



**An electrophysiological investigation of the extrinsic
modulation of ventral pallidum neurons by dopamine
and serotonin**

By

M Clark

A thesis submitted to the University of Sheffield in
fulfilment of the requirements for the degree of Doctor of
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Faculty of Science

Department of Psychology

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Summary

The ventral pallidum (VP) is a key output structure of the basal ganglia and has multiple connections with cortical and limbic regions. The VP is modulated by extrinsic neurotransmitters, including dopamine and serotonin, but the cellular mechanisms underlying this modulation are only partially understood. This thesis describes an *in vitro* electrophysiological investigation of such extrinsic modulation employing extracellular multi electrode recordings of the VP and pharmacological manipulations.

Our data provide novel information on how dopamine modulates VP neurons acting both pre- and post-synaptically. Presynaptic excitatory effects of dopamine are mediated by D1-like and D2-like receptors, through effects on glutamate release and subsequently ionotropic and metabotropic glutamate receptor activation. Postsynaptic, direct effects are mediated by D2-like receptors and induce decreases in firing frequency within the VP. Our data identifies two populations of neurons in the VP, which can be consistently separated by their spike half-width profile and their responses to D2-like receptor agonists.

With regard to serotonin, both excitatory and inhibitory responses to its application were observed in the VP. Our data suggests that excitatory effects of Serotonin (5HT) are presynaptic, while the inhibitory responses to 5HT are postsynaptic, direct effects. Our data also suggest that 5HT_{1a}, 5HT_{5A} and 5HT₇ receptors are responsible for increases in firing frequency to 5HT application and 5-HT_{2c} receptors are likely candidates for the decrease in firing frequency to 5HT application in the VP.

We also investigated the effects of electric activation of NAc inputs to the VP. Our data are consistent with the literature, showing that NAc inputs inhibit VP neurons.

Overall, these results cast light on the cellular mechanism by which dopamine and serotonin modulate VP neurons and have important implications for our understanding of the role the VP plays in reward processing and related dysfunctions.

This thesis is dedicated to Rachel Crosby, I couldn't have done it otherwise.

Chapter 3, and parts of chapter 2, of this thesis have been published by myself and Enrico Bracci as original research in the journal: *Frontiers in cellular neuroscience*. All authors have consented to its use in this thesis.

Clark, M., & Bracci, E. (2018). Dichotomous Dopaminergic Control of Ventral Pallidum Neurons. *Frontiers in Cellular Neuroscience*, *12*, 260.
<https://doi.org/10.3389/fncel.2018.00260>

Contents

Summary	5
Acknowledgements:.....	12
List of Abbreviations:	13
List of figures:.....	15
List of tables:.....	18
1 Chapter 1: General introduction.....	19
1.1 The Basal Ganglia:.....	20
1.2 Anatomy of the basal ganglia.....	20
1.3 Physiology of the basal ganglia:.....	21
1.4 Function of the basal ganglia	22
1.4.1 The basal ganglia and reward learning:	22
1.5 The ventral pallidum	23
1.5.1 Organisation of the VP:.....	23
1.6 Functional roles of the VP:.....	25
1.6.1 Role of the VP in reward processing:.....	25
1.6.2 Role of the VP in pleasure/liking responses:	27
1.6.3 Role of the VP in addiction to drugs of abuse:.....	28
1.7 Neurons of the VP:.....	30
1.7.1 Electrophysiological classification of VP neurons:.....	32
1.8 Neurochemicals and receptors in the VP.....	36
1.8.1 GABA (GABA _A and GABA _B receptors):.....	37
1.8.2 Glutamate	38
1.8.3 Opioids (enkephalin and dynorphin) and opioid receptors (δ , kappa and μ):....	41
1.8.4 Substance P	44
1.8.5 Dopamine:.....	46
1.8.6 Serotonin (5HT).....	50
1.9 Connections between the NAc and the VP	54
1.9.1 Anatomical connectivity:	54
1.9.2 Functional connectivity.....	55
1.9.3 Neurochemistry and receptors of the connections between the NacS and VP: 56	
1.9.4 Neuronal and receptor targets of the NAc input into the VP:.....	58
2 Chapter 2: Materials and methods.....	61
2.1 Animals:.....	62
2.2 Slice preparation:.....	63

2.3	pMEA (perforated Multi-Electrode Array) electrophysiological recordings:	64
2.4	Slice visualisation:.....	66
2.5	Stimulation methods and protocols:.....	67
2.6	Pharmacology:.....	69
2.7	Data Analysis for pharmacological experiments:	71
2.7.1	Data analysis for stimulation experiments:.....	73
3	Chapter 3: Dopaminergic modulation of VP neurons	74
3.1	Abstract:	75
3.2	Introduction:.....	76
3.3	Results:	78
3.3.1	Dopamine application has dual effects on VP neurons.....	78
3.3.2	Dopamine receptors responsible for its effects on VP neurons.....	80
3.3.3	Repeated application of dopamine and dopamine receptor agonists.....	84
3.3.4	Role of D1-like and D2-like receptors in the modulation of VP neurons	87
3.3.5	D1-like receptors exert their effects presynaptically, while D2-like receptors cause excitation presynaptically and inhibition postsynaptically.	91
3.3.6	D1-like receptors excite VP neurons through presynaptic modulation of GA. ...	95
3.3.7	Excitatory effects of D2-like receptor agonists require mGluRs	97
3.3.8	Inhibitory effects of D2-like receptor agonists persevere in the presence of mGluR receptor antagonists.....	100
3.3.9	Excitatory responses to D1-like receptor agonists are not effected by metabotropic glutamate receptor antagonists	103
3.4	Discussion	105
3.4.1	Presynaptic and postsynaptic effects of dopamine.....	105
3.4.2	Identity of VP neurons modulated by dopamine	108
3.4.3	Functional implications:.....	111
4	Chapter 4: Serotonergic modulation of VP neurons	112
4.1	Abstract:	113
4.2	Introduction:.....	114
4.3	Results:	117
4.3.1	Serotonin application has dual effects on VP neurons.....	117
4.3.2	Repeated application of serotonin produces no clear sensitisation effects in VP neurons	119
4.3.3	Serotonin produces excitatory effects on VP neurons through presynaptic modulation	122
4.3.4	Inhibitory responses to serotonin application persist in the presence of low Ca ²⁺ aCSF	124
4.3.5	5HT1a receptors are required for the excitatory responses of VP neurons to serotonin	126

4.3.6	5HT2c receptors have an inhibitory influence on VP neurons	129
4.3.7	5HT5a receptors contribute to the excitatory effects of serotonin	132
4.3.8	5HT7 receptors contribute to VP excitatory response to serotonin.....	135
4.4	Discussion:	138
4.4.1	Pre and postsynaptic effects of serotonin application	138
4.4.2	Contribution of different serotonin receptors to serotonin responses in VP neurons	139
4.4.3	Contribution of specific serotonin receptors to inhibitory serotonin responses in VP neurons.....	142
4.4.4	The 5HT1a receptor antagonist WAY100635 is also a D4 receptor agonists ...	142
4.4.5	Functional implications:.....	143
5	Chapter 5: Effects of electrical stimulation of NAc afferents on VP neurons' firing	144
5.1	Abstract:.....	145
5.2	Introduction:	146
5.3	Results:.....	148
5.3.1	Stimulation of the NAc inhibits tonic firing in some VP neurons.....	148
5.3.2	NAc stimulation induces pauses of different durations dorsally compared to ventrally in the VP.....	151
5.3.3	Picrotoxin abolishes VP neurons inhibition by NAc stimulation.....	153
5.3.4	Inhibition of VP neurons by NAc stimulation is not affected by Opioid agonists	156
5.3.5	NK-1 receptors modulate the effect of NAc stimulation on VP tonic firing	159
5.4	Discussion:	162
5.4.1	Difference in pause duration dorsal to ventral within the VP:	163
5.4.2	Neurotransmitters and neuropeptides modulating pause duration:.....	164
5.4.3	Methodological issues with stimulation protocols:.....	165
5.4.4	Substance P modulation of pause duration:.....	166
5.4.5	Implications:.....	167
6	Chapter 6: General Discussion:	168
6.1.1	Dopaminergic modulation of VP neurons and their cellular mechanism	169
6.1.2	Serotonin receptor subtypes role in the dichotomous effects of serotonin on VP neurons	171
6.1.3	Modulation of VP neurons tonic firing by stimulation of NAc afferents	172
6.1.4	Technical considerations.....	173
6.1.5	Future work.....	175
6.1.6	Concluding remarks	176
7	References:	178

Acknowledgements:

Thank you to Enrico who has continued to support me and provide invaluable advice despite being on leave, it is greatly appreciated. Thank you to Jason Berwick for his support and paperwork skills.

Thank you to Clare Howarth for the use of equipment and the supply of animals, it was, and is greatly appreciated.

Thank you to Rachel who has coped admirably with all the nonsense that comes out of my mouth.

And finally, thank you to all the people in the laboratory and in Psychology who have given me help and support over the years, including: Nico, Mariana, Llewelyn and Claire.

List of Abbreviations:

aCSF	Artificial cerebrospinal fluid
AHP	After-hyperpolarisation
BLA	Basal lateral amygdala
BOLD	Blood oxygen level dependent
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CNS	Central nervous system
CPP	Conditioned place preference
DA	Dopamine
DOR	δ -opioid receptor
DRN	Dorsal raphe nuclei
GA	Glutamate
GABA	Gamma-aminobutyric acid
GABA _a	Gamma-aminobutyric acid _a
GABA _b	Gamma-aminobutyric acid _b
GAD	Glutamate decarboxylase
GP	Globus Pallidus
GPI	Globus pallidus internal
GPe	Globus Pallidus external
HFS	High frequency stimulation
HH	Hedonic Hotspot
IP	Intraperitoneal
LTD	Long term depression
LTP	Long term potentiation

MEA	Multi-electrode array
mGluR	Metabotropic glutamate receptor
MOR	μ -opioid receptor
mPFC	Medial pre-frontal cortex
MSNs	Medium spiny neurons
NAc	Nucleus accumbens
NAaC	Nucleus accumbens Core
NAcS	Nucleus accumbens Shell
NK-1, 2 and 3	Neurokinin receptor
PIT	Pavlovian – instrumental transfer
pMEA	Perforated multi-electrode array
PV+	Parvalbumin positive
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SOM	Somatostatin
SP	Substance P
SPNs	Spiny projection neuron
STN	Subthalamic nucleus
TH	Thyrosine Hydroxylase
VP	Ventral Pallidum
VTA	Ventral Tegmental Area
5HT	Serotonin

List of figures:

1. Figure 1.1: A schematic representation of the VP's neuronal connectivity. Adapted from Root et al., (2015).
2. Figure 1.2: Ionotropic and metabotropic glutamate receptors and their subtypes
3. Figure 1.3 Comparison of the classical canonical view of NAc projections and the revised
4. Figure 2.1 MEA perfusion schematic
5. Figure 2.2 sagittal slice including VP and NAc
6. Figure 2.3: differential effects of low and high-frequency stimulation protocols
7. Figure 2.4: Spike half-width calculation
8. Figure 3.1: dopamine application has dual effects on VP neurons
9. Figure 3.2: D1-like receptor agonists excite while D2-like receptor agonists excite and inhibit VP neurons
10. Figure 3.3: repeated application of dopamine, D1-like receptor agonists and D2-like receptor agonists produce no clear sensitisation effects in VP neurons
11. Figure 3.4: D1-like and D2-like receptor antagonists modulate the effect of dopamine in the VP.
12. Figure 3.5: low Ca^{2+} aCSF blocks the excitatory effects of D1-like and D2-like receptor agonists but not the inhibitory effects of D2-like receptor agonists.
13. Figure 3.6: D1-like receptor mediated excitation requires ionotropic glutamate receptors
14. Figure 3.7: excitatory effects of D2-like receptor agonists require metabotropic glutamate receptors
15. Figure 3.8: inhibitory effects of D2-like receptor agonists continue in the presence of mGluR receptor antagonists and low Ca^{2+} aCSF.

16. Figure 3.9: D1-like receptor agonists continue to excite VP neurons in the presence of
17. Figure 3.10: A minimal model explaining the differential effects of D1-like and D2-like receptor activation on type I and type II VP neurons in the VP.
18. Figure 4.1: serotonin application has dual effects on VP neurons
19. Figure 4.2: repeated application of serotonin produces no clear sensitisation effects in VP neurons excited by serotonin application
20. Figure 4.3: Repeated application of serotonin has no clear sensitisation effects in VP neurons inhibited by serotonin application
21. Figure 4.4: Blocking synaptic transmission with low Ca^{2+} aCSF prevents the excitatory effect of serotonin application in the VP.
22. Figure 4.5: Inhibitory responses to serotonin application maintain in the presence of low Ca^{2+} aCSF
23. Figure 4.6: 5HT1a antagonists block excitatory responses to serotonin application but do not block inhibitory responses to serotonin application in the VP
24. Figure 4.7: 5HT2c antagonists modulate inhibitory responses to serotonin application, but do not block excitatory responses to serotonin application in the VP.
25. Figure 4.8: 5HT5a antagonists modulate the magnitude of excitatory responses in the VP.
26. Figure 4.9: 5HT7 antagonist modulates the increase in firing frequency seen to serotonin application in the VP
27. Figure 5.1: stimulation of the NAc results in inhibition of tonic firing in some VP neurons

28. Figure 5.2: NAc stimulation induces a longer pause duration in dorsal VP neurons than
29. Figure 5.3: picrotoxin substantially disinhibits VP neurons inhibited by NAc stimulation
30. Figure 5.4: Inhibition of VP neurons by NAc stimulation is not effected by opioid agonists or antagonists
31. Figure 5.5 Inhibition of VP neurons by NAc stimulation is modulated by NK-1 receptor antagonists

List of tables:

1. Table 1.1: Key electrophysiological characteristics of the neuron types identified by Lavin and Grace (1996)
2. Table 1.2: Electrophysiological characteristics of cholinergic and non-cholinergic neurons identified by Bengtson and Osborne (2000)
3. Table 1.3: morphological and electrophysiological characteristics of VP neuron types identified by Pang et al, (1998)
4. 2.1: Summary table of animals used and the related recorded units for each study.

1 Chapter 1: General introduction

1.1 The Basal Ganglia:

The aim of this sections is to give a brief overview of the anatomy, physiology and function of the basal ganglia, followed by an outline of the ventral pallidum in more detail.

1.2 Anatomy of the basal ganglia

Morphologically the basal ganglia consists of four main nuclei: the striatum, globus pallidus, substantia nigra and subthalamic nucleus. The striatum is the main input structure of the basal ganglia and is subdivided into ventral and dorsal extents. The dorsal striatum is split into the caudate and putamen, these are divided by the grey matter of the internal capsule (Voogd, 1985), which produces the characteristic striped pattern, and gives the striatum its name. The ventral striatum consists of the nucleus accumbens (NAc), which is further sub-divided into the nucleus accumbens core (NAcC) and nucleus accumbens shell (NAcS). The shell wraps around the core at its ventral and medial edges and borders on to the rostral edge of the amygdala. The core is therefore partially surrounded by the shell and is classically considered to be the main component of the ventral striatum (George & Paxinos, 1998). The basal ganglia also includes the globus pallidus (GP) which has a dorsal and ventral division. The dorsal division is known as the globus pallidus (GP) and is sub-divided into GPi and GPe (internal and external). The internal segments is the most medial and the external segment is more lateral. Ventral to the anterior commissure the GP becomes the ventral pallidum (VP) and lies within the substantia innominata (Bolam, et al., 2000; Yelnik, 2002). The basal ganglia also consists of the STN (subthalamic nucleus) and SN (substantia nigra). The SN is again subdivided into pars compacta (SNc) and pars

reticulate (SNr), with SNc being more dorsal and SNr being more ventral (Bolam et al., 2000).

1.3 Physiology of the basal ganglia:

The basal ganglia consists of two main output structures and two main input structures. As previously mentioned, the striatum is the principal input area of the basal ganglia (Zahm & Brog, 1992). It receives multiple excitatory glutamatergic inputs from the cerebral cortex and thalamus. It also has serotonergic inputs from the dorsal raphe nuclei (DRN), dopaminergic input from the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Redgrave et al., 2010) and shares reciprocal GABAergic connections with the GPe and VP (Kupchik & Kalivas, 2016; Tepper et al., 2007). The striatum's outputs are inhibitory and target the GPe and SNr.

The other input area of the basal ganglia is the STN that also receives major excitatory inputs from cortical and subcortical regions (thalamus and brainstem) and receives dopaminergic input from the SNc, serotonergic input from the DRN and GABAergic inputs from the GPe (Bolam et al., 2000). The STN's main output targets are the GPi and the SNr, where it sends excitatory glutamatergic outputs (Tepper et al., 2007). It also sends excitatory outputs to the GPe (Redgrave et al., 2010).

The basal ganglia also has two key output nuclei: the GPi and the SNr, these receive inhibitory inputs from the striatum and GPe, and excitatory inputs from the STN. They both send inhibitory, GABAergic outputs to the thalamus, which then feeds back to the striatum, STN and cortical regions with glutamatergic inputs (Gerfen & Bolam, 2010).

1.4 Function of the basal ganglia

The basal ganglia is involved in multiple functions, such as sensory processing, motor control and reward learning. It is generally considered that the basal ganglia consists of multiple looped circuits that are reciprocally connected (Redgrave, 2007). These circuits are believed to carry cortical information relating to these functions, which are integrated within the basal ganglia (Redgrave et al., 2011).

1.4.1 The basal ganglia and reward learning:

Several of the basal ganglia nuclei are considered to be involved in reward processing. Two key nuclei are the striatum (especially its ventral divisions) and the ventral division of the GPe, known as the VP. The basal ganglia integrates sensory information with memory and knowledge about reward values, before developing this information to produce a motor plan, which is output to motor control structures to enact actions. These selected actions are appropriate in light of the processing that has occurred in relation to the reward value of the sensory information in the basal ganglia (Delgado, 2007).

Drugs of abuse have also been shown to modulate the reward function of the basal ganglia by mimicking natural rewards. Drugs of abuse, such as cocaine, modulate the basal ganglia pathways involved in reward learning, resulting in LTP and LTD. This results in some of the behavioural aspects of addiction (Creed et al., 2016)

1.5 The ventral pallidum

As previously mentioned the VP is considered the ventral extent of the GP/GPe and is one of the main output structures of the basal ganglia (Heimer et al., 1982). It is implicated in aspects of learning, reward, pleasure and addiction (Berridge & Kringelbach, 2013; Creed et al., 2016; Itoga et al., 2016; Smith et al., 2009). It is perfectly placed to integrate information from cortical regions with information from forebrain and limbic regions (Smith et al., 2009) and as such has been postulated as a central region for the integration of signals related to reward and addiction (Ambroggi et al., 2016) and is suggested as a central region in converting motivational signals into appropriate behavioural responses (Kalivas et al., 1999). In the following section I will give an overview of what we currently know about the VP, including, connectivity, functional roles, neuron types and neurochemistry.

