz is an artefact likely to be from breathing motion.

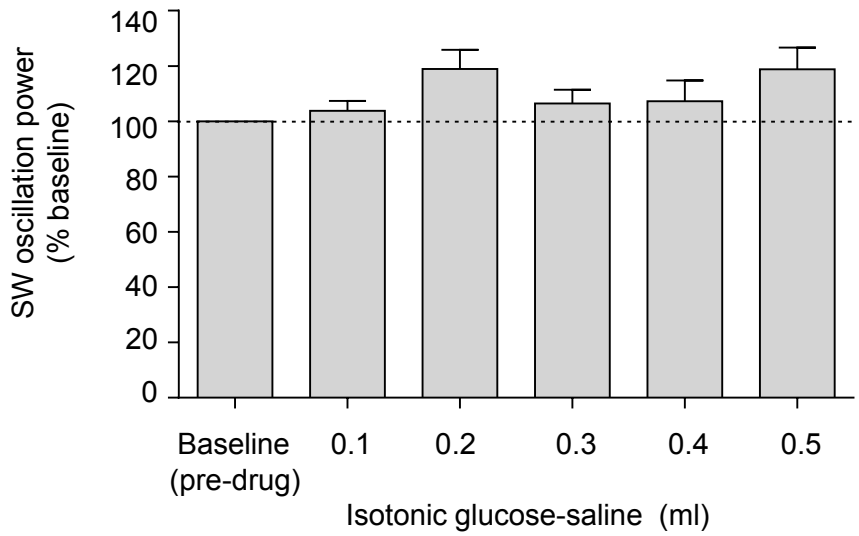


Figure 4.4
Group data demonstrating the effect of vehicle administration on mPFC SW oscillations. Vehicle was administered i.v. in accumulating volumes at 2 min intervals. Data expressed as mean ±SEM values (n=8 rats).

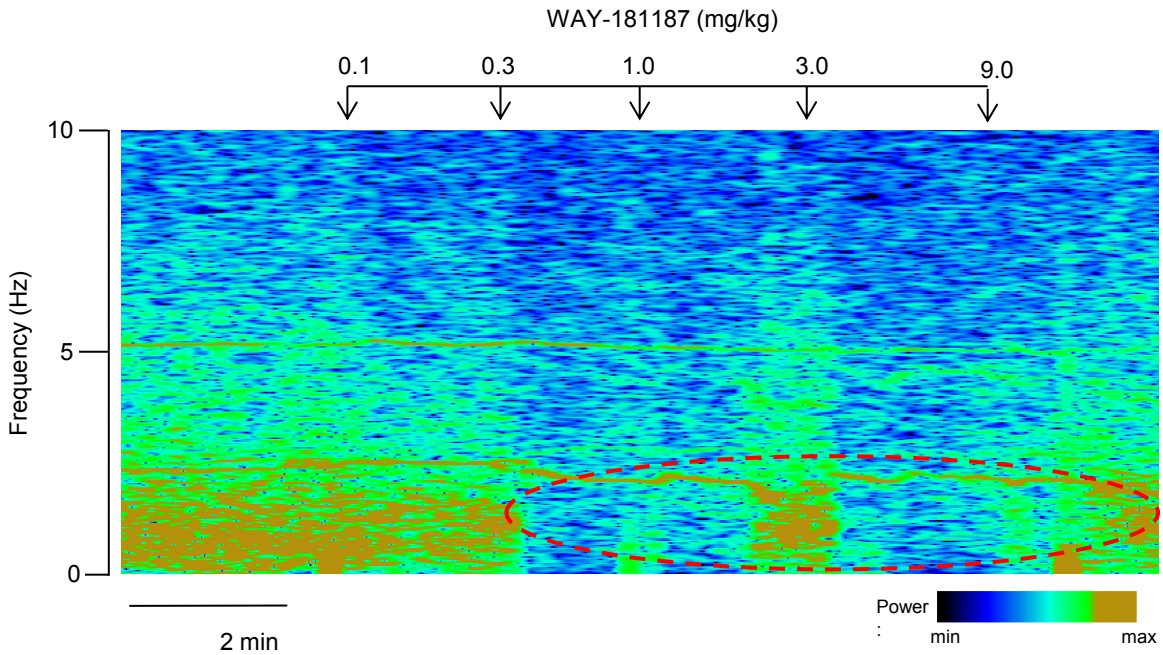


Figure 4.5
Effect of WAY-181187 administration on SW oscillations in the mPFC. A representative LFP spectrogram from a single rat during WAY-181187 administration. Key denotes power with high power orange, moderate power green, lower power blue. Note the high SW oscillation power between 0.5 and 1.5 Hz which is reduced after WAY-181187 3 mg/kg (circled in red).

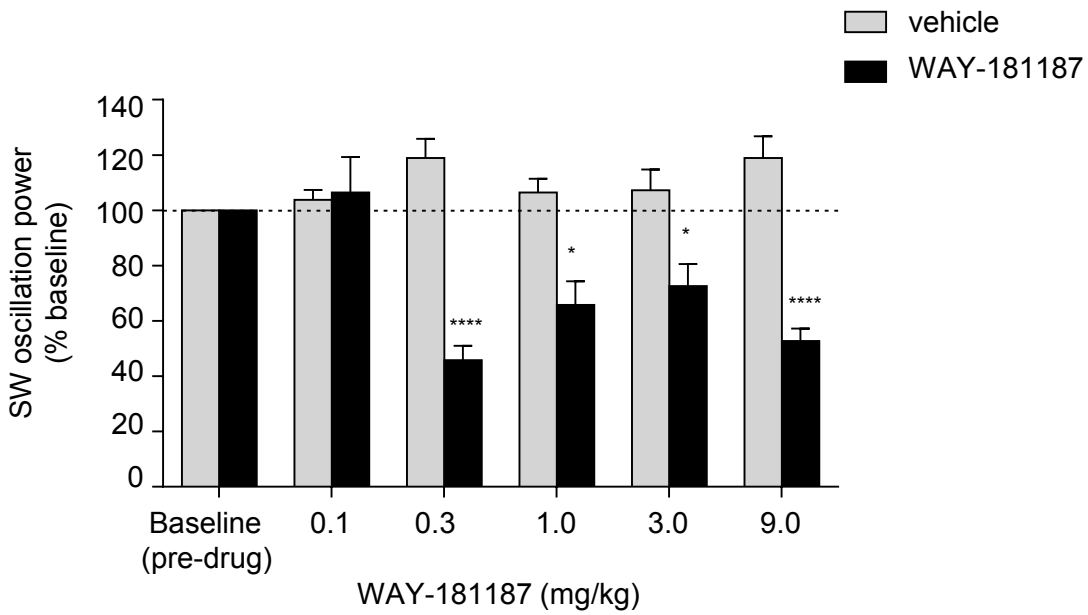


Figure 4.6
Group data demonstrating the effect of WAY-181187 administration on mPFC SW oscillations. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals. Data expressed as mean ±SEM values (n=10 rats). *p<0.05 ****p< 0.0001 versus vehicle (ANOVA with Bonferroni's post hoc test).

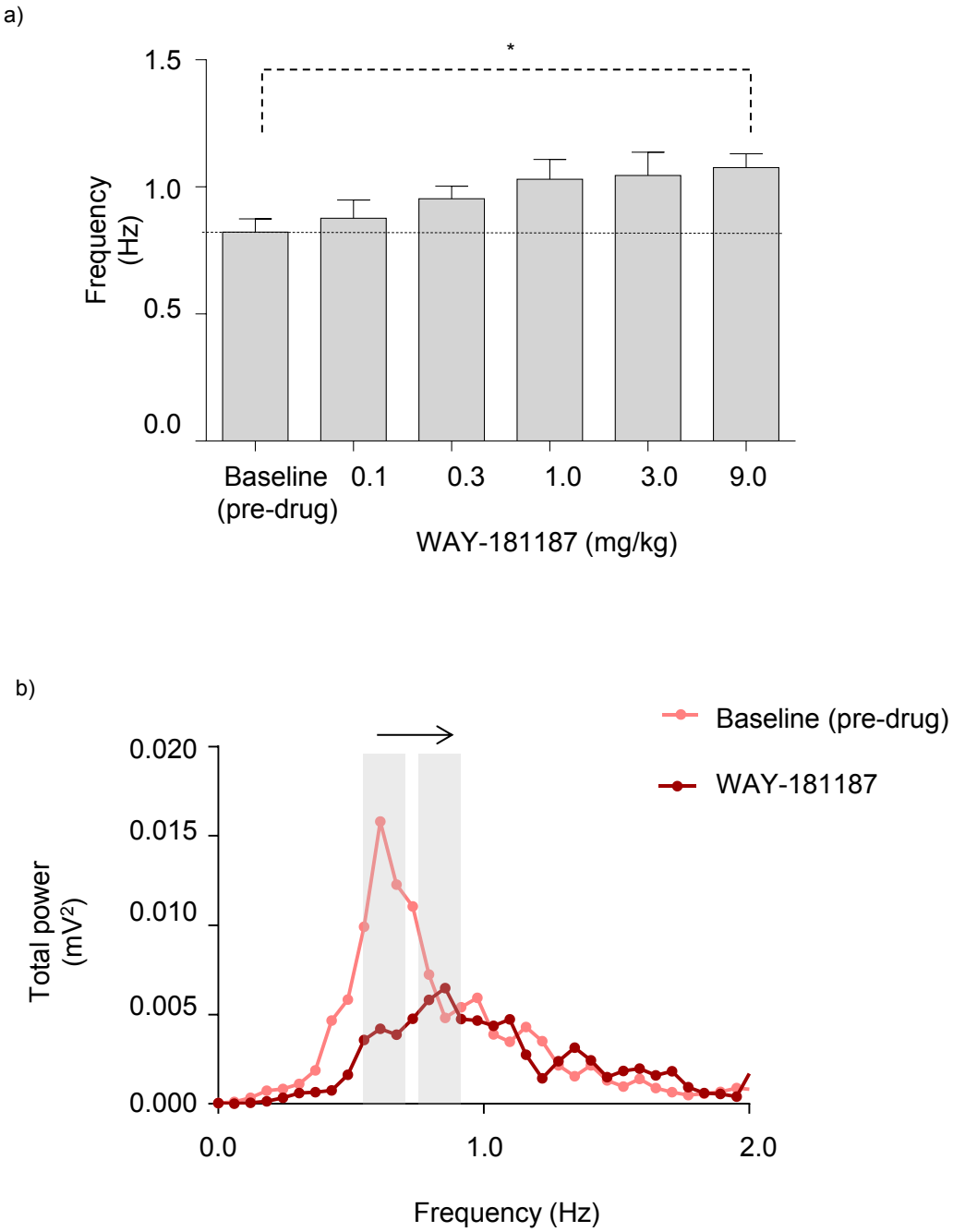


Figure 4.7
Effect of WAY-181187 on the frequency of mPFC SW oscillations. a) Group data demonstrating the effect of accumulating doses of WAY-181187 on the frequency of SW oscillations. *p< 0.05 versus pre-drug (ANOVA with Dunnet's post hoc test). Data expressed as mean ±SEM values (n=10 rats). b) Representative power spectra from a single LFP recording demonstrating the significant shift in the frequency of SW oscillations induced by the final dose of WAY-181187 (9 mg/kg). Areas shaded in grey highlight the peak of SW oscillation power and the arrow illustrates the direction of frequency shift.

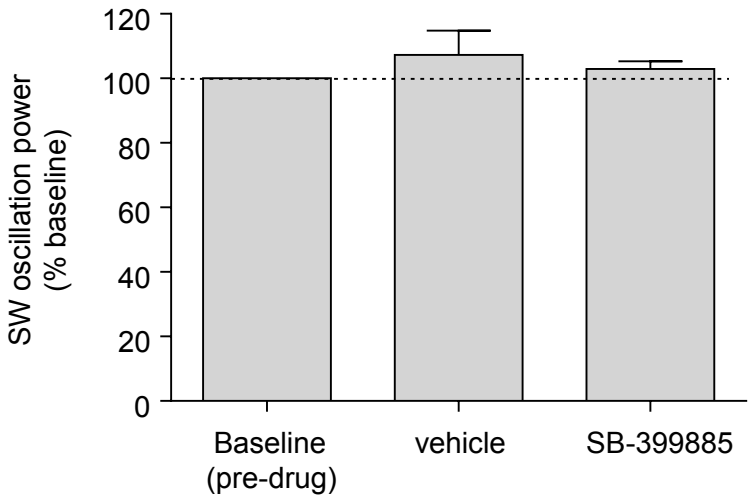


Figure 4.8
Lack of effect of SB-399885 alone on mPFC SW oscillations. Group data demonstrating the lack of effect of SB-399885 (1 mg/kg i.v.) on mPFC SW oscillation power compared to pre-drug values and vehicle controls; Student's paired t-test, p=0.24 and unpaired t-test, p=0.58, respectively. Data were collected from a 2 min recording period. Data expressed as mean ±SEM values (n=10 rats).

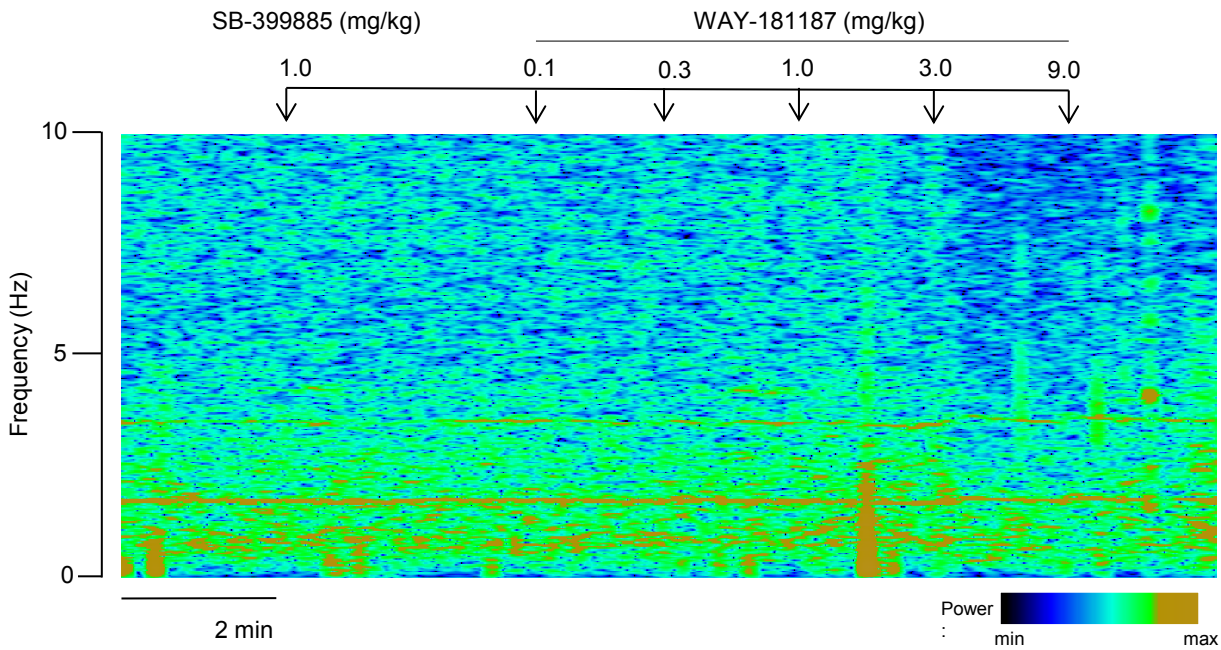


Figure 4.9
Effect of SB-399885 pre-treatment on WAY-181187-induced changes in SW oscillations in the mPFC. A representative LFP spectrogram from a single rat during SB-399885 and WAY-1811871 administration. Key denotes power with high power orange, moderate power green, lower power blue. Note the SW oscillation power between 0.5 and 1.5 Hz.

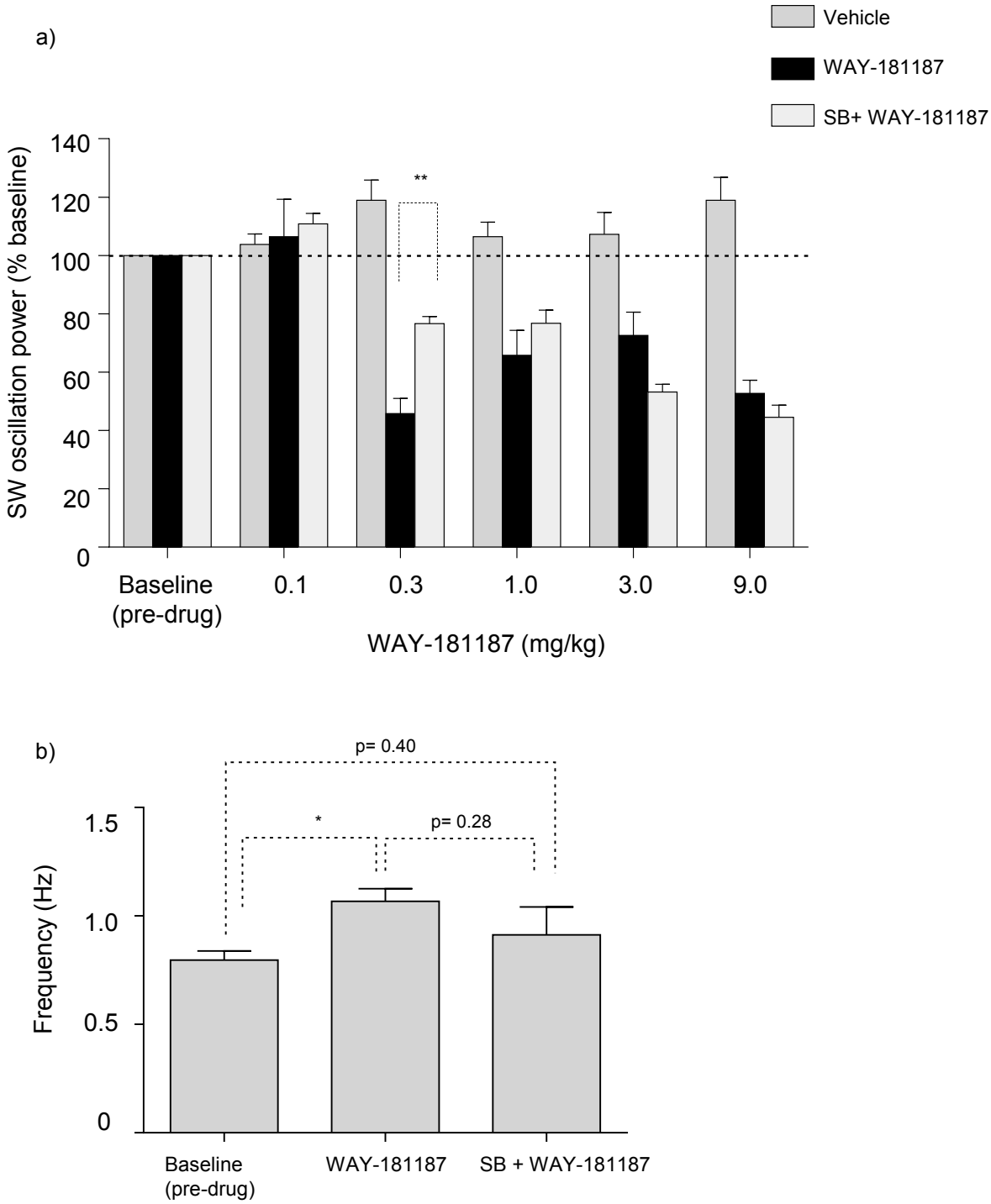


Figure 4.10
Effect of SB-399885 on WAY-181187-induced changes in mPFC SW oscillations. a) Group data demonstrating the effect of WAY-181187 following SB-399885 (1 mg/kg) on mPFC SW oscillation power **p<0.01 compared to WAY-181187 alone (ANOVA with Bonferroni's post hoc test). Data expressed as mean ±SEM. (n=10 rats). b) Group data demonstrating the frequency of SW oscillations following the final dose of WAY-181187 alone (9mg/kg; *p< 0.05 versus pre-drug) and following the final dose of WAY-181187 (9mg/kg) with SB-399885 pre-treatment (1 mg/kg; p=0.40 versus pre-drug; Student's paired t-test; p=0.28 versus to WAY-181187 alone; unpaired t-test). Data expressed as mean ±SEM values.

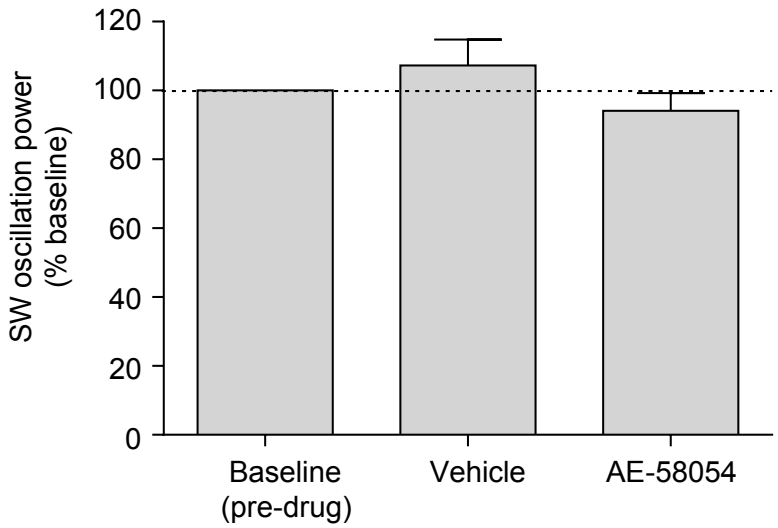


Figure 4.11
Lack of effect of AE-58054 on mPFC SW oscillations. Group data demonstrating the lack of effect of AE-58054 (2 mg/kg i.v.) on mPFC SW oscillation power compared to pre-drug values and vehicle controls; p=0.27, Student's paired t-test; p=0.16, unpaired t-test; respectively. Data were collected from a 2 min recording period. Data expressed as mean ±SEM (n=7 rats).

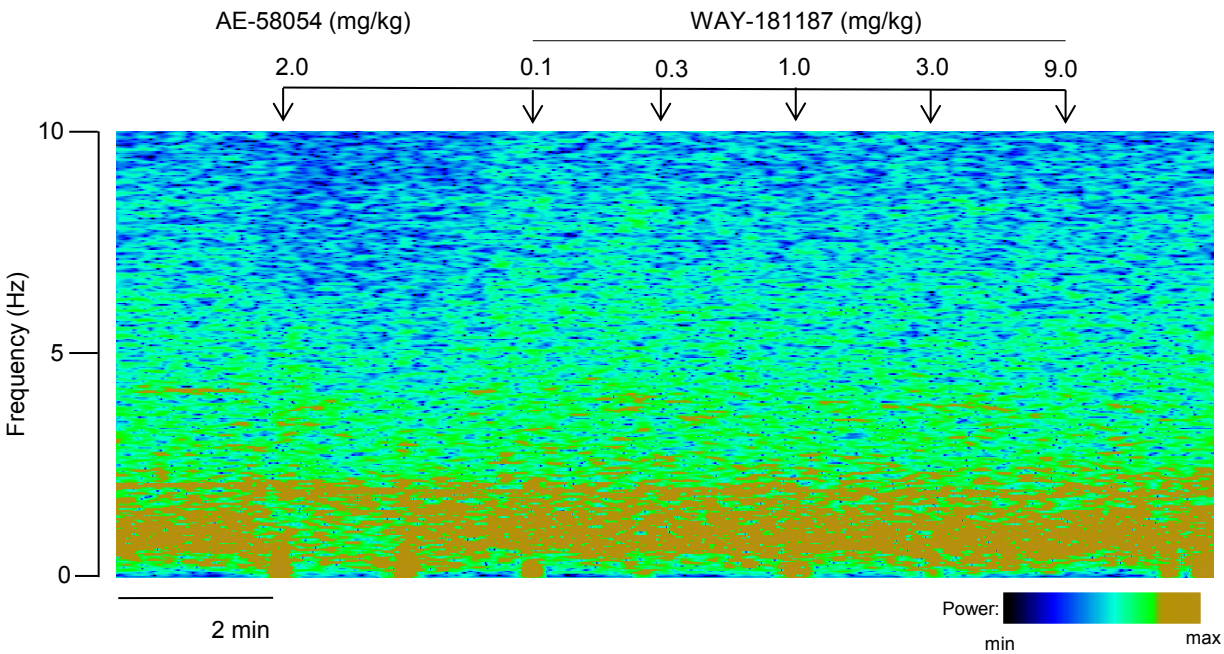


Figure 4.12
Effect of AE-58054 pre-treatment on WAY-181187-induced changes in SW oscillations in the mPFC. A representative LFP spectrogram from a single rat during AE-58054 and WAY-1811871 administration. Key denotes power with high power orange, moderate power green, lower power blue. Note the high SW oscillation power between 0.5 and 1.5 Hz which is maintained throughout WAY-181187 administration.

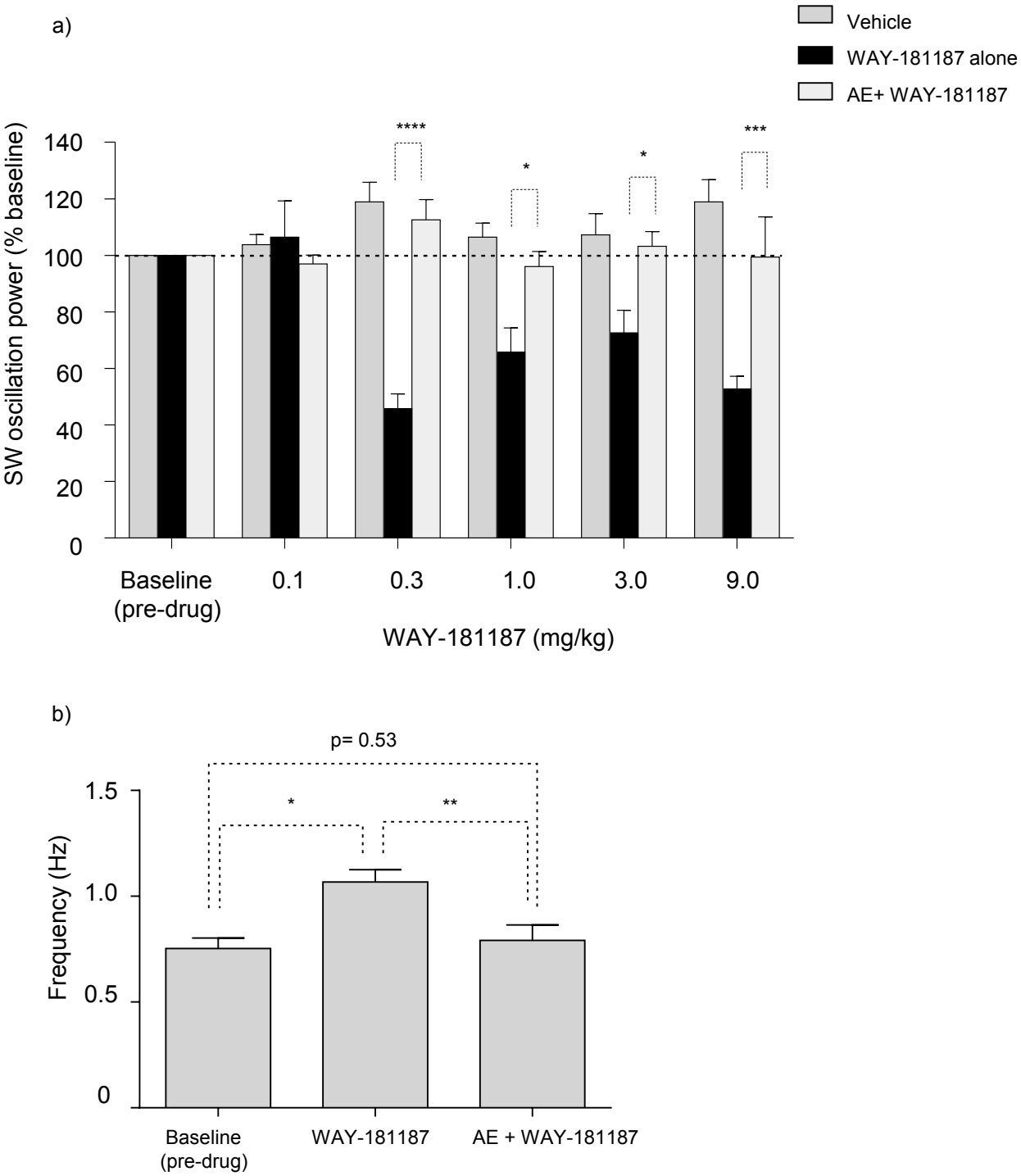


Figure 4.13
Effect of AE-58054 on WAY-181187-induced changes in mPFC SW oscillations. a) Group data demonstrating the effect of WAY-181187 following AE-58054 (2 mg/kg i.v) on mPFC SW oscillation power *p<0.05 ***p<0.001 ****p<0.0001 versus vehicle controls (ANOVA with Bonferroni's post hoc test). Data expressed as mean ±SEM values (n=7 rats). b) Group data demonstrating the frequency of SW oscillations following the final dose of WAY-181187 alone (9 mg/kg; *p< 0.05 versus pre-drug) and following the final dose of WAY-181187 (9mg/kg) with SB-399885 pre-treatment (1 mg/kg; p=0.53 versus pre-drug; Student's paired t-test; **p<0.01 versus WAY-181187 alone; unpaired t-test). Data expressed as mean ±SEM values.

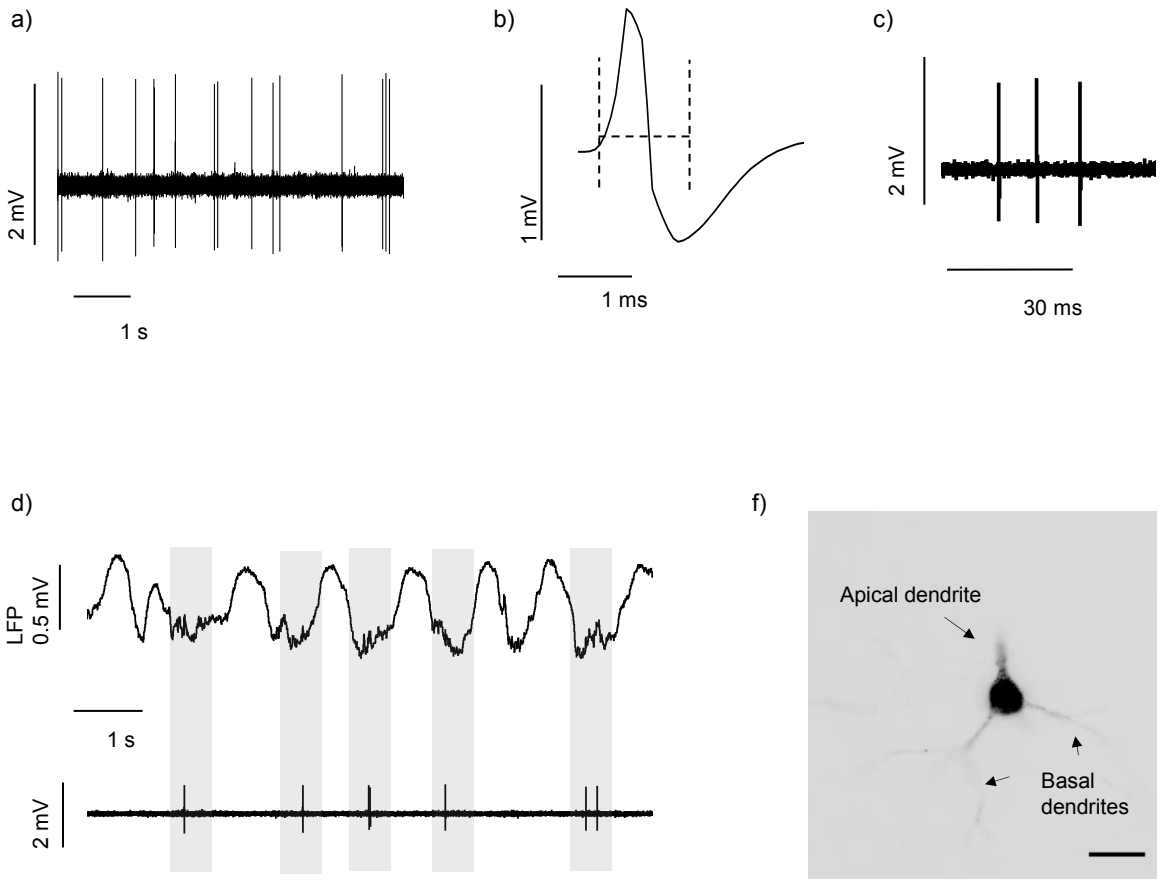


Figure 4.14

Electrophysiological and morphological properties of a representative pyramidal neuron recorded in the mPFC of an anaesthetised rat. The neuron exhibited a slow firing rate and broad spike waveform as illustrated in a) and b), respectively. The neurons exhibited burst firing periods (c) and fired most in the active phase of mPFC SW oscillations (d). Finally, the neuron was successfully juxtacellular labelled and found to have a pyramidal morphology (f). Scale bars= 20µm.

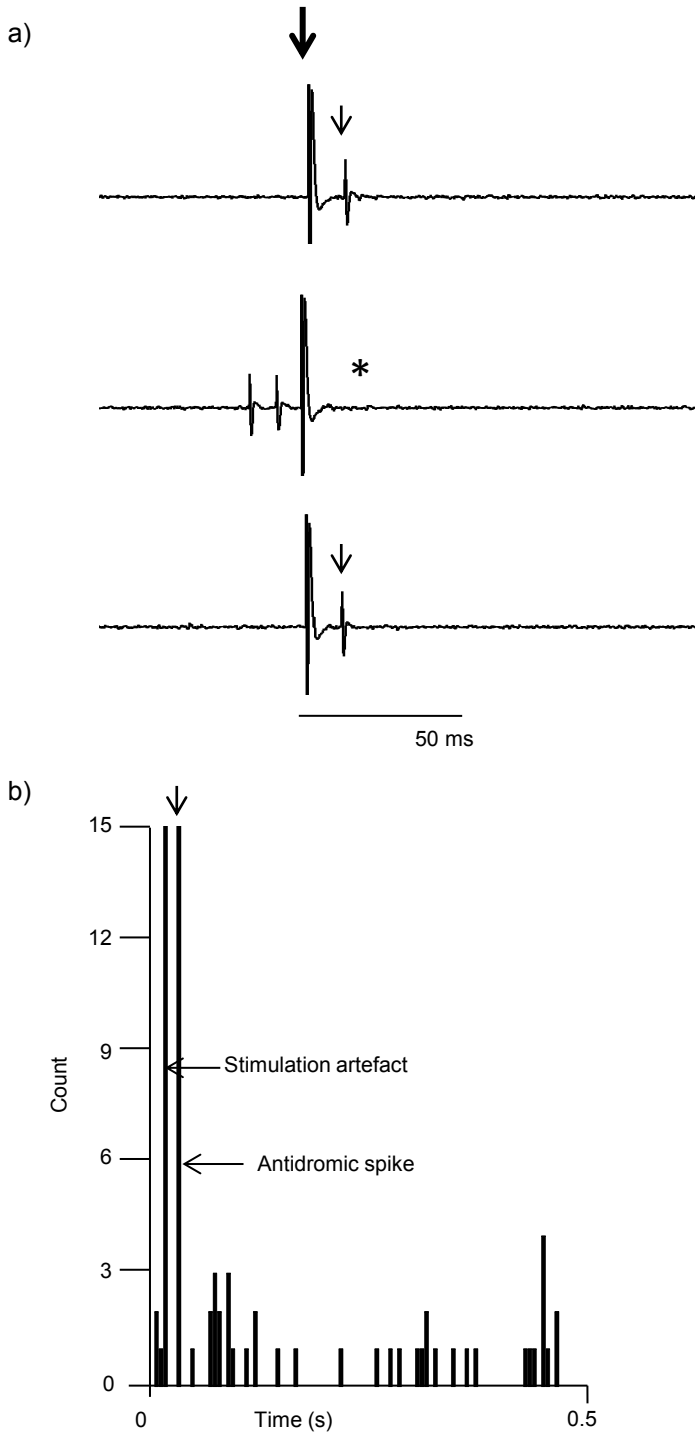


Figure 4.15

Example of antidromic spikes in mPFC a pyramidal neuron generated by DRN stimulation. a) Spike trains from a representative antidromically activated neuron. The larger arrow indicates the stimulation artefacts, smaller arrows indicate response to stimulation and the asterisk marks where the antidromic response was abolished due to collision with a spontaneously occurring spike. b) A representative peristimulus time histogram from an antidromically activated neuron demonstrating a fixed latency and high fidelity following stimulation. Response to stimulation indicated by arrow.

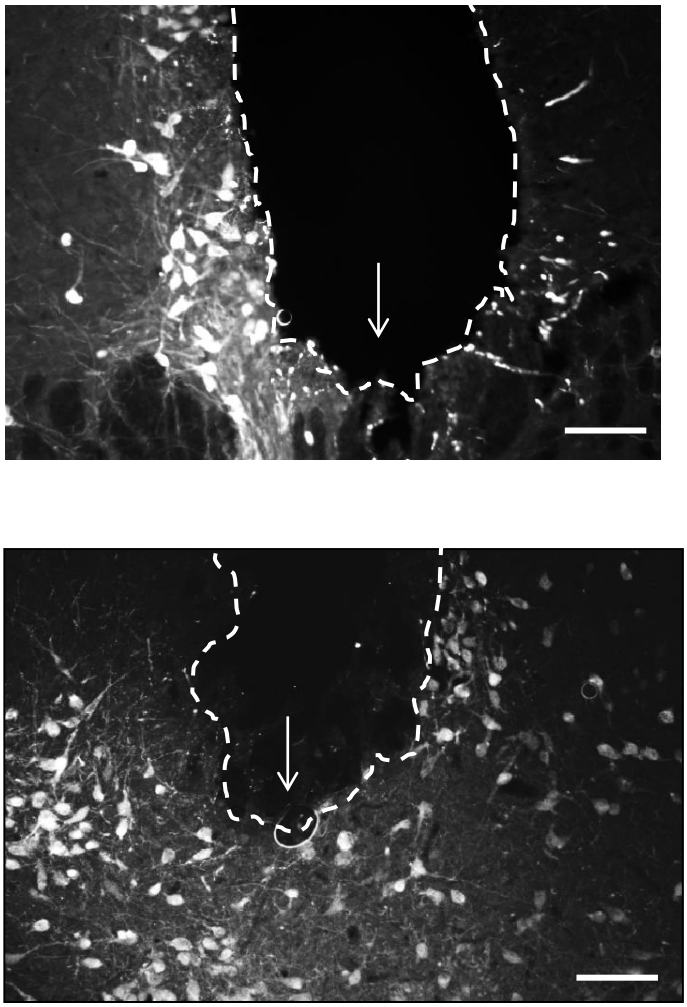


Figure 4.16
Localisation of stimulating electrode to the DRN. Representative images taken of the DRN from two separate experiments showing fluorescent cells which have been stained for 5-HT. The site of the stimulating electrode is outlined and the arrow marks the tip of the stimulating electrode. Scale bar = 60μm

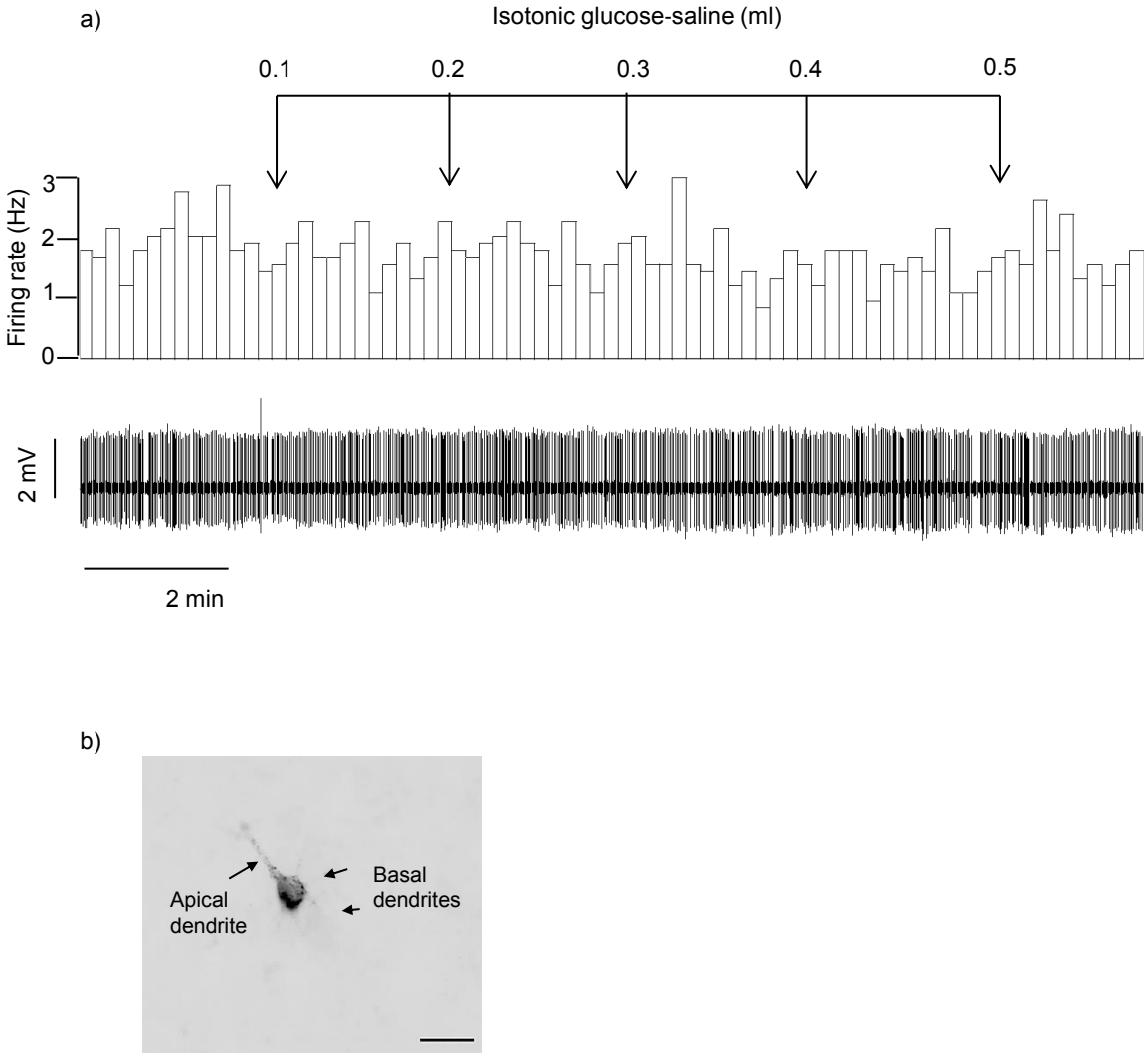


Figure 4.17
Effect of vehicle on the firing of a single pyramidal neuron in the rat mPFC. a) Rate meter (top) and spike train (bottom) recording during vehicle (isotonic glucose-saline) administration. b) The same cell was successfully labelled with neurobiotin and identified as pyramidal based on it's morphology. Scale bar= 20μm.

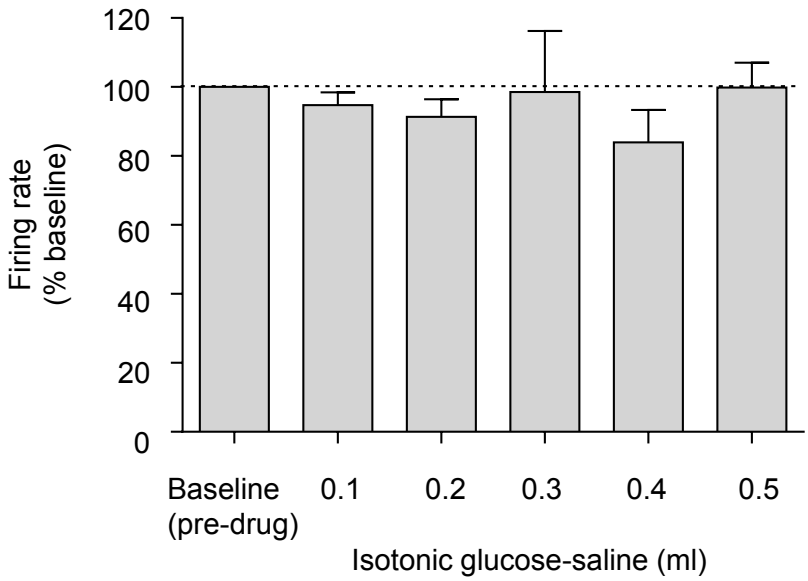


Figure 4.18
Effect of vehicle on mPFC pyramidal neuron firing. Group data demonstrating the effect of administration of accumulating doses (2 min intervals, i.v.) of vehicle (isotonic glucose-saline) on pyramidal neuron firing. Data expressed as mean ±SEM (n= 6 neurons).

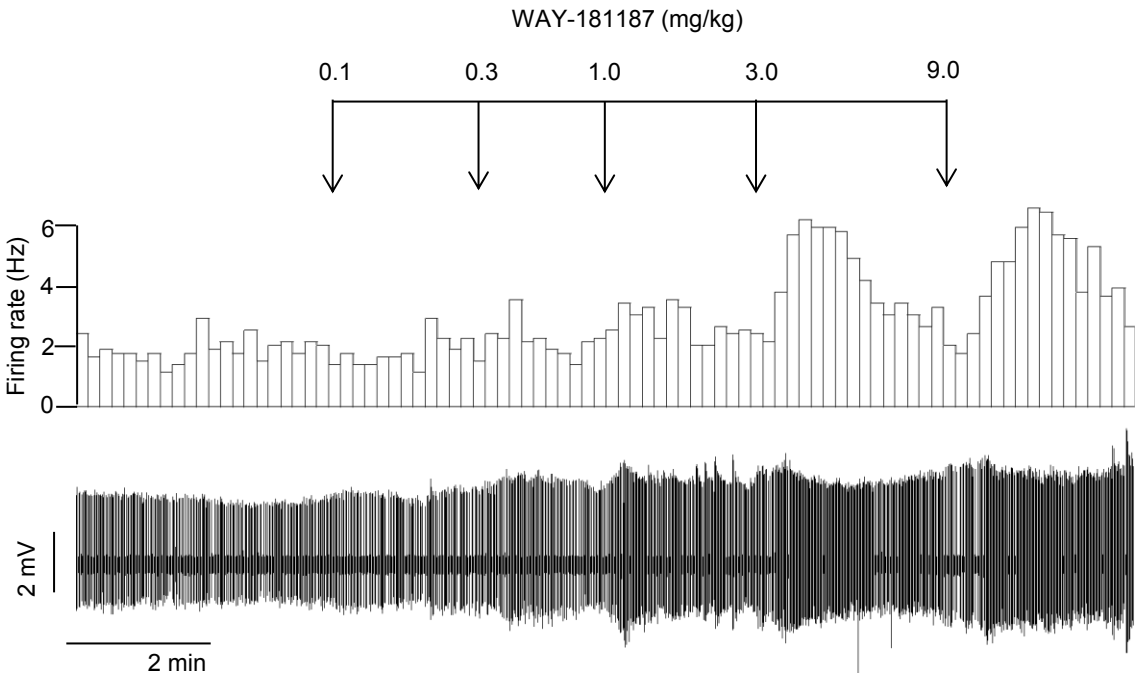


Figure 4.19
Effect of WAY-181187 on the firing of a single, excited pyramidal neuron in the rat mPFC. Rate meter (top) and spike train (bottom) recording during WAY-181187 administration. Scale bar= 20μm.

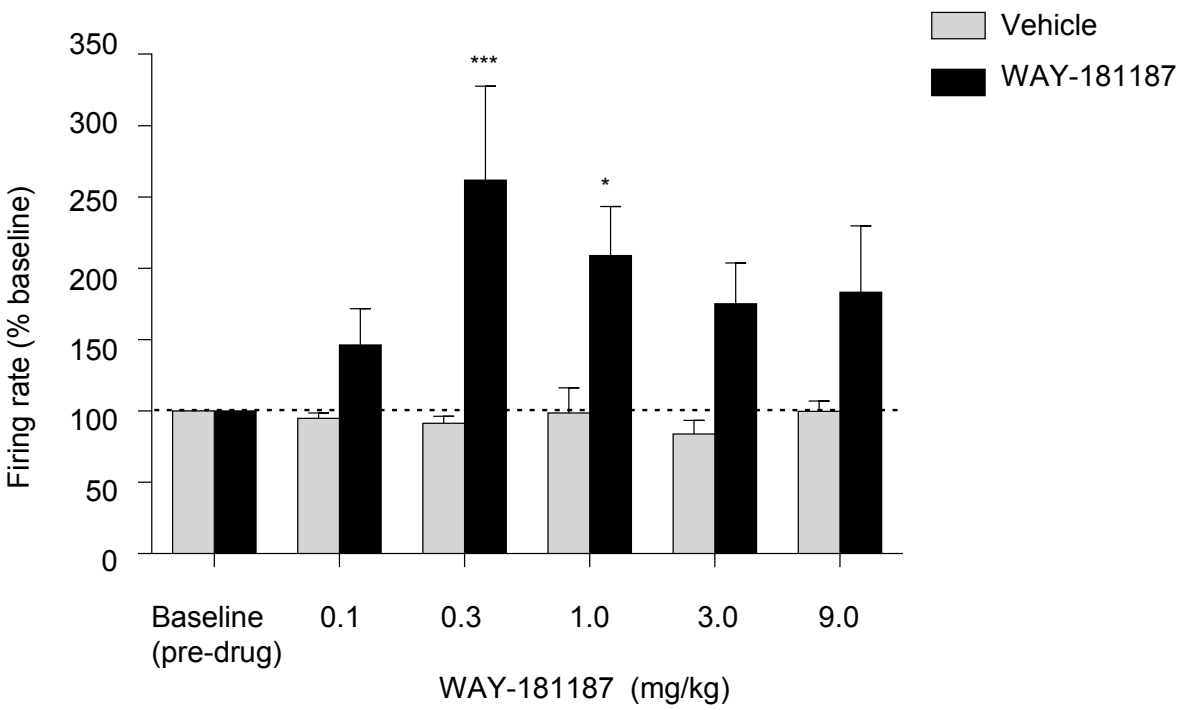


Figure 4.20
Group data demonstrating the effect of WAY-181187 administration on excited mPFC pyramidal neurons. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals, ***p= 0.001 *p<0.05 versus vehicle controls (unpaired t-test). Data expressed as mean ±SEM (WAY-181187 n=11, 11, 11, 10, 10, 9 neurons across the doses; vehicle n= 6, 6, 6, 6, 5, 5 neurons across the doses).

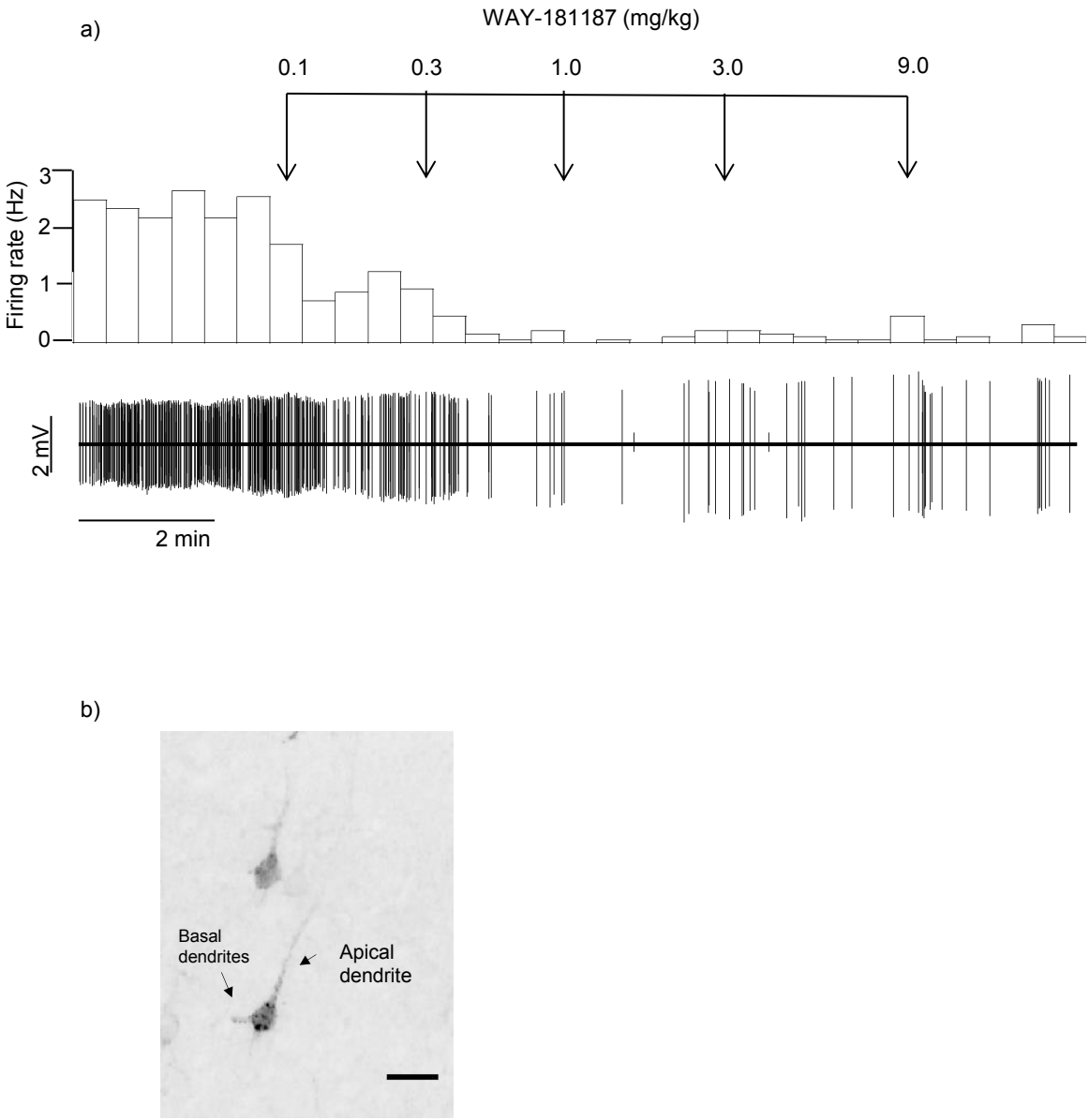


Figure 4.21
Effect of WAY-181187 on the firing of a single, inhibited pyramidal neuron in the rat mPFC. a) Rate meter (top) and spike train (bottom) recording during WAY-181187 administration. b) Two pyramidal cells were labelled inadvertently during juxtacellular labelling and both were identified as pyramidal based on morphology. It is not possible to determine which of the two was the recorded neuron. Scale bar= 20μm.