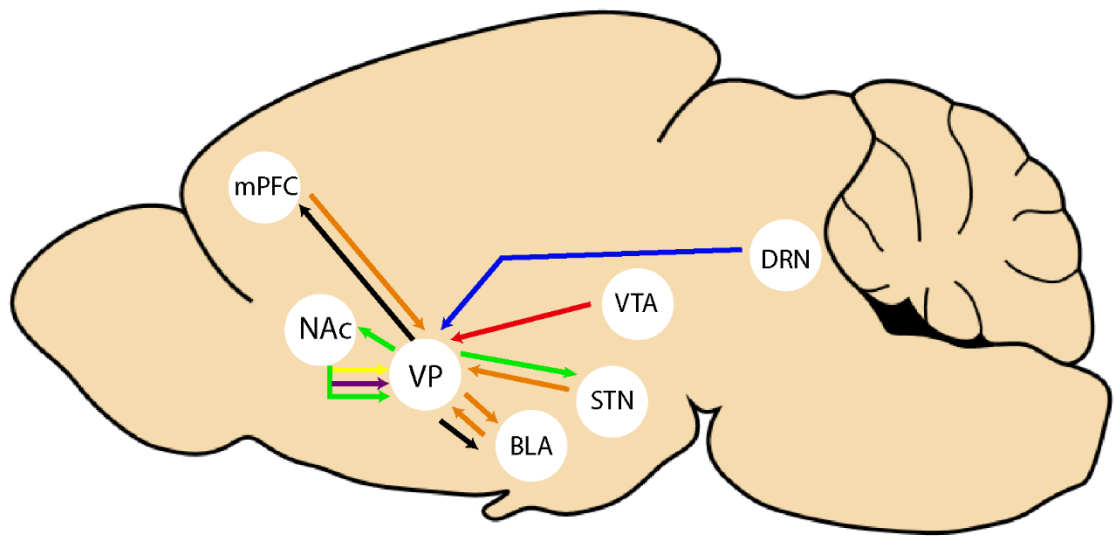
1.5.1 Organisation of the VP:

The VP was described first in the rodent by Heimer and Wilson (1975) (cited in: Bengtson & Osborne, 2000), who identified it as the ventral extent of the GPe, that resided below the anterior commissure. It is considered to be part of the basal forebrain (Duque et al., 2007; Pang et al., 1998; Root et al., 2015; Zaborszky et al., 2012), which is a heterogeneous set of structures including: the medial septum, BLA (basal lateral amygdala), substantia innominata, diagonal band nuclei and the peripallidial regions (Zaborszky et al., 2012). Because of this the VP is considered to express neurons common to other basal forebrain regions. On the other hand the VP is also considered to be part of the striatopallidal system of the basal ganglia and be a key output structure for the basal ganglia. It is therefore also believed to include neurons similar in morphology and electrophysiology to those found in the ventral striatum (Kupchik & Kalivas, 2013).

Just like the dorsal extent of the pallidum, the VP contains two distinct regions, delineated by their projections and by staining. One region stains for substance-p and projects chiefly to the thalamus, while the other stains for enkephalin and projects chiefly to the STN (Haber & Elde, 1981; Haber et al., 1985; Mai et al., 1986)

It is due to its connectivity that the VP is considered a key region for integration of information related to reward and the motivational salience of stimuli, as well as being a key region in relation to addiction and hedonic responses. This is because it integrates GABA, glutamate, opioid, serotonin and dopamine signals from multiple regions. It has glutamatergic inputs from limbic regions, such as the BLA, dopaminergic inputs from the SNc and VTA (Klitenick et al., 1992; Smith & Kieval, 2000), a large GABAergic input from the NAc (Bolam et al., 1986; Churchill et al., 1990a; Churchill & Kalivas, 1994), which recent evidence shows includes both D1 and D2 expressing MSNs that also release substance P and enkephalin into the VP (Creed et al., 2016; Kupchik et al., 2015). It also has glutamatergic input from the STN (Groenewegen et al., 1993; Zahm et al., 1996), Serotonergic inputs from the dorsal raphe nuclei (DRN) (Vertes, 1991) and glutamatergic inputs from the medial pre-frontal cortex (mPFC) (Yang et al., 2014).

The VP also outputs to all these regions, with most being either GABAergic or glutamatergic, bar its reciprocal connections with the BLA, thalamus and mPFC, where some of its outputs are considered to be Ch4 cholinergic neurons (McKinney et al., 1983). It also outputs to other regions, such as the lateral hypothalamus and Lateral Habenula (Haber et al., 1985). The VP is therefore well placed to drive ascending signals to the mPFC and thalamus as well as descending signals to the VTA and brainstem regions that drive reward related behaviour (Panagis et al., 1997).










BLA = basal lateral amygdala	 Acetylcholine
DRN = dorsal raphe nuclei	 Dopamine
mPFC = medial pre-frontal cortex	 Enkephalin
NAc = Nucleus accumbens	 GABA
STN = sub-thelamic	 Glutamate
VP = ventral pallidum	 Serotonin
VTA = ventral tegmental area	 Substance P

FIGURE 1.1: A Schematic representation of the VP's neuronal connectivity. Adapted from Root et al., (2015).

1.6 Functional roles of the VP:

1.6.1 Role of the VP in reward processing:

There are multiple lines of evidence to suggest the VP plays an integral role in reward processing and reward related behaviours, such as conditioned place preference and instrumental responses. Indeed Smith et al., (2009) suggests it may constitute a “limbic final common pathway” for the processing of reward.

The VP responds robustly to reward and reward predicting cues, changing its firing patterns to code Pavlovian cues that represent a predictive reward (Tindell et al., 2004; Tindell et al., 2005). Richard et al., (2016a) have also shown that activity in VP neurons is essential for cue-elicited reward seeking. Interestingly, Richard et al., (2016a) found that cue-elicited responses in VP neurons occur at a shorter latency than responses in NAc neurons, suggesting that they are not simply responding to the NAc input, highlighting the broader (mesolimbic) integrative nature of the VP in reward processing. Lesion studies combined with conditioned place preference paradigms have also shown that the VP is integral to reward related processing. McAlonan et al., (1993) found that animals lesioned in either the anterior or posterior VP displayed attenuated conditioned place preference responses. Self-stimulation studies, such as that by (Panagis et al., 1995) also support the involvement of the VP in reward processing as animals would actively self-stimulate, using a lever; in 98% of the sites the stimulation electrode was placed in the VP.

Farrar et al., (2008) has also shown that inhibition of VP circuitry via GABA_A infusion decreased the lever pressing behaviour of animals for a preferred food and instead switched their behaviour towards increased intake of a least preferred food that required less effort. This suggests that the VP may be key to effort and motivational drive to pursue rewarding stimuli.

There is also evidence from humans and other primates to support the role the VP plays reward. Neuroimaging studies have shown that the VP is active during reward tasks that require motivation (Beaver et al., 2006; Pessiglione et al., 2007). Others have also shown that single neurons in the VP code expected reward value, for example Tachibana & Hikosaka, (2012) provide evidence to suggest that VP neuronal activity increases in line with the size of the expected reward. They also show that the VP codes the information on expected reward value, which in turn is used to alter motor

responses. This fits well with Smith et al's., (2009) suggestion that the VP may constitute the “limbic final common pathway” for processing of reward and action appropriate motor responses.

1.6.2 Role of the VP in pleasure/liking responses:

Evidence also implicates the VP in the pleasure-processing aspects of reward.

Pleasurable stimuli, such as sucrose, have been shown to increase firing within the VP (Itoga et al., 2016; Tindell et al., 2006). The same increases in firing have also been seen in the VP in response to saline in salt depleted animals (Tindell et al., 2009), suggesting that it encodes and integrates information based on internal states and results in appropriate motor responses.

Lesions of the VP have been shown to reduce hedonic responses to rewarding stimuli (Cromwell & Berridge, 1993; Smith et al., 2009). Lesion studies also suggest that the VP is essential for normal pleasure responses, as damage to posterior regions of the VP result in elimination of positive orofacial responses to sweet tastes (Ho & Berridge, 2014). Neurochemical modulation of the VP has also been shown to modulate pleasure/liking responses. Shimura et al., (2006) found that GABA_A antagonists increased the ingestion of liked sucrose solutions, while GABA_A agonists injected into the VP decreased the ingestion of a liked sucrose solution. Inui and Shimura, (2014) have also shown that specific delta opioid receptor inhibition in the VP increases the palatability of saccharin and its ingestion. Further to this, there is evidence from human imaging studies that suggest that the VP responds to pleasurable stimuli. Calder et al., (2007) presented images of pleasurable food to participants and measured BOLD responses across brain regions, finding an increased response in certain sub regions of the VP. Other studies with human participants also support the notion that the VP plays

a key role in pleasure responses beyond the rodent. Miller et al., (2006) reports a human case study where an overdose of methadone induced lesions to the pallidum resulting in reduced craving for drugs of abuse and reduced pleasure responses, including depressive symptomology. Research suggests that it is perhaps not the whole of the VP that modulates pleasure responses to rewarding stimuli. Evidence suggests that there are distinct regional differences. For example, Smith and Berridge's., (2005) study maps the VP for hedonics and suggests the posterior VP, when stimulated with DAMGO, produces the greatest increase in pleasure responses to sucrose. This is supported by Peciña et al., (2006).

1.6.3 Role of the VP in addiction to drugs of abuse:

There are several lines of evidence implicating the VP in responses to drugs of abuse and the suggestion that the VP is a key site in the incentive salience theory of addiction (Tindell et al., 2009).

Drugs of abuse, such as cocaine are known to modulate the firing of VP neurons.

Johnson & Napier, (1996) found that intravenous cocaine administration altered the firing in 80% of VP neurons. Root et al., (2012) also found that this change in firing to administration of cocaine reduced as the level of cocaine decayed after administration.

Research has also shown that drugs of abuse alter neurochemical levels in the VP and the ability of neurochemicals to modulate activity in the VP. Stout et al., (2016) has shown that there are distinct neurochemical changes within the VP in response to cocaine sensitization. A cocaine challenge, after cocaine sensitization, produced dramatic increases in dopamine levels within the VP. There is also evidence to show that, as well as dopamine, serotonin levels increase as a result of cocaine self-administration within the VP (Sizemore et al., 2000), and McDaid et al., (2005) has

shown cocaine sensitisation promotes the ability of glutamate to increase firing in the VP and inhibits the ability of GABA to reduce firing .

The VP is also involved in drug seeking reinstatement, for example Tang et al., (2005) have shown that μ opioid-receptor antagonists, directly injected into the VP, blocked reinstatement of cocaine self-administration and stimulation of the VP with morphine reinstated this self-administration. Microinjections of μ opioid-receptor antagonists and D1-like dopamine receptor antagonists directly into the VP have also been shown to completely abolish sensitization to drugs of abuse, such as morphine (Johnson & Napier, 2000).

Further evidence of the VP's role in addictive responses comes from lesion studies of the VP. Hubner and Koob, (1990) have shown that Ibotenic acid lesions of the VP, after animals had been trained to self-administer cocaine or heroin, dramatically reduces self-administration of cocaine and heroin, this furthers the notion that the VP is essential for drug seeking behaviour. Hiroi and White, (1993) also carried out lesioning studies of the VP. Animals were given NMDA lesions in the VP, either pre or post training in a conditioned place preference task for amphetamine. Animals with a lesions pre-conditioning did not acquire a conditioned place preference, those that were lesioned post conditioning did. This suggests that the VP is involved in acquisition of conditioned place preferences to amphetamine and not in the execution once conditioned. Chemogenetic silencing of the VP has also been shown to reduce the reinstatement of drug seeking (Mahler et al., 2014). McFarland and Kalivas, (2001) also showed that chemogenetic silencing of the VP via administration of GABA agonists reduced drug seeking behaviour in cocaine sensitized animals.

1.7 Neurons of the VP:

The VP is commonly considered to be part of the basal forebrain (Duque et al., 2007; Pang et al., 1998; Root et al., 2015; Zaborszky et al., 2012), which is a heterogeneous set of structures that includes: the medial septum, BLA (basal lateral amygdala), substantia innominata, diagonal band nuclei and the peripallidial regions (Zaborszky et al., 2012). Because of this it contains neurons of the basal forebrain, such as the CH4 cholinergic neurons (Gritti et al., 1993; McKinney et al., 1983) . However it is also considered to be part of the striatopallidal system of the basal ganglia and is believed to also include neurons similar in morphology and electrophysiology to those found in the ventral striatum (Kupchik & Kalivas, 2013b). Because of this intriguing mix of cell types many studies that have focused broadly on the basal forebrain may draw false conclusion about cell types found within the VP as they may well be over generalisations, based upon cell types found within the wider basal forebrain territories.

The main population of neurons in the VP express markers for GABA, these are thought to constitute approximately 80% (Gritti et al., 1993; Root et al., 2015). It is believed that these probably make up several subpopulations, some being spiny projection neurons (SPNs), projecting to areas outside the VP, such as the lateral hypothalamus, thalamus and STN (Bevan et al., 1997; Haber et al., 1993; Mariotti et al., 2001), and others that are believed to be interneurons (Bengtson & Osborne, 2000; Gritti et al., 1993) the distinction between SPNs and interneurons is mainly based on ionotropic filling of neurons, which has revealed neurons with sparse intranuclear branches, akin to projection neurons, and those with a dense plexus of intranuclear branches, akin to interneurons (Pang et al., 1998). These subpopulations can be further divided based upon their expression of other neurochemicals and neuropeptides (Root et al., 2015). Immunohistochemical studies suggest VP neurons express calretinin, calbindin, PV, NPY and SOM (Zaborszky, 1989; Zaborszky et al., 2012).

As well as the presence of a number of GABAergic neurons, there is also the existence of cholinergic neurons, (with no evidence of colocalisation, between GAD and CHAT) within the VP (Gritti et al., 1993). These cholinergic neurons are a constituent part of the basal forebrain, magnocellular, cholinergic system (Kupchik & Kalivas, 2013a; Mallet et al., 2012; Zaborszky & Cullinan, 1992). Although they are considered to be extremely sparse in number, (Gritti et al., 1993). Others suggest they make up approximately 23% of the neurons in the VP and have been shown to have extensive collaterals with other neurons within the VP (Bengtson & Osborne, 2000; Duque et al., 2007; Gritti et al., 1993). The cholinergic neurons of the VP are interesting as they are known to be heavily innervated by projections from several regions, including GABAergic projections from the NAc (Zaborszky & Cullinan, 1992) and dopaminergic inputs from the VTA (Smith & Kieval, 2000; Zaborszky et al., 2012). They are also known to express NK1 receptors (Chen et al., 2001; Gerfen, 1991), and therefore maybe implicated in the effects of substance P expressed by Nac projections to the VP (Mengual et al., 2008). Cholinergic neurons in the VP are also seen to be hyperpolarised by serotonin, while non cholinergic neurons in the VP are depolarized (Bengtson et al., 2004).

Cholinergic neurons of the VP also project outside the VP, to both the cortex and amygdala (Duque et al., 2007; Lavin & Grace, 1996; McKinney et al., 1983), and therefore provide the intriguing potential for integrating information from basal forebrain regions, with ventral striatal regions and then providing a common final mechanism for the delivery of this information to cortical and subcortical regions. There local collaterals (Zaborszky & Duque, 2000) also provide the intriguing prospect that they may well interact with the other main population of neurons in the VP that are GABAergic, which have also been shown to project outside the VP. However the question remains as to whether they interact and modulate all the GABAergic neurons

within the VP or specific types, such as the NPY expressing type or the SOM expressing type.

There is also evidence for the presence of glutamatergic neurons within the VP with immunohistochemical labelling for VGLUT1, VGLUT2 and VGLUT3 (Geisler et al., 2007), however it is considered most likely that VGLUT1 and VGLUT2 consists of labelled projections emanating from the VTA (Root et al., 2015). There is however some labeling for VGLUT3 in the VP and this is thought to be labelling mainly cholinergic neurons within the VP (Poulin et al., 2006).

1.7.1 Electrophysiological classification of VP neurons:

There have been several attempts to electrophysiologically characterise and group neurons within the VP *in vivo* and *in vitro*. One of the key *in vivo* studies was carried out by (Lavin & Grace, 1996), they focused on several key electrophysiological characteristics of VP neurons spike discharge. They found these to be able to reliably place all VP neurons studied into one of three groups, referred to as type, A, B and C.

Type of neuron (Lavin & Grace, 1996)	discharge	Action potential	Afterhyperpolarisation (AHP)
A (most common:29/55)	Tonic (8.7Hz)	Long duration	No AHP
B	14.5Hz	Ramp-like depolarisation pre- discharge. Short duration spike.	Post-spike AHP
C	Fired in burst or couplets.	Accommodation shown. Subsequent spikes lasted longer, but had reduced amplitude.	No AHP

TABLE 1.1: Key electrophysiological characteristics of the neuron types identified by Lavin and Grace (1996)

Lavin and Grace, (1996) believe that type A and B are non-cholinergic neurons, therefore most likely GABAergic, while type C are believed to most likely be cholinergic neurons as they share firing patterns very similar to cholinergic neurons identified in other areas of the basal forebrain, which are part of the same magnocellular projection pathway (Bengtson & Osborne, 2000; Griffith & Matthews, 1986). Lavin and Grace, (1996) also suggest that there is a strong similarity between the neurons they have categorised as type A, B, C with those categorised as 3,2 and 1, respectively by Nambu and Llinas, (1994) in the GPe, with type C of Lavin and Grace, (1996) considered to be most like type 1 of Nambu and Llinas, (1994), as they shared a

bursting firing pattern. Interestingly, Lavin and Grace, (1996) also found that type A and C responded to NAc stimulation with excitation, and were suggested by Lavin and Grace, (1996) to be mediated by substance P, which is known to be expressed by the projections between NAc and VP.

Bengtson and Osborne, (2000) also investigated the electrophysiological properties of neurons in the VP. They grouped neurons in the VP as cholinergic and non-cholinergic. They found that the cholinergic neurons had a very similar electrophysiological profile to cholinergic neurons found elsewhere in the basal forebrain. Some key features of these neurons include:

Types	Tonic firing	Spike accommodation	Inward rectification	Resting membrane potential	After hyperpolarisation (AHP)
Cholinergic	No	Strong spike accommodation	Fast and large	Hyperpolarised resting membrane potential	Prominent and long duration
Non-cholinergic	yes	No spike accommodation	Time dependent. Large h currents		

Those in bold are considered the distinguishing features of VP cholinergic neurons by Bengtson and Osborne, (2000)

TABLE 1.2: Electrophysiological characteristics of non-cholinergic and cholinergic neurons identified by Bengtson and Osborne, (2000)

Because of the above profiles, Bengtson and Osborne, (2000) suggest that Lavin and Grace, (1996) assertion that there type C neurons in the VP are cholinergic is incorrect. One key feature difference is the lack of an AHP in the type C of Lavin and Grace, (1996), while Bengtson and Osborne, (2000) find cholinergic neurons to have a prominent AHP. Another difference is that type C of Lavin and Grace, (1996) have a bursting firing pattern, while Bengtson and Osborne, (2000) suggest cholinergic neurons in the VP to not tonically fire in a bursting pattern. There is therefore some controversy over the electrophysiological features of VP cholinergic and non-cholinergic (likely GABAergic) neurons.