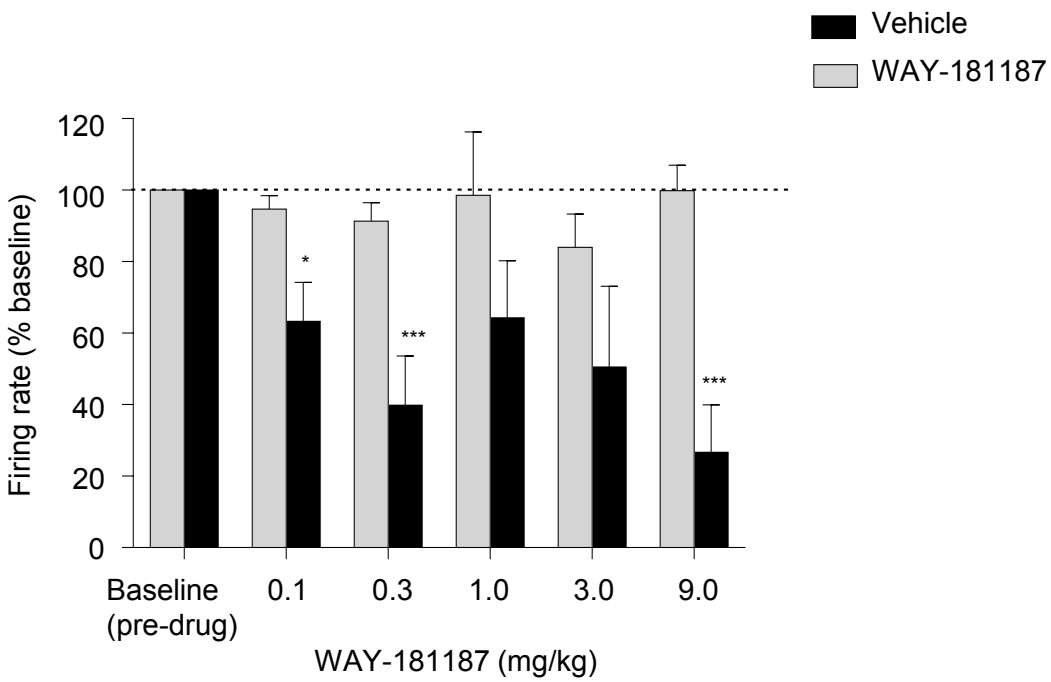


Figure 4.22
Group data demonstrating the effect of WAY-181187 administration on inhibited mPFC pyramidal neurons. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals, *p< 0.05 ***p< 0.001 versus vehicle controls (unpaired t-tests). Data expressed as mean ±SEM (WAY-181187 n=8, 8, 7, 7, 5, 5 neurons across the doses, n= 6, 6, 6, 6, 5, 5 neurons across the doses).

Chapter 5

Effect of 5-HT_6 receptor agonist administration on DRN 5-HT neuron firing following mPFC lesion

5.1 Introduction

This thesis had the overarching aim of investigating the influence of 5-HT_6 receptors on DRN 5-HT neurons. It was hypothesised that 5-HT_6 receptors control the firing of 5-HT neurons in the DRN, and that this represents a novel homeostatic feedback pathway. Experiments described in Chapter 3 demonstrated that the 5-HT_6 agonists WAY-181187 and WAY-208466 increased the firing of DRN 5-HT neurons, via a putatively 5-HT_6-dependent mechanism (Chapter 3). EEG recordings provided evidence for a role of the prefrontal cortex in this action of the 5-HT_6 agonists since both WAY-181187 and WAY-208466 reduced frontal cortex SW oscillations (Chapter 3). Further experiments described in Chapter 4 revealed evidence that 5-HT_6 agonist administration influenced the mPFC including mPFC-DRN connectivity; thus, WAY-181187 modulated the firing of pyramidal neurons in the mPFC that projected to the DRN (Chapter 4). Overall, the data presented in Chapters 3 and 4 are consistent with the idea that 5-HT_6 receptor agonists activate 5-HT neurons in the DRN via an mPFC-dependent mechanism.

The mPFC has previously been shown to modulate 5-HT neuron activity in the DRN. Moreover, recent evidence supports the localisation of 5-HT_6 receptors on a large number of mPFC pyramidal neurons (Helboe et al., 2015). Experiments in the current chapter aimed to investigate the role of the mPFC in the effects of 5-HT_6 receptor agonist administration on 5-HT neuron firing in the DRN. To this end, electrophysiological recordings were obtained from DRN 5-HT neurons in rats with neurotoxic lesions of the mPFC.

5.1.1 Evidence for mPFC influence on the DRN

Evidence supports a role of mPFC neurons in the control of 5-HT neuron firing and 5-HT release. Specifically, pathway tracing studies in rats have identified bilateral projections from the mPFC to the DRN (Sesack et al., 1989; Hajos et al. 1998; Peyron et al., 1998), arising predominantly from deeper mPFC layers, particularly layer 5 (Hajos et al. 1998; Peyron et al., 1998). Until recently, the majority of the mPFC inputs to DRN 5-HT neurons were considered indirect, via DRN GABA neurons (Hajos et al., 1998; Peyron et al., 1998; Varga et al., 2001). However, recent studies using virally-mediated pathway tracing techniques in mice suggest that a number of mPFC inputs to 5-HT neurons are direct (Ogawa et al., 2014; Pollack Doroic et al., 2014). Further evidence for this 'hyperdirect' pathway comes from recent optogenetic experiments which demonstrated that light-induced activation of axons derived from the mPFC elicited excitation of 5-HT neurons *in vitro* in an AMPA receptor-dependent manner (Pollak Doroic et al., 2014).

In addition to the evidence mentioned above, electrophysiology and microdialysis studies provide further support for functional connections from the mPFC to DRN. In particular, several studies have demonstrated that electrical stimulation of the mPFC leads to inhibition in the majority of 5-HT neurons recorded; however, the firing of 10-20% of 5-HT neurons was activated by mPFC stimulations (Hajos et al., 1998; Varga et al., 2001; Celada et al., 2001). In addition, in microdialysis studies electrical stimulation of the mPFC has been shown to increase 5-HT release in the DRN (Celada et al., 2001). Together with data from anatomical studies, these findings provide evidence of an influence of the mPFC over DRN 5-HT neurons.

5.1.2 Evidence for 5-HT receptor control of mPFC-DRN connectivity

5-HT receptors in the mPFC are thought to control mPFC-DRN connections to form homeostatic 5-HT feedback pathways. These pathways can drive either inhibition or excitation of 5-HT neuron firing and are likely to exist in a number of configurations. In particular, DRN 5-HT neurons are thought to be inhibited by 5-HT_{1A} receptors localised on mPFC GABAergic neurons which input to mPFC glutamatergic pyramidal neurons; these in turn input to DRN GABAergic interneurons and then 5-HT neurons (Sharp et al., 2007; Chapter 1, Figure 1.3). Thus, intra-mPFC injection of selective 5-HT_{1A} agonist 8-OH-DPAT has been shown to inhibit the firing of DRN 5-HT neurons, and to reduce 5-HT release in the mPFC and DRN (Celada et al., 2001). In addition, mPFC 5-HT_{2A} receptors putatively located on mPFC pyramidal neurons are thought to mediate inhibition of 5-HT neuron firing by increasing excitatory drive to DRN GABA neurons (Sharp et al., 2007; Chapter 1, Figure 1.3). Thus, systemic administration of 5-HT_2 agonist DOI inhibited 5-HT neuron firing, an effect which was reversed by systemic administration of the 5-HT_{2A} antagonist MDL-100907 and $GABA_A$ antagonist picrotoxin (Martin-Ruiz et al., 2001; Boothman et al., 2003). Moreover, the involvement of DRN GABA neurons is evident in the observation that administration of the 5-HT_2 agonist induced Fos expression in DRN GABA neurons, an effect also reversed by MDL-100907 (Boothman and Sharp, 2005).

Finally, 5-HT neurons are thought to receive inputs from 5-HT_4 receptor-expressing mPFC pyramidal neurons, forming a putative excitatory 5-HT feedback pathway (Lucas and Debonnel, 2002; Lucas et al., 2005; Sharp et al., 2007; Chapter 1, Figure 1.3). Thus, both the 5-HT_4 agonist cisapride and virally-induced overexpression of 5-HT_4 receptors in the mPFC were found to increase 5-HT neuron firing in the DRN (Lucas and Debonnel, 2002; Lucas et al., 2005). On the other hand, 5-HT_4 receptor blockade reduced 5-HT neuron firing (Lucas and Debonnel, 2002; Lucas et al.,

2005), suggesting that 5-HT_4-mediated feedback was tonically active. Taken together, these findings suggest that various 5-HT receptors located in the mPFC act in an inhibitory or excitatory way to control 5-HT neuron firing in the DRN. Evidence provided in this thesis supports the hypothesis that 5-HT_6 receptors located in the mPFC may also act to control 5-HT neurons in the DRN in a manner akin to the above-mentioned 5-HT_4 receptor-mediated excitatory pathway.

5.1.3 Aims of chapter

Experiments presented in Chapters 3 and 4 provide evidence for a role of 5-HT_6 receptors in the excitatory control of 5-HT neurons, via a mechanism involving the mPFC. Therefore, experiments in the current chapter aimed to test the hypothesis that the excitatory effect of the 5-HT_6 receptor agonist WAY-181187 on 5-HT neuron firing may be dependent on the mPFC. To this end, electrophysiological recordings were made from DRN 5-HT neurons during WAY-181187 administration in rats with mPFC neurotoxic lesions, which allowed the effects of WAY-181187 to be investigated in animals with disrupted mPFC-DRN connectivity.

5.2 Methods

5.2.1 Animals

Male Sprague Dawley rats (250-420g) were group housed at 20°C ± 2°C under 12 hour light-dark cycle (lights on 0800 h) in an enriched environment with water and food given ad libitum. Rats were left to acclimatise for at least 1 week following delivery to the housing facility. On experiment days rats were taken from the housing facility to the experiment room and left to acclimatise for 30 min prior to induction of anaesthesia.

5.2.2 Lesioning protocol

A detailed protocol for neurotoxic mPFC lesions is described in Chapter 2. In brief, rats were anaesthetised with isoflurane (4% isoflurane and 4 l/min oxygen) and a craniotomy was performed bilaterally over the mPFC. Intracranial injections of neurotoxin quinolinic acid (0.09M, or deionised water in sham surgeries) were made into the mPFC ((from bregma = i) +3.3 mm anterior-posterior (AP), +0.5 mm medio-lateral (ML), ii) +3.3 mm AP,–0.5 mm ML, iii) +2.6 mm AP,+0.5 mm ML, iv) +2.6 mm AP, –0.5 mm ML). At each of the four sites an intracranial injection of quinolinic acid or deionised water was administered in 150 nl volumes at two depths within the mPFC (-3.0mm and -3.5 from *dura mata*). The scalp was sutured, and rats were administered isotonic glucose-saline and analgesia. Recovery was monitored closely and rats were left for 2-3 weeks before undergoing electrophysiological recordings.

5.2.3 Electrophysiology protocol

In vivo electrophysiological recordings in the DRN were carried out as described in Chapter 2. In brief, anaesthesia was induced with isoflurane and maintained with urethane (1.3 mg/kg i.p.) and supplemental doses of ketamine (30 mg/kg i.m.) and

xylazine (3 mg/kg i.m). A craniotomy was performed over the DRN (from bregma= -7.4-7.8 mm AP, and midline) and the recording electrode (filled with neurobiotin) was lowered into the DRN using a microdrive (-4.0 mm from *dura mata*). Once a neuron was detected the spike amplitude was allowed to increase to around 1 mV and then recorded for 2-3 min to establish a stable baseline. WAY-181187 was then administered in 4 accumulating doses at 2 min intervals via a lateral tail vein cannula. If a neuron was lost after no more than one dose of drug an alternative neuron was sought and the WAY-181187 dose regime was recommenced. Attempts were then made to juxtacellular label the recorded neurons. Rats were perfused with 4% PFA and brains were extracted and processed for 5-HT and neurobiotin immunohistochemistry. Alternatively rats were culled with pentobarbital overdose. In all cases brains were extracted to validate the site of the lesion. Figure 5.1 illustrates the time course of these experiments.

Experiments in this chapter examined the effects of the 5-HT_6 agonist WAY-181187 on 5-HT neuron firing following neurotoxic or sham lesion of the mPFC. During some experiments EEG recordings were monitored using a skull screw placed above the frontal cortex. Table 5.1 provides a summary of the experiments carried out in this chapter.

Treatment	**Drugs doses (i.v.)**	**Number of neurons or rats**
1) mPFC neurotoxic lesion		12 rats
2) mPFC sham lesion		6 rats
3) Effect of WAY-181187 on 5-HT neuron firing in PFC lesioned rats	1.0 - 4.0 mg/kg (1.0 – 4.1 ml/kg)	6 neurons (rats from Experiment 1)
4) WAY-181187 on 5-HT neuron firing in sham-lesioned rats	1.0 – 4.0 mg/kg (1.0 – 4.1 ml/kg)	5 neurons (rats from Experiment 2)
5) Effect of WAY-181187 on cortical SW oscillations	1.0 – 4.0 mg/kg (1.0 – 4.1 ml/kg)	5 rats (rats from Experiments 1 and 2)

Table 5.1
Experiments carried out in Chapter 5.

5.2.4 Data analysis

Recordings from individual DRN neurons were acquired online using Spike2 (version 8.03) software. Details regarding the filter and conversion of raw data using Spike2 software are given in Chapter 2. Spike2 was also used offline to sort spikes, measure spike width, and determine firing coherence with EEG recordings. Data were exported into Microsoft Excel to determine firing rates (Hz) and regularity (COV_{ISI}). For final analysis of drug effects, firing rates were normalised to pre-drug, baseline periods (taken as 100%). Data are expressed as mean ± SEM values. Neurons included in the final data analysis exhibited a stable baseline firing rate defined as firing during the pre-drug period which did not fall outside of 2x S.D. of the mean pre-drug firing rate for more than two consecutive 10 second bins.

EEG data were analysed offline in Spike2. Spectrograms were generated to illustrate the power, i.e. relative dominance, of a frequency band within a given time frame. To obtain robust measurements of power, at least 60 seconds of uninterrupted (i.e. no artefacts) EEG trace was required. Bleeding around the EEG screw sites was found to be a common cause of artefacts which hindered EEG analysis.

5.2.5 Identification of 5-HT neurons

Putative 5-HT neurons were selected according to the following firing characteristics: slow firing rate (typically ≤ 2 Hz), broad waveform width (typically ≥ 1msec) and a regular firing pattern defined by a COV_{ISI} of <0.5, which typifies clock-like 5-HT neurons (Allers and Sharp, 2003; Schweimer et al., 2010; Schweimer et al., 2011). Burst-firing 5-HT neurons had similar firing rate and waveform characteristics to clock-like 5-HT neurons but had a higher COV_{ISI} (Kocsis et al., 2006; Schweimer et al. 2011).

5.2.6 Lesion validation

Coronal sections of the mPFC from sham and lesioned rats were cut at -20^{o}C on a cryostat, sections were mounted onto glass slides and cresyl violet staining was carried out. Sections from lesion and sham brains were examined under a light microscope to assess the extent of the lesion with the observer blind to treatment. Later, the area of mPFC damage in lesioned rats was drawn manually onto diagrammatic coronal sections taken from Paxinos and Watson (2007). These images were then overlaid to produce an illustration of the lesioned area across the group of rats (Figure 5.2). Rats were included in the final analysis if lesions were found to be bilateral, and encompassing part or all of the following mPFC regions: the

anterior cingulate cortex, prelimbic cortex, infralimbic cortex and dorsal peduncular cortex. Rats were excluded only if they exhibited unilateral lesions, lesions extending outside the mPFC or absence of a lesion. The electrophysiological data from these rats was excluded from the final analysis.

5.2.7 Statistical analysis

The baseline electrophysiological data (e.g. firing rate, spike width) from mPFC lesioned rats were compared with sham-injected controls, and tested statistically using a Student's unpaired t-test (two-tailed). The effect of WAY-181187 on 5-HT neuron firing in mPFC-lesioned rats was compared to the effect in sham-lesioned rats using Student's unpaired t-test (two-tailed), corrected for multiple comparisons using the Sidak-Bonferroni method. The effect of WAY-181187 on DRN 5-HT neuron firing in sham- and mPFC-lesioned rats was also tested statistically by comparing post- and pre-drug values using a Student's paired t-tests (two-tailed) corrected for multiple comparisons (Sidak-Bonferroni method). Finally, the effect of WAY-181187 in sham and mPFC-lesioned rats was compared to a vehicle-injected group of treatment-naïve rats. This vehicle group is the same as that used in Chapter 3 sections 3.3.2, where vehicle (isotonic glucose-saline) was administered to non-lesioned, non-sham anaesthetised rats. The effect of WAY-181187 on 5-HT neuron firing was compared to this vehicle control group using Student's unpaired t-test (two-tailed) correcting for multiple comparisons (Sidak-Bonferroni method). ANOVA analysis was not used for the above comparisons because group sizes varied between drug doses.

The effect of WAY-181187 on SW oscillations was analysed statistically comparing pre-drug and post-drug values using Student's paired t-test (two-tailed). Also, the

effect of WAY-181187 on mPFC- and sham-lesioned rats was compared to the effect of vehicle in non-lesioned, non-sham rats, using Student's unpaired t-test. This vehicle group were reported in Chapter 3 sections 3.3.5, where vehicle (isotonic glucose-saline) was administered to non-lesioned non-sham anaesthetised rats.

Probability values of 0.05 or less were considered statistically significant.

5.3. Results

5.3.1 mPFC lesion validation

A total of 12 rats underwent surgery for neurotoxic lesion of the mPFC and 6 rats underwent sham lesion surgery. Four rats were excluded from the lesion group due to lesions being unilateral, the lesion being absent (probably due to a blocked injection needle), or, in one case, a rat was excluded because the lesion was positioned too laterally, in the secondary motor cortex. Electrophysiological recordings from these rats were not included in the final analysis. Two further rats did not recover from lesion surgery, so electrophysiological recordings were not carried out on these animals. The final dataset for electrophysiological recordings comprised 6 lesioned and 5 sham rats. Histological analysis confirmed that in sham rats the mPFC was non-lesioned. By contrast, in the group of lesioned rats the damaged areas included the dorsal mPFC regions (anterior cingulate cortex and prelimbic cortex) and ventral mPFC regions (infralimbic cortex and part of the dorsal peduncular cortex), as illustrated in Figure 5.2.

5.3.2 Effect of mPFC lesion on the firing of 5-HT neurons

In sham-lesioned rats a total of 8 putative 5-HT neurons were recorded. Six of these neurons exhibited baseline firing properties previously reported for clock-like 5-HT neurons, specifically a slow firing rate (1.27±0.39 Hz), broad spike width (1.52 ±0.09 ms), and regular firing pattern (COV_{ISI}= 0.29±0.09) (Figure 5.3). In addition, 2 neurons were recorded with characteristics of burst firing 5-HT neurons: a slow firing rate (0.56 ±0.02 Hz), broad spike width (1.52±0.10 ms), and periods of short burst firing (doublets or triplets) but an otherwise regular firing pattern (COV_{ISI} of 0.41 ±0.04).

Ten putative 5-HT neurons were recorded from mPFC-lesioned rats. Seven of these neurons exhibited baseline firing properties previously reported for clock-like 5-HT neurons, specifically a slow firing rate (1.51±0.2 Hz), broad spike width (1.37±0.09 ms) and regular firing pattern (COV_{ISI}= 0.29±0.03) (Figure 5.3). In addition, 3 neurons were recorded with characteristics of burst firing 5-HT neurons: a slow firing rate (1.15±0.23 Hz), broad spike width (1.25±0.14 ms) with periods of short burst firing (doublets or triplets; COV_{ISI}= 0.74±0.20).

Overall, there was no statistically significant difference in firing rate, spike width or COV_{ISI} between putative clock-like and bursting 5-HT neurons from sham or lesioned rats (Table 5.2).

	Sham (number of cells as % of total)	Lesion (number of cells as % of total)	Sham versus lesion (Student's unpaired t-test)
Clock-like			
Firing rate (Hz)	1.27 ± 0.39 (75%)	1.51 ± 0.2 (70%)	p= 0.62
Spike width (ms)	1.52 ± 0.09 (75%)	1.37 ± 0.09 (70%)	p= 0.28
COV_{ISI}	0.29 ± 0.09 (75%)	0.29 ± 0.03 (70%)	p= 0.91
Bursting			
Firing rate (Hz)	0.56 ± 0.02 (25%)	1.15 ± 0.23 (30%)	p= 0.16
Spike width (ms)	1.52 ± 0.10 (25%)	1.25 ± 0.14 (30%)	p= 0.28
COV_{ISI}	0.41 ± 0.04 (25%)	0.74 ± 0.20 (30%)	p= 0.29

Table 5.2 Firing properties of putative 5-HT neurons in the DRN of sham and mPFC-lesioned rats. Data are mean ± SEM values.

Analysis of spike timing with cortical SW oscillations, as measured through EEG recordings, was investigated in recorded putative 5-HT neurons from sham rats. It

was found that neurons exhibited firing in significant coherence with SW oscillations ($p< 0.05$, n=4), with spikes predominantly in the inactive phase of oscillations (Figure 5.3). In mPFC-lesioned rats 6 putative 5-HT neurons also exhibited firing in significant coherence with SW oscillations ($p< 0.05$; Figure 5.3). However, of these only 50% (n=3) exhibited spiking during the inactive phase of oscillations, with the other half appearing to fire at other times during the SW oscillations (Figure 5.3).

5.3.3 Effect of WAY-181187 on 5-HT neuron firing in sham and mPFC-lesioned rats

WAY-181187 (1.0, 2.0, 3.0, 4.0 mg/kg i.v.) was administered to sham and mPFC-lesioned rats. In sham-lesioned rats WAY-181187 caused an increase in the firing of 5-HT neurons, with a maximal increase of 24% above baseline firing at 2.0 mg/kg WAY-181187 (Figure 5.4 and 5.5). In comparison, this excitatory effect of WAY-181187 was reduced in mPFC-lesioned rats, where WAY-181187 caused a maximal increase of 5% above baseline firing at 3.0 mg/kg (Figure 5.4 and 5.5). This difference in the effect of WAY-181187 on 5-HT neurons in lesioned compared to sham rats was statistically significant at the dose 2.0 mg/kg (unpaired t-test: $p= 0.005$; Figure 5.5).

WAY-181187 did not cause a statistically significant increase in 5-HT neuron firing in either sham- or mPFC-lesioned animals when comparing post-drug with pre-drug values (sham: $p> 0.1$ at all doses post-drug versus pre-drug; lesion: $p>0.3$ at all doses post-drug versus pre-drug; Student's paired t-test). However, when compared to the effect of vehicle in treatment naïve rats, WAY-181187 caused a significant increase in firing rate in sham rats ($p=0.0005$ for 2.0 mg/kg; $p=0.003$ for 3.0 mg/kg; $p=0.005$ for 4 mg/kg; unpaired t-test; Figure 5.6). In contrast, WAY-181187 did not cause a statistically significant effect on the firing of 5-HT neurons in rats with mPFC

lesions when compared to this vehicle group ($p > 0.07$ for all WAY-181187 doses versus vehicle; unpaired t-test; Figure 5.6).

5.3.4 Effect of WAY-181187 on SW oscillations in sham- and mPFC-lesioned rats

Quantifiable EEG recordings were obtained from 4 mPFC-lesioned rats during WAY-181187 administration (4 mg/kg). In these rats, WAY-181187 had no statistically significant effect on SW oscillation power when comparing pre-drug and post-drug values ($p=0.33$; Student's paired t-test: Figure 5.7 and 5.8). If a comparison was made with the effect of vehicle in naïve rats WAY-181187 (4.0 mg/kg) caused a statistically significant decrease in SW oscillation power ($p=0.0002$; Unpaired t-test; Figure 5.8). Unfortunately, EEG data was only quantifiable in one sham rat where WAY-181187 (4.0 mg/kg, i.v.) decreased SW oscillation power by approximately 30% compared to pre-drug power (Figure 5.7 and 5.8). Overall, due to the paucity of data, it was difficult to make firm conclusions regarding the effect of mPFC lesion on the decrease in cortical SW oscillations induced by WAY-181187.

5.4. Discussion

Experiments in this chapter aimed to investigate the involvement of the mPFC in the excitatory effect of the 5-HT_6 agonist WAY-181187 on 5-HT neuron firing in the DRN. To this end, electrophysiological recordings of DRN 5-HT neurons were carried out in rats with sham or neurotoxic lesions of the mPFC. The key finding was that the excitatory effect of WAY-181187 on 5-HT neuron firing was reduced in mPFC-lesioned compared to sham-lesioned rats. This finding supports a role of the mPFC in the excitatory effect of WAY-181187 on 5-HT neuron firing in the DRN. Together with data from previous chapters this implicates the involvement of the mPFC in 5-HT_6- receptor-mediated feedback control of 5-HT neurons.

5.4.1 Electrophysiological properties of 5-HT neurons in mPFC-lesioned rats

Since the mPFC is a source of input to the DRN, it may have been anticipated that mPFC lesion would result in changes to the baseline firing properties of 5-HT neurons. However, the firing rate, regularity and spike width of the recorded, putative 5-HT neurons did not significantly differ between sham- and mPFC-lesioned animals. Since the firing characteristics of these neurons were not distinguishable from those of DRN neurons that were juxtacellular-labelled and found to be 5-HT-containing (Chapter 3, and Allers and Sharp, 2003, Schweimer et al., 2010, Schweimer et al., 2011), it is highly likely that neurons included in this chapter were 5-HT neurons. These data suggest that the mPFC does not exert significant tonic control over the firing rate or pattern of DRN 5-HT neurons under the present experimental conditions (i.e. anaesthetised rats). This agrees with a previous study that found no effect of cortical ablation on the baseline firing properties of DRN 5-HT neurons (Hajos et al., 1999). Interestingly, it was also noted that the proportion of clock-like and bursting

neurons recorded in experiments in this chapter were consistent with those reported in Chapter 3 from DRN recordings in non-lesioned, non-sham anaesthetised rats.

In contrast, the current experiments found evidence that an mPFC lesion alters the spike timing of 5-HT neurons in relation to cortical SW oscillations. Thus, only 3 of the putative 5-HT neurons tested in mPFC-lesioned rats fired in the inactive phase of SW oscillations, whereas in sham-lesioned rats all putative 5-HT neurons showed firing in synchrony with the inactive phase of SW oscillations. The latter is a consistent property of DRN 5-HT neurons in treatment-naïve rats (Chapter 3; Schweimer et al., 2011). Thus, while an mPFC lesion did not alter the baseline firing properties of DRN neurons, it did disrupt the timing of 5-HT neuron firing in relation to SW oscillations. In other words, the mPFC lesions did affect mPFC-DRN connectivity in the current experiments, as perhaps might be expected.

5.4.2 mPFC as a substrate for 5-HT_6 receptor-mediated feedback control of 5-HT neurons

Previous experiments in this thesis showed that administration of WAY-181187, as well as another 5-HT_6 agonist WAY-208466, increased the firing of DRN 5-HT neurons (Chapter 3). This effect is likely to be 5-HT_6 receptor-mediated since it was blocked (in the case of WAY-181187) by 5-HT_6 antagonists SB-271046 and SB-258585. Moreover, WAY-181187 modulated the firing of mPFC pyramidal neurons projecting to the DRN (Chapter 4). Experiments in the current chapter found that a neurotoxic lesion of the mPFC reduced the excitatory effect of WAY-181187 on 5-HT neuron firing. Thus, WAY-181187 increased the firing of DRN 5-HT neurons in sham-lesioned rats but the effect was significantly smaller in rats with an mPFC lesion. This difference was even more striking when data from naïve rats were incorporated in the

analysis; WAY-181187 increased 5-HT neuron firing in sham rats compared to vehicle controls but had no effect in mPFC-lesioned rats. This suggests that the mPFC is an important substrate for mediating the excitatory effect of 5-HT_6 receptor agonists on 5-HT neurons.

It is not possible to identify from the current data whether the 5-HT_6 receptors mediating the increases in 5-HT neuron firing are directly located on mPFC neurons, or whether the 5-HT_6 receptors are located elsewhere and evoke actions which are dependent on the mPFC. However, 5-HT_6 receptors are known to be predominantly expressed by pyramidal neurons in the mPFC (Helboe et al., 2015). Thus, it is conceivable that the mPFC is the site of 5-HT_6 receptor activation by WAY-181187. This could be further tested using intra-mPFC injections of WAY-181187 while recording the firing rate of DRN 5-HT neurons or by virally-mediated overexpression of 5-HT_6 receptors as used to link mPFC 5-HT_4 receptors to 5-HT neuron control (Lucas et al., 2005).

Despite the above arguments, 5-HT_6 receptor modulation of input to the mPFC via other regions cannot be ruled out. In particular, the hippocampus has a high level of 5-HT_6 receptor expression on pyramidal neurons which could influence mPFC neural activity via the hippocampal-mPFC pathway (Jay and Witter, 1991; Jay et al., 1992; Helboe et al., 2015). Alternatively, the current experiments do not rule out a role for 5-HT_6 receptors located in the DRN itself. Indeed, evidence suggests 5-HT_6 receptors are expressed on non-5-HT neurons in the DRN, as shown by in situ hybridisation and RT-PCR (Gerard et al. 1996; Helboe et al., 2015). Moreover, intra-DRN injection of 5-HT_6 agonist WAY-208466 and EMD-386088 has been shown to alter sleeping and waking in rats which could indicate direct effects on 5-HT transmission (Monti et al. 2013; Ly et al., 2013). However, it is difficult to understand why such a mechanism would depend on the mPFC.

5.4.3 Relevance of SW oscillations induced by WAY-181187 in lesioned rats

In experiments described in Chapter 3 WAY-181187 reduced cortical SW oscillations in treatment-naïve rats. Also, although more numbers are needed before firm conclusions can be drawn, in the current experiments WAY-181187 reduced SW oscillation power in the one tested sham rat whereas this effect was absent in mPFC-lesioned rats, at least when post-drug data was compared to pre-drug date. These data are likely to indicate a role of the mPFC in generating the decrease in in cortical SW oscillations induced by WAY-18187, which might indirectly link to the interaction between the mPFC and DRN.

5.4.4 Conclusion

The current chapter found that neurotoxic lesion of the mPFC reduced the excitatory effect of the 5-HT_6 agonist WAY-181187 on 5-HT neuron firing. Data in Chapter 3 showed that the WAY-181187-induced increase in 5-HT neuron firing was likely mediated by 5-HT_6 receptors. Together with findings from Chapters 3 and 4 this provides support for the hypothesis that 5-HT_6 receptors exert an excitatory influence over DRN 5-HT neurons in an mPFC-dependent feedback mechanism. Moreover, since 5-HT_6 receptors are located on pyramidal neurons in the mPFC (Helboe et al., 2015), and since there is a strong 5-HT projection from the DRN to the mPFC (Descarries et al., 1975, Azmitia and Segal, 1978, Steinbusch and Nieuwenhuys, 1981), the data are consistent with the excitatory feedback control of DRN 5-HT neurons mediated by 5-HT_6 receptors in the mPFC.

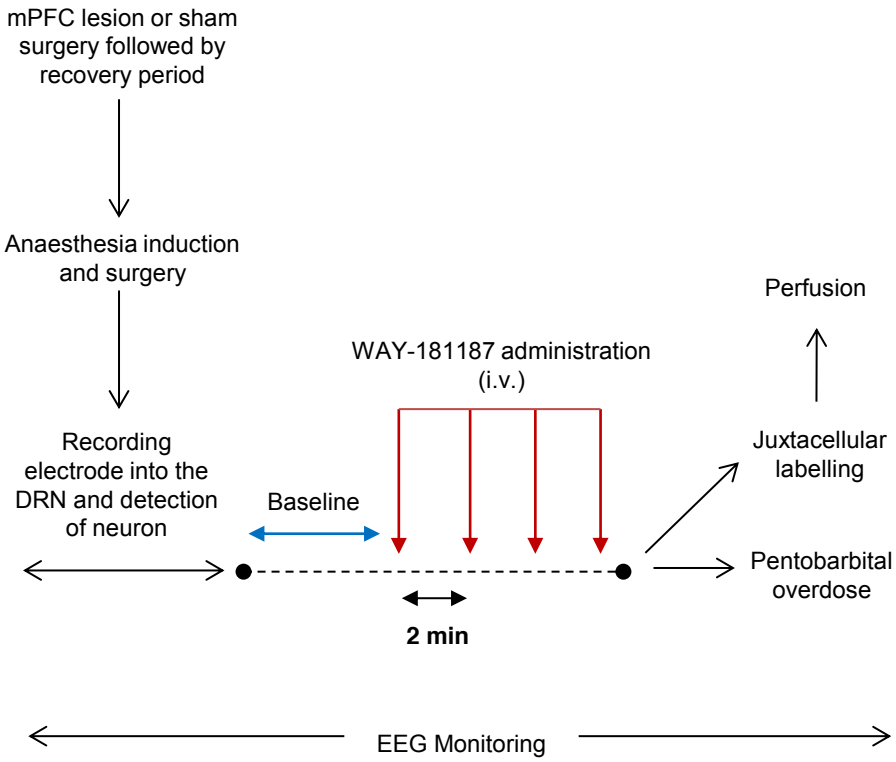


Figure 5.1
General protocol for experiments described in Chapter 5. Rats underwent recovery surgery involving neurotoxic or sham lesion of the mPFC. Following a 2-3 weeks recovery period rats were anaesthetised and a recording electrode was lowered into the DRN. Neurons were recorded for 2-3 min to establish a stable baseline followed by administration of drugs or vehicle in 2 min intervals. Then, either recorded neurons were juxtacellular labelled followed by perfusion, or otherwise rats were culled with pentobarbital overdose.

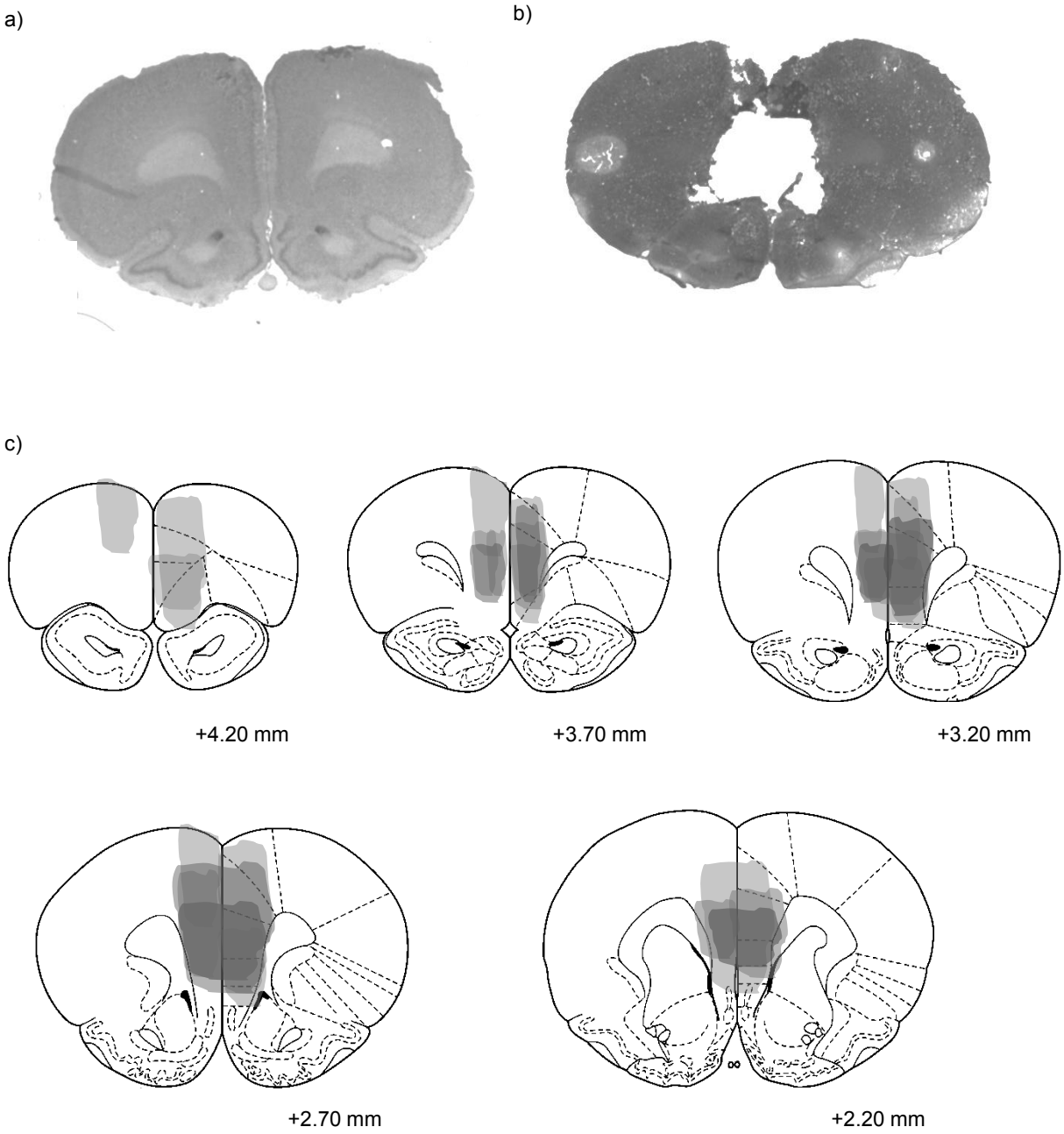


Figure 5.2

Localisation of lesion sites in the rat mPFC. Representative images show cresyl violet stained sections from a) sham and b) neurotoxic lesioned brains, respectively. Diagrams in c) show coronal sections of the mPFC illustrating the areas covered by lesion across the group of rats, with darkest areas demonstrating regions of greatest lesion overlap between animals (Paxinos and Watson, 2007) Distance is indicated from bregma according to Paxinos and Watson, 2007.

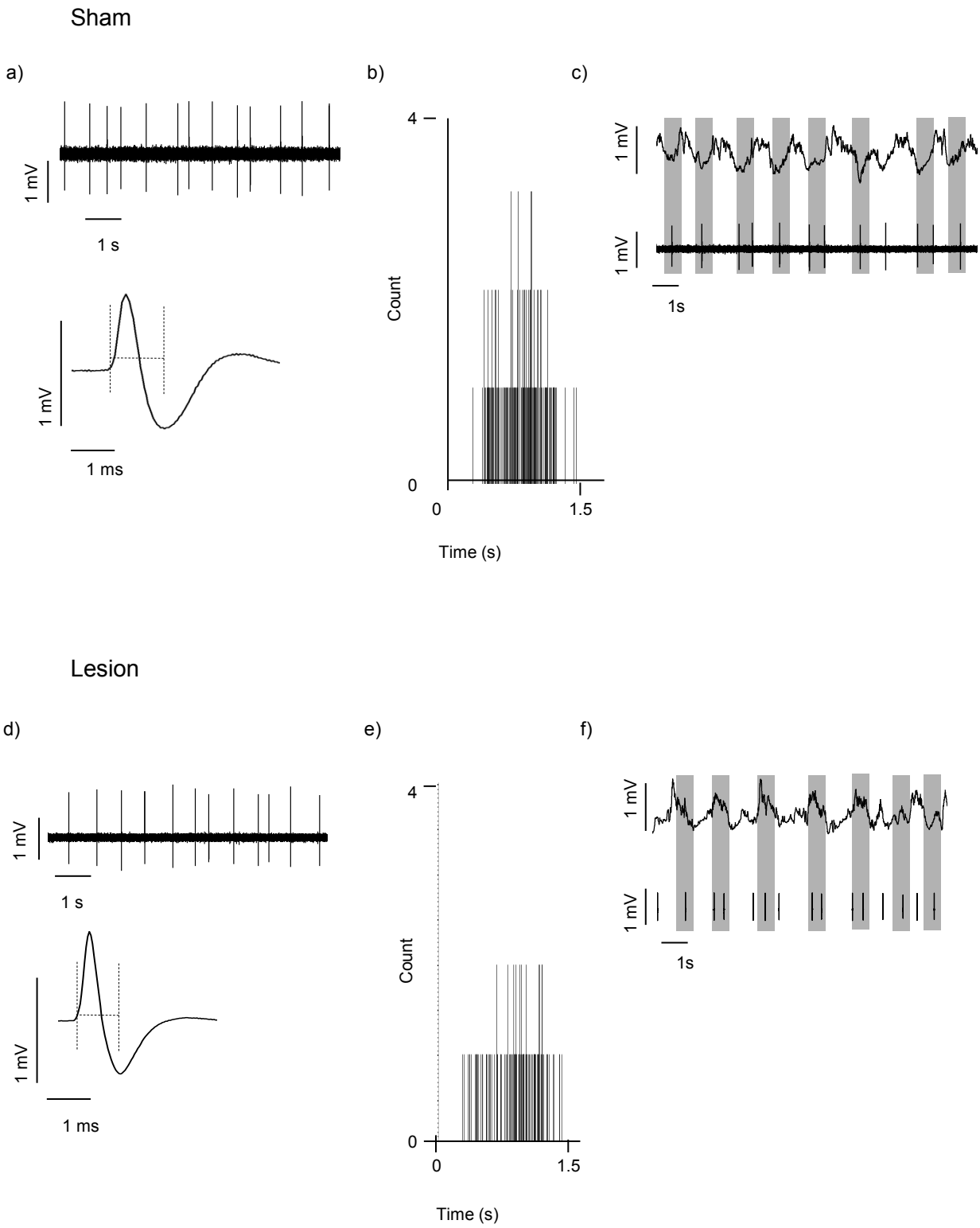


Figure 5.3

Electrophysiological properties of representative 5-HT neurons in rats with a sham (top) or neurotoxic (bottom) lesioned mPFC. Neurons exhibited a slow firing rate and broad spike waveform (a and d). The neurons had a highly regular firing pattern, illustrated by the interspike-interval histogram (b and e), and exhibited coherence with cortical SW oscillations(c and f).

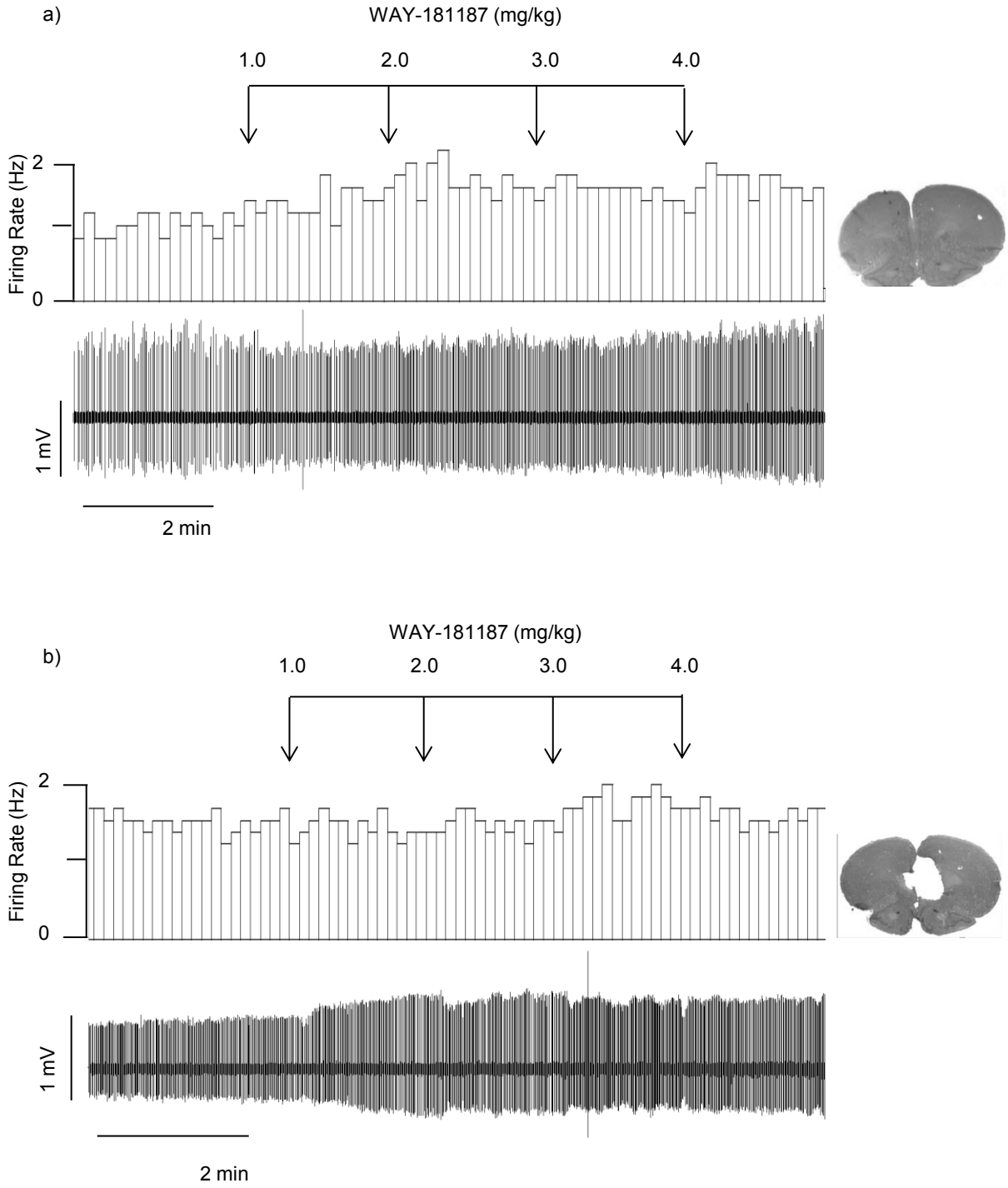


Figure 5.4
Electrophysiological traces illustrating the effect of WAY-181187 on 5-HT neurons in rats with either a) sham or b) neurotoxic lesion of the mPFC. Representative rate meter (top traces) and spike train (bottom traces) recordings. Images of cresyl violet stained coronal sections taken from the mPFC of these rats.

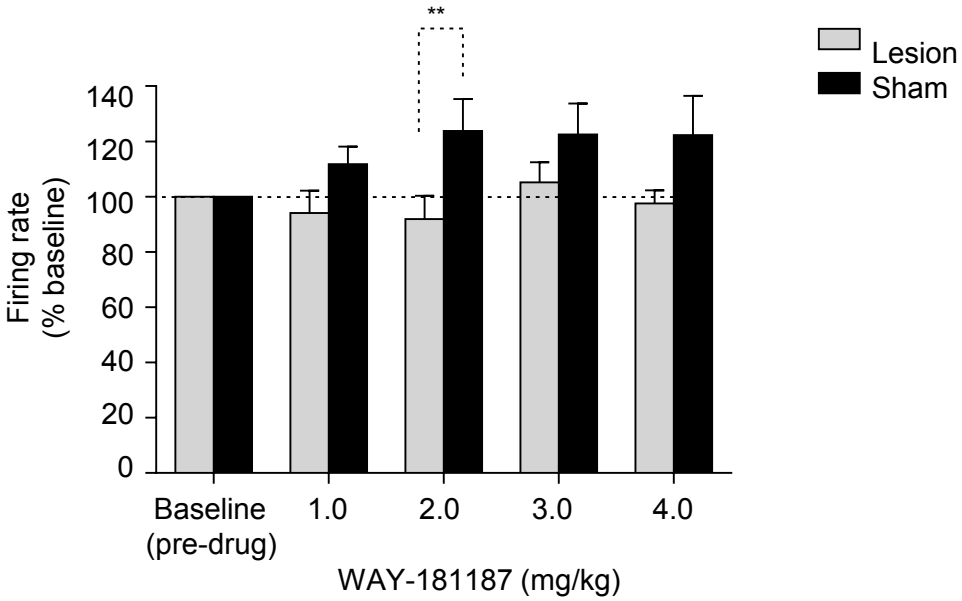


Figure 5.5
Group data demonstrating the effect of WAY-181187 administration on 5-HT neuron firing rate in sham and lesioned rats. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals, **p< 0.01 sham versus lesion (unpaired t-tests). Data expressed as mean ±SEM (sham: n= 5,5,5,4,4; lesion n=6,6,6,4,4 neurons across the doses).

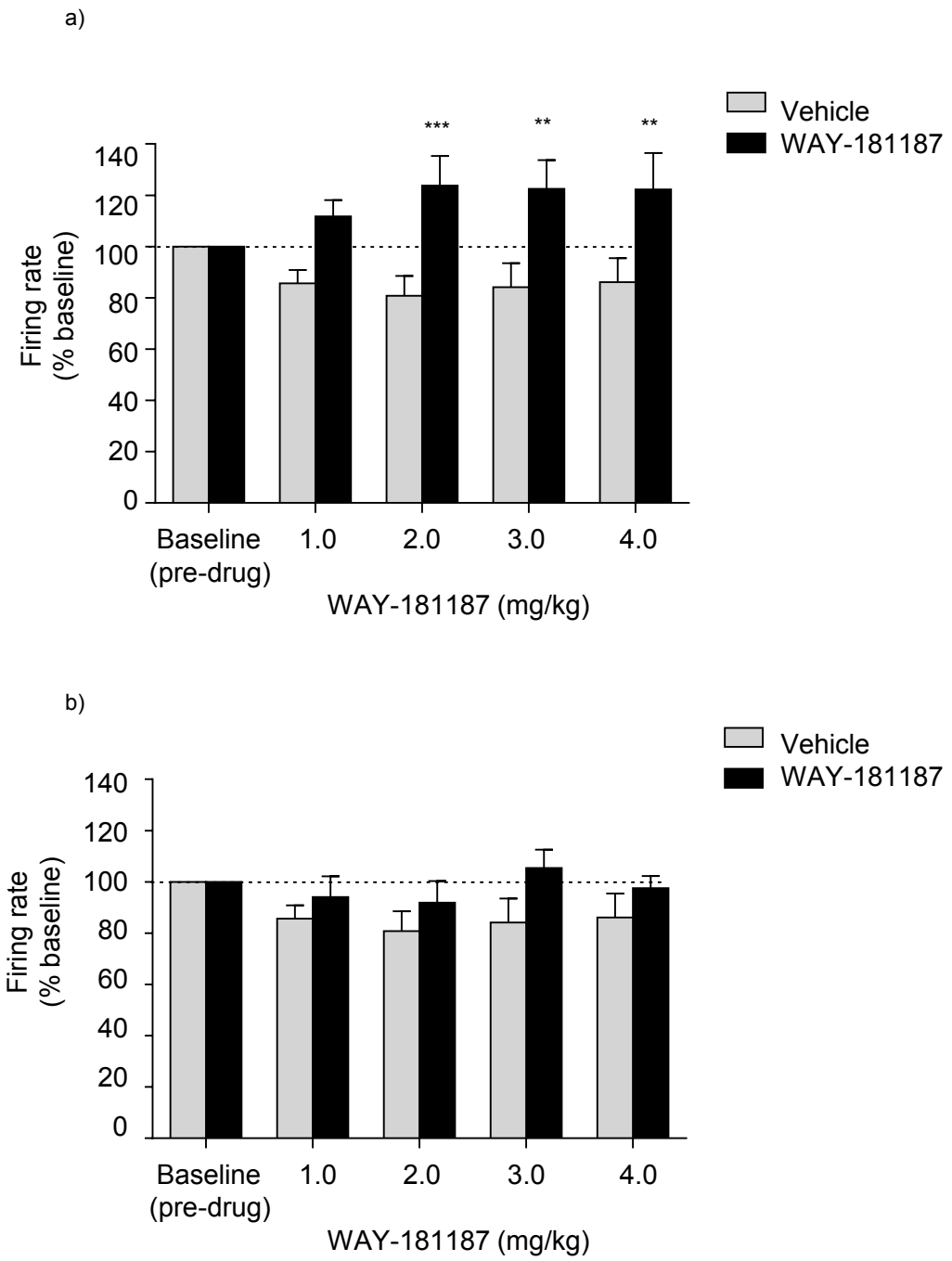


Figure 5.6
Group data demonstrating the effect of WAY-181187 administration on 5-HT neuron firing rate in a) sham and b) lesioned rats compared to a vehicle group. WAY-181187 was administered i.v. in accumulating doses at 2 min intervals, **p< 0.01 ***p<0.001 versus vehicle (unpaired t-tests). Data expressed as mean ± SEM. a) sham: n= 5,5,5,4,4 and b) lesion n=6,6,6,4,4 neurons across the doses; vehicle: n=8 neurons.