Another study that tried to group VP neurons based on their electrophysiological characteristics was carried out by Pang et al., (1998). As well as electrophysiological measures, Pang et al., (1998) also carried out immunohistochemically procedures and intracellular filling to morphologically examine the neurons.

Type	morphology	Spontaneous firing rates	Action potential duration	Action potential waveform
I	Rarely branched	Low (13.2+/- 7.3 Hz)	2.4+/-0.2 ms	Triphasic :Positive-negative-positive
II	Branched extensively	Higher (35.5+/- 8.4 Hz)	2.5+/-0.3 ms	Triphasic

TABLE 1.3: morphological and electrophysiological characteristics of VP neuron types identified by Pang et al, (1998)

Pang et al, (1998) suggest that their type I neurons are likely the same as Lavin and Grace's, (1996) type B neurons, and that they may well represent GABAergic projection neurons. They also suggest that type II neurons, in their study, are likely to correspond to Lavin and Grace's, (1996) type A neurons and that the type II neurons are likely GABAergic interneurons, due to their large arbors.

Recent research by Kupchik and Kalivas, (2013b) suggests there maybe regional difference within the VP as to the type of neurons present, therefore the electrophysiological profiles of neurons across the VP may vary. They identified two electrophysiologically distinct types of neurons (type 1 and type 2). Type one shared many characteristics with neurons already identified in the VP by Lavin and Grace, (1996) and Pang et al, (1998). These type 1 neurons were reminiscent of Lavin and Grace's, (1996) type B neurons and of Pang et al's (1998) type 1 neurons. They were also Immunoreactive for GAD, therefore are believed to be GABAergic. Type 2 neurons however were distinct from anything previously identified in the VP, and shared more in common with the known electrophysiological profile of NAcS neurons. Because of their predominantly rostral location in the VP, bordering the NacS, Kupchik and Kalivas, (2013b) suggest they may be NacS neurons that have invaded the VP. This highlights the variety of potential neuronal types in the VP and suggests that the VP is not a homogenous structure.

1.8 Neurochemicals and receptors in the VP

In order to fully understand how the variety of neurons identified in the VP interact as well as the likely effects of its various inputs, it is important to consider the different neurotransmitters released into the VP and their known receptors, so we can fully understand the function of the VP as an integrative area.

1.8.1 GABA (GABA_A and GABA_B receptors):

GABA is the main inhibitory neurotransmitter expressed throughout the CNS. There are both GABA_A and GABA_B receptors. GABA_B receptors are thought to mediate a slower inhibition and GABA_A is thought to mediate a fast inhibition (Kemppainen et al., 2012a). Both GABA_A and GABA_B receptors are known to be expressed within the VP (June et al., 2003). GABA_A receptors have several isoforms, referred to as GABA_{A1-6} (Barnard et al., 1998). These are all known to be expressed within the VP with the dominant expression being GABA_{A1} (June et al., 2003).

The VP receives an extensive GABAergic input from the NAc (Churchill et al., 1990; Groenewegen & Russchen, 1984). There is also the suggestion of a number of potentially GABAergic interneurons within the VP (Root et al., 2015). Therefore GABA and its receptors are a likely contributory factor in the VP's known functions and cellular responses to various neurotransmitters.

1.8.1.1 Functional role of GABA in VP

GABA levels in the VP are believed to be involved in its role in drug seeking behaviour. Research has found (Kemppainen et al., 2012a; McFarland & Kalivas, 2001) that GABA agonists, injected into the VP, significantly reduced animals drug seeking behaviour. This seems to be largely mediated by GABA_A as GABA_B agonists have been shown to have no effect on motor activity when injected into the VP (Austin & Kalivas, 1990). A GABA_A isoform (GABA_{A1}) has also been shown to reduce alcohol seeking behaviours when injected into the VP (June et al., 2003). GABA levels in the VP have also been shown to modulate motivation, Farrar et al., (2008) have shown that the GABA_A agonist muscimol reduced animals drive towards a preferred food and switched

their consumption to a less preferred food that was easier to obtain, suggesting that GABA_A may play an integral role in the modulation of motivational behaviour towards reward stimuli via its modulation of the VP. Other neurotransmitters released into the VP, and by VP neurons, may also modulate these responses by modulation of pre-synaptic receptors found on GABAergic inputs from the NAc into the VP.

1.8.2 Glutamate

Glutamate is an important neurotransmitter in the VP and, as previously discussed, glutamatergic inputs to the VP arrive from several locations and these are: the BLA, mPFC and the STN.

There are two main groups of glutamate receptors. These are: ionotropic glutamate receptors and metabotropic glutamate receptors. Ionotropic receptors form ion-gated channels and are believed to control the majority of fast excitatory transmission in the CNS (Testa et al., 1994). Metabotropic glutamate receptors are C family, G protein coupled receptors (Niswender & Conn, 2010) and mediate their effects through modulation of internal secondary messengers, such as cAMP. They are considered to be involved in more long term processes, such long-term potentiation, synaptic plasticity and long term depression (Calabresi et al., 1992).

Ionotropic receptors split into three subtypes: NMDA, AMPA and Kinate. Metabotropic glutamate receptors also spilt into three subtypes, however only one is considered excitatory (group 1) while two subtypes (group 2 and 3) are considered inhibitory. Testa et al's., (1994) data shows the presence of all the MGLuR receptors in the VP with high levels of mGluR1 and 5, compared to the NAc and GP.

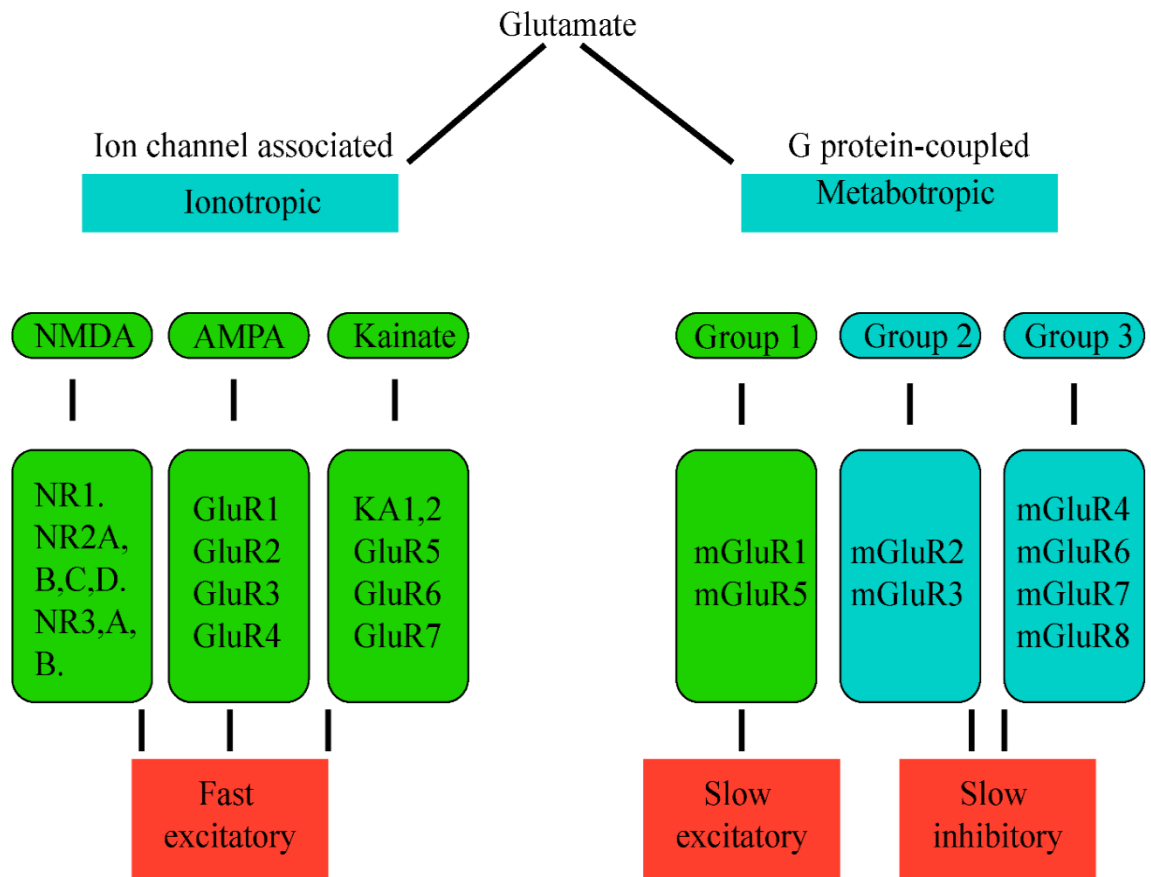


FIGURE 1.2: Ionotropic and metabotropic glutamate receptors and their subtypes. The multiple receptor subtypes found in both the metabotropic glutamate receptor family and the ionotropic glutamate receptor family. All Ionotropic glutamate receptors are believed to be Excitatory, while only group one metabotropic glutamate receptors are believed to be excitatory. Adapted from Blackshaw et al., (2011).

1.8.2.1 Group 1: mGluR 1 and 5

mGluR1 are mainly considered to be located in extrasynaptic and perisynaptic locations at the edge of the synaptic cleft and are thought to be modulated by glutamate that has escaped the synapse (Ferraguti et al., 2008; Ferraguti & Shigemoto, 2006). Little in the literature suggests they play a role in the modulation of functions the VP is known to be implicated in.

mGluR5 are considered to be located postsynaptically and can also be found on non-neuronal cells, such as astrocytes and microglia (Nicoletti et al., 2011). They are known

to be heavily involved in both LTP and LTD and have been shown to enhance ionotropic glutamate receptor functions, specifically NMDA receptor function (Pisani et al., 2001). mGluR5 is involved in modulation of the basal ganglia circuitry and is well known in the cocaine literature as a modulator of cocaine seeking and withdrawal, indeed research (Kenny et al., 2005; Kenny & Markou, 2004) suggests that mGluR5 receptors activation is essential for incentive motivation towards drugs of abuse and mGluR5 antagonists are considered promising treatments for tobacco addiction (Kenny, 2009). This fits well with the known involvement of the VP in drug seeking and sensitization, therefore mGluR5 receptors maybe a key mechanism underpinning the VP's neuronal responses to drugs of abuse.

1.8.2.2 Group 2: mGluR 2 and 3

mGluR 2 and 3 are located at both pre and post synaptic locations (Nicoletti et al., 2011; Petralia et al., 1996) and are considered to have inhibitory effects through inhibition of cAMP formation, excitation of potassium channels and inhibition of calcium channels (Pin & Duvoisin, 1995). Ohishi et al., (1994) and Ohishi et al., (1993) Mrna studies show that there is labelling for mGluR2 in the VP.

These receptors are also implicated in behavioural functions similar to the VP, such as hedonic responses and reinforcing effects of drugs of abuse. For example mGluR2/3 antagonists have been shown to elicit antidepressant effects (Yoshimizu et al., 2006) and mGluR2 receptors are implicated in regulating the reward pathway as cocaine is more reinforcing in mGluR2 knockout mice and mGluR2/3 agonists reduce the self-administration of cocaine (Adewale et al., 2006). These receptors have also been implicated in other regions of the basal ganglia, especially areas upstream from the VP, such as the NAc. Kalivas, (2009) suggests that it is dysfunction of these receptors in the

NAc that results in the increased chance of relapse in addiction to drugs of abuse. Their role in the modulation of the VP circuitry therefore might be insightful.

1.8.2.3 Group 3: mGluR 4, 6, 7 and 8

mGluR 4,7 and 8 receptors are known to be located presynaptically in the active zone of the synaptic cleft, this combined with their inhibitory function, suggests they provide fast negative feedback, inhibiting further release of glutamate from the presynaptic neuron (Niswender & Conn, 2010).

mGluR 6 receptors are exclusively found in the dendrites of on-polar cells of the retina (Nicoletti et al., 2011) and there is little evidence implicating mGluR 4 and 8 in any of the functions of the VP. However recent evidence suggests that mGluR 7 receptors may play some functional role in mediating drug seeking behaviour, something the VP is strongly linked with (Gong et al., 1996; Gong et al., 1997). Li et al., (2013) have also shown that a specific mGluR7 agonists modulates the GABAergic connections between the NAc and the VP and are able to inhibit cocaine induced reinstatement of cocaine seeking when directly injected into the VP. These effects were also shown to be reversed with mGluR7 antagonists.

1.8.3 Opioids (enkephalin and dynorphin) and opioid receptors (δ , kappa and μ):

One of the main inputs of the VP is from the NAc and these projections are GABAergic (Bolam et al., 1986; Churchill et al., 1990). These studies also show that the inputs from the NAc also release enkephalin and substance P into the VP. Recent evidence also shows that the projections from the NAc include D1 and D2 MSNs associated with the direct and indirect pathway respectively. Both D1 and D2 MSNs are also known to express substance P and enkephalin, respectively. The ventral striatum is therefore the

likely main source of enkephalin in the VP, especially as it is considered that very few neurons in the VP actually synthesise enkephalin (Mansour et al., 1993).

There are two main opioid-receptors for enkephalin. These are the δ -opioid receptors (DORs) and the μ -opioid receptors (MORs).

1.8.3.1 MORs in the VP

MOR are considered to be one of the best markers for delineating the VP from other structures in the striatopallidial circuit and basal forebrain, especially in the rostradorsal extent of the VP, where they are expressed in moderate levels (Mansour et al., 1994; Mansour et al., 1995; Olive et al., 1997). Mansour et al, (1997) also show that MORs are located presynaptically in the VP and therefore are likely to act as inhibitory autoreceptors. This fits well with Kupchik et al's., (2014) data, which shows that MORs activation with agonists inhibits the release of GABA from SPNs innervating the VP, most likely inhibiting the release of GABA from the inputs of the NAc. However, MORs are known to have dichotomous effects on interneurons within other striatal territories (Elghaba and Bracci, 2017) and Olive et al's, (1997) data shows that MORs are in fact located at both pre and post synaptic locations in the VP, therefore their effects may be broader than reducing GABAergic input into the VP.

1.8.3.2 DORs in the VP:

Research has shown (Mansour et al., 1988; Mansour et al., 1993) that DORs are expressed in the VP. More specifically, Olive et al., (1997) have shown that DORs are found at postsynaptic locations, in the cell bodies of neurons in the VP. This postsynaptic location was found to be opposite enkephalin labelled fibre terminals.

Olive et al's, (1997) data also suggests that DORs may well be expressed on fibres projecting back to the NAc from the VP as an inhibitory feedback loop. This certainly fits with Churchill and Kalivas, (1994) identification of a GABAergic input from the VP to the NAc.

1.8.3.3 Functional roles of MORs and DORs in the VP:

There is a strong link between MORs activation in the VP and consumption of drugs of abuse. For example Kemppainen et al., (2012b) study shows that MOR agonists injected into the VP reduce ethanol intake dose dependently and MOR antagonists increase ethanol intake. Research also suggest other effects of MOR modulation in the VP. Skoubis and Maidment, (2003) found that blockade of VP opioid receptors with antagonists induced a place aversion, while reducing the conditioned place preference to cocaine. Further to this Tang et al., (2005) found that reinstatement of drug seeking correlated with reduced levels of extracellular GABA in the VP, and reinstatement of drug seeking requires MORs in the VP to be recruited, as MOR antagonists blocked reinstatement of lever pressing caused by intravenous cocaine administration. Indeed Kupchik et al., (2014) suggests that cocaine acts by dysregulating opioid gating of GABA inputs of the VP. Kupchik et al., (2014) found, in cocaine extinguished animals, MOR agonists no longer reduced GABA release into the VP, but MOR antagonists, in these animals, still increased GABA release. They suggest this is due to cocaine increasing tone on the MOR receptors, they therefore continue to tonically suppress GABA transmission in cocaine sensitized animals. This all suggests that opioid receptors in the VP are essential for expression of drug related behaviour, however their exact effect may differ depending upon the drug of abuse.

With DORs the research points towards a role in the modulation of hedonic responses. Inui and Shimura, (2014) found that antagonists for DORs in the VP increased the palatability and consumption of saccharin. However, DOR's have also been shown to modulate how rewarding self-stimulation is, with Johnson and Stellar, (1994) finding increases of up to 31% in reward responses when DOR agonists were self-administered into the caudal VP.

1.8.4 Substance P

Substance P is part of a class of neuromodulators known as neurokinins. Neurokinins also include: neurokinin a, neurokinin b, substance K (SK), and neuromedin K (NK,) in the mammalian brain (Chen et al., 2001). Substance P is known to be in high concentration in the VP and modulation of it in the VP, mainly via the use of NK-1 receptor antagonists, effects behaviour related to the VP, including: affective/anxiety responses as well as aspects of conditioning, such as responses to CPP tasks (Nikolaus et al., 1999a; Nikolaus et al., 1999b).

The main source of substance P in the VP is thought be the D1 (direct) pathway inputs from the NAc (Bolam et al., 1986; Kupchik et al., 2014; Kupchik et al., 2015; Napier et al., 1995b) and there is evidence to suggest these inputs might directly target VP cholinergic neurons, which are known to express NK-1 receptors (Bolam et al., 1986; Gerfen, 1991).

Research has shown that VP neurons are excited by substance P application. Napier et al, (1995a) found 40% of VP neurons were excited by ionotropic direct application of substance P or an agonist analog of substance P. Napier et al, (1995) suggest the type of excitation they observed as a result of substance P application was significantly different from glutamate induced excitation, with a slower onset and a longer duration.

However, Substance P has also been shown to modulate glutamatergic inputs from the amygdala into the VP (Mitrovic & Napier, 1998).

1.8.4.1 NK receptor expression in the VP

There are two key neurokinin receptors (NK) known to be expressed in the VP, these are NK1, and NK3 (Chen et al., 2001; Furuta et al., 2004). Substance P is considered the preferred ligand for NK1 receptors, while neurokinin B is for NK3 receptors (Furuta et al., 2004; Mantyh, 2002; Quirion & Dam, 1988).

1.8.4.2 NK1 receptors:

NK1 receptors have been shown to modulate the effects of substance P injections into the VP on both anxiolytic responses and conditioned place preferences (Nikolaus et al., 1999a; Nikolaus et al., 1999b). These effects may be modulated by cholinergic neurons in the VP, as NK1 receptors in the VP are expressed by cholinergic neurons. In fact research (Chen et al., 2001; Gerfen, 1991; Mengual et al., 2008) suggests they are the main neurons in the VP express NK1 receptors. Cholinergic neurons of the VP are also therefore the likely target for substance P induced excitatory responses seen in 40% of VP neurons by Napier et al., (1995a). However Mengual et al., (2008) data also suggests that NK1 receptors are found in non-cholinergic neurons in the VP and the glutamatergic terminal inputs to the VP, therefore possibly playing a role in modulation of other VP neuron populations and increasing glutamate levels within the VP. NK1 receptors therefore have an intriguing role to play in the modulation of VP neurons and their inputs and therefore are an interesting target for future research.