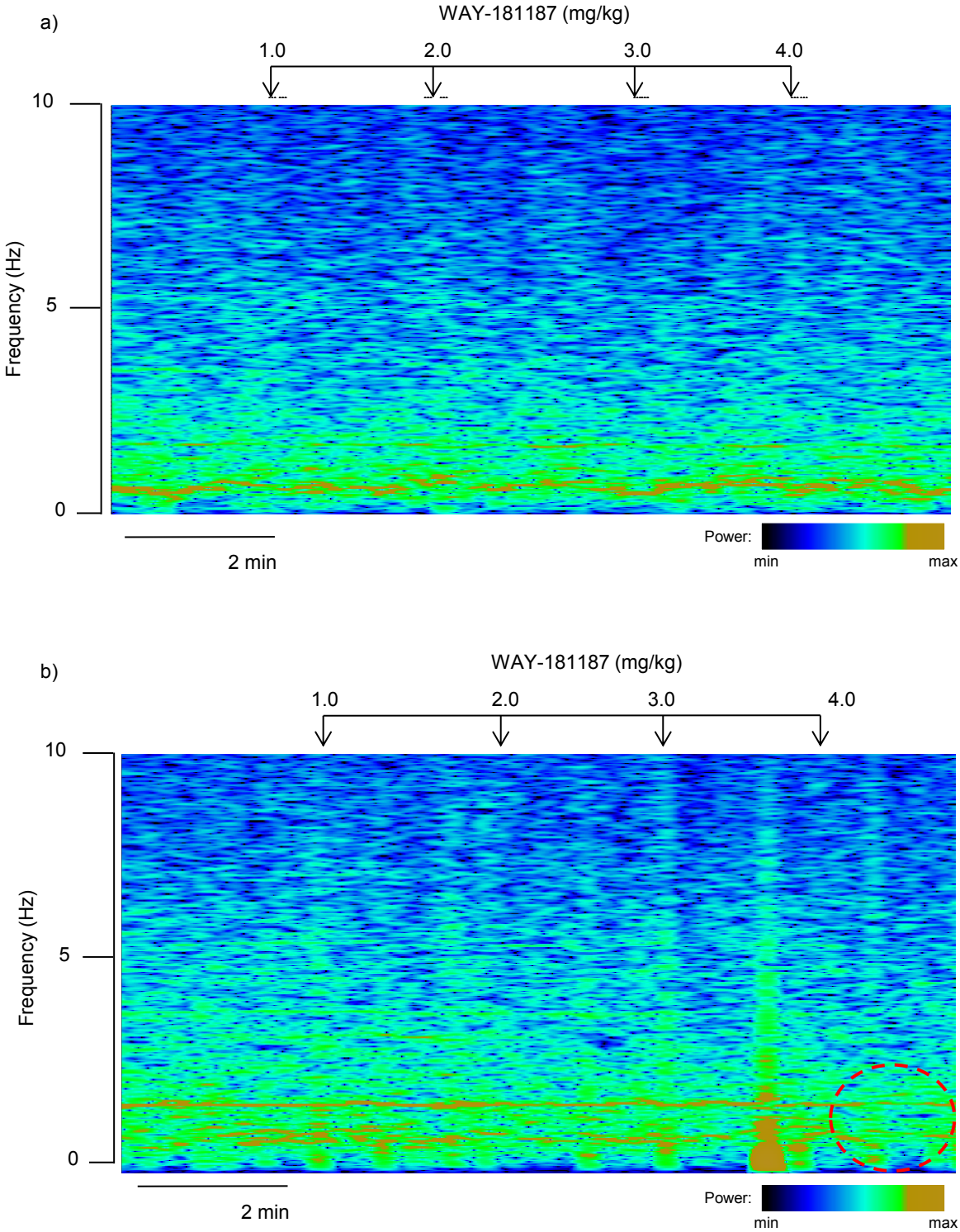


Figure 5.7
Effect of WAY-181187 on cortical SW oscillations in rats with mPFC neurotoxic lesion or sham lesion. Representative spectrograms from single EEG recordings over the frontal cortex during WAY-181187 administration in a) a lesioned rat and b) a sham lesioned rat. Key denotes power with high power orange, moderate power green, lower power blue. Note the higher SW oscillation power between 0.5 and 1.5 Hz which is reduced after WAY-181187 4 mg/kg in b) sham lesioned rat (circled in red).

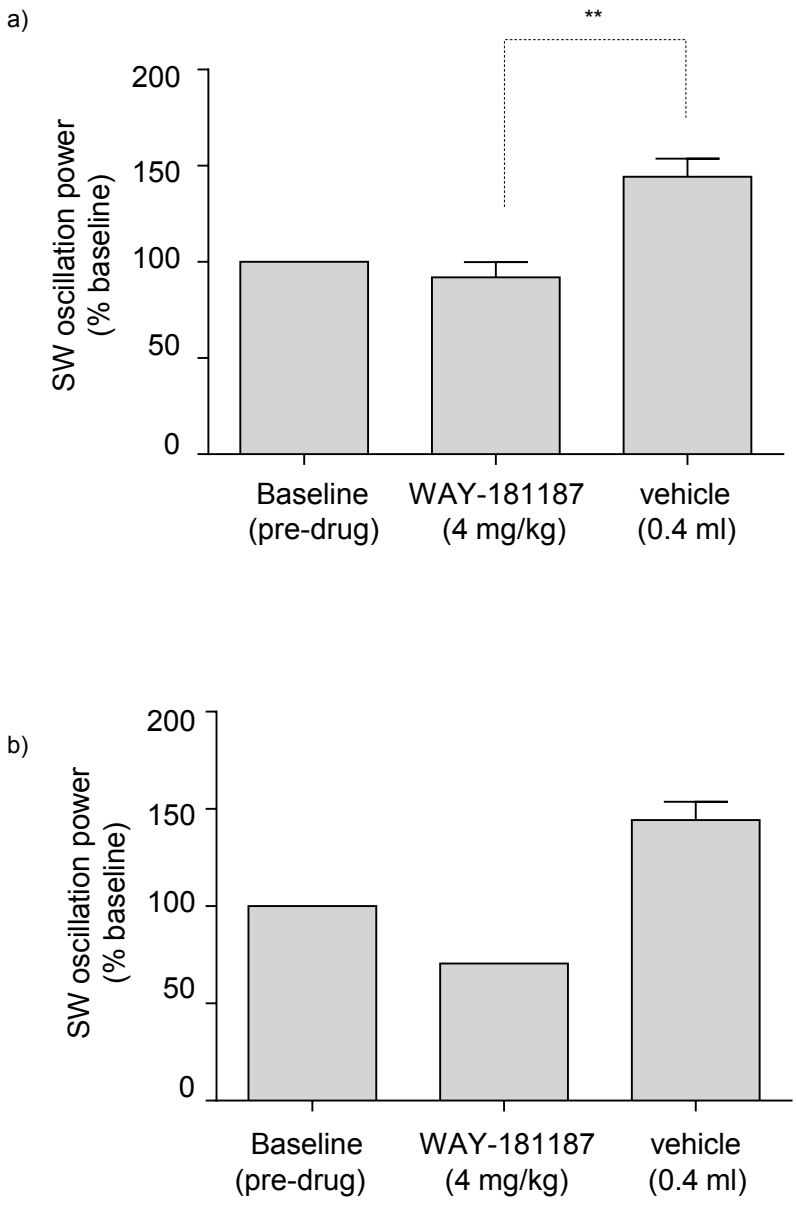


Figure 5.8

Effect of WAY-181187 on cortical SW oscillation in rats with mPFC a) neurotoxic lesion or b) sham lesion. a) Group data demonstrating the effect of WAY-181187 on SW oscillation power in neurotoxin lesioned rats, n=4 rats, ***p< 0.001 versus vehicle groups (n=8 rats). b) Data demonstrating the effect of WAY-181187 on SW oscillation power in a sham lesioned rat (n=1 rat). Data expressed as mean ±SEM.

Chapter 6

General discussion

6.1 Review of thesis background and aims

Evidence suggests that the firing of DRN 5-HT neurons is under strict homeostatic control from 5-HT receptors located on 5-HT neurons themselves, as well as on post-synaptic neurons. Certain 5-HT receptors and their pathways (e.g. 5-HT_{1A}, and 5-HT_{2A} receptors) mediate feedback inhibition of 5-HT neuron activity, whereas others (i.e. 5-HT_4 receptors) mediate feedback excitation. Feedback control of 5-HT neurons originates from 5-HT receptors located in the DRN itself. For instance, 5-HT_{1A} autoreceptors located on 5-HT neurons inhibit 5-HT neuron firing and release (Sprouse and Aghajanian, 1987, Hjorth and Sharp, 1991). In addition, evidence suggests DRN GABA neurons express 5-HT_{2C} receptors which also mediate feedback inhibition of 5-HT neurons (Boothman et al., 2006, Quérée et. al 2009). By contrast, a number of 5-HT feedback pathways originate outside the DRN and some appear to be located in the mPFC. In particular, pathways controlled by postsynaptic 5-HT_{1A} and 5-HT_{2A} receptors in the mPFC are thought to bring about feedback inhibition of DRN 5-HT neurons (Ceci et al., 1994: Hajos et al., 1999; Martin-Ruiz t al., 2001; Boothman et al., 2003; Boothman and Sharp, 2005), whereas 5-HT_4 receptors located in the mPFC are thought to mediate feedback excitation of DRN 5-HT neurons (Ge and Barnes, 1996; Lucas and Debonnel, 2002; Lucas et al., 2005).

At the start of this thesis, preliminary evidence suggested that 5-HT_6 receptors may also exert an influence over 5-HT neuron firing. Thus, administration of the 5-HT_6 receptor antagonist SB-399885 was found to reduce 5-HT neuron firing, whereas the 5-HT_6 receptor agonist WAY-181187 reversed this effect (K. Burnham and P.

Quérée, unpublished data). In addition, experiments in other areas supported the general concept of an interaction between 5-HT_6 receptors and 5-HT neurons (Chapter 1, section 1.6.8). The initial aim of this thesis was to confirm these electrophysiological findings and then extend the pharmacological characterisation of the effect. These experiments also attempted to establish the identity of the recorded neurons using a juxtacellular labelling technique combined with 5-HT-immunocytochemistry. Further work was then planned to investigate the neural substrates involved.

6.2 Summary of key findings

6.2.1 Effect of 5-HT_6 receptor agonist and antagonist administration on 5-HT neuron firing

In support of the earlier findings mentioned above experiments described in Chapter 3 found that the 5-HT_6 receptor agonists WAY-181187 and WAY-208466 increased 5-HT neuron firing, while 5-HT_6 receptor antagonist SB-399885 inhibited firing. A number of neurons were successfully juxtacellular-labelled and found to be 5-HT-immunopositive, confirming the 5-HT identity of the DRN neurons being investigated. It was also found that the 5-HT_6 receptor antagonists SB-271046 and SB-258585 blocked the effect of WAY-181187, indicating that the agonists were acting via a 5-HT_6 receptor-dependent mechanism. However, the fact that the latter 5-HT_6 receptor antagonists, as well as AE-58054, had no effect on 5-HT neuron firing when administered alone, suggested that the decrease in firing indicated by SB-399885 may not involve 5-HT_6 receptors. Taken together, these data support the hypothesis that 5-HT_6 receptors exert an excitatory influence over 5-HT neuron firing, but that the mechanism is not tonically active under the present experimental conditions.

6.2.2 Effect of 5-HT_6 receptor agonist administration on neural activity in the mPFC

Interestingly, it was observed by chance in EEG recordings that simultaneous with the increase in 5-HT neuron firing, both WAY-181187 and WAY-208466 reduced SW oscillations in the frontal cortex. It is known from previous studies that the frontal cortex and the mPFC in particular is rich in 5-HT_6 receptors and exerts an influence over DRN neurons. Thus, experiments in Chapter 4 investigated the effect of 5-HT_6 receptor agonist administration on mPFC activity at both a network and single neuron level, and specifically tested whether this manipulation altered the firing of mPFC neurons projecting to the DRN.

These experiments found that WAY-181187 reduced SW oscillations in the mPFC in a 5-HT_6-dependent manner, since this effect was blocked by AE-58054 and partially blocked by SB-399885. Following these findings it was observed that WAY-181187 altered the firing of individual pyramidal neurons in the mPFC; one population of neurons responded with an increase in firing, while another population responded with a decrease. Juxtacellular labelling confirmed the pyramidal identity of these neurons. Importantly, it was also demonstrated that some of the mPFC pyramidal neurons that responded to WAY-181187 projected to the DRN, as identified through antidromic activation. Thus, it was hypothesised that 5-HT_6 receptor agonist administration not only impacted on neural activity in the mPFC, but also on the mPFC pathway to the DRN.

6.2.3 Effect of 5-HT_6 receptor agonist administration on 5-HT neuron firing following mPFC lesion

A final set of experiments described in Chapter 5 tested the effect of WAY-181187 on the firing of DRN 5-HT neurons in rats with a neurotoxic lesion of the mPFC. It was

found that when compared to sham lesion controls, the excitatory effect of WAY-181187 on 5-HT neuron firing was less in mPFC lesioned animals. This difference was even more marked if data obtained from sham and mPFC lesioned animals were compared with data obtained from vehicle-treated naïve animals. Thus, WAY-181187 clearly increased 5-HT neuron firing in sham lesioned animals compared to vehicle controls, whereas the drug had little effect on 5-HT neuron firing in mPFC lesioned rats compared to vehicle controls.

Overall, the findings reported in Chapter 3, 4 and 5 are consistent with the control of DRN 5-HT neurons by 5-HT_6 receptors acting via an mPFC-dependent mechanism.

6.3 Discussion

Experiments in this thesis provide evidence for the existence of a previously unreported 5-HT_6 receptor-mediated excitatory feedback mechanism governing the activity of DRN 5-HT neurons. Moreover, the data suggest that the mPFC is a key neural substrate in this mechanism. The discussion following addresses some of the wider issues arising from this work.

6.3.1 Limitations of the experimental model in the current study

Electrophysiological recordings in this thesis were obtained in rats under urethane general anaesthesia with maintenance doses of ketamine and xylazine. While this anaesthetic combination is often used during *in vivo* electrophysiology studies of DRN 5-HT neurons, mPFC neurons, and other neuron populations (Klausberger et al., 2003; Ungless et al., 2004; Hartwich et al., 2009; Schweimer et al., 2010;

Schweimer et al., 2011), an important question is whether the anaesthetics influence mPFC or DRN neuronal activity in a way which could confound the effects of 5-HT_6 receptor ligands. There is some evidence to indicate that general anaesthesia can have an impact on 5-HT neuron physiology. For example, *in vitro* recordings from rat DRN slices showed that bath application of urethane, at concentrations equivalent to the doses used to induce general anaesthesia, increased the firing rates of 5-HT neurons. On the other hand, chloral hydrate, another commonly used anaesthetic, enhanced the response of 5-HT neurons to 5-HT (McCardle and Gartside, 2012). Unfortunately, similar work has not been published for mPFC neurons. Nevertheless, this work suggests caution is required when extrapolating the effects observed in anaesthetised rats to awake models.

A number of studies have reported that putative 5-HT neurons in non-anaesthetised rats and a number of other species such as mice and cats, exhibited a slow and regular firing pattern with a broad spike width, as well as sensitivity to 5-HT_{1A} agonists which inhibited firing (Rasmussen et al., 1984; Fornal et al., 1989; Levine and Jacobs, 1992; Gervasoni et al., 2000; Sakai and Crochet, 2001; Guzman-Marin et al., 2001; Sakai, 2011). These findings are very much consistent with the firing properties of 5-HT neurons in rats under anaesthesia reported here. A few studies have reported the firing properties of putative pyramidal neurons in the neocortex of awake rats (Luczak et al., 2007; Vyazovskiy et al., 2009), and they also appear very similar to those in anaesthetised rats described in this thesis and by others (Tierney et al., 2004; Tseng et al., 2006). However, descriptions of the firing properties of pyramidal neurons firing in awake rats are hindered by the uncertainty of the neuron type being recorded (i.e. pyramidal versus interneuron), since awake recordings typically use metal electrodes which do not allow juxtacellular labelling and confirmation of the cell recorded. It may be worth noting that the firing properties of

pyramidal neurons *in vitro* (in the absence of general anaesthetic) are similar to those reported in this thesis. For example, rat and guinea pig neocortical pyramidal neurons showed slow firing rates, broad spike widths, and action potentials in clusters of 5-7 spikes (Connors et al., 1982; McCormick et al., 1985; Amitai and Connors, 1995; Contreras, 2004). Together these studies suggest that the general anaesthetics used in the current experiments have not greatly altered the baseline firing properties of either mPFC pyramidal neurons or DRN 5-HT neurons.

Finally, preliminary data from the current laboratory using chloral hydrate anaesthetised rats (Chapter 1, Figure 1.4; K. Burnham and P. Quérée) found that WAY-181187 and SB-399885 influenced 5-HT neuron firing in a manner similar to that reported in this thesis using urethane anaesthetised rats. This at least shows that the excitatory effect of WAY-181187 on 5-HT neuron firing occurred independently of the type of general anaesthetic used.

6.3.2 Evidence that 5-HT_6 receptor agonists alter mPFC and DRN neural activity in the absence of general anaesthesia

Another consideration is whether the effects of 5-HT_6 receptor agonist administration on DRN and mPFC neural activity would be observed in the absence of general anaesthesia. In support of this idea, WAY-181187 increased Fos immunoreactivity in pyramidal neurons in the mPFC of awake rats (Burnham et al., 2010). This suggests that in the absence of anaesthesia WAY-181187 excites mPFC pyramidal neurons, as observed here in anaesthetised rats. In addition, another study reported that WAY-208466 decreased slow wave sleep measured by frontal cortex EEG recordings in non-anaesthetised rats (Monti et al., 2013). Slow wave sleep is characterised by cortical oscillations around 1 Hz (Azmica and Steriade, 1998), which

are analogous to the SW oscillations occurring under anaesthesia (0.5-1.5 Hz; Chapter 3) that are considered to mimic slow wave sleep (Azmica and Steriade, 1998). Therefore, the findings of Monti et al. support the idea that the decrease in frontal cortex SW oscillations induced by 5-HT_6 receptor agonist administration (Chapters 3 and 4) occur in non-anaesthetised rats.

The effect of WAY-181187 or WAY-208466 on 5-HT neuron firing in awake rats needs to be investigated. Although indirect, it is reported that both WAY-181187 and WAY-208466 have antidepressant effects in rats (Carr et al., 2011), which could reflect increased 5-HT neuron firing. However, a microdialysis study reports that WAY-181187 decreased extracellular 5-HT in the frontal cortex of awake rats (Schechter et al., 2008), which is not consistent with experiments in Chapter 3 showing that WAY-181187 and WAY-208466 increased 5-HT neuron firing. One explanation for this discrepancy is the differences in timescale between the microdialysis and electrophysiological measurements. In particular, the microdialysis study sampled every 20 min and WAY-181187 decreased extracellular 5-HT in the cortex an hour after administration (Schechter et al., 2008). By contrast, WAY-181187 increased 5-HT neuron firing 1-2 min after administration. Thus it is conceivable that the microdialysis study missed a short-lasting increase in 5-HT. It is also conceivable that the increase in 5-HT neuron firing is specific to subpopulations of 5-HT neurons that do not project to the cortex, or that the decrease in extracellular 5-HT is a compensatory response to increased 5-HT neuron firing.

Finally, there is evidence to support the idea that other feedback pathways controlling 5-HT neurons are active in both anaesthetised and awake rats. For example, 5-HT_{1A} receptor agonists inhibit 5-HT neuron firing in anaesthetised rats (Sprouse and

Aghajanian, 1986; Blier and de Montigny, 1987; Sprouse and Aghajanian, 1987; Lum and Piercey, 1988) and decrease extracellular 5-HT levels in forebrain regions in freely-moving rats (Sharp et al., 1989; Casanovas and Artigas, 1996). Moreover, 5-HT_4 receptor agonist administration increases 5-HT neuron firing in anaesthetised rats (Lucas and Debonnel, 2002), as well as extracellular 5-HT in freely-moving rats (Ge and Barnes, 1996).

6.3.3 Neural circuits involved in 5-HT_6 receptor control of 5-HT neurons

Experiments in this thesis support the idea that 5-HT_6 receptors mediate feedback excitation of 5-HT neurons in an mPFC-dependent mechanism. This idea is consistent with evidence that the 5-HT_6 receptors are located on mPFC pyramidal neurons. In particular, *in situ* hybridisation studies demonstrated that 5-HT_6 receptor mRNA is commonly co-localised with vesicular glutamate transporter vGluT1, which is a marker of mPFC pyramidal neurons (Helboe et al., 2015). Moreover, this co-localisation was found across all mPFC layers (Helboe et al., 2015), including deeper layers which project to 5-HT neurons in the DRN (Aghajanian and Wang, 1977; Hajos et al., 1998; Peyron et al., 1998; Vertes, 2004; Ogawa et al., 2014; Pollak Dorocic et al., 2014). Thus, 5-HT_6 receptors located on mPFC pyramidal neurons which make monosynaptic excitatory synapses with 5-HT neurons in the DRN (Pollak Dorocic et al., 2014) might be the circuit through which 5-HT_6 receptors control 5-HT neurons (Figure 6.1).

This putative circuit would be akin to that proposed for 5-HT_4 receptor-mediated excitatory feedback control of 5-HT neurons (Lucas et al., 2005; Sharp et al., 2007). It is even possible that 5-HT_4 and 5-HT_6 receptors co-localise on mPFC pyramidal neurons to jointly control DRN 5-HT neurons. Indeed, the expression of these

receptors in the mPFC overlaps, with 5-HT_4 receptors being located in intermediate layers of the mPFC, and 5-HT_6 receptors are also expressed in this layer (Vilaro et al., 2005; Helboe et al., 2015). It is possible that other 5-HT feedback mechanisms originate from the same populations of mPFC pyramidal neurons. For instance it is reported that all 5-HT_4 receptor-expressing mPFC pyramidal neurons tested (n=16) co-expressed 5-HT_{2A} receptors (Feng et al., 2001). Moreover, 15-30% of mPFC pyramidal neurons expressed three or more 5-HT receptors, including 5-HT_{1A}, 5-HT_{2A}, 5-HT_{1B} and 5-HT_4 receptors (Feng et al., 2001). These findings would suggest that negative and positive feedback mechanisms act through the same mPFC pyramidal neurons to provide a balanced inhibitory and excitatory drive to 5-HT neurons (Feng et al., 2001; Lucas and Debonnel, 2005).

The number of 5-HT_6 receptor-expressing mPFC pyramidal neurons that project directly to DRN 5-HT neurons is likely to be small (~15%, Pollak Dorocic et al., 2014). However, 5-HT_6 receptor-mediated feedback may involve additional circuitry. For example, 5-HT_6 receptors in the mPFC are located on approximately 15% of interneurons. Thus, 5-HT_6 receptor-mediated facilitation of 5-HT neuron firing could be mediated via the mPFC but occur via disinhibition (Figure 6.1). In this scenario, 5-HT_6 receptor-expressing GABA interneurons would provide an inhibitory input to DRN projecting pyramidal neurons (Figure 6.1). Thus, when this population of 5-HT_6 receptors are activated it could lead to reduced excitatory input to DRN GABA neurons (Hajos et al., 1998; Peyron et al., 1998; Varga et al., 2001), and disinhibit DRN 5-HT neurons.

Finally, it is conceivable that 5-HT_6 receptor feedback involves 5-HT_6 receptors located in the DRN itself. 5-HT_6 receptors are thought to reside on non-5-HT neurons

in the DRN (Gerard et al., 1996; Helboe et al., 2015). Moreover, intra-DRN injection of WAY-208466 increased waking in rats, an effect which may involve altered 5-HT neurotransmission (for review see Monti, 2010). However, it is unclear how such a circuitry would be dependent on the mPFC. Alternatively, other 5-HT_6 receptor-expressing forebrain regions may relay effects to the DRN via the mPFC. For example, the hippocampus and striatum are both rich in 5-HT_6 receptors (Helboe et al., 2015). However, such circuitry would involve multiple synapses, which would make for an inefficient feedback control mechanism.

6.3.4 5-HT_6 receptor-mediated feedback in humans

An important question is whether the proposed 5-HT_6 receptor-mediated feedback mechanism is present in humans. Radioligand binding studies in humans reported that the regional distribution of 5-HT_6 receptor is very similar to that in the rat (East et al., 2002; Marazziti et al., 2012; Marazziti et al., 2013). Interestingly, a high level of 5-HT_6 receptor expression is found on mPFC pyramidal neurons in humans, as in the rat (Lorke et al., 2006; Marazziti et al., 2013; Helboe et al., 2015). Moreover, evidence suggests that mPFC-DRN projections are present in non-human primates (Freedman et al., 2000; Chiba et al., 2001), and that DRN 5-HT neurons in humans innervate the prefrontal cortex (Stone et al., 1990; Tork et al., 1999). Thus, there is good support for the idea that a 5-HT_6 receptor-mediated feedback mechanism involving the mPFC is present in the human brain.

6.3.5 5-HT_6 receptor-mediated feedback in neuropsychiatric disease pathogenesis

Abnormal function of receptors involved in feedback control of 5-HT neurons has previously been implicated in the pathogenesis of neuropsychiatric disorders. For

example, mice overexpressing 5-HT_{1A} autoreceptors, where 5-HT_{1A} heteroceptor expression is left unchanged, display depressive-like symptoms such as increased immobility in the forced swim test (Richardson-Jones et al., 2010). Others show that reduced 5-HT_{1A} autoreceptor expression in mice during development leads to increased anxiety and social isolation in adulthood (Donaldson et al., 2014). Finally, evidence from human studies suggests bipolar disorder patients have a higher 5-HT_{1A} autoreceptor density, as shown by PET imaging (Sullivan et al., 2009). Using such work as an exemplar, disruption of a feedback mechanism involving 5-HT_6 receptors could have a role in neuropsychiatric disease pathogenesis.

In this regard, it is interesting that reduced 5-HT_6 receptor expression has been reported by several studies in the frontal and temporal cortices of deceased Alzhemier's patients (Garcia-Alloza et al., 2004; Lorke et al., 2006; Marcos et al., 2008). Moreover, these studies reported an association between reduced cortical 5-HT_6 receptor expression and behavioural symptoms such as aggression and psychosis (Garcia-Alloza et al., 2004; Marcos et al., 2008). Marcos et al. also reported reduced cortical 5-HT levels in Alzheimer's disease patients with reduced 5-HT_6 expression (Marcos et al., 2008). Therefore, it is plausible that reduced cortical 5-HT_6 receptor expression in Alzheimer's disease leads to disruption of feedback control of 5-HT neurons which then contributes to behavioural symptoms. Little is known regarding cortical 5-HT_6 receptors in other diseases, but this may change with the imminent clinical application of 5-HT_6 receptor PET ligands (Tang et al., 2007; Liu et al., 2011; Parker et al., 2012; Becker et al., 2015).

6.3.6 Therapeutic potential of targeting 5-HT_6 receptor-mediated control of 5-HT neurons

Feedback pathways controlling 5-HT release provide an attractive pharmacological target for modulating 5-HT neuronal activity for therapeutic benefits. For example, 5-HT_{1A} receptor antagonists have been shown to be efficacious as adjuvants to SSRIs therapy in depression (Ballesteros et al., 2004; Portella et al., 2011). This mechanism probably involves prevention of feedback inhibition of 5-HT_{1A} autoreceptors mediated by elevated extracellular 5-HT with SSRI treatment, which otherwise is thought to slow down the onset of therapeutic effects of SSRIs (Ballesteros et al., 2004; Portella et al., 2011). The current work suggests that 5-HT_6 receptor agonists could be useful adjuvants to SSRI therapy. In particular, such drugs might counteract the inhibitory effect of SSRIs on 5-HT neuron firing in the early stages of SSRI administration, thereby hastening the onset of antidepressant effects. This idea is further supported by the finding that 5-HT_4 receptor agonists, which also increase 5-HT neuron firing, potentiated the antidepressant effect of SSRIs citalopram and fluvoxamine in antidepressant models (Lucas et al., 2010).

6.4 Future Studies

6.4.1 Determining the site of 5-HT_6 receptors influencing 5-HT neuron firing

Experiments in this thesis identified the mPFC as a key substrate in mediating 5-HT_6 receptor feedback control of 5-HT neurons. However, it is currently unclear whether this control originates in the mPFC, or whether the mPFC is an intermediate. To determine this, in future studies intra-mPFC administration of 5-HT_6 receptor agonist WAY-181187 could be carried out during electrophysiological recording of DRN 5-HT neurons. If the feedback control originated in the mPFC, an increase in 5-HT neuron firing may be expected with intra-mPFC administration of WAY-181187. Retrograde

tracing of monosynaptic DRN inputs, combined with localisation of CNS 5-HT_6 receptors, could subsequently establish whether the control is directly mediated from DRN projecting 5-HT_6 receptor-expressing mPFC pyramidal neurons. Retrograde tracing techniques have been employed previously to map inputs from forebrain regions, including the mPFC to the DRN (Hajos et al., 1998, Peyron et al., 1998). In addition, a recent study provided evidence for the use of *in situ* hybridisation to identify 5-HT_6 receptor mRNA (Helboe et al., 2015).

Alternatively to the above, virally-induced overexpression of 5-HT_4 receptors has previously been used to help identify the site of 5-HT_4 receptors mediating feedback control over 5-HT neurons (Lucas et al., 2005). In this study, herpes simplex virus constructs encoding 5-HT_4 receptors under an immediate early gene promotor were injected into candidate regions involved in feedback control, specifically the mPFC, hippocampus and striatum (Lucas et al., 2005). Overexpression of 5-HT_4 receptors in the mPFC resulted in a large (>70%) increase in 5-HT neuron firing compared to sham controls (Lucas et al., 2005). Accordingly, a similar approach could be used to identify the site of 5-HT_6 receptors which mediate feedback excitation of 5-HT neurons. Thus, a 5-HT_6 receptor-expressing virus construct might be initially directed to the mPFC. The expression of the 5-HT_6 receptors under a promotor such as Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) would restrict expression to pyramidal neurons (Benson et al., 1992; Jones et al., 1994). Subsequent injection sites could include the hippocampus and striatum, which also have high levels of 5-HT_6 receptor expression, as well as the DRN itself, where non-5-HT neurons are thought to express 5-HT_6 receptors (Gerard et al., 1996; Helboe et al., 2015). One limitation of this technique is that the extent to which receptor overexpression impacts on 5-HT neuron firing may not be an accurate reflection of the pathway influence on 5-HT neuron firing under normal receptor expression levels.

6.4.2 Pharmacogenetic manipulation of 5-HT_6 receptor-mediated control of 5-HT neurons

Once the location of 5-HT_6 receptors mediating feedback control is elucidated, it would be interesting to assess the consequences of selective manipulation of the 5-HT_6-DRN feedback mechanism on behaviour. DREADD (designer receptors exclusively activated by designer drugs) technology might be useful in this regard. These receptors are mutated muscarinic receptors engineered to couple to G_i, G_s or G_q proteins, which can be virally expressed in neurons *in vivo*. They are activated by their ligand, clozapine-n-oxide (CNO), which is otherwise pharmacologically inert (Conklin et al., 2008; Pei et al., 2008). Unlike other tools for manipulating neural circuits, such as optogenetics, DREADD manipulation would better resemble the physiology of a putative 5-HT_6-DRN pathway, since DREADDs would activate intracellular signalling mechanisms, and work over a timescale, relevant to GPCR function (Armbruster et al., 2007).

These experiments might use $G_{i/o}$-coupled DREADDs to determine the behavioural consequences of selective abolition of the proposed 5-HT_6 feedback pathway; for example, experiments could determine the effect of such a manipulation on the antidepressant and wake-promoting effects of 5-HT_6 receptor agonists WAY-181187 and WAY-208466. DREADDs have previously been employed for such a purpose. In particular, activation of inhibitory DREADDs expressed in the lateral habenula resulted in an antidepressant-like effect in a behavioural paradigm in rats (Nair et al., 2013). The authors suggested this effect was the consequence of an elevation in 5-HT neuron firing resulting from loss of inhibitory inputs from the LHb, which are

considered to be controlled by 5-HT_{2C} receptor feedback (Nair et al., 2013, Sharp et al., 2007).

6.5 Conclusion

This thesis investigated the control of DRN 5-HT neurons by 5-HT_6 receptors, with the aim of establishing evidence for a novel 5-HT_6 receptor mediated feedback mechanism. Experiments in Chapter 3 found that the 5-HT_6 receptor agonists WAY-181187 and WAY-208466 increased 5-HT neuron firing via a mechanism likely to be 5-HT_6 receptor-mediated. These findings are consistent with the hypothesis that 5-HT_6 receptors exert an excitatory feedback control over 5-HT neurons. During these experiments, simultaneous EEG recordings showed WAY-181187 and WAY-208466 reduced SW oscillations in the frontal cortex. This suggested this region may be involved with the effect of the 5-HT_6 receptor agonists on 5-HT neuron firing. In Chapter 4 recordings in the mPFC revealed that WAY-181187 reduced SW oscillations, an effect blocked by AE-58054 and SB-399885, indicating the involvement of 5-HT_6 receptors. It was also found that WAY-181187 modulated the firing of mPFC pyramidal neurons antidromically activated from the DRN. This provided direct evidence that WAY-181187 acted on mPFC neurons that projected to the DRN, and were likely to then impact on 5-HT neurons.

A final set of experiments described in Chapter 5 found that neurotoxic lesion of the mPFC reduced the excitatory effect of WAY-181187 on 5-HT neurons, compared to sham controls.

Taken together, experiments in this thesis provide evidence in support of the role of a 5-HT_6 receptor-mediated excitatory control of DRN 5-HT neurons via an mPFC-dependent mechanism. This appears to share characteristics with the reported excitatory 5-HT feedback mediated by 5-HT_4 receptors. Moreover, these excitatory feedback mechanisms may act to balance the inhibitory feedback control of 5-HT neurons exerted via 5-HT_{1A}-, 5-HT_{2A}- and 5-HT_{2C} receptors. 5-HT_6 receptor-mediated feedback control of 5-HT neurons may have relevance to the pathophysiology and treatment of various neuropsychiatric disorders.

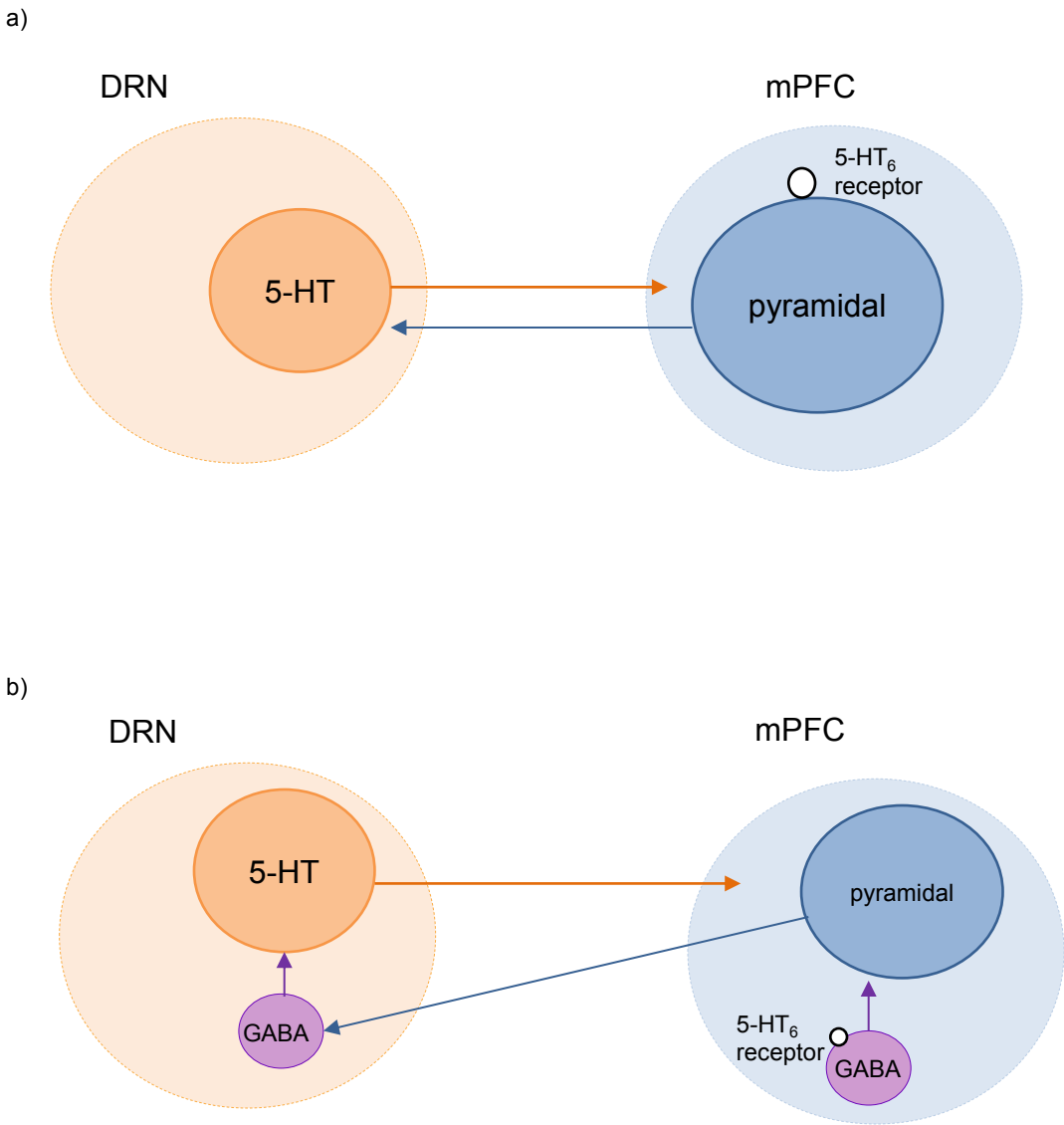


Figure 6.1
Putative mechanisms of mPFC-dependent 5-HT_6 receptor-mediated control of 5-HT neurons. a) 5-HT_6 receptors may be expressed on mPFC pyramidal neurons which form monosynaptic, excitatory connections with DRN 5-HT neurons. b) 5-HT_6 receptors on mPFC GABAergic interneurons may influence DRN 5-HT neurons via a disinhibition mechanism involving mPFC pyramidal neurons and DRN GABAergic interneurons as intermediates.

Bibliography

Adell, A., Carceller, A., & Artigas, F. (1993). In vivo brain dialysis study of the somatodendritic release of serotonin in the Raphe nuclei of the rat: effects of 8-hydroxy-2-(di-n-propylamino)tetralin. *J Neurochem, 60*(5), 1673-1681.

Aghajanian, G. K. (1972). Influence of drugs on the firing of serotonin-containing neurons in brain. *Fed Proc, 31*(1), 91-96.

Aghajanian, G. K. (1982). Regulation of serotonergic neuronal activity: autoreceptors and pacemaker potentials. *Adv Biochem Psychopharmacol, 34*, 173-181.

Aghajanian, G. K. (1985). Modulation of a transient outward current in serotonergic neurones by alpha 1-adrenoceptors. *Nature, 315*(6019), 501-503.

Aghajanian, G. K., Foote, W. E., & Sheard, M. H. (1968). Lysergic acid diethylamide: sensitive neuronal units in the midbrain raphe. *Science, 161*(3842), 706-708.

Aghajanian, G. K., Graham, A. W., & Sheard, M. H. (1970). Serotonin-containing neurons in brain: depression of firing by monoamine oxidase inhibitors. *Science, 169*(3950), 1100-1102.

Aghajanian, G. K., & Haigler, H. J. (1974). L-Tryptophan as a selective histochemical marker for serotonergic neurons in single-cell recording studies. *Brain Res, 81*(2), 364-372.

Aghajanian, G. K., & Vandermaelen, C. P. (1982). Intracellular recording in vivo from serotonergic neurons in the rat dorsal raphe nucleus: methodological considerations. *J Histochem Cytochem, 30*(8), 813-814.

Aghajanian, G. K., & Wang, R. Y. (1977). Habenular and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique. *Brain Res, 122*(2), 229-242.

Aghajanian, G. K., Wang, R. Y., & Baraban, J. (1978). Serotonergic and non-serotonergic neurons of the dorsal raphe: reciprocal changes in firing induced by peripheral nerve stimulation. *Brain Res, 153*(1), 169-175.

Aitken, A. R., & Törk, I. (1988). Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. *J Comp Neurol, 274*(1), 32-47. doi: 10.1002/cne.902740105

Allers, K. A., & Sharp, T. (2003). Neurochemical and anatomical identification of fast- and slow-firing neurones in the rat dorsal raphe nucleus using juxtacellular labelling methods in vivo. *Neuroscience, 122*(1), 193-204.

Arnt, J., Bang-Andersen, B., Grayson, B., Bymaster, F. P., Cohen, M. P., DeLapp, N. W., . . . Witten, L. M. (2010). Lu AE58054, a 5-HT6 antagonist, reverses cognitive impairment induced by subchronic phencyclidine in a novel object recognition test in rats. *Int J Neuropsychopharmacol, 13*(8), 1021-1033. doi: 10.1017/S1461145710000659

Asaoka, N., Nagayasu, K., Nishitani, N., Yamashiro, M., Shirakawa, H., Nakagawa, T., & Kaneko, S. (2015). Olanzapine augments the effect of selective serotonin reuptake inhibitors by suppressing GABAergic inhibition via antagonism of 5-HT6 receptors in the dorsal raphe nucleus. *Neuropharmacology*. doi: 10.1016/j.neuropharm.2015.03.032

Azmitia, E. C., & Segal, M. (1978). An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. *J Comp Neurol, 179*(3), 641-667. doi: 10.1002/cne.901790311

Bagdy, E., Solyom, S., & Harsing, L. G. (1998). Feedback stimulation of somatodendritic serotonin release: a 5-HT3 receptor-mediated effect in the raphe nuclei of the rat. *Brain Res Bull, 45*(2), 203-208.

Baker, K. G., Halliday, G. M., & Törk, I. (1990). Cytoarchitecture of the human dorsal raphe nucleus. *J Comp Neurol, 301*(2), 147-161. doi: 10.1002/cne.903010202

Ballesteros, J., & Callado, L. F. (2004). Effectiveness of pindolol plus serotonin uptake inhibitors in depression: a meta-analysis of early and late outcomes from

randomised controlled trials. *J Affect Disord, 79*(1-3), 137-147. doi: 10.1016/S0165-0327(02)00404-4

Baraban, J. M., & Aghajanian, G. K. (1981). Noradrenergic innervation of serotonergic neurons in the dorsal raphe: demonstration by electron microscopic autoradiography. *Brain Res, 204*(1), 1-11.

Barnes, N. M., & Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology, 38*(8), 1083-1152.

Becker, G., Colomb, J., Sgambato-Faure, V., Tremblay, L., Billard, T., & Zimmer, L. (2015). Preclinical evaluation of [18F]2FNQ1P as the first fluorinated serotonin 5-HT6 radioligand for PET imaging. *Eur J Nucl Med Mol Imaging, 42*(3), 495-502. doi: 10.1007/s00259-014-2936-y

Becquet, D., Faudon, M., & Hery, F. (1990). The role of serotonin release and autoreceptors in the dorsalis raphe nucleus in the control of serotonin release in the cat caudate nucleus. *Neuroscience, 39*(3), 639-647.

Behzadi, G., Kalén, P., Parvopassu, F., & Wiklund, L. (1990). Afferents to the median raphe nucleus of the rat: retrograde cholera toxin and wheat germ conjugated horseradish peroxidase tracing, and selective D-[3H]aspartate labelling of possible excitatory amino acid inputs. *Neuroscience, 37*(1), 77-100.

Belin, M. F., Aguera, M., Tappaz, M., McRae-Degueurce, A., Bobillier, P., & Pujol, J. F. (1979). GABA-accumulating neurons in the nucleus raphe dorsalis and periaqueductal gray in the rat: a biochemical and radioautographic study. *Brain Res, 170*(2), 279-297.

Benhamú, B., Martín-Fontecha, M., Vázquez-Villa, H., Pardo, L., & López-Rodríguez, M. L. (2014). Serotonin 5-HT6 Receptor Antagonists for the Treatment of Cognitive Deficiency in Alzheimer's Disease. *J Med Chem*. doi: 10.1021/jm5003952

Benson, D. L., Isackson, P. J., Gall, C. M., & Jones, E. G. (1992). Contrasting patterns in the localization of glutamic acid decarboxylase and Ca2+/calmodulin protein kinase gene expression in the rat central nervous system. *Neuroscience, 46*(4), 825-849.

Berger, B., Trottier, S., Verney, C., Gaspar, P., & Alvarez, C. (1988). Regional and laminar distribution of the dopamine and serotonin innervation in the macaque cerebral cortex: a radioautographic study. *J Comp Neurol, 273*(1), 99-119. doi: 10.1002/cne.902730109

Blier, P., & Bouchard, C. (1993). Functional characterization of a 5-HT3 receptor which modulates the release of 5-HT in the guinea-pig brain. *Br J Pharmacol, 108*(1), 13-22.

Blier, P., & Bouchard, C. (1994). Modulation of 5-HT release in the guinea-pig brain following long-term administration of antidepressant drugs. *Br J Pharmacol, 113*(2), 485-495.

Blier, P., & de Montigny, C. (1994). Current advances and trends in the treatment of depression. *Trends Pharmacol Sci, 15*(7), 220-226.

Blier, P., Lista, A., & De Montigny, C. (1993). Differential properties of pre- and postsynaptic 5-hydroxytryptamine1A receptors in the dorsal raphe and hippocampus: I. Effect of spiperone. *J Pharmacol Exp Ther, 265*(1), 7-15.

Boothman, L. J., Allers, K. A., Rasmussen, K., & Sharp, T. (2003). Evidence that central 5-HT2A and 5-HT2B/C receptors regulate 5-HT cell firing in the dorsal raphe nucleus of the anaesthetised rat. *Br J Pharmacol, 139*(5), 998-1004. doi: 10.1038/sj.bjp.0705328

Boothman, L. J., & Sharp, T. (2005). A role for midbrain raphe gamma aminobutyric acid neurons in 5-hydroxytryptamine feedback control. *Neuroreport, 16*(9), 891-896.

Boothman, L., Raley, J., Denk, F., Hirani, E., & Sharp, T. (2006). In vivo evidence that 5-HT(2C) receptors inhibit 5-HT neuronal activity via a GABAergic mechanism. *Br J Pharmacol, 149*(7), 861-869. doi: 10.1038/sj.bjp.0706935

Borsini, F., Bordi, F., Poggi, A., & Di Matteo, V. (2015). Effects of ST1936, a selective serotonin-6 agonist, on electrical activity of putative mesencephalic dopaminergic neurons in the rat brain. *J Psychopharmacol*. doi: 10.1177/0269881115573804

Borsini, F., Bordi, F., & Riccioni, T. (2011). 5-HT6 pharmacology inconsistencies. *Pharmacol Biochem Behav, 98*(2), 169-172. doi: 10.1016/j.pbb.2010.12.021

Borsini, F., Ceci, A., Bietti, G., & Donetti, A. (1995). BIMT 17, a 5-HT1A receptor agonist/5-HT2A receptor antagonist, directly activates postsynaptic 5-HT inhibitory responses in the rat cerebral cortex. *Naunyn Schmiedebergs Arch Pharmacol, 352*(3), 283-290.

Boschert, U., Amara, D. A., Segu, L., & Hen, R. (1994). The mouse 5-hydroxytryptamine1B receptor is localized predominantly on axon terminals. *Neuroscience, 58*(1), 167-182.

Brinschwitz, K., Dittgen, A., Madai, V. I., Lommel, R., Geisler, S., & Veh, R. W. (2010). Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. *Neuroscience, 168*(2), 463-476. doi: 10.1016/j.neuroscience.2010.03.050

Bruinvels, A. T., Landwehrmeyer, B., Gustafson, E. L., Durkin, M. M., Mengod, G., Branchek, T. A., . . . Palacios, J. M. (1994). Localization of 5-HT1B, 5-HT1D alpha, 5-HT1E and 5-HT1F receptor messenger RNA in rodent and primate brain. *Neuropharmacology, 33*(3-4), 367-386.

Bunin, M. A., & Wightman, R. M. (1998). Quantitative evaluation of 5-hydroxytryptamine (serotonin) neuronal release and uptake: an investigation of extrasynaptic transmission. *J Neurosci, 18*(13), 4854-4860.

Burnham, K. E., Baxter, M. G., Bainton, J. R., Southam, E., Dawson, L. A., Bannerman, D. M., & Sharp, T. (2010). Activation of 5-HT(6) receptors facilitates attentional set shifting. *Psychopharmacology (Berl), 208*(1), 13-21. doi: 10.1007/s00213-009-1701-6

Buzsáki, G. (2006). *Rhythms of the brain*. Oxford: Oxford University Press.

Carr, G. V., Schechter, L. E., & Lucki, I. (2011). Antidepressant and anxiolytic effects of selective 5-HT6 receptor agonists in rats. *Psychopharmacology (Berl), 213*(2-3), 499-507. doi: 10.1007/s00213-010-1798-7

Casanovas, J. M., & Artigas, F. (1996). Differential effects of ipsapirone on 5-hydroxytryptamine release in the dorsal and median raphe neuronal pathways. *J Neurochem, 67*(5), 1945-1952.

Ceci, A., Baschirotto, A., & Borsini, F. (1994). The inhibitory effect of 8-OH-DPAT on the firing activity of dorsal raphe serotoninergic neurons in rats is attenuated by lesion of the frontal cortex. *Neuropharmacology, 33*(5), 709-713.

Celada, P., Puig, M. V., Casanovas, J. M., Guillazo, G., & Artigas, F. (2001). Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. *J Neurosci, 21*(24), 9917-9929.

Chaput, Y., de Montigny, C., & Blier, P. (1986). Effects of a selective 5-HT reuptake blocker, citalopram, on the sensitivity of 5-HT autoreceptors: electrophysiological studies in the rat brain. *Naunyn Schmiedebergs Arch Pharmacol, 333*(4), 342-348.

Chaput, Y., de Montigny, C., & Blier, P. (1991). Presynaptic and postsynaptic modifications of the serotonin system by long-term administration of antidepressant treatments. An in vivo electrophysiologic study in the rat. *Neuropsychopharmacology, 5*(4), 219-229.

Ciranna, L. (2006). Serotonin as a modulator of glutamate- and GABA-mediated neurotransmission: implications in physiological functions and in pathology. *Curr Neuropharmacol, 4*(2), 101-114.

Clemett, D. A., Punhani, T., Duxon, M. S., Blackburn, T. P., & Fone, K. C. (2000). Immunohistochemical localisation of the 5-HT2C receptor protein in the rat CNS. *Neuropharmacology, 39*(1), 123-132.

Contreras, D., & Steriade, M. (1995). Cellular basis of EEG slow rhythms: a study of dynamic corticothalamic relationships. *J Neurosci, 15*(1 Pt 2), 604-622.

Corradetti, R., Pugliese, A. M., Le Poul, E., Laaris, N., Hamon, M., & Lanfumey, L. (1996). Electrophysiological effects of WAY 100635, a new 5-HT1A receptor antagonist, on dorsal raphe nucleus serotoninergic neurones and CA1 pyramidal cells in vitro. *Acta Physiol Hung, 84*(4), 407-409.

Cummings, J. L. (1993). Frontal-subcortical circuits and human behavior. *Arch Neurol, 50*(8), 873-880.

Czachura, J. F., & Rasmussen, K. (2000). Effects of acute and chronic administration of fluoxetine on the activity of serotonergic neurons in the dorsal raphe nucleus of the rat. *Naunyn Schmiedebergs Arch Pharmacol, 362*(3), 266-275.

DAHLSTROEM, A., & FUXE, K. (1964). EVIDENCE FOR THE EXISTENCE OF MONOAMINE-CONTAINING NEURONS IN THE CENTRAL NERVOUS SYSTEM. I. DEMONSTRATION OF MONOAMINES IN THE CELL BODIES OF BRAIN STEM NEURONS. *Acta Physiol Scand Suppl*, SUPPL 232:231-255.

Dawson, L. A., Nguyen, H. Q., & Li, P. (2001). The 5-HT(6) receptor antagonist SB-271046 selectively enhances excitatory neurotransmission in the rat frontal cortex and hippocampus. *Neuropsychopharmacology, 25*(5), 662-668. doi: 10.1016/S0893-133X(01)00265-2

de Bruin, N. M., Kloeze, B. M., & McCreary, A. C. (2011). The 5-HT_6 serotonin receptor antagonist SB-271046 attenuates the development and expression of nicotine-induced locomotor sensitisation in Wistar rats. *Neuropharmacology, 61*(3), 451-457. doi: 10.1016/j.neuropharm.2011.01.045

de Oliveira, R. C., de Oliveira, R., Biagioni, A. F., Falconi-Sobrinho, L. L., & Coimbra, N. C. (2016). Dorsal raphe nucleus acetylcholine-mediated neurotransmission modulates post-ictal antinociception: The role of muscarinic and nicotinic cholinergic receptors. *Brain Res, 1631*, 80-91. doi: 10.1016/j.brainres.2015.11.014

Descarries, L., Audet, M. A., Doucet, G., Garcia, S., Oleskevich, S., Séguéla, P., . . . Watkins, K. C. (1990). Morphology of central serotonin neurons. Brief review of quantified aspects of their distribution and ultrastructural relationships. *Ann N Y Acad Sci, 600*, 81-92.

Descarries, L., Beaudet, A., & Watkins, K. C. (1975). Serotonin nerve terminals in adult rat neocortex. *Brain Res, 100*(3), 563-588.

Descarries, L., Watkins, K. C., Garcia, S., & Beaudet, A. (1982). The serotonin neurons in nucleus raphe dorsalis of adult rat: a light and electron microscope radioautographic study. *J Comp Neurol, 207*(3), 239-254. doi: 10.1002/cne.902070305

Doucet, E., Pohl, M., Fattaccini, C. M., Adrien, J., Mestikawy, S. E., & Hamon, M. (1995). In situ hybridization evidence for the synthesis of 5-HT1B receptor in serotoninergic neurons of anterior raphe nuclei in the rat brain. *Synapse, 19*(1), 18-28. doi: 10.1002/syn.890190104

Dougalis, A. G., Matthews, G. A., Bishop, M. W., Brischoux, F., Kobayashi, K., & 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*(10), 3322-3332. doi: 10.1111/j.1460-9568.2012.08255.x

Dray, A., Davies, J., Oakley, N. R., Tongroach, P., & Vellucci, S. (1978). The dorsal and medial raphe projections to the substantia nigra in the rat: electrophysiological, biochemical and behavioural observations. *Brain Res, 151*(3), 431-442.