1.8.4.3 NK3 receptors:

Nikolaus et al's., (1999a) data suggests that substance P, via the administration of a substance P analog, was able to increase place preference responses when injected into the VP, however selective NK1 receptor antagonists did not abolish this increase in place preference, suggesting that substance P may also be having an impact at other receptor sites. In fact the substance P analog used in this study is known to have a high affinity for NK3 receptors. Therefore there is an interesting possibility that NK3 receptors, known to be expressed in the VP (Furuta et al., 2004), may also be modulating the effect of substance P input from NAc fibres. In fact Furuta et al, (2004) found that NK3 receptors were expressed in both cholinergic and PV+ neurons in the VP. Further, tentative support for this comes from Ciccocioppo et al., (1998) who found intravenous injections of a NK3 agonist could have rewarding effect in a place preference paradigm, and that it could reduce the intake of ethanol when microinjected into the nucleus basalis magnocellularis (a subdivision of the substantia innominata) of alcohol preferring rats.

1.8.5 Dopamine:

Dopamine belongs to the catecholamine group of neurotransmitters and constitutes approximately 80% of the catecholamines in the brain (Vallone et al., 2000). The areas that synthesise dopamine give rise to 4 neuronal pathways: the nigro-striatal (mainly projecting from the substantia nigra pars compacta to the striatum), the tuberoinfundibular (from the arcuate nuclei of the hypothalamus and periventricular to the median eminence of the hypothalamus), the mesocortical (from the VTA to pre-frontal cortical regions) and the mesolimbic (from the VTA to parts of the limbic system, ventral striatum and VP).

Dopaminergic projections into the VP are mainly from the VTA (Klitenick et al., 1992b), although there is evidence to suggest that there is some input from the substantia nigra pars compacta (Beckstead et al., 1979; Root et al., 2015), this is limited compared to the dopaminergic input from the VTA.

Stimulation of dopaminergic fibres from the VTA has been shown to both excite and inhibit neurons within the VP (Napier & Potter, 1989) and local iontophoretic application of dopamine, into the VP, also produces this characteristic excitations and inhibitions in VP neurons (Napier & Maslowski-Cobuzzi, 1994; Napier & Potter, 1989)

Other research has also shown that dopamine can modulate the effects of other neurotransmitters in the VP. Johnson and Napier, (1997) used iontophoretic injections of DA with glutamate or GABA into the VP. These data suggest that dopamine modulates the effect of both GA and GABA in the VP, although this was often seen at a sub-optimal level for the modulation of firing frequency. There is also evidence to suggest that dopamine modulates inputs from other regions in the VP. Maslowski-Cobuzzi and Napier, (1994) for example have shown that dopamine attenuates responses evoked by amygdala inputs in VP neurons.

1.8.5.1 Dopamine receptors and the VP:

Evidence shows that D1, D2, D3 and D4 receptors are found within the VP (Beckstead et al., 1988; Mansour et al., 1990; Noain et al., 2006), although there is suggestion that the majority of dopamine receptors in the VP are D1-like (Root et al., 2015).

Dopamine receptors are subdivided into two main groups. The D1-like, which comprises of D1 and D5 dopamine receptors and the D2-like groups, which consist of D2, D3 and D4 dopamine receptors (Vallone et al., 2000).

Classically D1-like receptors are considered to be excitatory and D2-like receptors inhibitory (Maslowski & Napier, 1991a). D1-like receptors are excitatory and have their effect by being positively coupled to adenylate cyclase. D2-like receptors are inhibitory and have their effect by being negatively coupled to adenylate cyclase and therefore reducing the secondary messenger, cAMP. Mengual and Pickel, (2002) carried out an ultrastructural electron microscopy study of the VP, which suggests that the majority of D2 receptors in the VP are located presynaptically and act as autoreceptors inhibiting the input to the VP from other regions. They suggest that they most likely modulate the GABAergic inputs from the NAc. Little else is known about the location of dopamine receptors within the VP.

1.8.5.2 Effects of selective dopamine receptor agonists on the VP:

Dopamine D1-like and D2-like receptors are thought to contribute to excitations and inhibitions seen in response to dopamine application in the VP (Napier & Potter, 1989). Maslowski and Napier, (1991) found D2-like receptor agonists reduced activity in 59% of neurons that responded to them and that D1-like receptor agonists excited 69% of neurons in the VP that responded to their application. This is also supported by Heidenreich et al's., (1995) data which shows a rate increase in VP neurons to the application of D1-like agonists by approximately 161-178%. However, Napier and Maslowski-Cobuzzi's, (1994) data suggest that D1-like receptor agonists largely produce inhibitions in VP neurons and D2-like receptor agonist largely produce excitation. These differences may be a results of the techniques used to administer the agonists, as the research (Heidenreich et al., 1995; Maslowski & Napier, 1991a) involved systemic application of the agonists, therefore potentially resulting in modulation of areas upstream of the VP. On the other hand Napier and Maslowski-

Cobuzzi, (1994) applied the specific receptor agonist directly into the VP via microiontophoretic injections, therefore reducing the impact of upstream modulation.

1.8.5.3 Functional effects of dopamine, D1-like agonists and D2-like agonists in the VP on behaviour:

Dopamine has been shown to modulate motor output via its effects in the VP.

Experiments suggest that dopamine produces robust changes in motor behaviour when injected into the VP (Austin & Kalivas, 1991; Gong et al., 1999; Klitenick et al., 1992b). Gong et al., (1999) also found that small doses of D1-like agonists injected directly into the VP increase locomotion, while D2-like agonists suppressed locomotion. Specific D1-like and D2-like antagonists directly injected into the VP have also been shown to block increases in motor activity induced by opioid agonists application (Napier, 1992), therefore suggesting an interaction between the effect of opioids and dopamine in the VP.

Dopamine is also known to play an essential role in the reward circuits (Schultz, 2002; Wise, 2002). Consistently, there are multiple studies suggesting that dopamine and dopaminergic inputs from the VTA modulate reward related behaviour in the VP.

Lesioning studies of dopaminergic VTA inputs into the VP, such as that of Gong et al., (1997) shows that dopamine is involved in conditioned place preference (CPP) responses to drugs of abuse, as lesions of dopaminergic inputs reduced the CPP to I.P injections of amphetamine. Research also shows, that drugs of abuse, such as cocaine, increase dopamine levels in the VP (Sizemore et al., 2000; Stout et al., 2016), they therefore likely play a role in the reinforcing effects of drugs of abuse. Other researchers have found that inhibition of D2-like receptors, in the VP increases the seeking/anticipatory behaviour for a preferred substance (in this case ethanol) and

exciting, with D2-like agonists reduced the seeking/anticipatory behaviour for ethanol in ethanol preferring animals (Melendez et al., 2004). The self-stimulation literature also supports the role of dopamine and its receptors subtypes in the VP in reward. Research has shown (Panagis et al., 1995; Panagis & Spyraiki, 1996) that D1-like and D2-like antagonists modulate the amount animals self-stimulate the VP, therefore suggesting that activation of dopamine receptors modulates how reinforcing self-stimulation is. There is also evidence to suggest D1-like and D2-like receptors are involved in negative reinforcement processes, learning and memory consolidation. Péczely et al., (2014) showed that D1-like agonist could dose-dependently enhance inhibitory avoidance learning, increasing the step through latencies in an inhibitory avoidance paradigm. Lénárd et al., (2017) also used this paradigm and found similar effects for D2-like receptor activation in the VP, suggesting that both D1-like and D2-like receptors are recruited in the VP during negative reinforcement.

All these data point towards dopamine and its receptors having a profound effect on VP circuitry and the functional output of the VP. However, surprisingly little is known about the cellular mechanisms underlying dopamine's effects in the VP. These therefore requires further investigation.

1.8.6 Serotonin (5HT)

Serotonin is a key monoamine found at the terminal of nerve cells. It belongs to a group of neurochemicals, including melatonin and tryptamine, sharing a similar chemical structure and known as indolalkylamines (Doggrell, 2003). The main source of serotonin in the VP is from the terminals of the dorsal raphe nuclei neurons (Vertes, 1991).

There are multiple different serotonin receptor subtypes and their distribution is heterogeneous across the brain (Pytliak et al., 2011). It is generally accepted that there are seven subtypes (5HT₁₋₇), with many being further divided. All but one are G protein coupled, with 5HT₃ being a ligand gated ion channel. Five out of the seven are excitatory with two (5HT₁ and 5HT₅) being inhibitory and decreasing the levels of cAMP (Berger et al., 2009).

Studies have shown that serotonin differentially modulates different populations of neurons across basal ganglia territories (Blomeley and Bracci, 2005; Blomeley and Bracci, 2009; Cains et al., 2012). Bengtson et al., (2004) has shown it specifically modulates neurons within the VP, with the suggestion being that it hyperpolarises cholinergic neurons and depolarises GABAergic neurons within the VP. It may well be that these opposing effects are modulated by different serotonin receptor subtypes in the VP. In fact several serotonin receptor subtypes have been detected within the VP, with some found on GABAergic terminals within the VP, where their function is believed to be inhibitory, reducing the release of GABA from these neurons onto cholinergic terminals (Nishijo et al., 2016). Indeed, In the dorsal extent of the pallidum (GPe) Hashimoto & Kita, (2008) have found that serotonin mainly has its effects through pre-synaptic mechanism, modulating both GABAergic and Glutamatergic inputs. Although there is some suggestion of post synaptic, direct effects. The precise location of all the serotonin receptor subtypes in the VP is not however clear and therefore requires investigation, as it would provide novel insights into the cellular mechanism underpinning serotonin's effects within the VP, as well as providing potential novel pharmacological targets.

1.8.6.1 Different classes of 5HT receptors:

As previously mentioned, there are commonly considered to be seven subtypes of the serotonin receptor (Berger et al., 2009) many of which have further subtypes (Barnes & Sharp, 1999; Nichols & Nichols, 2008). Autoradiographic studies have identified the presence of many of these receptor subtypes within the architecture of the VP, although with differing degrees of expression, these include: 5ht2 receptors (Appel et al., 1990) 5ht7 (To et al., 1995) 5ht4 (Vilaro et al., 1996) and 5ht1a, (Wright et al., 1995).

1.8.6.1.1 5HT7:

5HT7 receptors are G_s protein coupled (Pytliak et al., 2011), their mechanism of action is to increase intracellular concentrations of cAMP. Studies shows 5ht7 receptors are found in the ventral pallidum, although only at low-moderate levels (To et al., 1995).

Hauser et al., (2014) suggests that the 5HT7 receptor may be key to mediating craving and reinforcing effects of drugs, such as alcohol. Something the VP is also heavily associated with (Perry & McNally, 2013; Tang et al., 2005). 5HT7 has also been implicated in the dichotomous responses seen in the VP to serotonin. Bengtson et al., (2004) suggest this as they found these dichotomous actions in response to 5-Carboxamidotryptamine (5CT) application, which has high affinity at 5HT1, 5HT5a and 5HT7 receptor sites.

1.8.6.1.2 5HT2c

5HT2 receptors are a G_{q11} protein coupled receptor and are largely considered to have excitatory effects, through increasing intracellular concentration of *IP3* and DAG (Berger et al., 2009). A subpopulation of these receptors; 5HT_{2c} are found in high concentration in the GP and VP (Appel et al., 1990). Agonists for 5HT_{2c} receptors have also recently been shown to modulate the firing rate in a significant number of VP

neurons, producing increases in 58% of those neurons that responded (Napier & Istre, 2008).

This subpopulation are heavily associated with psychiatric disorders, particularly affective disorders. Agomelatine, which is a 5HT_{2c} antagonist has been found to be an effective antidepressant (Goodwin et al., 2009; San & Arranz, 2008), and research by (Cryan & Lucki, 2000) also suggests a role for 5HT_{2c} antagonism to modulate rat models of depression. This fits well with the VP's known role in the modulation of pleasure responses and motivation.

Recent research has shown definitively that 5HT_{2c} receptors in the VP do indeed modulate some of its functional roles, including motor activity and sensitization to drugs of abuse. Specific 5HT_{2c} agonists directly injected into the VP have been shown to attenuate motor activity (symptom of depression) (Graves et al., 2013) and Napier & Istre, (2008) have also recently shown that sensitization to methamphetamine produces an upregulation of 5HT_{2c} receptors in the VP.

Further elucidation of the cellular mechanisms underpinning 5HT_{2c} receptors' modulatory roles in the VP may well prove insightful and provide novel targets for pharmacological interventions.

1.8.6.1.3 5HT_{1a}

5HT_{1A} receptors are expressed in the rat brain (Wright et al., 1995), and are known to be both present and modulate firing rates within the VP. Heidenreich and Napier, (2000) found that 9/26 neurons activity was enhanced by 5HT_{1a} agonist while 8/26 of the neurons activity was suppressed. Other research suggests that 5HT_{1a} receptors are inextricably linked to major depressive disorder (Bhagwagar et al., 2004; Drevets et al., 1999). Therefore their modulation of the VP may play some part in its known involvement in affective/pleasure responses. Little else is known about the location of

5HT1a receptors or the cellular mechanisms they modulate. This is therefore pertinent for further investigation.

1.8.6.1.4 5HT5a

Rodents express two subtypes of 5HT5 receptors, however in humans the 5HT5b has been shown to be non-functional, therefore the only one of relevance to humans is the 5HT5a receptor (Pytliak et al., 2011). It is thought to be inhibitory and have its modulatory effects through the reduction of intracellular cAMP levels in the same manner as 5HT1 receptors. 5HT5a has been found to be expressed in moderate levels in the ventral pallidum of rats via immunohistochemical techniques (Oliver et al., 2000). Until recently little research had been carried out on 5HT5a receptors, due largely to the lack of a selective antagonists. However the highly selective antagonists SB699551 (Thomas et al., 2006) is now available and recent research suggest 5HT5a may become a novel therapeutic target (Thomas, 2006). Therefore elucidation of its cellular effects within the VP will provide invaluable new knowledge.

1.9 Connections between the NAc and the VP

1.9.1 Anatomical connectivity:

The VP is major output structure of the striatum and as such is innervated by afferents from its ventral division (NAc). Many consider the NAc afferents to be the main input of the VP (Bolam et al., 1986; Haber et al., 1985). Lesioning studies for instance suggest a strong area to area connection between the Nac and VP (Chrobak & Napier, 1993; Churchill et al., 1990; Kupchik et al., 2014; Root et al., 2015) and anterograde/reterograde tract tracing research supports these conclusion (Groenewegen & Russchen, 1984; Thompson & Swanson, 2010; Zaborszky & Cullinan, 1992). Not

only are the NAc and VP heavily connected, but tract tracing studies find their connection pattern to be very specific, with extremely localised areas in the NAc targeting extremely localised, and topographically organised areas within the VP. Zahm and Brog, (1992) carried out extensive research, which shows that the projection patterns are different between the NAcC and the NAcS, with the shell providing the majority of input into the ventro-medial VP, whereas the NAcC projections are predominantly localised to the dorsolateral portions of the VP. This pattern of connectivity was also seen in the anterograde tracing work of Heimer et al., (1991). It is less clear if these differences in projection patterns confer to any differences in the modulatory effect of NAc inputs on to VP neurons.

1.9.2 Functional connectivity

The connections between the NAc and VP have become major targets for research, with much emphasising the fact these areas are not only anatomically connected, but also functionally connected. These connections are implicated in a variety of behaviours. For example Berridge and colleagues (Peciña et al., 2006; Smith & Berridge, 2007; Smith & Berridge, 2005; Tindell et al., 2006) have shown that these connections are essential for experience of pleasure-related behaviour. For example Smith and Berridge., (2007) found that stimulation of specific areas within both the VP and NAc, with the opioid agonist DAMGO, results in increased orofacial pleasure responses to sucrose ingestion. However, they also found that this increase in pleasure responses to DAMGO application, in either the NAc or VP, was abolished if the opioid antagonist naloxone was administered simultaneously in the other area, suggesting that the connections between both areas are reciprocal and both areas must be recruited for elevation of pleasure responses. Much research also suggests that these connections play an

instrumental part in responses to drugs of abuse. Tang et al's., (2005) research suggests the projection from the NAc to the VP regulate the reinstatement of cocaine seeking in animals that had been extinguished from self-administering cocaine. More recently Creed et al., (2016) have shown that D1 and D2 connections between the NAc and VP are effected differently by cocaine, and result in different aspects of cocaine withdrawal and anhedonic aspects of motivational behaviour. Heinsbroek et al., (2016) also draws similar conclusions, suggesting that cocaine modulates the D1 but not the D2 projections and occludes LTD in D1 inputs to the VP. Research on the connections between the NAc and VP also points to these connections playing a key functional role in learning responses. Leung and Balleine's, (2013) study investigated the connections between the NacS and VP with animals that had undergone the Pavlovian transfer task (PIT). They found that there was a significant increase in activity in the medial VP (Cfos expression) and in the activity of regions known to project to this area from the NacS in animals that had undergone the PIT task, but not controls. This suggests that these connections mediate predictive learning and choice.

1.9.3 Neurochemistry and receptors of the connections between the NacS and VP:

The pharmacology of these inputs, and the neurons/receptors they target in the VP, is essential knowledge as understanding the pharmacology of these connections may provide novel ways of modulating affective responses, learning responses and certain aspects of behaviour towards drugs of abuse. The NAc inputs into the VP are considered to be largely inhibitory. For example, recent research using optogenetics by Wang et al., (2014) found that optogenetic activation of SPNs in the NAc, produced a reduced activation in the VP. With a resultant, decrease in cFos expression in the VP.

Much of the research implementing immunohistochemical techniques and electrolytic/acid lesioning methodologies suggest that the connection between the NAc and VP are almost definitely GABAergic (Churchill et al., 1990b; Churchill & Kalivas, 1994; Kitamura et al., 2001; Mogenson et al., 1983; Reiner & Anderson, 1990; Walaas & Fonnum, 1979; Yang & Mogenson, 1985; Zaborszky & Cullinan, 1992). As well as releasing GABA into the VP these connections have also been found to release other neuromodulators. Haber et al., (1985) found that approximately 40% of the connections between the NAc and VP express preproenkephalin, suggesting they release enkephalin. Substance P is also expressed in high levels within the VP. Indeed for many years staining for substance P has been used as a key way of delineating the VP from other anatomical regions. It was suggested that the NAc afferents of the VP may provide some of this substance P. Research has shown (Mitrovic & Napier, 1998; Napier et al., 1995a) that a significant proportion of the substance P in the VP is from the NAc afferents. This is supported by *In situ* hybridization studies (Lu et al., 1997) which confirm that a significant proportion of the connections between the NAc and VP express beta-preprotachykinin, which is a marker for substance P expression. The release of enkephalin and substance P by NAc afferents into the VP has also recently been supported by definitive evidence that the NAc innervates the VP with both D2 expressing MSNs (indirect pathway) and D1 expressing (direct pathway) MSN's (Creed et al., 2016; Kupchik & Kalivas, 2016). This supports the idea that the inputs from the NAc express enkephalin and substance P, as indirect pathway neurons express enkephalin and direct pathway neurons express substance P.