Duncan, M. J., Jennes, L., Jefferson, J. B., & Brownfield, M. S. (2000). Localization of serotonin(5A) receptors in discrete regions of the circadian timing system in the Syrian hamster. *Brain Res, 869*(1-2), 178-185.

Dupuis, D. S., Mannoury la Cour, C., Chaput, C., Verrièle, L., Lavielle, G., & Millan, M. J. (2008). Actions of novel agonists, antagonists and antipsychotic agents at recombinant rat 5-HT6 receptors: a comparative study of coupling to G alpha s. *Eur J Pharmacol, 588*(2-3), 170-177. doi: 10.1016/j.ejphar.2008.04.039

East, S. Z., Burnet, P. W., Leslie, R. A., Roberts, J. C., & Harrison, P. J. (2002). 5-HT6 receptor binding sites in schizophrenia and following antipsychotic drug administration: autoradiographic studies with [125I]SB-258585. *Synapse, 45*(3), 191-199. doi: 10.1002/syn.10097

ERSPAMER, V., & ASERO, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature, 169*(4306), 800-801.

Eskenazi, D., Brodsky, M., & Neumaier, J. F. (2015). Deconstructing 5-HT6 receptor effects on striatal circuit function. *Neuroscience, 299*, 97-106. doi: 10.1016/j.neuroscience.2015.04.046

Farnebo, L. O., & Hamberger, B. (1971). Drug-induced changes in the release of 3 H-monoamines from field stimulated rat brain slices. *Acta Physiol Scand Suppl, 371*, 35-44.

Feighner, J. P. (1987). Buspirone in the long-term treatment of generalized anxiety disorder. *J Clin Psychiatry, 48 Suppl*, 3-6.

Ferguson, S. M., Mitchell, E. S., & Neumaier, J. F. (2008). Increased expression of 5-HT6 receptors in the nucleus accumbens blocks the rewarding but not psychomotor activating properties of cocaine. *Biol Psychiatry, 63*(2), 207-213. doi: 10.1016/j.biopsych.2007.02.018

Fornal, C. A., Litto, W. J., Morilak, D. A., & Jacobs, B. L. (1989). Single-unit responses of serotonergic neurons to glucose and insulin administration in behaving cats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 257*(6), R1345-R1353.

Frazer A, Hensler JG. (1999). Serotonin. In A. B. Siegel GJ, Albers RW, et al. (Ed.), *Basic Neurochemistry: Molecular, Cellular and Medical Aspects.* (6th edition ed.). Philadelphia: Lippincott-Raven.

Gallager, D. W., & Aghajanian, G. K. (1976). Effect of antipsychotic drugs on the firing of dorsal raphe cells. I. Role of adrenergic system. *Eur J Pharmacol, 39*(2), 341-355.

Garratt, J. C., Kidd, E. J., Wright, I. K., & Marsden, C. A. (1991). Inhibition of 5-hydroxytryptamine neuronal activity by the 5-HT agonist, DOI. *Eur J Pharmacol, 199*(3), 349-355.

Gartside, S. E., Umbers, V., & Sharp, T. (1997). Inhibition of 5-HT cell firing in the DRN by non-selective 5-HT reuptake inhibitors: studies on the role of 5-HT1A autoreceptors and noradrenergic mechanisms. *Psychopharmacology (Berl), 130*(3), 261-268.

Ge, J., & Barnes, N. M. (1996). 5-HT4 receptor-mediated modulation of 5-HT release in the rat hippocampus in vivo. *Br J Pharmacol, 117*(7), 1475-1480.

Gervasoni, D., Peyron, C., Rampon, C., Barbagli, B., Chouvet, G., Urbain, N., . . . Luppi, P. H. (2000). Role and origin of the GABAergic innervation of dorsal raphe serotonergic neurons. *J Neurosci, 20*(11), 4217-4225.

Gravius, A., Laszy, J., Pietraszek, M., Sághy, K., Nagel, J., Chambon, C., . . . Gyertyán, I. (2011). Effects of 5-HT6 antagonists, Ro-4368554 and SB-258585, in tests used for the detection of cognitive enhancement and antipsychotic-like activity. *Behav Pharmacol, 22*(2), 122-135. doi: 10.1097/FBP.0b013e328343d804

Guilloux, J. P., David, D. J., Xia, L., Nguyen, H. T., Rainer, Q., Guiard, B. P., . . . Gardier, A. M. (2011). Characterization of 5-HT(1A/1B)-/- mice: an animal model sensitive to anxiolytic treatments. *Neuropharmacology, 61*(3), 478-488. doi: 10.1016/j.neuropharm.2011.02.009

Gustafson, E. L., Durkin, M. M., Bard, J. A., Zgombick, J., & Branchek, T. A. (1996). A receptor autoradiographic and in situ hybridization analysis of the distribution of the 5-ht7 receptor in rat brain. *Br J Pharmacol, 117*(4), 657-666.

Guzmán-Marín, Rubén, Alam, Md Noor, Mihailescu, Stefan, Szymusiak, Ron, McGinty, Dennis, & Drucker-Colín, René. (2001). Subcutaneous administration of nicotine changes dorsal raphe serotonergic neurons discharge rate during REM sleep. *Brain Research, 888*(2), 321-325. doi: http://dx.doi.org/10.1016/S0006-8993(00)03104-8

Gérard, C., el Mestikawy, S., Lebrand, C., Adrien, J., Ruat, M., Traiffort, E., . . . Martres, M. P. (1996). Quantitative RT-PCR distribution of serotonin 5-HT6 receptor mRNA in the central nervous system of control or 5,7-dihydroxytryptamine-treated rats. *Synapse, 23*(3), 164-173. doi: 10.1002/(SICI)1098-2396(199607)23:3<164::AID-SYN5>3.0.CO;2-6

Gérard, C., Martres, M. P., Lefèvre, K., Miquel, M. C., Vergé, D., Lanfumey, L., . . . el Mestikawy, S. (1997). Immuno-localization of serotonin 5-HT6 receptor-like material in the rat central nervous system. *Brain Res, 746*(1-2), 207-219.

Göthert, M. (2013). Serotonin discovery and stepwise disclosure of 5-HT receptor complexity over four decades. Part I. General background and discovery of serotonin as a basis for 5-HT receptor identification. *Pharmacol Rep, 65*(4), 771-786.

Göthert, M., & Weinheimer, G. (1979). Extracellular 5-hydroxytryptamine inhibits 5-hydroxytryptamine release from rat brain cortex slices. *Naunyn Schmiedebergs Arch Pharmacol, 310*(1), 93-96.

Hajós, M., Allers, K. A., Jennings, K., Sharp, T., Charette, G., Sík, A., & Kocsis, B. (2007). Neurochemical identification of stereotypic burst-firing neurons in the rat dorsal raphe nucleus using juxtacellular labelling methods. *Eur J Neurosci, 25*(1), 119-126. doi: 10.1111/j.1460-9568.2006.05276.x

Hajós, M., Gartside, S. E., Varga, V., & Sharp, T. (2003). In vivo inhibition of neuronal activity in the rat ventromedial prefrontal cortex by midbrain-raphe nuclei: role of 5-HT1A receptors. *Neuropharmacology, 45*(1), 72-81.

Hajós, M., Gartside, S. E., Villa, A. E., & Sharp, T. (1995). Evidence for a repetitive (burst) firing pattern in a sub-population of 5-hydroxytryptamine neurons in the dorsal and median raphe nuclei of the rat. *Neuroscience, 69*(1), 189-197.

Hajós, M., Hajós-Korcsok, E., & Sharp, T. (1999). Role of the medial prefrontal cortex in 5-HT1A receptor-induced inhibition of 5-HT neuronal activity in the rat. *Br J Pharmacol, 126*(8), 1741-1750. doi: 10.1038/sj.bjp.0702510

Hajós, M., Richards, C. D., Székely, A. D., & Sharp, T. (1998). An electrophysiological and neuroanatomical study of the medial prefrontal cortical projection to the midbrain raphe nuclei in the rat. *Neuroscience, 87*(1), 95-108.

Hajós, M., & Sharp, T. (1996). Burst-firing activity of presumed 5-HT neurones of the rat dorsal raphe nucleus: electrophysiological analysis by antidromic stimulation. *Brain Res, 740*(1-2), 162-168.

Harsing, L. G., Prauda, I., Barkoczy, J., Matyus, P., & Juranyi, Z. (2004). A 5-HT7 heteroreceptor-mediated inhibition of [3H]serotonin release in raphe nuclei slices of the rat: evidence for a serotonergic-glutamatergic interaction. *Neurochem Res, 29*(8), 1487-1497.

Hauser, S. R., Hedlund, P. B., Roberts, A. J., Sari, Y., Bell, R. L., & Engleman, E. A. (2014). The 5-HT7 receptor as a potential target for treating drug and alcohol abuse. *Front Neurosci, 8*, 448. doi: 10.3389/fnins.2014.00448

Heal, D. J., Smith, S. L., Fisas, A., Codony, X., & Buschmann, H. (2008). Selective 5-HT6 receptor ligands: progress in the development of a novel pharmacological approach to the treatment of obesity and related metabolic disorders. *Pharmacol Ther, 117*(2), 207-231. doi: 10.1016/j.pharmthera.2007.08.006

Heisler, L. K., Chu, H. M., Brennan, T. J., Danao, J. A., Bajwa, P., Parsons, L. H., & Tecott, L. H. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proc Natl Acad Sci U S A, 95*(25), 15049-15054.

Helboe, L., Egebjerg, J., & de Jong, I. E. (2015). Distribution of serotonin receptor 5-HT6 mRNA in rat neuronal subpopulations: A double in situ hybridization study. *Neuroscience, 310*, 442-454. doi: 10.1016/j.neuroscience.2015.09.064

Hery, F., Faudon, M., & Fueri, C. (1986). Release of serotonin in structures containing serotoninergic nerve cell bodies: dorsalis raphe nucleus and nodose ganglia of the cat. *Ann N Y Acad Sci, 473*, 239-255.

Higgs, S., Cooper, A. J., & Barnes, N. M. (2016). The 5-HT_2C receptor agonist, lorcaserin, and the 5-HT_6 receptor antagonist, SB-742457, promote satiety; a microstructural analysis of feeding behaviour. *Psychopharmacology (Berl), 233*(3), 417-424. doi: 10.1007/s00213-015-4112-x

Hirano, K., Piers, T. M., Searle, K. L., Miller, N. D., Rutter, A. R., & Chapman, P. F. (2009). Procognitive 5-HT6 antagonists in the rat forced swimming test: potential

therapeutic utility in mood disorders associated with Alzheimer's disease. *Life Sci, 84*(15-16), 558-562. doi: 10.1016/j.lfs.2009.01.019

Hirst, W. D., Abrahamsen, B., Blaney, F. E., Calver, A. R., Aloj, L., Price, G. W., & Medhurst, A. D. (2003). Differences in the central nervous system distribution and pharmacology of the mouse 5-hydroxytryptamine-6 receptor compared with rat and human receptors investigated by radioligand binding, site-directed mutagenesis, and molecular modeling. *Mol Pharmacol, 64*(6), 1295-1308. doi: 10.1124/mol.64.6.1295

Hirst, W. D., Minton, J. A., Bromidge, S. M., Moss, S. F., Latter, A. J., Riley, G., . . . Price, G. W. (2000). Characterization of [(125)I]-SB-258585 binding to human recombinant and native 5-HT(6) receptors in rat, pig and human brain tissue. *Br J Pharmacol, 130*(7), 1597-1605. doi: 10.1038/sj.bjp.0703458

Hirst, W. D., Stean, T. O., Rogers, D. C., Sunter, D., Pugh, P., Moss, S. F., . . . Upton, N. (2006). SB-399885 is a potent, selective 5-HT6 receptor antagonist with cognitive enhancing properties in aged rat water maze and novel object recognition models. *Eur J Pharmacol, 553*(1-3), 109-119. doi: 10.1016/j.ejphar.2006.09.049

Hjorth, S., & Sharp, T. (1991). Effect of the 5-HT1A receptor agonist 8-OH-DPAT on the release of 5-HT in dorsal and median raphe-innervated rat brain regions as measured by in vivo microdialysis. *Life Sci, 48*(18), 1779-1786.

Hjorth, S., & Tao, R. (1991). The putative 5-HT1B receptor agonist CP-93,129 suppresses rat hippocampal 5-HT release in vivo: comparison with RU 24969. *Eur J Pharmacol, 209*(3), 249-252.

Hornung, J. P., Fritschy, J. M., & Törk, I. (1990). Distribution of two morphologically distinct subsets of serotoninergic axons in the cerebral cortex of the marmoset. *J Comp Neurol, 297*(2), 165-181. doi: 10.1002/cne.902970202

Innis, R. B., & Aghajanian, G. K. (1987). Pertussis toxin blocks 5-HT1A and GABAB receptor-mediated inhibition of serotonergic neurons. *Eur J Pharmacol, 143*(2), 195-204.

Jacobs, B. L., & Azmitia, E. C. (1992). Structure and function of the brain serotonin system. *Physiol Rev, 72*(1), 165-229.

Jakab, R. L., & Goldman-Rakic, P. S. (1998). 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. *Proc Natl Acad Sci U S A, 95*(2), 735-740.

Jankowski, M. P., & Sesack, S. R. (2004). Prefrontal cortical projections to the rat dorsal raphe nucleus: ultrastructural features and associations with serotonin and gamma-aminobutyric acid neurons. *J Comp Neurol, 468*(4), 518-529. doi: 10.1002/cne.10976

Jastrzębska-Więsek, M., Siwek, A., Partyka, A., Kubacka, M., Mogilski, S., Wasik, A., . . . Wesołowska, A. (2014). Pharmacological evaluation of the anxiolytic-like effects of EMD 386088, a partial 5-HT6 receptor agonist, in the rat elevated plus-maze and Vogel conflict tests. *Neuropharmacology, 85*, 253-262. doi: 10.1016/j.neuropharm.2014.05.036

Jay, T. M., Thierry, A. M., Wiklund, L., & Glowinski, J. (1992). Excitatory Amino Acid Pathway from the Hippocampus to the Prefrontal Cortex. Contribution of AMPA Receptors in Hippocampo-prefrontal Cortex Transmission. *Eur J Neurosci, 4*(12), 1285-1295.

Jay, T. M., & Witter, M. P. (1991). Distribution of hippocampal CA1 and subicular efferents in the prefrontal cortex of the rat studied by means of anterograde transport of Phaseolus vulgaris-leucoagglutinin. *J Comp Neurol, 313*(4), 574-586. doi: 10.1002/cne.903130404

Jones, E. G., Huntley, G. W., & Benson, D. L. (1994). Alpha calcium/calmodulin-dependent protein kinase II selectively expressed in a subpopulation of excitatory neurons in monkey sensory-motor cortex: comparison with GAD-67 expression. *J Neurosci, 14*(2), 611-629.

Judge, S. J., Ingram, C. D., & Gartside, S. E. (2004). GABA receptor modulation of 5-HT neuronal firing: characterization and effect of moderate in vivo variations in

glucocorticoid levels. *Neurochem Int, 45*(7), 1057-1065. doi: 10.1016/j.neuint.2004.05.003

Kalén, P., Karlson, M., & Wiklund, L. (1985). Possible excitatory amino acid afferents to nucleus raphe dorsalis of the rat investigated with retrograde wheat germ agglutinin and D-[3H]aspartate tracing. *Brain Res, 360*(1-2), 285-297.

Kalén, P., Skagerberg, G., & Lindvall, O. (1988). Projections from the ventral tegmental area and mesencephalic raphe to the dorsal raphe nucleus in the rat. Evidence for a minor dopaminergic component. *Exp Brain Res, 73*(1), 69-77.

Kang, H., Lee, W. K., Choi, Y. H., Vukoti, K. M., Bang, W. G., & Yu, Y. G. (2005). Molecular analysis of the interaction between the intracellular loops of the human serotonin receptor type 6 (5-HT6) and the alpha subunit of GS protein. *Biochem Biophys Res Commun, 329*(2), 684-692. doi: 10.1016/j.bbrc.2005.02.040

Kendall, I., Slotten, H. A., Codony, X., Burgueño, J., Pauwels, P. J., Vela, J. M., & Fone, K. C. (2011). E-6801, a 5-HT6 receptor agonist, improves recognition memory by combined modulation of cholinergic and glutamatergic neurotransmission in the rat. *Psychopharmacology (Berl), 213*(2-3), 413-430. doi: 10.1007/s00213-010-1854-3

Kia, H. K., Brisorgueil, M. J., Hamon, M., Calas, A., & Vergé, D. (1996). Ultrastructural localization of 5-hydroxytryptamine1A receptors in the rat brain. *J Neurosci Res, 46*(6), 697-708. doi: 10.1002/(SICI)1097-4547(19961215)46:6<697::AID-JNR7>3.0.CO;2-A

King, M. A., Louis, P. M., Hunter, B. E., & Walker, D. W. (1989). Biocytin: a versatile anterograde neuroanatomical tract-tracing alternative. *Brain Res, 497*(2), 361-367.

Knobelman, D. A., Kung, H. F., & Lucki, I. (2000). Regulation of extracellular concentrations of 5-hydroxytryptamine (5-HT) in mouse striatum by 5-HT(1A) and 5-HT(1B) receptors. *J Pharmacol Exp Ther, 292*(3), 1111-1117.

Kocsis, B., Varga, V., Dahan, L., & Sik, A. (2006). Serotonergic neuron diversity: identification of raphe neurons with discharges time-locked to the hippocampal theta rhythm. *Proc Natl Acad Sci U S A, 103*(4), 1059-1064. doi: 10.1073/pnas.0508360103

Kohen, R., Metcalf, M. A., Khan, N., Druck, T., Huebner, K., Lachowicz, J. E., . . . Hamblin, M. W. (1996). Cloning, characterization, and chromosomal localization of a human 5-HT6 serotonin receptor. *J Neurochem, 66*(1), 47-56.

Lacroix, L. P., Dawson, L. A., Hagan, J. J., & Heidbreder, C. A. (2004). 5-HT6 receptor antagonist SB-271046 enhances extracellular levels of monoamines in the rat medial prefrontal cortex. *Synapse, 51*(2), 158-164. doi: 10.1002/syn.10288

Lakoski, J. M., & Aghajanian, G. K. (1985). Effects of ketanserin on neuronal responses to serotonin in the prefrontal cortex, lateral geniculate and dorsal raphe nucleus. *Neuropharmacology, 24*(4), 265-273.

Levine, E. S., & Jacobs, B. L. (1992). Neurochemical afferents controlling the activity of serotonergic neurons in the dorsal raphe nucleus: microiontophoretic studies in the awake cat. *J Neurosci, 12*(10), 4037-4044.

Li, Y. Q., Li, H., Kaneko, T., & Mizuno, N. (2001). Morphological features and electrophysiological properties of serotonergic and non-serotonergic projection neurons in the dorsal raphe nucleus. An intracellular recording and labeling study in rat brain slices. *Brain Res, 900*(1), 110-118.

Lidov, H. G., & Molliver, M. E. (1982). Immunohistochemical study of the development of serotonergic neurons in the rat CNS. *Brain Res Bull, 9*(1-6), 559-604.

Liu, F., Majo, V. J., Prabhakaran, J., Milak, M. S., John Mann, J., Parsey, R. V., & Kumar, J. S. (2011). Synthesis and in vivo evaluation of [O-methyl-11C] N-[3,5-dichloro-2-(methoxy)phenyl]-4-(methoxy)-3-(1-piperazinyl)benzenesulfonamide as an imaging probe for 5-HT6 receptors. *Bioorg Med Chem, 19*(17), 5255-5259. doi: 10.1016/j.bmc.2011.06.090

Liu, R., Jolas, T., & Aghajanian, G. (2000). Serotonin 5-HT(2) receptors activate local GABA inhibitory inputs to serotonergic neurons of the dorsal raphe nucleus. *Brain Res, 873*(1), 34-45.

Liu, Z., Zhou, J., Li, Y., Hu, F., Lu, Y., Ma, M., . . . Luo, M. (2014). Dorsal raphe neurons signal reward through 5-HT and glutamate. *Neuron, 81*(6), 1360-1374. doi: 10.1016/j.neuron.2014.02.010

Lorke, D. E., Lu, G., Cho, E., & Yew, D. T. (2006). Serotonin 5-HT2A and 5-HT6 receptors in the prefrontal cortex of Alzheimer and normal aging patients. *BMC Neurosci, 7*, 36. doi: 10.1186/1471-2202-7-36

Lucas, G., Compan, V., Charnay, Y., Neve, R. L., Nestler, E. J., Bockaert, J., . . . Debonnel, G. (2005). Frontocortical 5-HT4 receptors exert positive feedback on serotonergic activity: viral transfections, subacute and chronic treatments with 5-HT4 agonists. *Biol Psychiatry, 57*(8), 918-925. doi: 10.1016/j.biopsych.2004.12.023

5-HT4 receptors exert a frequency-related facilitatory control on dorsal raphé nucleus 5-HT neuronal activity, 16 C.F.R. (2002).

Lucas, G., Du, J., Romeas, T., Mnie-Filali, O., Haddjeri, N., Piñeyro, G., & Debonnel, G. (2010). Selective serotonin reuptake inhibitors potentiate the rapid antidepressant-like effects of serotonin4 receptor agonists in the rat. *PLoS One, 5*(2), e9253. doi: 10.1371/journal.pone.0009253

Lucas, G., Rymar, V. V., Du, J., Mnie-Filali, O., Bisgaard, C., Manta, S., . . . Debonnel, G. (2007). Serotonin(4) (5-HT(4)) receptor agonists are putative antidepressants with a rapid onset of action. *Neuron, 55*(5), 712-725. doi: 10.1016/j.neuron.2007.07.041

Luczak, A., Barthó, P., Marguet, S. L., Buzsáki, G., & Harris, K. D. (2007). Sequential structure of neocortical spontaneous activity in vivo. *Proc Natl Acad Sci U S A, 104*(1), 347-352. doi: 10.1073/pnas.0605643104

Lum, J. T., & Piercey, M. F. (1988). Electrophysiological evidence that spiperone is an antagonist of 5-HT1A receptors in the dorsal raphe nucleus. *Eur J Pharmacol, 149*(1-2), 9-15.

Ly, S., Pishdari, B., Lok, L. L., Hajos, M., & Kocsis, B. (2013). Activation of 5-HT6 receptors modulates sleep-wake activity and hippocampal theta oscillation. *ACS Chem Neurosci, 4*(1), 191-199. doi: 10.1021/cn300184t

Maher-Edwards, G., Zvartau-Hind, M., Hunter, A. J., Gold, M., Hopton, G., Jacobs, G., . . . Williams, P. (2010). Double-blind, controlled phase II study of a 5-HT6 receptor antagonist, SB-742457, in Alzheimer's disease. *Curr Alzheimer Res, 7*(5), 374-385.

Mantz, J., Godbout, R., Tassin, J. P., Glowinski, J., & Thierry, A. M. (1990). Inhibition of spontaneous and evoked unit activity in the rat medial prefrontal cortex by mesencephalic raphe nuclei. *Brain Res, 524*(1), 22-30.

Marazziti, D., Baroni, S., Pirone, A., Giannaccini, G., Betti, L., Schmid, L., . . . Lucacchini, A. (2012). Distribution of serotonin receptor of type 6 (5-HT_6) in human brain post-mortem. A pharmacology, autoradiography and immunohistochemistry study. *Neurochem Res, 37*(5), 920-927. doi: 10.1007/s11064-011-0684-y

Marazziti, D., Baroni, S., Pirone, A., Giannaccini, G., Betti, L., Testa, G., . . . Lucacchini, A. (2013). Serotonin receptor of type 6 (5-HT6) in human prefrontal cortex and hippocampus post-mortem: an immunohistochemical and immunofluorescence study. *Neurochem Int, 62*(2), 182-188. doi: 10.1016/j.neuint.2012.11.013

Marcos, B., Cabero, M., Solas, M., Aisa, B., & Ramirez, M. J. (2010). Signalling pathways associated with 5-HT6 receptors: relevance for cognitive effects. *Int J Neuropsychopharmacol, 13*(6), 775-784. doi: 10.1017/S146114570999054X

Martin, K. F., Hannon, S., Phillips, I., & Heal, D. J. (1992). Opposing roles for 5-HT1B and 5-HT3 receptors in the control of 5-HT release in rat hippocampus in vivo. *Br J Pharmacol, 106*(1), 139-142.

Martín-Ruiz, R., Puig, M. V., Celada, P., Shapiro, D. A., Roth, B. L., Mengod, G., & Artigas, F. (2001). Control of serotonergic function in medial prefrontal cortex by serotonin-2A receptors through a glutamate-dependent mechanism. *J Neurosci, 21*(24), 9856-9866.

Martín-Ruiz, R., Ugedo, L., Honrubia, M. A., Mengod, G., & Artigas, F. (2001). Control of serotonergic neurons in rat brain by dopaminergic receptors outside the dorsal raphe nucleus. *J Neurochem, 77*(3), 762-775.

Mason, J. P., Dring, L. G., & Caldwell, J. (1995). Pharmacokinetics of the 5-hydroxytryptamine1A agonist 8-hydroxy-2-(N,N-di-n-propylamino)tetralin (8-OHDPAT) in the rat after intravenous and oral administration. *Xenobiotica, 25*(12), 1371-1380.

Matthews, G. A., Nieh, E. H., Vander Weele, C. M., Halbert, S. A., Pradhan, R. V., Yosafat, A. S., . . . Tye, K. M. (2016). Dorsal Raphe Dopamine Neurons Represent the Experience of Social Isolation. *Cell, 164*(4), 617-631. doi: 10.1016/j.cell.2015.12.040

Meffre, J., Chaumont-Dubel, S., Mannoury la Cour, C., Loiseau, F., Watson, D. J., Dekeyne, A., . . . Marin, P. (2012). 5-HT(6) receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia. *EMBO Mol Med, 4*(10), 1043-1056. doi: 10.1002/emmm.201201410

Meltzer, H. Y. (1994). An overview of the mechanism of action of clozapine. *J Clin Psychiatry, 55 Suppl B*, 47-52.

Meneses, A. (1999). 5-HT system and cognition. *Neurosci Biobehav Rev, 23*(8), 1111-1125.

Michelsen, K. A., Schmitz, C., & Steinbusch, H. W. (2007). The dorsal raphe nucleus--from silver stainings to a role in depression. *Brain Res Rev, 55*(2), 329-342. doi: 10.1016/j.brainresrev.2007.01.002

Miller, E. K. (2000). The prefrontal cortex and cognitive control. *Nat Rev Neurosci, 1*(1), 59-65. doi: 10.1038/35036228

Miyamoto, S., Duncan, G. E., Marx, C. E., & Lieberman, J. A. (2005). Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol Psychiatry, 10*(1), 79-104. doi: 10.1038/sj.mp.4001556

Molliver, M. E. (1987). Serotonergic neuronal systems: what their anatomic organization tells us about function. *J Clin Psychopharmacol, 7*(6 Suppl), 3S-23S.