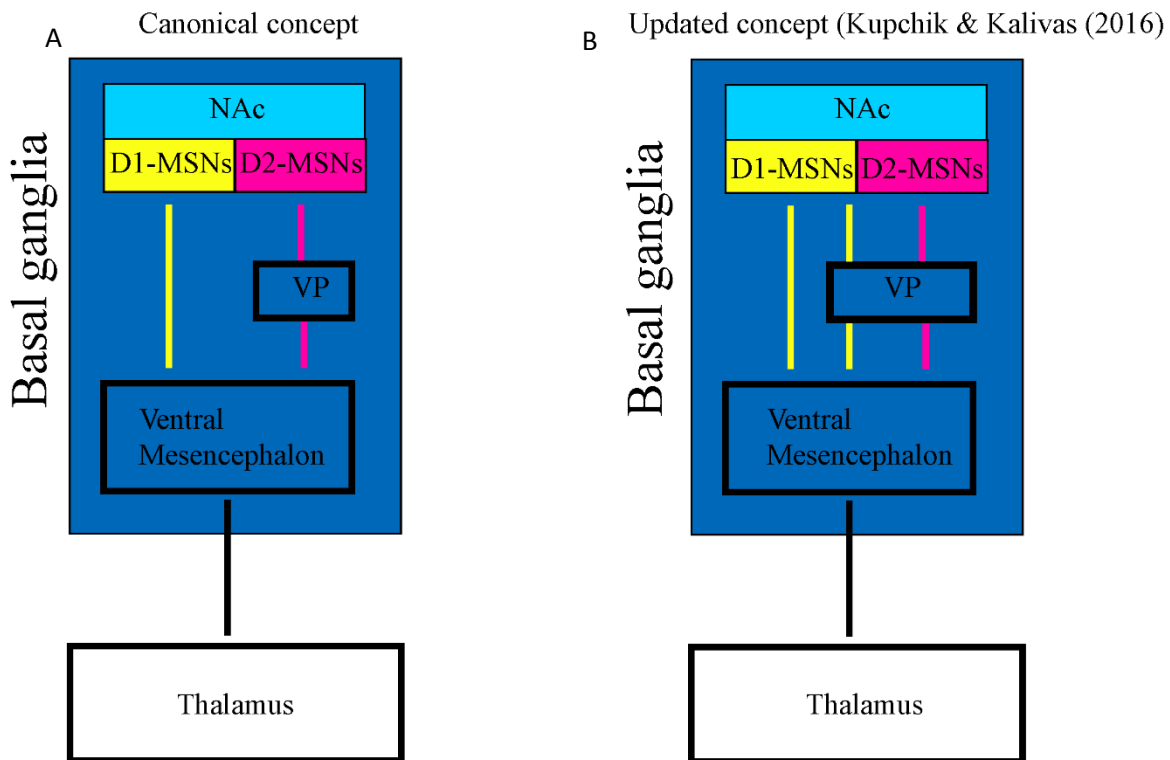


FIGURE 1.3: Comparison of the classical canonical view of NAc projections and the revised view. **(A)** The canonical view of striatal projections sees only D2 MSNs projecting to the VP **(B)** The updated view, based upon recent data suggests both D1 and D2 MSNs project to the VP. Adapted from Kupchik and Kalivas., (2016).

1.9.4 Neuronal and receptor targets of the NAc input into the VP:

Questions remain as to what neurons the afferent connections of the NAc target in the VP. A number of double immunohistochemical labelling studies (Grove et al., 1986; Zaborszky & Cullinan, 1992; Zaborszky et al., 1986) suggest GABA projections from the NAc directly target Cholinergic expressing neurons in the VP. There is less evidence supporting NAc afferents targeting other neuron populations in the VP, such as GABAergic neurons and PV+ neurons, although recent research by Root et al., (2015) suggests NAc afferents do directly innervate GABAergic neurons of the VP. Further research is needed to elucidate how NAc inputs modulate the VP neurons they

innervate, and whether the modulatory effects of NAc afferent stimulation differs between the neuron populations found in the VP.

Stimulation of NAc afferents have been shown to inhibit VP neurons and this inhibition has been shown to be blocked via local application of GABA antagonists (Chrobak & Napier, 1993; Mogenson et al., 1980). However stimulation of these NAc afferents has also been shown to excite some neurons In the VP (Chrobak & Napier, 1993). This could well relate to the type of neuron they innervate and the receptors expressed by these neurons. As previously discussed the NAc afferents of the VP are known to release substance P and Enkephalin as well as GABA. Substance p is a known excitatory neurotransmitter and has been shown to excite VP neurons (Napier et al., 1995a). This combined with the known innervation of VP cholinergic neurons by NAc afferents and the known expression of NK1 receptors on VP cholinergic neurons, suggests cholinergic neurons may be the main neuron population responding with excitation from stimulation of NAc inputs, as seen by Chrobak and Napier (1993). However substance P is also known to modulate glutamatergic inputs from the amygdala in the VP (Mitrovic & Napier, 1998) and in other basal ganglia structures (Blomeley & Bracci, 2008; Blomeley et al., 2009). This requires further investigation and clarification. Opioids have also been shown to modulate striatal projection neurons (Blomeley & Bracci, 2011) and have specifically been shown to modulate the inhibitory response induced by NAC afferent stimulation in the VP. This is because naloxone has been shown to reduce the inhibitions evoked by NAc afferent stimulation into pallidal regions (Napier et al., 1992; Napier et al., 1983), therefore suggesting that enkephalin may contribute to the inhibitory response seen as a result of NAc afferent stimulation in the VP. However MOR activation has been found to have dichotomous effects on neurons within other striatal regions (Elghaba and Bracci, 2017), therefore the effect of

enkephalin may be more complex than simple facilitation of the inhibition induced by GABA release within the VP.

The NAc afferents of the VP are also known to express receptors for various different neurotransmitters, such as inhibitory 5HT1B serotonin receptors (Nishijo et al., 2016) , inhibitory mGluR7 metabotropic glutamate receptors (Li et al., 2013), D1-like receptors and D2-like receptors (Kupchik et al., 2015). This suggests that serotonergic, glutamatergic and dopaminergic inputs into the VP may provide a source of modulation for NAc afferent of the VP. Therefore considering their known role in aspects of addiction, focus on how these neurotransmitters modulate the inhibitory and excitatory effects of NAc stimulation in the VP would be pertinent.

2 Chapter 2: Materials and methods

2.1 Animals: All experiments were carried out in line with the 1986 Animals (Scientific procedures) Act, and with full approval from the UK Home Office. Extracellular *in vitro* recordings were obtained from C57 mice. In some instances, due to availability, mice from the transgenic line ChR2-NNos (Jackson Laboratory, USA) were used. No differences were found in the electrophysiological activity of VP neurons between these animals and the C57 mice.

Chapter	Section	Experiments	Number of animals	Number of slices used	Number of recorded units	Number of analysed units
3	3.3.1	6	6	6	47	35
	3.3.2	11	8	11	82	55
	3.3.3	6	5	6	41	26
	3.3.4	4	4	4	38	34
	3.3.5	6	4	6	28	19
	3.3.6	2	2	2	12	7
	3.3.7	5	5	5	33	24
	3.3.8	4	3	4	17	8
	3.3.9	2	2	2	14	11
4	4.3.1	10	8	10	105	76
	4.3.2	1	1	1	13	10
	4.3.3	3	3	3	11	9
	4.3.4	1	1	1	10	6
	4.3.5	3	2	3	16	12
	4.3.6	2	2	2	14	13
	4.3.7	1	1	1	10	8
	4.3.8	2	2	2	13	9
5	5.3.1	13	10	13	67	52
	5.3.2	10	6	10	31	17
	5.3.3	12	8	12	41	26
	5.3.4	8	8	8	22	16
	5.3.5	3	3	3	15	8

TABLE 2.1: Summary table of animals used and the related recorded units for each study

2.2 Slice preparation:

Mice aged between 28 and 42 days were killed by cervical dislocation and death confirmed by decapitation. The brain was rapidly removed from the skull and parasagittal slices of 400 μ m obtained using a vibroslicer (Cambden instruments) immersed in ice cold (5°C), oxygenated (saturated 95 % O₂ and 5 % CO₂) Sucrose cutting solution. This solution was made up fresh daily and contained (in mM): Sucrose (184), NaH₂PO₄ (1.2), KCl (2.5), NaHCO₃ (30), Glucose (25), sodium ascorbate (5), Thiourea (2), HEPES (20) MgSO₄.7H₂O (10), sodium pyruvate (3), CaCl₂.2H₂O (0.5).

Once cut, slices were immediately transferred to a recovery chamber maintained at 26°C, containing a Tris recovery solution, which was continuously aerated with a carbogen mixture of 95 % O₂ and 5 % CO₂ gas. The Tris recovery solution was made up fresh daily and contained (in mM): Tris HCl (76), Tris base (19.5), KCl (2.5), Thiourea (2), Glucose (25), NaHCO₃ (30), HEPES (20), CaCl₂.2H₂O (0.5), NaH₂PO₄ (1.2), sodium pyruvate (3), sodium ascorbate (5), MgSO₄.7H₂O (10). The slices remained in this chamber for 30 minutes, before being transferred to another chamber for storage. This chamber was also maintained at 26°C and contained standard aCSF, which was continuously aerated with a carbogen mixture of 95 % O₂ and 5 % CO₂ gas. The standard aCSF was also made up fresh daily and contained (in mM): KCl (3), CaCl₂ (2), NaHCO₃ (26), Glucose (15), MgSO₄.7H₂O (2), NaH₂PO₄ (1.2), NaCl (124). Slices were then left for a minimum of 60 minutes to equilibrate and recover before electrophysiological recordings commenced.

2.3 pMEA (perforated Multi-Electrode Array) electrophysiological recordings:

Neural network activity was monitored and recorded using a perforated multi-electrode array (pMEA, Multi-Channel Systems, Reutlingen, Germany). The pMEA contained 60 embedded electrodes constructed of titanium nitrite. Each of these electrodes has a diameter of 30 μm and they are spaced at 200 μm . Recorded electrical activity for selected channels of interest was digitized at a sampling rate of 10 kHz using a MEA1060-Up-BC amplifier and MC Rack version(version: 4.6.2) software (Multi Channel Systems, Reutlingen, Germany).

For recording slices were transferred to the pMEA chamber, already in position in the amplifier. Once the slice was moved into position over the electrodes on the MEA, the bottom flow was switched on to produce suction and fix the slice into position. A mesh harp was then placed on top of the slice and a top flow applied as quickly as possible to maintain healthy slices. Both the top and bottom flow (perfusion) contained continuously aerated aCSF. The bottom flow rate was maintained at 0.65 – 1 ml per minute and top flow was maintained at 3-5 ml/per minute. This imbalance in the top and bottom flow rate was maintained to produce suction, so the slice was firmly fixed to the pMEA (Figure 2.1 cross section). Activity was monitored for 1 hour before recordings commenced.

During recording aCSF was perfused and removed from the recording chamber, via the top flow, with a Gilson Minipuls 3 peristaltic pump. Inlet and outlet bath perfusion mounts, for the top flow perfusion system, were obtained from Scientifica (www.scientifica.uk.com). Insulated wire was soldered on to their bodies so they could be grounded to the microscopes mounting plate. The bottom inflow was gravity fed while aCSF was removed from the bottom flow with a Watson Marlow 120s peristaltic pump. All experiments was performed inside a grounded faraday cage open on one side.

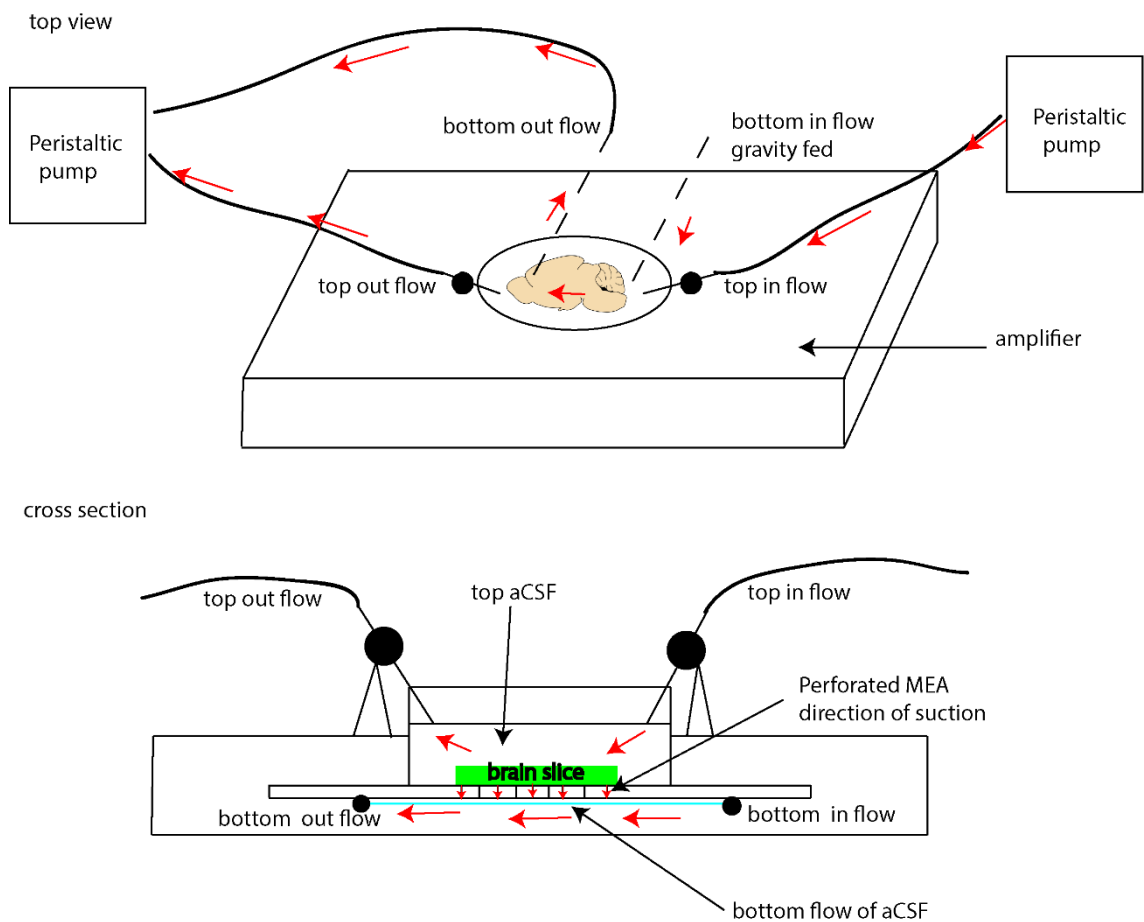


FIGURE 2.1: Schematic of the multi-electrode array perfusion set up. (**Top view**) illustration of the upper perfusion flow over the slice. (**Cross section**) illustration of the upper perfusion flow direction, the direction of the bottom perfusion flow and the movement of the aCSF through the slice to produce suction

Depending upon the experimental protocol, two types of aCSF were used to perfuse the slice once in the MEA recording chamber. One referred to as standard aCSF/control and a second with low Ca^{2+} levels referred to as low Ca^{2+} aCSF. The standard aCSF contained: (in mM): KCl (3), NaH_2PO_4 (1.2), NaHCO_3 (26), Glucose (15), NaCl (124), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2). The low Ca^{2+} aCSF was the same as the “normal” aCSF, bar a reduced quantity of CaCl_2 (0.2mM).

2.4 Slice visualisation:

To identify the correct area of the mouse slice for recording of the VP neurons and stimulation of the NAc, the PMEAs were placed in the amplifier and then under an Olympus BX51 microscope, with a 4 x lens. The slice was viewed via a Tucson digital microscope camera, which was sending a live feed to a Viglen computer (4gb of memory and an i5 processor) running IS capture software.

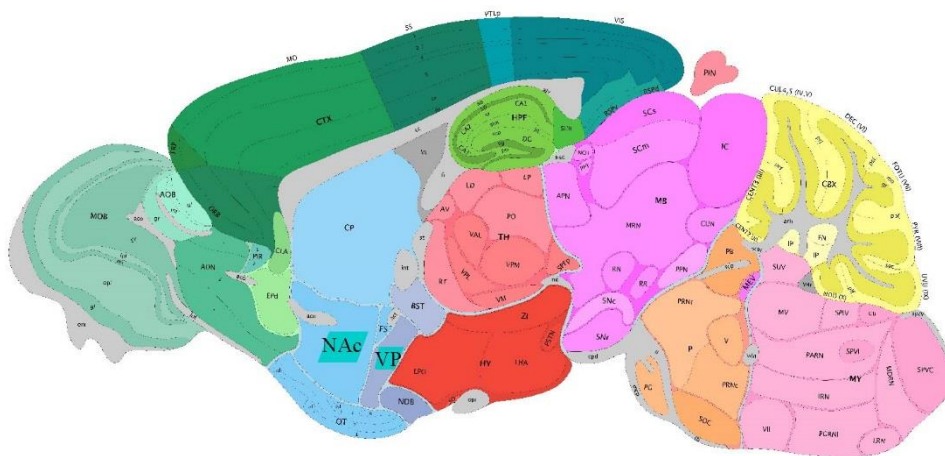


FIGURE 2.2: Adapted from, Allen mouse brain atlas

The VP was considered as any area ventral to the caudal anterior commissure and before the caudal edge of the rostral anterior commissure as it subdivides the striatum into its dorsal and ventral extents (figure 2.2) . The NAc was considered as any area directly ventral to the rostral extent of the anterior commissure and to the left of the identified VP region. For stimulation experiments (chapter 5) identification of the dorsal and ventral extents of the VP were necessary. The dorsal VP was defined as any electrode, located within the VP, which was within 500 μm of the caudal anterior commissure. The Ventral VP was defined as any region, located in the identified VP, over 600 μm away from the ventral edge of the caudal anterior commissure.

2.5 Stimulation methods and protocols:

Stimulation was performed using a STG 1002 (Multichannel systems), which was programmable via MC_stimulus software (version 2.1.5). Stimulation was delivered via the internal electrodes of the MEA chip, the electrode to be used for stimulation was selected based upon the location of the slice in the MEA chip chamber, which was observed via the video stream from the camera mounted on the optic of the microscope. Electrodes were always chosen for stimulation that were at least 400microns away from the rostral edge of the identified VP. Once the correct area and the corresponding electrodes in this area were identified, MC_select software (version 1.3.0), which allows you to allocate the embedded electrodes as stimulating channels, was used to administer a biphasic stimulation. Two electrodes adjacent to one another in the MEA were always selected for the delivery of stimulation. The stimulation was always bipolar, with a negative followed by positive polarity, in line with multichannel systems suggestions (Wagenaar et al., 2005; Wagenaar et al., 2004). The voltage of stimulation never exceeded +/- 3000 mv and lasted for a duration of:

- 1 stimulation of 200 μ s duration followed by a 10 second gap, before the next trial.
- HFS which was at 100 Hz: 5 stimulations of 300 μ s duration with 9700 μ s gap between each, giving a total stimulation time of 50 ms. Each trial was 10 seconds apart.

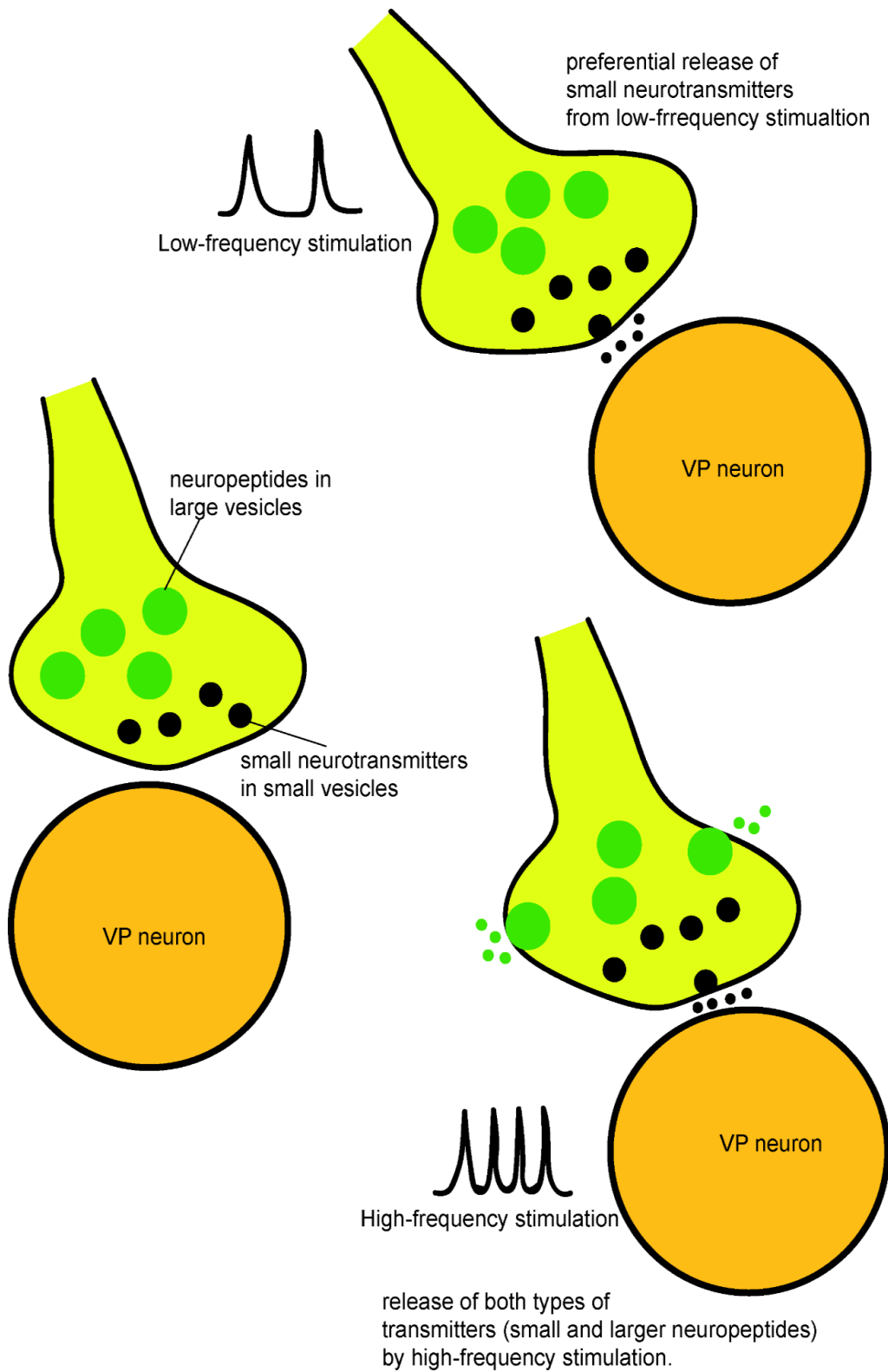


FIGURE 2.3: differential effects of high and low-frequency stimulation protocols. Stimulation of neurons with a low frequency stimulation protocol is known to result in the preferential release of small neurotransmitters, while high frequency stimulation protocols are known to result in the release of both small neurotransmitters and larger neuropeptides. Figure adapted from Purves., 2001.

The HFS stimulation protocol was used for experiments using NK-1 receptor antagonists, as it had been shown to promote the release of neuropeptides (figure 2.3), such as SP (Purves, 2001). The low frequency stimulation was used in experiments involving, opioids and GABA_a antagonists as it is known to favour the release of smaller neurotransmitters, such as GABA.

2.6 Pharmacology:

All drugs were obtained from either Tocris Biosciences (UK) or Sigma Aldrich and were bath applied into the header reservoir feeding the top perfusion flow of the pMEA at the following concentrations:

1. (4a*R*-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline hydrochloride: (-)-Quinpirole hydrochloride (quinpirole), 20 μM.
2. (±)-6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine hydrobromide: (SKF81297), 20 μM.
3. (*S*)-(-)-5-Aminosulfonyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide (sulpiride), 20 μM.
4. (6a*S*-trans)-11-Chloro-6,6a,7,8,9,13b-hexahydro-7-methyl-5*H*-benzo[*d*]naphth[2,1-*b*]azepin-12-ol hydrobromide (SCH39166), 20 μM.
5. 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX), 20 μM
6. D-(-)-2-Amino-5-phosphonopentanoic acid (AP5) 20 μM
7. (*RS*)-α-Methyl-4-carboxyphenylglycine (MCPG), 20 μM
8. (*RS*)-α-Methyl-4-sulfonophenylglycine (MSPG), 10 μM
9. 3,4-Dihydroxyphenethylamine hydrochloride: (dopamine), 30 μM
10. 5-Hydroxytryptamine hydrochloride (serotonin) 30 μM, as per (Bengtson et al., 2004)

11. *N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate: 5HT1a antagonist (WAY100635) 10 nM, as per (Corradetti et al., 1996).
12. 8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl)-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride: 5HT2c antagonists (RS102221) 1-3 μ M, as per (Bonsi et al., 2007).
13. *N*-[2-(Dimethylamino)ethyl]-*N*-[[4'-[[2-phenylethyl)amino]methyl][1,1'-biphenyl]-4-yl]methyl]cyclopentanepropanamide dihydrochloride: 5HT5a antagonists (SB699551) 1 μ M.
14. (2*R*)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride: 5HT7 antagonist (SB269970) 1-3 μ M, as per (Bonsi et al., 2007).
15. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide: (NBQX) 20 μ M.
16. 2-APV, D-APV, D-2-amino-5-phosphonovalerate: D-AP5 (AP5) 20 μ m
17. Picrotoxin: (Picrotoxin) 20 μ M.
18. *N*-Acetyl-L-tryptophan 3,5-*bis*(trifluoromethyl)benzyl ester: (L732,138) 20 μ m
19. Substance P: (SP) 20 μ M.
20. (5 α)-4,5-Epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride: (Naloxone) 20 μ M.
21. [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin: (DAMGO) 20 μ M.

Washout of any pharmacological treatment was considered complete when firing frequency recorded over a period of 1200 s was not significantly different from that observed over a 1200 s period just before drug application.

2.7 Data Analysis for pharmacological experiments:

Data was acquired using Mc_rack software (version: 4.6.2) and a MEA1060-Up-BC amplifier (Multi-Channel Systems, Reutlingen, Germany). These files (.mcd) were then converted to .ced files using multichannel data manager software (version: 1.9.7, Multi-Channel Systems, Reutlingen, Germany) for off-line analysis using Spike 2 software (C.E.D)

For all recordings, spike sorting was carried out offline using dedicated Spike2 (C.E.D.) software. This software uses an automated waveform matching system to construct waveform templates and allows the user to set an appropriate threshold for detection of individual units. When multiple units were detected in a trace, discrete clustering of waveforms within a template was verified through principal components analysis, also implemented by Spike2.

See figure legends for information on result expression. All error bars are expressed as SEM.

In order to assess differences in a neuron's firing frequency in different pharmacological conditions, we measured consecutive inter-spike intervals (ISIs) during the final 1200seconds of each condition. Average ISIs for relevant conditions were then compared using a Student's t-test. A statistically significant difference was considered to be present if $P < 0.05$. If a treatment caused a significant increase in ISI, we refer to this observation in the results as a significant decrease in firing frequency and an inhibitory effect of the treatment. If a treatment caused a significant decrease in ISI, we refer to this observation as a significant increase in firing frequency and an excitatory effect of the treatment.

Coefficient of variation (CoV) was calculated as a measure of spike train variability in different pharmacological conditions and as a potential way of identifying different neuronal types in the VP. It was calculated as: standard deviation (ISI) / Mean ISI.

Threshold for spike detection was considered to be reached when the recorded voltage departed from baseline (0 mV in AC recording mode) by more than the standard deviation of the voltage recorded for that channel during an apparently quiescent period (of at least 3 s). Spikes consisted of a biphasic negative-positive waveform. Spike amplitude was defined as the difference between the negative voltage peak and the spike threshold level defined above. Spike half-width was defined as the time the value of the recorded voltage (measured from the threshold level) remained more negative than half of the spike amplitude (Figure 2.4) (Pettersen & Einevoll, 2008).

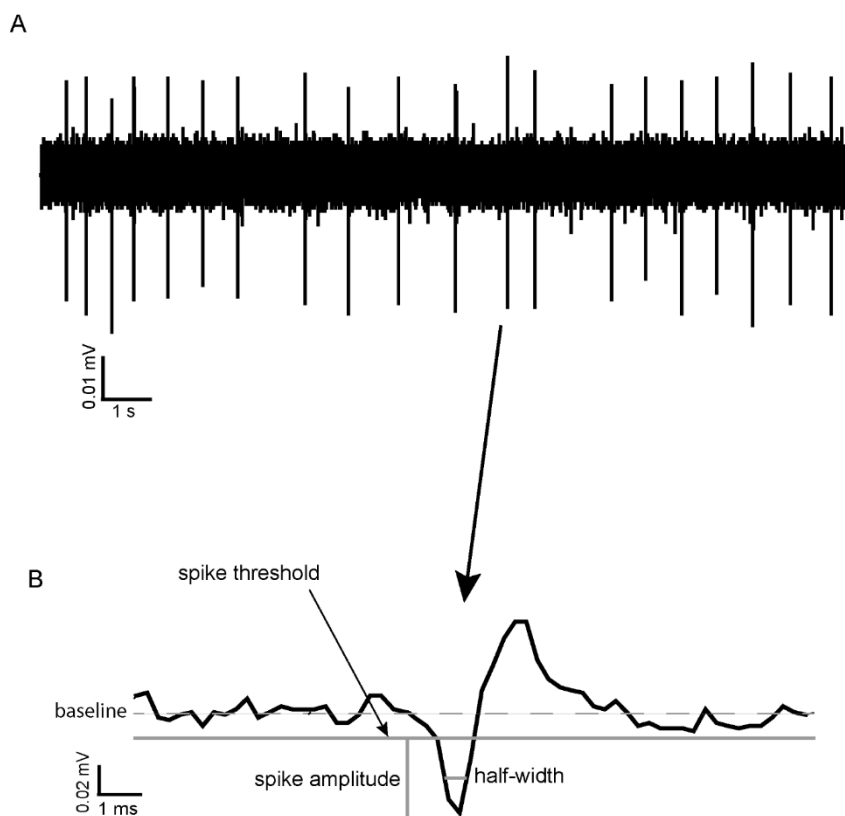


FIGURE 2.4: Spike half-width calculation. **(A)** A raw data trace of tonic neuronal activity measured in the VP. **(B)** An example of a typical spike recorded in the VP with the reference point used for calculation of its spike half-width, halfway between baseline and maximum negative amplitude.

2.7.1 Data analysis for stimulation experiments:

Pause duration was calculated as the amount of time taken for the neuron to return to 90% of the pre-stimulation firing rate. If this occurred in < 100 ms these neurons were considered as non-pauses. If the pause duration was >100 ms these neurons were considered pauses. This was done to ensure the HFS, which lasted for 50 ms, was not falsely accepted as a pause in firing

In order to assess differences in a neuron's firing frequency in different pharmacological treatments. We measured consecutive inter-spike intervals (ISIs) at the end of the stimulation protocol and during the final 60 s of each pharmacological condition. Average ISIs for relevant conditions were then compared using a Student's t-test. A statistically significant difference was considered to be present if $P < 0.05$. If a treatment caused a significant increase in ISI, we refer to this observation in the results as a significant decrease in firing frequency and an inhibitory effect of the treatment. If a treatment caused a significant decrease in ISI, we refer to this observation as a significant increase in firing frequency and an excitatory effect of the treatment. Coefficient of variation and spike half-width were also calculated in different pharmacological treatments during the final 60 s of each pharmacological condition. These were calculated in the same manner as for pharmacological experiments (pg68-69).

3 Chapter 3: Dopaminergic modulation of VP neurons

3.1 Abstract:

The ventral pallidum (VP) is crucially involved in reward processing. Dopaminergic afferents reach the VP from the ventral tegmental area (VTA). Recent *in vivo* studies suggest systemic dopamine application increase the firing frequency of neuronal populations within the VP. However, little is known about the cellular effects of dopamine within the VP. The current study aimed to address this paucity of data using brain slices containing the VP and multi-electrode array (MEA) recordings. Dopamine significantly affected firing in 86 % of spontaneously active VP neurons. Among the affected neurons, 84 % were excited, while 16 % were inhibited. The selective D1-like receptor agonist SKF81297 also had modulatory effects on the majority of VP neurons, but its effects were universally excitatory. However, the D2-like receptor agonist quinpirole had modulatory effects on 87 % of VP neurons studied. It caused significant inhibitory effects in 33 % of the cases and excitatory effects in the remaining 67 %. The effects of D1-like receptor activation were presynaptic as blocking synaptic transmission with low Ca^{2+} abolished the effects of SKF81297 application. Furthermore, SKF81297 effects were abolished by blocking ionotropic glutamate receptors, suggesting that D1-like receptors boost glutamate release, which in turn excites VP neurons through postsynaptic glutamate receptors. Effects caused by D2-like receptor activation were found to involve pre and postsynaptic mechanisms, as low Ca^{2+} abolished the excitatory effects of quinpirole but not the inhibitory ones. Increases in firing frequency to quinpirole application were abolished by a group 2/3 mGluR antagonist, suggesting that D2-like receptors cause presynaptic inhibition of glutamate release, resulting in reduced postsynaptic activation of inhibitory mGluRs. Conversely, the inhibitory effects of quinpirole persisted in low Ca^{2+} and therefore can be attributed to postsynaptic D2-like receptor activation. VP neurons excited by dopamine have shorter spike half-widths and are excited by D1-like receptors (presynaptically) and by

D2-like receptors (postsynaptically). VP neurons inhibited by dopamine have longer spike half-widths and while D1-like receptor activation has a presynaptic excitatory influence on them, D2-like receptor activation has a postsynaptic inhibitory effect that prevails, on balance. These data provide novel insights into the cellular mechanisms by which dopamine controls information processing within the VP.

3.2 Introduction:

The ventral pallidum (VP) is a key output structure for the ventral striatum (Smith, et al., 2009). It also forms multiple feedback loops with some of the key structures involved in reward signaling, including the nucleus accumbens (NAc), the medial prefrontal cortex, the basal lateral amygdala, the subthalamic nucleus and the ventral tegmental area (Root, et al. 2015). The VP is known to have populations of GABAergic interneurons and cholinergic neurons that belong to the forebrain magnocellular cholinergic system (Bengtson & Osborne, 2000; Gritti, et al., 1993; Pang, et al., 1998).

Interest in the VP has increased recently as the VP, and its connective circuitry, is heavily associated with reward, aspects of reinforcement and motivational salience (Cromwell & Berridge, 1993; Itoga, et al., 2016; Richard, et al., 2016). Indeed Smith et al., (2009) conclude that it is an essential integrative region for reward and reward learning and may well be a “limbic final common pathway”. The VP also plays a key role in addictive behaviour towards drugs of abuse (Fletcher, et al.,1998; Gong, et al., 1996; Hiroi & White, 1993; Hubner & Koob, 1990; Robledo & Koob, 1993; Tang, et al., 2005),

The VP has a substantial dopaminergic projection arising from the ventral tegmental area (VTA) (Klitenick, et al., 1992; Root et al., 2015; Smith & Kieval, 2000) and

dopamine receptor subtypes (D1, D2, D3) are known to be expressed within the VP (Mansour et al., 1990; Richtand et al., 1995). However, little is known about the effects of dopamine in the VP. Heidenreich et al., (1995) have shown that, *in vivo*, D1-like receptor agonists increase the firing frequency in approximately 45 % of VP neurons. Napier & Maslowski-Cobuzzi, (1994) found that D2-like receptor agonists also modulated the firing frequency in the VP *in vivo* and Johnson & Napier, (1997) suggested that dopamine effects depend on modulation of GABA inputs into VP. However, the cellular mechanisms activated by dopamine in the VP are still obscure. *In vivo* experiments are not ideal to clarify this issue, as the concentrations of ligands cannot be precisely controlled and because the presence of multiple active inputs to both the VP and the dopaminergic neurons in the VTA, greatly complicate the interpretation of the results.

Elucidating the cellular mechanisms underlying the dopamine action in the VP is an extremely important aim, as there is a strong connection between dopamine and the role of the VP in reward and addictive behaviour. Recently, manipulation of dopamine in the VP has been shown to dramatically affect avoidance learning. Lénárd et al., (2017) and Péczely et al., (2014) have shown that D1-like and D2-like receptors within the VP are involved in the formation and retention of avoidance learning. Further, research (Maslowski & Napier, 1991b; Stout et al., 2016) has illustrated the role of dopamine in the VP on the effects of drugs of abuse. Creed, et al., (2016) have suggested that targeted research on the VP is essential as it may provide novel strategies to treat addiction/addictive disorders.

3.3 Results:

3.3.1 Dopamine application has dual effects on VP neurons

From 6 experiments 35 VP neurons were selected for analysis as they responded to dopamine application. 29/35 neurons displayed significant ($P < 0.05$) increases in firing frequency to application of dopamine (Figure 3.1B) while 5/35 displayed significant ($P < 0.05$) decreases in firing frequency (Figure 3.1D). In the neurons that were excited by dopamine, the average increase in firing frequency was $78 \pm 14\%$ (Figure 3.1E). In the neurons that were inhibited by dopamine, the average decrease in firing frequency was $-17 \pm 3\%$ (Figure 3.1E).

Baseline firing frequency and spike half-width were calculated for all neurons. Two clusters of neurons, with different spike half-width profiles can be identified in figure 3.1F, those with a shorter spike half-width (cluster I) in the range 0.12 – 0.24 ms and those with a longer spike half-width (cluster II), in the range 0.28 – 0.36 ms. Those neurons with a shorter spike half-width (cluster I) are all excited by dopamine, while neurons with a longer spike half-width (cluster II) show both excitatory and inhibitory responses to dopamine. The baseline firing frequency of VP neurons excited by dopamine was not significantly different ($P > 0.05$) from that of those inhibited by dopamine (Figure 3.1G). However, spike half-width was significantly ($P < 0.01$) larger 0.32 ± 0.04 ms in neurons inhibited by dopamine than in those excited by dopamine 0.19 ± 0.01 ms as illustrated in figure 3.1H.