Monsma, F. J., Shen, Y., Ward, R. P., Hamblin, M. W., & Sibley, D. R. (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol Pharmacol, 43*(3), 320-327.

Monti, J. M., & Jantos, H. (2008). Activation of the serotonin 5-HT3 receptor in the dorsal raphe nucleus suppresses REM sleep in the rat. *Prog Neuropsychopharmacol Biol Psychiatry, 32*(4), 940-947. doi: 10.1016/j.pnpbp.2007.12.024

Monti, J. M., & Jantos, H. (2011). Effects of the 5-HT_6 receptor antagonists SB-399885 and RO-4368554 and of the 5-HT(2A) receptor antagonist EMD 281014 on sleep and wakefulness in the rat during both phases of the light-dark cycle. *Behav Brain Res, 216*(1), 381-388. doi: 10.1016/j.bbr.2010.08.020

Monti, J. M., Jantos, H., & Schechter, L. E. (2013). The effects of systemic and local microinjection into the central nervous system of the selective serotonin 5-HT6 receptor agonist WAY-208466 on sleep and wakefulness in the rat. *Behav Brain Res, 249*, 65-74. doi: 10.1016/j.bbr.2013.04.024

Morairty, S. R., Hedley, L., Flores, J., Martin, R., & Kilduff, T. S. (2008). Selective 5HT2A and 5HT6 receptor antagonists promote sleep in rats. *Sleep, 31*(1), 34-44.

Nelson, D. L. (2004). 5-HT5 receptors. *Curr Drug Targets CNS Neurol Disord, 3*(1), 53-58.

Nikiforuk, A., Kos, T., & Wesołowska, A. (2011). The 5-HT6 receptor agonist EMD 386088 produces antidepressant and anxiolytic effects in rats after intrahippocampal administration. *Psychopharmacology (Berl), 217*(3), 411-418. doi: 10.1007/s00213-011-2297-1

Ogawa, S. K., Cohen, J. Y., Hwang, D., Uchida, N., & Watabe-Uchida, M. (2014). Organization of monosynaptic inputs to the serotonin and dopamine neuromodulatory systems. *Cell Rep, 8*(4), 1105-1118. doi: 10.1016/j.celrep.2014.06.042

Oliver, K. R., Kinsey, A. M., Wainwright, A., & Sirinathsinghji, D. J. (2000). Localization of 5-ht(5A) receptor-like immunoreactivity in the rat brain. *Brain Res, 867*(1-2), 131-142.

Papadopoulos, G. C., Parnavelas, J. G., & Buijs, R. (1987). Monoaminergic fibers form conventional synapses in the cerebral cortex. *Neurosci Lett, 76*(3), 275-279.

Paris, J. M., & Cunningham, K. A. (1994). Habenula lesions decrease the responsiveness of dorsal raphe serotonin neurons to cocaine. *Pharmacol Biochem Behav, 49*(3), 555-560.

Parker, C. A., Gunn, R. N., Rabiner, E. A., Slifstein, M., Comley, R., Salinas, C., . . . Martarello, L. (2012). Radiosynthesis and characterization of 11C-GSK215083 as a PET radioligand for the 5-HT6 receptor. *J Nucl Med, 53*(2), 295-303. doi: 10.2967/jnumed.111.093419

Parks, C. L., Robinson, P. S., Sibille, E., Shenk, T., & Toth, M. (1998). Increased anxiety of mice lacking the serotonin1A receptor. *Proc Natl Acad Sci U S A, 95*(18), 10734-10739.

Paxinos G, Watson C. (2007). *The Rat Brain in Stereotaxic Coordinates.* Amsterdam: Elsevier.

Peyron, C., Luppi, P. H., Fort, P., Rampon, C., & Jouvet, M. (1996). Lower brainstem catecholamine afferents to the rat dorsal raphe nucleus. *J Comp Neurol, 364*(3), 402-413. doi: 10.1002/(SICI)1096-9861(19960115)364:3<402::AID-CNE2>3.0.CO;2-8

Peyron, C., Luppi, P. H., Kitahama, K., Fort, P., Hermann, D. M., & Jouvet, M. (1995). Origin of the dopaminergic innervation of the rat dorsal raphe nucleus. *Neuroreport, 6*(18), 2527-2531.

Peyron, C., Petit, J. M., Rampon, C., Jouvet, M., & Luppi, P. H. (1998). Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience, 82*(2), 443-468.

Pinault, D. (1996). A novel single-cell staining procedure performed in vivo under electrophysiological control: morpho-functional features of juxtacellularly labeled thalamic cells and other central neurons with biocytin or Neurobiotin. *J Neurosci Methods, 65*(2), 113-136.

Pollak Dorocic, I., Fürth, D., Xuan, Y., Johansson, Y., Pozzi, L., Silberberg, G., . . . Meletis, K. (2014). A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei. *Neuron, 83*(3), 663-678. doi: 10.1016/j.neuron.2014.07.002

Pompeiano, M., Palacios, J. M., & Mengod, G. (1994). Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors. *Brain Res Mol Brain Res, 23*(1-2), 163-178.

Portella, M. J., de Diego-Adeliño, J., Ballesteros, J., Puigdemont, D., Oller, S., Santos, B., . . . Pérez, V. (2011). Can we really accelerate and enhance the selective serotonin reuptake inhibitor antidepressant effect? A randomized clinical trial and a meta-analysis of pindolol in nonresistant depression. *J Clin Psychiatry, 72*(7), 962-969. doi: 10.4088/JCP.09m05827blu

Puig, M. V., Celada, P., Díaz-Mataix, L., & Artigas, F. (2003). In vivo modulation of the activity of pyramidal neurons in the rat medial prefrontal cortex by 5-HT2A receptors: relationship to thalamocortical afferents. *Cereb Cortex, 13*(8), 870-882.

Pytliak, M., Vargová, V., Mechírová, V., & Felšöci, M. (2011). Serotonin receptors - from molecular biology to clinical applications. *Physiol Res, 60*(1), 15-25.

Quérée, P., Peters, S., & Sharp, T. (2009). Further pharmacological characterization of 5-HT(2C) receptor agonist-induced inhibition of 5-HT neuronal activity in the dorsal raphe nucleus in vivo. *Br J Pharmacol, 158*(6), 1477-1485. doi: 10.1111/j.1476-5381.2009.00406.x

Ramboz, S., Oosting, R., Amara, D. A., Kung, H. F., Blier, P., Mendelsohn, M., . . . Hen, R. (1998). Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. *Proc Natl Acad Sci U S A, 95*(24), 14476-14481.

RAPPORT, M. M., GREEN, A. A., & PAGE, I. H. (1948). Serum vasoconstrictor, serotonin; isolation and characterization. *J Biol Chem, 176*(3), 1243-1251.

Rasmussen, K., Heym, J., & Jacobs, B. L. (1984). Activity of serotonin-containing neurons in nucleus centralis superior of freely moving cats. *Exp Neurol, 83*(2), 302-317. doi: 10.1016/S0014-4886(84)90100-6

Riad, M., Garcia, S., Watkins, K. C., Jodoin, N., Doucet, E., Langlois, X., . . . Descarries, L. (2000). Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. *J Comp Neurol, 417*(2), 181-194.

Riccioni, T., Bordi, F., Minetti, P., Spadoni, G., Yun, H. M., Im, B. H., . . . Borsini, F. (2011). ST1936 stimulates cAMP, Ca2+, ERK1/2 and Fyn kinase through a full activation of cloned human 5-HT6 receptors. *Eur J Pharmacol, 661*(1-3), 8-14. doi: 10.1016/j.ejphar.2011.04.028

Richardson-Jones, J. W., Craige, C. P., Guiard, B. P., Stephen, A., Metzger, K. L., Kung, H. F., . . . Leonardo, E. D. (2010). 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. *Neuron, 65*(1), 40-52. doi: 10.1016/j.neuron.2009.12.003

Richer, M., Hen, R., & Blier, P. (2002). Modification of serotonin neuron properties in mice lacking 5-HT1A receptors. *Eur J Pharmacol, 435*(2-3), 195-203.

Roberts, C., Thomas, D. R., Bate, S. T., & Kew, J. N. (2004). GABAergic modulation of 5-HT7 receptor-mediated effects on 5-HT efflux in the guinea-pig dorsal raphe nucleus. *Neuropharmacology, 46*(7), 935-941. doi: 10.1016/j.neuropharm.2004.01.010

Roberts, J. C., Reavill, C., East, S. Z., Harrison, P. J., Patel, S., Routledge, C., & Leslie, R. A. (2002). The distribution of 5-HT(6) receptors in rat brain: an autoradiographic binding study using the radiolabelled 5-HT(6) receptor antagonist [(125)I]SB-258585. *Brain Res, 934*(1), 49-57.

Romero, G., Sánchez, E., Pujol, M., Pérez, P., Codony, X., Holenz, J., . . . Pauwels, P. J. (2006). Efficacy of selective 5-HT6 receptor ligands determined by monitoring 5-HT6 receptor-mediated cAMP signaling pathways. *Br J Pharmacol, 148*(8), 1133-1143. doi: 10.1038/sj.bjp.0706827

Ruat, M., Traiffort, E., Arrang, J. M., Tardivel-Lacombe, J., Diaz, J., Leurs, R., & Schwartz, J. C. (1993). A novel rat serotonin (5-HT6) receptor: molecular cloning, localization and stimulation of cAMP accumulation. *Biochem Biophys Res Commun, 193*(1), 268-276.

Sakai, K. (2011). Sleep-waking discharge profiles of median preoptic and surrounding neurons in mice. *Neuroscience, 182*, 144-161. doi: 10.1016/j.neuroscience.2011.03.010

Sakai, K., & Crochet, S. (2001). Differentiation of presumed serotonergic dorsal raphe neurons in relation to behavior and wake-sleep states. *Neuroscience, 104*(4), 1141-1155.

Sakai, K., Salvert, D., Touret, M., & Jouvet, M. (1977). Afferent connections of the nucleus raphe dorsalis in the cat as visualized by the horseradish peroxidase technique. *Brain Res, 137*(1), 11-35.

Salgado, S., & Kaplitt, M. G. (2015). The Nucleus Accumbens: A Comprehensive Review. *Stereotact Funct Neurosurg, 93*(2), 75-93. doi: 10.1159/000368279

Sanchez, C., Asin, K. E., & Artigas, F. (2015). Vortioxetine, a novel antidepressant with multimodal activity: review of preclinical and clinical data. *Pharmacol Ther, 145*, 43-57. doi: 10.1016/j.pharmthera.2014.07.001

Saunders, A., Oldenburg, I. A., Berezovskii, V. K., Johnson, C. A., Kingery, N. D., Elliott, H. L., . . . Sabatini, B. L. (2015). A direct GABAergic output from the basal ganglia to frontal cortex. *Nature, 521*(7550), 85-89. doi: 10.1038/nature14179

Sawyer, S. F., Tepper, J. M., Young, S. J., & Groves, P. M. (1985). Antidromic activation of dorsal raphe neurons from neostriatum: physiological characterization and effects of terminal autoreceptor activation. *Brain Res, 332*(1), 15-28.

Schechter, L. E., Lin, Q., Smith, D. L., Zhang, G., Shan, Q., Platt, B., . . . Beyer, C. E. (2008). Neuropharmacological profile of novel and selective 5-HT6 receptor agonists: WAY-181187 and WAY-208466. *Neuropsychopharmacology, 33*(6), 1323-1335. doi: 10.1038/sj.npp.1301503

Scheggi, S., Marchese, G., Borsini, F., Bordi, F., & De Montis, M. G. (2011). Effects of the 5-HT(6) receptor agonist ST 1936 on depression- and anhedonia-like experimental models. *Behav Brain Res, 224*(1), 35-43. doi: 10.1016/j.bbr.2011.05.019

Schweimer, J. V., Coullon, G. S., Betts, J. F., Burnet, P. W., Engle, S. J., Brandon, N. J., . . . Sharp, T. (2014). Increased burst-firing of ventral tegmental area dopaminergic neurons in D-amino acid oxidase knockout mice in vivo. *Eur J Neurosci, 40*(7), 2999-3009. doi: 10.1111/ejn.12667

Schweimer, J. V., Mallet, N., Sharp, T., & Ungless, M. A. (2011). Spike-timing relationship of neurochemically-identified dorsal raphe neurons during cortical slow oscillations. *Neuroscience, 196*, 115-123. doi: 10.1016/j.neuroscience.2011.08.072

Schweimer, J. V., & Ungless, M. A. (2010). Phasic responses in dorsal raphe serotonin neurons to noxious stimuli. *Neuroscience, 171*(4), 1209-1215. doi: 10.1016/j.neuroscience.2010.09.058

Sebben, M., Ansanay, H., Bockaert, J., & Dumuis, A. (1994). 5-HT6 receptors positively coupled to adenylyl cyclase in striatal neurones in culture. *Neuroreport, 5*(18), 2553-2557.

Serrats, J., Mengod, G., & Cortés, R. (2005). Expression of serotonin 5-HT2C receptors in GABAergic cells of the anterior raphe nuclei. *J Chem Neuroanat, 29*(2), 83-91. doi: 10.1016/j.jchemneu.2004.03.010

Setola, V., & Roth, B. L. (2003). Why mice are neither miniature humans nor small rats: a cautionary tale involving 5-hydroxytryptamine-6 serotonin receptor species variants. *Mol Pharmacol, 64*(6), 1277-1278. doi: 10.1124/mol.64.6.1277

Sharp, T., Boothman, L., Raley, J., & Quérée, P. (2007). Important messages in the 'post': recent discoveries in 5-HT neurone feedback control. *Trends Pharmacol Sci, 28*(12), 629-636. doi: 10.1016/j.tips.2007.10.009

Sharp, T., Bramwell, S. R., Hjorth, S., & Grahame-Smith, D. G. (1989). Pharmacological characterization of 8-OH-DPAT-induced inhibition of rat hippocampal 5-HT release in vivo as measured by microdialysis. *Br J Pharmacol, 98*(3), 989-997.

Shen, Y., Monsma, F. J., Metcalf, M. A., Jose, P. A., Hamblin, M. W., & Sibley, D. R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. *J Biol Chem, 268*(24), 18200-18204.

Shikanai, H., Yoshida, T., Konno, K., Yamasaki, M., Izumi, T., Ohmura, Y., . . . Yoshioka, M. (2012). Distinct neurochemical and functional properties of GAD67-containing 5-HT neurons in the rat dorsal raphe nucleus. *J Neurosci, 32*(41), 14415-14426. doi: 10.1523/JNEUROSCI.5929-11.2012

Soiza-Reilly, M., & Commons, K. G. (2014). Unraveling the architecture of the dorsal raphe synaptic neuropil using high-resolution neuroanatomy. *Front Neural Circuits, 8*, 105. doi: 10.3389/fncir.2014.00105

Sotelo, C., Cholley, B., El Mestikawy, S., Gozlan, H., & Hamon, M. (1990). Direct Immunohistochemical Evidence of the Existence of 5-HT1A Autoreceptors on Serotoninergic Neurons in the Midbrain Raphe Nuclei. *Eur J Neurosci, 2*(12), 1144-1154.

Sprouse, J. S., & Aghajanian, G. K. (1987). Electrophysiological responses of serotoninergic dorsal raphe neurons to 5-HT1A and 5-HT1B agonists. *Synapse, 1*(1), 3-9. doi: 10.1002/syn.890010103

Stamp, J. A., & Semba, K. (1995). Extent of colocalization of serotonin and GABA in the neurons of the rat raphe nuclei. *Brain Res, 677*(1), 39-49.

Steinbusch, H. W. (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience, 6*(4), 557-618.

Steinbusch, H. W., & Nieuwenhuys, R. (1981). Localization of serotonin-like immunoreactivity in the central nervous system and pituitary of the rat, with special references to the innervation of the hypothalamus. *Adv Exp Med Biol, 133*, 7-35.

Steinbusch, H. W., Nieuwenhuys, R., Verhofstad, A. A., & Van der Kooy, D. (1981). The nucleus raphe dorsalis of the rat and its projection upon the caudatoputamen. A combined cytoarchitectonic, immunohistochemical and retrograde transport study. *J Physiol (Paris), 77*(2-3), 157-174.

Steriade, M. (2000). Corticothalamic resonance, states of vigilance and mentation. *Neuroscience, 101*(2), 243-276.

Svenningsson, P., Tzavara, E. T., Qi, H., Carruthers, R., Witkin, J. M., Nomikos, G. G., & Greengard, P. (2007). Biochemical and behavioral evidence for antidepressant-like effects of 5-HT6 receptor stimulation. *J Neurosci, 27*(15), 4201-4209. doi: 10.1523/JNEUROSCI.3110-06.2007

Svartengren, B. Öhman, N. Edling, M. Svensson, K. Fhölenhag, P. Axelsson-Lendin et al. (2003). ***The serotonin 5-HT$_6$ receptor antagonist BVT.5182 reduces body weight of high fat diet-induced mice***. Paper presented at the Proceedings Of The 12th European Congress Of Obesity, Helsinki, 29 May–31 June

Svartengren, P. Axelsson-Lendin, N. Edling, K. Fhölenhag, R. Isacson, V. Hillegaart, *et al.* (2004). ***The selective serotonin 5-HT$_6$ receptor antagonist BVT5182 decreases food intake and body weight in both rats and mice***. Paper presented at the Society for Neuroscience, Washington, DC

Takahashi, H., Nakashima, S., Ohama, E., Takeda, S., & Ikuta, F. (1986). Distribution of serotonin-containing cell bodies in the brainstem of the human fetus determined with immunohistochemistry using antiserotonin serum. *Brain Dev, 8*(4), 355-365.

Tang, S., Verdurand, M., Joseph, B., Lemoine, L., Daoust, A., Billard, T., . . . Zimmer, L. (2007). Synthesis and biological evaluation in rat and cat of [18F]12ST05 as a potential 5-HT6 PET radioligand. *Nucl Med Biol, 34*(8), 995-1002. doi: 10.1016/j.nucmedbio.2007.07.002

Thomas, D. R. (2006). 5-ht5A receptors as a therapeutic target. *Pharmacol Ther, 111*(3), 707-714. doi: 10.1016/j.pharmthera.2005.12.006

Thomas, D. R., Soffin, E. M., Roberts, C., Kew, J. N., de la Flor, R. M., Dawson, L. A., . . . Hagan, J. J. (2006). SB-699551-A (3-cyclopentyl-N-[2-(dimethylamino)ethyl]-N-[(4'-{[(2-phenylethyl)amino]methyl}-4-biphenylyl)methyl]propanamide dihydrochloride), a novel 5-ht5A receptor-selective antagonist, enhances 5-HT neuronal function: Evidence for an autoreceptor role for the 5-ht5A receptor in guinea pig brain. *Neuropharmacology, 51*(3), 566-577. doi: 10.1016/j.neuropharm.2006.04.019

Tierney, P. L., Dégenètais, E., Thierry, A. M., Glowinski, J., & Gioanni, Y. (2004). Influence of the hippocampus on interneurons of the rat prefrontal cortex. *Eur J Neurosci, 20*(2), 514-524. doi: 10.1111/j.1460-9568.2004.03501.x

To, Z. P., Bonhaus, D. W., Eglen, R. M., & Jakeman, L. B. (1995). Characterization and distribution of putative 5-ht7 receptors in guinea-pig brain. *Br J Pharmacol, 115*(1), 107-116.

Tseng, K. Y., Mallet, N., Toreson, K. L., Le Moine, C., Gonon, F., & O'Donnell, P. (2006). Excitatory response of prefrontal cortical fast-spiking interneurons to ventral tegmental area stimulation in vivo. *Synapse, 59*(7), 412-417. doi: 10.1002/syn.20255

TWAROG, B. M., & PAGE, I. H. (1953). Serotonin content of some mammalian tissues and urine and a method for its determination. *Am J Physiol, 175*(1), 157-161.

Törk, I. (1990). Anatomy of the serotonergic system. *Ann N Y Acad Sci, 600*, 9-34; discussion 34-35.

van Gaalen, M. M., Schetters, D., Schoffelmeer, A. N., & De Vries, T. J. (2010). 5-HT6 antagonism attenuates cue-induced relapse to cocaine seeking without affecting cocaine reinforcement. *Int J Neuropsychopharmacol, 13*(7), 961-965. doi: 10.1017/S1461145710000428

Varga, V., Kocsis, B., & Sharp, T. (2003). Electrophysiological evidence for convergence of inputs from the medial prefrontal cortex and lateral habenula on single neurons in the dorsal raphe nucleus. *Eur J Neurosci, 17*(2), 280-286.

Varga, V., Székely, A. D., Csillag, A., Sharp, T., & Hajós, M. (2001). Evidence for a role of GABA interneurones in the cortical modulation of midbrain 5-hydroxytryptamine neurones. *Neuroscience, 106*(4), 783-792.

Vasudeva, R. K., Lin, R. C., Simpson, K. L., & Waterhouse, B. D. (2011). Functional organization of the dorsal raphe efferent system with special consideration of

nitrergic cell groups. *J Chem Neuroanat, 41*(4), 281-293. doi: 10.1016/j.jchemneu.2011.05.008

Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse, 51*(1), 32-58. doi: 10.1002/syn.10279

Vilaró, M. T., Cortés, R., Gerald, C., Branchek, T. A., Palacios, J. M., & Mengod, G. (1996). Localization of 5-HT4 receptor mRNA in rat brain by in situ hybridization histochemistry. *Brain Res Mol Brain Res, 43*(1-2), 356-360.

Vilaró, M. T., Cortés, R., & Mengod, G. (2005). Serotonin 5-HT4 receptors and their mRNAs in rat and guinea pig brain: distribution and effects of neurotoxic lesions. *J Comp Neurol, 484*(4), 418-439. doi: 10.1002/cne.20447

Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., & Stork, P. J. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell, 89*(1), 73-82.

Vyazovskiy, V. V., Olcese, U., Lazimy, Y. M., Faraguna, U., Esser, S. K., Williams, J. C., . . . Tononi, G. (2009). Cortical firing and sleep homeostasis. *Neuron, 63*(6), 865-878. doi: 10.1016/j.neuron.2009.08.024

Wang, L., Lv, Y., Deng, W., Peng, X., Xiao, Z., Xi, Z., . . . Wang, X. (2015). 5-HT6 Receptor Recruitment of mTOR Modulates Seizure Activity in Epilepsy. *Mol Neurobiol, 51*(3), 1292-1299. doi: 10.1007/s12035-014-8806-6

Wang, Q. P., Guan, J. L., Ochiai, H., & Nakai, Y. (1998). An electron microscopic observation of the vesicular acetylcholine transporter-immunoreactive fibers in the rat dorsal raphe nucleus. *Brain Res Bull, 46*(6), 555-561.

Wang, Q. P., & Nakai, Y. (1993). Enkephalinergic innervation of GABAergic neurons in the dorsal raphe nucleus of the rat. *Brain Res Bull, 32*(3), 315-320.

Wang, R. Y., & Aghajanian, G. K. (1977). Antidromically identified serotonergic neurons in the rat midbrain raphe: evidence for collateral inhibition. *Brain Res, 132*(1), 186-193.

Wang, R. Y., & Aghajanian, G. K. (1978). Collateral inhibition of serotonergic neurones in the rat dorsal raphe nucleus: pharmacological evidence. *Neuropharmacology, 17*(10), 819-825.

Ward, R. P., Hamblin, M. W., Lachowicz, J. E., Hoffman, B. J., Sibley, D. R., & Dorsa, D. M. (1995). Localization of serotonin subtype 6 receptor messenger RNA in the rat brain by in situ hybridization histochemistry. *Neuroscience, 64*(4), 1105-1111.

Wesołowska, A. (2007). Study into a possible mechanism responsible for the antidepressant-like activity of the selective 5-HT6 receptor antagonist SB-399885 in rats. *Pharmacol Rep, 59*(6), 664-671.

Wesołowska, A., & Nikiforuk, A. (2007). Effects of the brain-penetrant and selective 5-HT6 receptor antagonist SB-399885 in animal models of anxiety and depression. *Neuropharmacology, 52*(5), 1274-1283. doi: 10.1016/j.neuropharm.2007.01.007

Wesołowska, A., Nikiforuk, A., & Stachowicz, K. (2007). Anxiolytic-like and antidepressant-like effects produced by the selective 5-HT6 receptor antagonist SB-258585 after intrahippocampal administration to rats. *Behav Pharmacol, 18*(5-6), 439-446. doi: 10.1097/FBP.0b013e3282d28f9c

Wilkinson, D., Windfeld, K., & Colding-Jørgensen, E. (2014). Safety and efficacy of idalopirdine, a 5-HT6 receptor antagonist, in patients with moderate Alzheimer's disease (LADDER): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol, 13*(11), 1092-1099. doi: 10.1016/S1474-4422(14)70198-X

Woods, S., Clarke, N. N., Layfield, R., & Fone, K. C. (2012). 5-HT(6) receptor agonists and antagonists enhance learning and memory in a conditioned emotion response paradigm by modulation of cholinergic and glutamatergic mechanisms. *Br J Pharmacol, 167*(2), 436-449. doi: 10.1111/j.1476-5381.2012.02022.x

Woolley, D. W., & Shaw, E. (1954). A BIOCHEMICAL AND PHARMACOLOGICAL SUGGESTION ABOUT CERTAIN MENTAL DISORDERS. *Proc Natl Acad Sci U S A, 40*(4), 228-231.

Woolley, M. L., Bentley, J. C., Sleight, A. J., Marsden, C. A., & Fone, K. C. (2001). A role for 5-ht6 receptors in retention of spatial learning in the Morris water maze. *Neuropharmacology, 41*(2), 210-219.

Wright, I. K., Garratt, J. C., & Marsden, C. A. (1990). Effects of a selective 5-HT2 agonist, DOI, on 5-HT neuronal firing in the dorsal raphe nucleus and 5-HT release and metabolism in the frontal cortex. *Br J Pharmacol, 99*(2), 221-222.

Yun, H. M., Kim, S., Kim, H. J., Kostenis, E., Kim, J. I., Seong, J. Y., . . . Rhim, H. (2007). The novel cellular mechanism of human 5-HT6 receptor through an interaction with Fyn. *J Biol Chem, 282*(8), 5496-5505. doi: 10.1074/jbc.M606215200

Yun, H. M., & Rhim, H. (2011). 5-HT6 receptor ligands, EMD386088 and SB258585, differentially regulate 5-HT6 receptor-independent events. *Toxicol In Vitro, 25*(8), 2035-2040. doi: 10.1016/j.tiv.2011.08.004

Zhang, Z. W., & Deschênes, M. (1997). Intracortical axonal projections of lamina VI cells of the primary somatosensory cortex in the rat: a single-cell labeling study. *J Neurosci, 17*(16), 6365-6379.

Zuo, W., Zhang, Y., Xie, G., Gregor, D., Bekker, A., & Ye, J. H. (2016). Serotonin stimulates lateral habenula via activation of the post-synaptic serotonin 2/3 receptors and transient receptor potential channels. *Neuropharmacology, 101*, 449-459. doi: 10.1016/j.neuropharm.2015.10.012