We concluded that there are two electrophysiologically distinct populations of VP neurons that could be consistently differentiated by their spike half-widths. Those with a

shorter half-width (cluster I) were consistently excited by dopamine, however, for cluster II, some neurons were excited by dopamine while others were inhibited by dopamine.

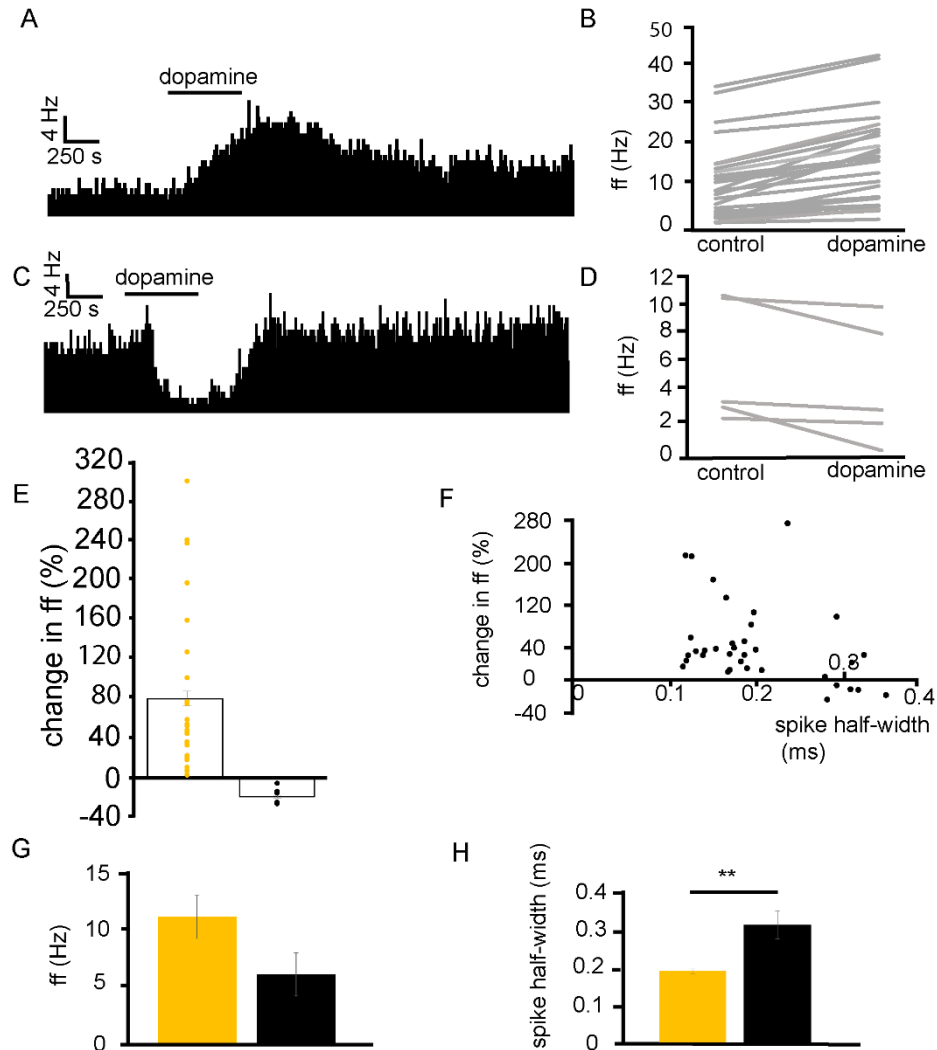


FIGURE 3.1: dopamine application has dual effects on VP neurons. **(A)** Excitatory response to dopamine application in a VP neuron. **(B)** Changes in firing frequency (ff) for 30 VP neurons in experiments similar to that illustrated in panel A. Firing frequency was measured before and during the application of dopamine. **(C)** Inhibitory response to dopamine application in a VP neuron. **(D)** Changes in firing frequency for 5 VP neurons in experiments similar to those illustrated in Panel C. Firing frequency was measured before and during the application of dopamine **(E)** Distribution of excitatory (yellow) and inhibitory (black) responses (% change compared to baseline) to dopamine application in VP neurons with their corresponding average values. **(F)** Relationship between change in firing frequency induced by dopamine (expressed as percentage of control) and spike half-widths (ms) for each neuron tested. **(G)** Average firing frequency for VP neurons excited by dopamine (yellow) and those inhibited (black). **(H)** Average spike half-width (ms) for VP neurons excited by dopamine application (yellow) and those inhibited (black). In this and following figures * $P < 0.05$ and ** $P < 0.01$

3.3.2 Dopamine receptors responsible for its effects on VP neurons.

Mansour et al., (1990) found that D1, D2 and D3 receptors are present within the VP.

To identify the dopamine receptors involved in excitatory and inhibitory VP neurons responses, we carried out experiments using D1-like and D2-like receptor agonists.

In 5 experiments 34 VP neurons were selected for analysis as they responded to application of the D2-like receptor agonist quinpirole. 28/34 of these neurons displayed significant ($P < 0.05$) changes in firing frequency in response to quinpirole application with, 9/34 displaying significant ($P < 0.05$) decreases in firing frequency and 19/34 displaying significant ($P < 0.05$) increases in firing frequency in response to quinpirole application. In a further 4 experiments 29 VP neurons were selected for analysis as they responded to application of the D1-like receptor agonist SKF81297. 22/29 of these neurons displayed significant ($P < 0.05$) increases in firing frequency in response to SKF81297 application.

In order to further explore the role of dopamine receptor types, we carried out experiments in which quinpirole and SKF81297 were applied sequentially (SKF81297 was applied after complete washout of quinpirole).

From 6 experiments 21 VP neurons were identified for analysis as they responded to quinpirole and SKF81297 application. Sequential application of quinpirole and SKF81297 resulted in 13/21 neurons displaying significant ($P < 0.05$) increases in firing frequency to both SKF81297 and quinpirole (Figure 3.2B). 6/21 neurons displayed significant ($P < 0.05$) decreases in firing frequency to quinpirole and significant ($P < 0.05$) increases, in firing frequency, to SKF81297 (Figure 3.2D). This means that 62% of the

neurons in the VP were excited by both SKF81297 and quinpirole, while 29% of neurons were inhibited by quinpirole but excited by SKF81297 (Figure 3.2E).

In order to ascertain if the responses to quinpirole and SKF81297 were produced by distinct types of neurons in the VP, baseline firing frequency (Figure 3.2F), coefficient of variation (Figure 3.2G) and spike half-width (Figure 3.2H) were measured for neurons that were excited by both quinpirole and SKF81297 and for those excited by SKF81297 but inhibited by quinpirole. Baseline firing frequency and coefficient of variation (Figure 3.2F, G) were not significantly ($P > 0.05$) different in the two populations. On the other hand there was a significant ($P < 0.01$) differences in spike half-width (Figure 3.2H). Neurons excited by both quinpirole and SKF81297 had a spike half-width of 0.15 ± 0.08 ms, while those excited by SKF81297 but inhibited by quinpirole had a spike half-width of 0.37 ± 0.03 ms. The range of spike half-widths for these populations was similar to those of cluster I and cluster II (Figure 3.1F). Cluster I had a spike half-width range of 0.12 -0.24 ms (Figure 3.1F), which compares well to neurons that were excited by both quinpirole and SKF81297 that had a spike half-width range of 0.10 –0.22 ms. On the other hand, cluster II had a spike half-width range of 0.28-0.36 ms (Figure 3.1F), which compared well to neurons that were excited by SKF81297 but inhibited by quinpirole, which had a spike half-width range of 0.27 – 0.42 ms. This suggests that cluster I in Figure 3.1F correspond to those neurons excited by both quinpirole and SKF81297 (Figure 3.2B), while cluster II in Figure 3.1F correspond to those neurons excited by SKF81297, but inhibited by quinpirole (Figure 3.2D).

We concluded that D1-like and D2-like receptors can cause, independently, an increase in firing frequency in the majority of VP neurons, but that an electrophysiologically

distinct minority of neurons, characterised by longer spike half-width durations, are inhibited by D2-like receptors. Therefore, it appeared likely the effects of dopamine in the neurons inhibited by D2-like receptors is depended on the net balance between the excitatory influence of D1-like receptors and the inhibitory influence of D2-like receptors.

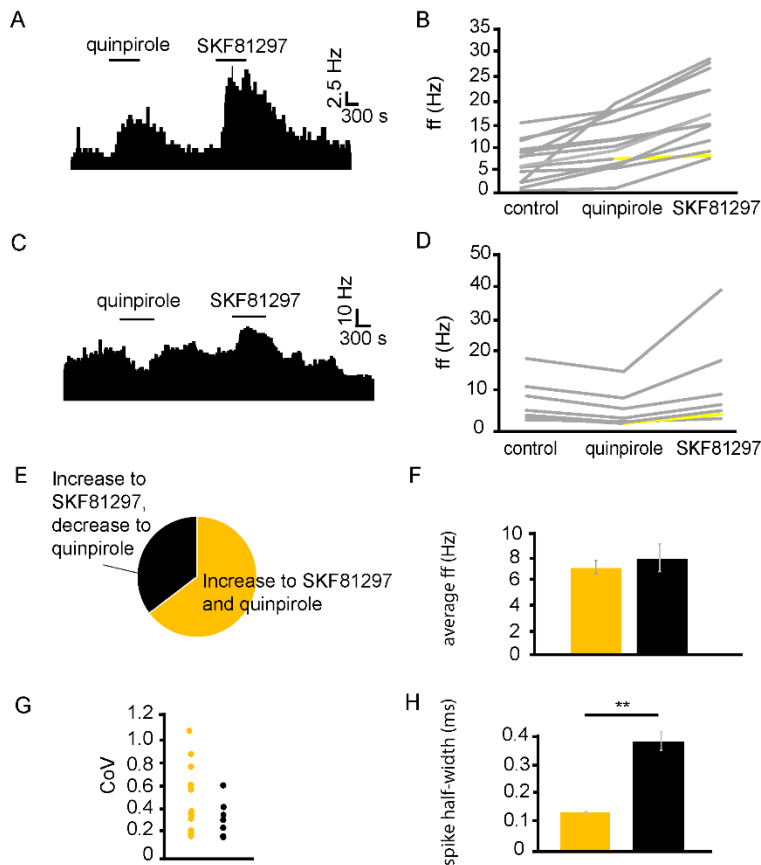


FIGURE 3.2: D1-like receptor agonists excite while D2-like receptor agonists excite and inhibit VP neurons. **(A)** Excitatory response to the application of quinpirole and SKF81297 (separated by complete washout). **(B)** Changes in firing frequency for 14 VP neurons induced by quinpirole application and SKF81297 application similar to that illustrated in panel A. Firing frequency was measured; before the application of quinpirole, after the application of quinpirole and subsequently, after a period of wash out, for the application of SKF81297. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Inhibitory response to the application of quinpirole and excitatory response to the application of SKF81297 (separated by complete washout). **(D)** Changes in firing frequency for 7 VP neurons induced by quinpirole application and SKF81297 application similar to that illustrated in Panel C. Firing frequency was measured; before the application quinpirole, after the application of quinpirole and subsequently, after a period of wash out, for the application of SKF81297. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(E)** The majority of neurons analysed responded with increases in firing frequency in response to both quinpirole and SKF81297 (yellow), but a minority decreased their firing frequency in response to quinpirole and increased their firing frequency in response to SKF81297 (black). **(F)** Firing frequency for those neurons excited by both quinpirole and SKF81297 (Yellow) and those neurons that were inhibited by quinpirole (black). **(G)** Coefficient of variation (CoV) for those neurons excited by both quinpirole and SKF81297 (yellow) and those inhibited by quinpirole (black). **(H)** Significant ($** = P < 0.01$) differences in spike half width (ms) for those neurons excited by both quinpirole and SKF81297 (yellow) and those that were inhibited by quinpirole (black).

3.3.3 Repeated application of dopamine and dopamine receptor agonists

Preliminary experiments showed that application of dopamine caused strong increases or decreases in firing frequency in VP neurons. As the following experiments involved pharmacological protocols with repeated applications of dopamine (or dopamine receptor agonists) in the presence of different ligands, it was important to establish whether neuronal responses to subsequent short applications of dopamine were similar, or whether significant sensitisation or desensitisation (Calabresi et al., 2007; Chen et al., 1996; Otani, et al., 1998) were observed.

Dopamine was applied twice (the second application was carried out after complete washout of the first). In 2 experiments, 10 VP neurons were identified for analysis, as they responded to dopamine. In 7 of these neurons, dopamine significantly ($P < 0.05$) increased firing, while in the other 3 dopamine significantly ($P < 0.05$) decreased firing. In all cases, the second application of dopamine produced similar effects to the first one, as the firing frequencies measured during the first and the second application of dopamine were not significantly different for any of the 10 neurons (Figure 3.3D, E). We concluded that repeated exposure to dopamine elicited similar effects in VP neurons.

Having established that repeated application of dopamine caused similar responses in VP neurons, we carried out the same protocol for D1-like and D2-like receptor agonists, to ensure the responses to these agonists did not undergo sensitisation or desensitisation upon repeated exposure.

Next quinpirole was applied twice (the second application was carried out after complete washout of the first). In 2 experiments, 9 VP neurons were identified for analysis, as they responded to quinpirole. In 6 of these neurons, quinpirole significantly ($P < 0.05$) increased firing, while in the other 3 quinpirole significantly ($P < 0.05$) decreased firing. In all cases, the second application of quinpirole produced similar effects to the first one, as the firing frequencies measured during the first and the second application of quinpirole were not significantly different for any of the 9 neurons (Figure 3.3I, J). We concluded that repeated exposure to D2-like receptor agonists elicited similar effects in VP neurons.

Next SKF81297 was repeatedly applied (with the second application after complete wash out of the first) to test if differences were seen due to repeated exposure. In 2 experiments 7 neurons in the VP were identified for analysis as they responded to SKF81297. 7/7 neurons in the VP studied showed a statistically significant ($P < 0.05$) increase in firing frequency in response to SKF81297 application. In all cases, the second application of SKF81297 produced similar effects to the first one, as the firing frequencies measured during the first and the second application of SKF81297 were not significantly different for any of the 7 neurons (Figure 3.3M). We concluded that repeated exposure to D1-like receptor agonists elicited similar effects in VP neuron.

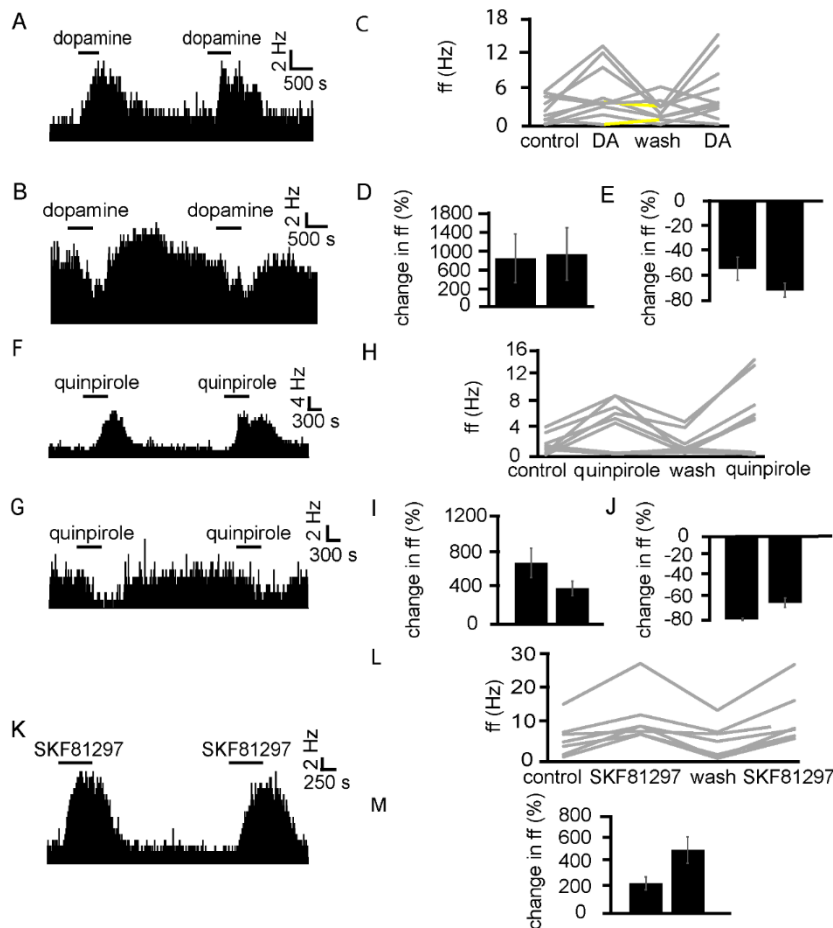


FIGURE 3.3: repeated application of dopamine, D1-like receptor agonists and D2-like receptor agonists produce no clear sensitisation effects in VP neurons **(A)** Excitatory responses to two similar dopamine applications (separated by complete washout) in a VP neuron. **(B)** Inhibitory responses to two dopamine applications (separated by complete washout) in a different VP neuron. **(C)** Changes in firing frequency for 10 VP neurons induced by repeated dopamine applications similar to that illustrated in panel A and B. Firing frequency was measured before the first application of dopamine, during the first application of dopamine, after dopamine wash out and finally during the second application of dopamine. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(D)** Firing frequency during first and second dopamine application for neurons excited by dopamine. **(E)** Firing frequency during first and second dopamine application for neurons inhibited by dopamine. **(F)** Excitatory responses to two quinpirole applications (separated by complete washout) in a VP neuron. **(G)** Inhibitory response to two quinpirole applications in a different neuron. **(H)** Changes in firing frequency for 9 VP neurons to the repeated application of quinpirole in experiments similar to that illustrated in panel A and B. Firing frequency was measured before the first application of quinpirole, during the application of quinpirole, after wash out, before the second application of quinpirole and finally during the second application of quinpirole. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow **(I)** Firing frequency changes during first and second application of quinpirole in neurons excited by quinpirole. **(J)** Firing frequency changes during first and second application of quinpirole in neurons inhibited by quinpirole application. **(K)** Excitatory responses to two applications of SKF81297 (separated by complete washout) in a VP neuron. **(L)** Changes in firing frequency for 7 VP neurons in response to repeated SKF81297 application in experiments similar to that illustrated in panel A. Firing frequency was measured; before the first application of SKF81297, during the application of SKF81297, after wash out, before the second application of SKF81297 and finally during the second application of SKF81297. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow **(M)** Firing frequency changes during first and second application of SKF81297 for neurons excited by SKF81297 application.

3.3.4 Role of D1-like and D2-like receptors in the modulation of VP neurons

To dissect out the modulatory effects of D1-like and D2-like receptors on VP neurons and to confirm the ability to identify the neuron types based upon spike half-width, dopamine was applied in the presence of D1-like and D2-like receptor antagonists.

Dopamine was applied twice, first alone and second (after complete washout of the first application) in the presence of the D2-like receptor antagonist sulpiride. In 2 experiments, 23 VP neurons were identified for analysis, as they responded to dopamine. 16/ 23 of these neurons had a significantly ($P < 0.05$) smaller increase in firing in response to dopamine in the presence of sulpiride than to dopamine alone (Figure 3.4C, D). On the other hand 7/23 of these neurons had a significantly ($P < 0.05$) larger increase in firing in response to dopamine in the presence of sulpiride than to dopamine alone (Figure 3.4A, B).

In order to ascertain if this effect of sulpiride on the excitatory effects of dopamine was related to classes of neurons within the VP, baseline firing frequency (Figure 3.4E), coefficient of variation (Figure 3.4F) and spike half-width (Figure 3.4G) were calculated. There was no significant ($P > 0.05$) difference in average baseline firing frequency for those neurons that had a larger increase in firing in response to dopamine in the presence of sulpiride, than those that had a smaller increase in firing in response to dopamine in the presence of sulpiride. There was also no significant ($P > 0.05$) difference in the coefficient of variation for those neurons that had a larger increase in firing in response to dopamine in the presence of sulpiride, than those that had a smaller increase in firing in response to dopamine in the presence of sulpiride. On the other hand, there was a significant ($P < 0.01$) difference in the spike half-width for those

neurons that had larger increase in firing in response to dopamine in the presence of sulpiride 0.29 ± 0.01 ms, with a range of 0.25 –0.34 ms (Figure 3.4G), than those that had a smaller increase in firing in response to dopamine in the presence of sulpiride 0.19 ± 0.01 ms, with a range of 0.13 -0.24 ms. These results proved consistent with those seen in figure 3.1F, G, H and figure 3.2H.

Next the same experimental protocol was applied as in figure 3.4A, C, but with the D1-like receptor antagonist SCH39166. In 2 experiments, 11 VP neurons were identified for analysis, as they responded to dopamine. 5/11 of these neurons had a significantly ($P < 0.05$) smaller increase in firing in response to dopamine in the presence of SCH39166 than to dopamine alone (Figure 3.4H, I). Moreover, 6/11 of these neurons had a significantly ($P < 0.05$) inhibitory response to dopamine in the presence of SCH39166 while being excited by dopamine alone (Figure 3.4J, K).

In order to ascertain if the responses to dopamine in the presence of SCH39166 were related to different classes of neurons within the VP, baseline firing frequency (Figure 3.4L), coefficient of variation (Figure 3.4M) and spike half-width (Figure 3.4N) were calculated.

There was no significant ($P > 0.05$) difference in average baseline firing frequency (Figure 3.4L) for those neurons that had a smaller increase in firing (yellow) in response to dopamine in the presence of SCH39166 compared to those that displayed an inhibitory response to dopamine in the presence of SCH39166 (black). There was also no significant difference in coefficient of variation (Figure 3.4M) for those neurons that had a smaller increase in firing (yellow) in response to dopamine in the presence of SCH39166 compared to those that displayed an inhibitory response to dopamine in the

presence of SCH39166 (black). On the other hand, there was a statistically significant ($P < 0.01$) difference in spike half-width (Figure 3.4N) between those neurons that had a smaller increase in firing (yellow) in response to dopamine in the presence of SCH39166 0.18 ± 0.01 ms, with a range of 0.12 – 0.23 ms, compared to those that displayed an inhibitory response to dopamine in the presence of SCH39166 0.34 ± 0.01 ms (black), with a range of 0.28 – 0.39 ms. Again, these results proved consistent with those seen in figure 3.1F, G, H and figure 3.2H.

We can conclude that D2-like receptors are responsible for both excitation and inhibition of VP neurons, and that these responses are in distinct groups of neurons, displaying different spike half-width profiles. We can confirm that these data supports the conclusion that those neurons with a longer spike half-width profile are excited by D1-like receptor agonists and inhibited by D2-like receptors agonists, while those with a shorter spike half-width profile are excited by both D1-like and D2-like receptor agonists. Those neurons excited by both D1 and D2 agonists and with a shorter spike half-width profile are referred to as type I neurons, while those neurons inhibited by D2 agonists with a longer spike half-width profile are referred to as type II neurons.

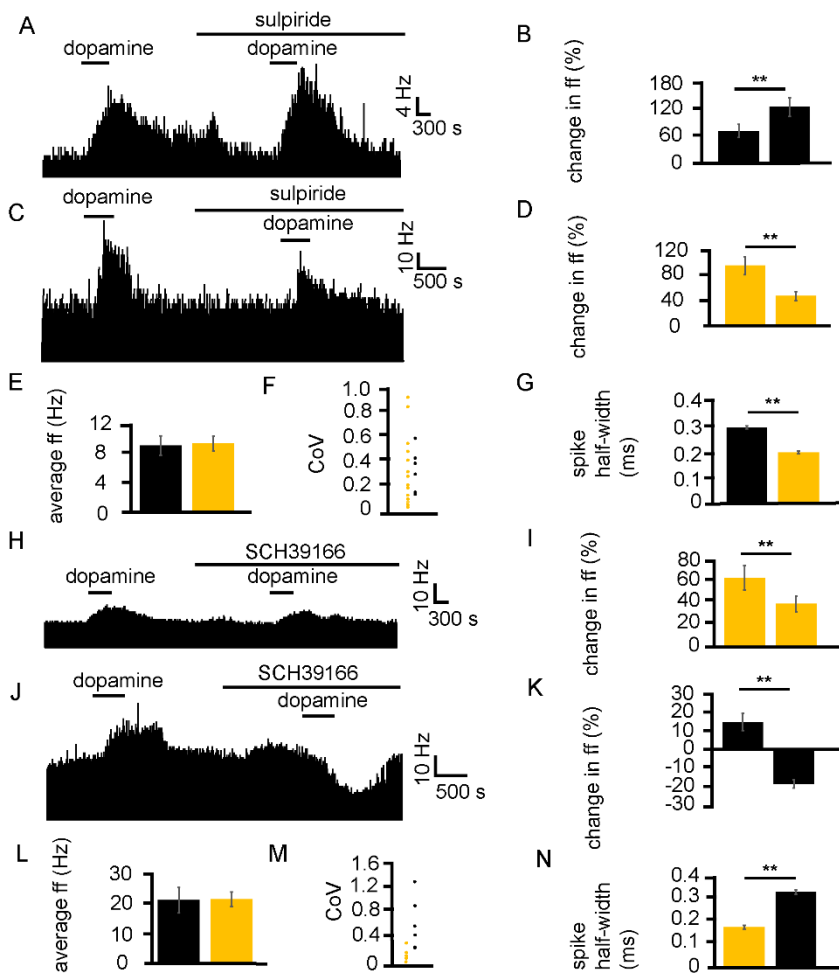


FIGURE 3.4: D1-like and D2-like receptor antagonists modulate the effect of dopamine in the VP. (A) For some neurons, when dopamine was applied in the presence of sulpiride, it produced a larger increase in firing than when it was applied in the absence of sulpiride. (B) Firing frequency changes to dopamine application in control and in the presence of sulpiride, for neurons similar to that illustrated in panel (A). (C) For some neurons, when dopamine was applied in the presence of sulpiride, it produced a smaller increase in firing than when it was applied in the absence of sulpiride. (D) Firing frequency changes to dopamine application in control and in the presence of sulpiride, for neurons similar to that illustrated in panel C. (E) Firing frequency changes to dopamine application for neurons that showed a larger increase in firing to dopamine application (black) in the presence of sulpiride and neurons that showed a smaller increase in firing to dopamine application in the presence of sulpiride (yellow). (F) Coefficient of variation for neurons that showed a larger increase in firing to dopamine in the presence of sulpiride (black) and those that showed a smaller increase in firing to dopamine in the presence of sulpiride (yellow). (G) Significant differences ($P < 0.01$) in spike half width (ms) for neurons that showed a larger increase in firing to dopamine in sulpiride (black) compared to those that showed a smaller increase in firing to dopamine in sulpiride (yellow). (H) For some neurons, when dopamine was applied in the presence of SCH39166, it produced a smaller increase in firing than when it was applied in the absence of SCH39166. (I) Firing frequency changes during dopamine application and dopamine in the presence of SCH39166, for neurons similar to that illustrated in panel (H). (J) Excitatory responses to dopamine application followed (after complete washout of dopamine) by inhibitory responses to dopamine application in the presence of SCH39166. (K) Firing frequency changes during application of dopamine and dopamine in the presence of SCH39166, for neurons similar to those illustrated in panel J. (L) Firing frequency changes to dopamine application for those neurons that showed a smaller increase in firing to the application of dopamine in the presence of SCH39166 (yellow), and those that showed a decrease in firing frequency to dopamine in the presence SCH39166 (black). (M) Coefficient of variation for neurons that showed a smaller increase in firing to dopamine in the presence of SCH39166 (yellow), and those that showed a decrease in firing frequency to dopamine in the presence of SCH39166 (black). (N) Significant ($P < 0.01$) differences in spike half width (ms) for those neurons that showed a smaller increase in firing to dopamine in the presence of SCH39166 (yellow), compared to those that showed a decrease in firing frequency to dopamine in the presence of SCH39166 (black).

3.3.5 D1-like receptors exert their effects presynaptically, while D2-like receptors cause excitation presynaptically and inhibition postsynaptically.

Because of the GABAergic and Glutamatergic inputs of the VP (Root et al., 2015) and the number of different interneurons, including cholinergic neurons (Pang et al., 1998; Zaborszky & Duque, 2000). It could be that D1-like receptors have their modulatory effects, presynaptically, postsynaptically or at both points. To identify whether D1-like receptors caused the increase in firing frequency in VP neurons acting pre- or postsynaptically, SKF81297 was applied in a solution of aCSF with low levels of Ca^{2+} , used to block presynaptic transmission. This was compared to application of SKF81297 in standard aCSF.

SKF81297 was applied twice, first in the presence of low Ca^{2+} aCSF and second (after complete washout of the first) in standard aCSF. In 2 experiments 7/7 spontaneously active neurons in the VP showed no significant change in firing frequency in response to SKF81297 in low Ca^{2+} conditions (Figure 3.5B). However, 5/7 neurons, showed strong significant ($P < 0.05$) increases in excitation 2856 ± 1079 % in response to SKF81297 when subsequently applied in standard aCSF (Figure 3.5B). These 5 neurons also showed statistically significant ($P < 0.05$) decreases in firing in response to wash-in of standard aCSF after prolonged exposure to low Ca^{2+} .

We can therefore conclude that D1-like receptors largely have their effects presynaptically. However the question remains as to whether this is through disinhibition, facilitation, or through another neurochemical mechanism.

Next the D2-like receptor mediated responses were investigated. D2-like receptor agonists have been shown in Figure 3 to produce both excitation and inhibition in the firing of VP neurons. Mengual & Pickel, (2002) have shown that some D2-like receptors are located presynaptically in the VP. Little else is known about their site of action within the VP. It was therefore imperative to investigate this further. To clarify the mechanism of action of D2-like receptors in the VP, and to investigate how this relates to their dichotomous effects on firing frequency, the effects of quinpirole in standard aCSF were compared to those in low Ca^{2+} aCSF, which was used to block presynaptic transmission.

From 4 experiments 12 neurons were identified in the VP for analysis that responded to quinpirole application. 8/12 of these neurons responded with significant ($P < 0.05$) increases in firing frequency to the application of quinpirole and 4/12 of these neurons responded with significant ($P < 0.05$) decreases in firing frequency to the application of quinpirole.

Of the 8 that were excited by quinpirole application 6/8 showed non-significant responses to quinpirole application in the presence of low Ca^{2+} aCSF, and 2/8 show significant ($P < 0.05$) increases in firing frequency to quinpirole application in the presence of low Ca^{2+} . For these 8 neurons, the application of quinpirole alone produced an average percentage increase in firing frequency of 111 ± 34 % compared to 42 ± 11 % for the application of quinpirole in the presence of low Ca^{2+} (Figure 3.5D). In 6/8 cases the first application of quinpirole (alone) produced significantly ($P < 0.05$) different responses to the second application (in low Ca^{2+}) as the firing frequencies measured during the first (alone) and second (in the presence of low Ca^{2+}) application of quinpirole were significantly different (Figure 3.5D). In 2/8 cases the second application

of quinpirole (in low Ca^{2+}) produced similar effects to the first one (alone) as the firing frequencies measured during the first and second application of quinpirole, for these 2 neurons, were not significantly different.

Of the 4 that were inhibited by quinpirole application all 4 continued to show significant ($P < 0.05$) decreases in firing frequency to quinpirole application in the presence of low Ca^{2+} . For these 4 neurons, the average decrease in firing frequency was $-65 \pm 13\%$ in response to quinpirole application alone and $-67 \pm 10\%$ to quinpirole application in the presence of low Ca^{2+} (Figure 3.5F). In all 4 cases the second application of quinpirole (in low Ca^{2+}) produced similar effects to the first one (alone) as the firing frequencies measured during the first and second application of quinpirole were not significantly different (Figure 3.5F).

We conclude that D2-like receptors mediate an increase in firing frequency, acting presynaptically in the majority of VP neurons, while D2-like receptors mediate a decrease in firing frequency acting postsynaptically in a minority of VP neurons.

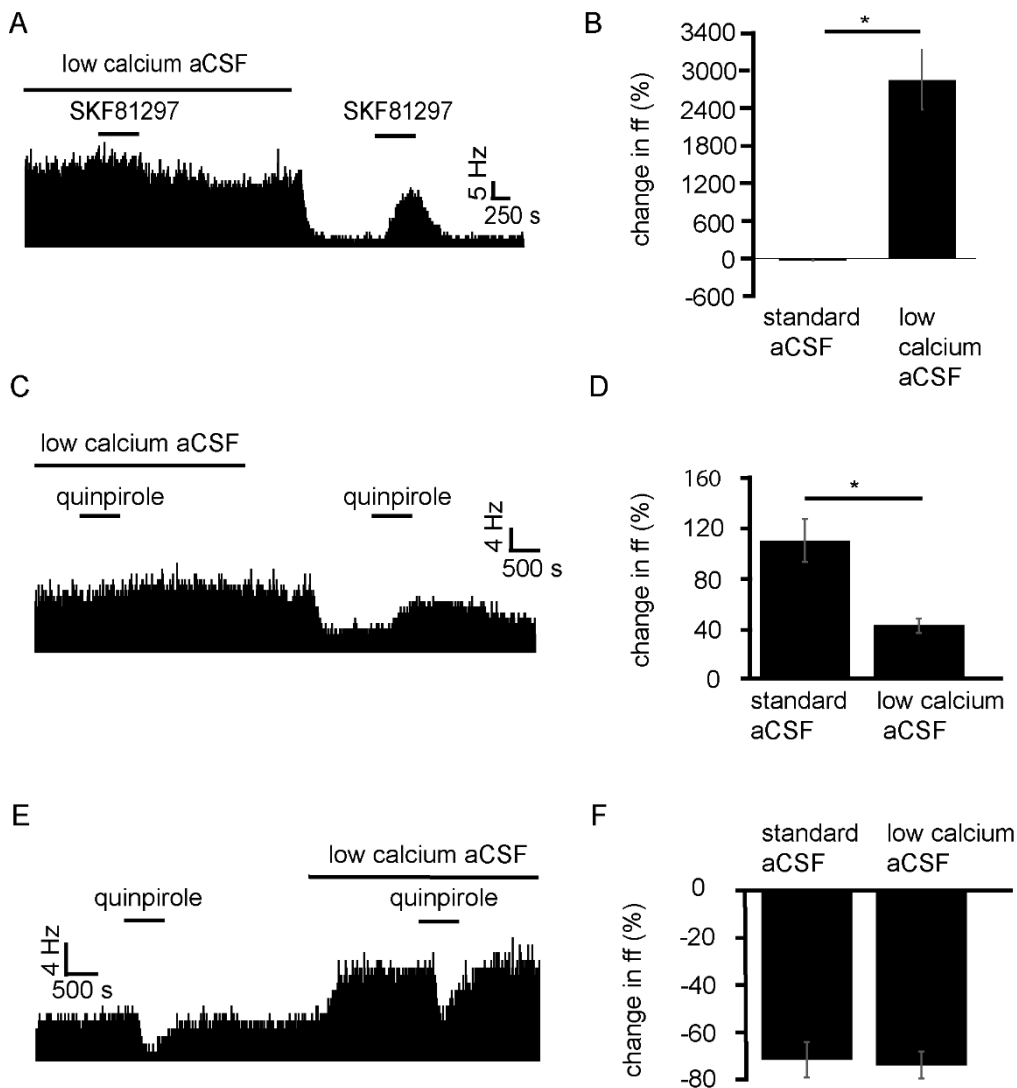


FIGURE 3.5: low Ca^{2+} aCSF blocks the excitatory effects of D1-like and D2-like receptor agonists but not the inhibitory effects of D2-like receptor agonists. **(A)** Excitatory response to SKF81297 application in standard aCSF, which is not present in low calcium. **(B)** Significant ($*=P<0.05$) differences in firing frequency for SKF81297 application in low calcium compared to the application of SKF81297 in standard aCSF. **(C)** Excitatory responses to quinpirole application alone were occluded in response to quinpirole application in low calcium aCSF. **(D)** Significant ($*=P<0.05$) difference in firing frequency for quinpirole application alone compared to quinpirole application in low calcium aCSF. **(E)** Inhibitory responses to quinpirole application persevere to quinpirole application in the presence of low calcium aCSF. **(F)** Firing frequency responses to quinpirole alone compared to quinpirole application in low calcium aCSF.

3.3.6 D1-like receptors excite VP neurons through presynaptic modulation of GA.

Because the VP has both GABAergic and glutamatergic inputs (Root, et al., 2015), these transmitters were potentially involved in the D1-like receptor agonist action in the VP. As current research suggests that D1 receptors are located presynaptically in the VP, and D1-like receptors are largely considered to be excitatory (Vallone, et al., 2000), it seemed possible that they were modulating glutamatergic inputs and therefore increasing neuronal firing in the VP via increased release of GA (Figure 1.1). To test this hypothesis, SKF81297 was applied twice, first alone and then in the presence of ionotropic glutamate receptor antagonists (NBQX and AP5). The second application was carried out after complete washout of the first.

In 2 experiments 7 spontaneously active VP neurons were identified for analysis as they responded to SKF81297. All 7 neurons studied in the VP showed strong significant ($P < 0.05$) increases in firing frequency in response to SKF81297 (Figure 3.6B).

However, all 7 showed no significant change in firing frequency in response to NBQX and AP5 application, and no significant change in firing frequency in response to subsequent SKF81297 application in the presence of NBQX and AP5 (Figure 3.6B). In all cases the firing frequencies measured during the first and the second application of SKF81297 were significantly ($P < 0.05$) different. The first application of SKF81297 produced an average increase in excitation of 439 ± 144 % compared to 6 ± 6 % for the second application of SKF81297 (Figure 3.6C), in the presence of NBQX and AP5.

We can therefore conclude that the increase in firing frequency produced in the VP as a result of D1-like receptor agonists application is a result of facilitation of presynaptic

glutamate terminals and the subsequent increase in activation of ionotropic receptors on VP neurons

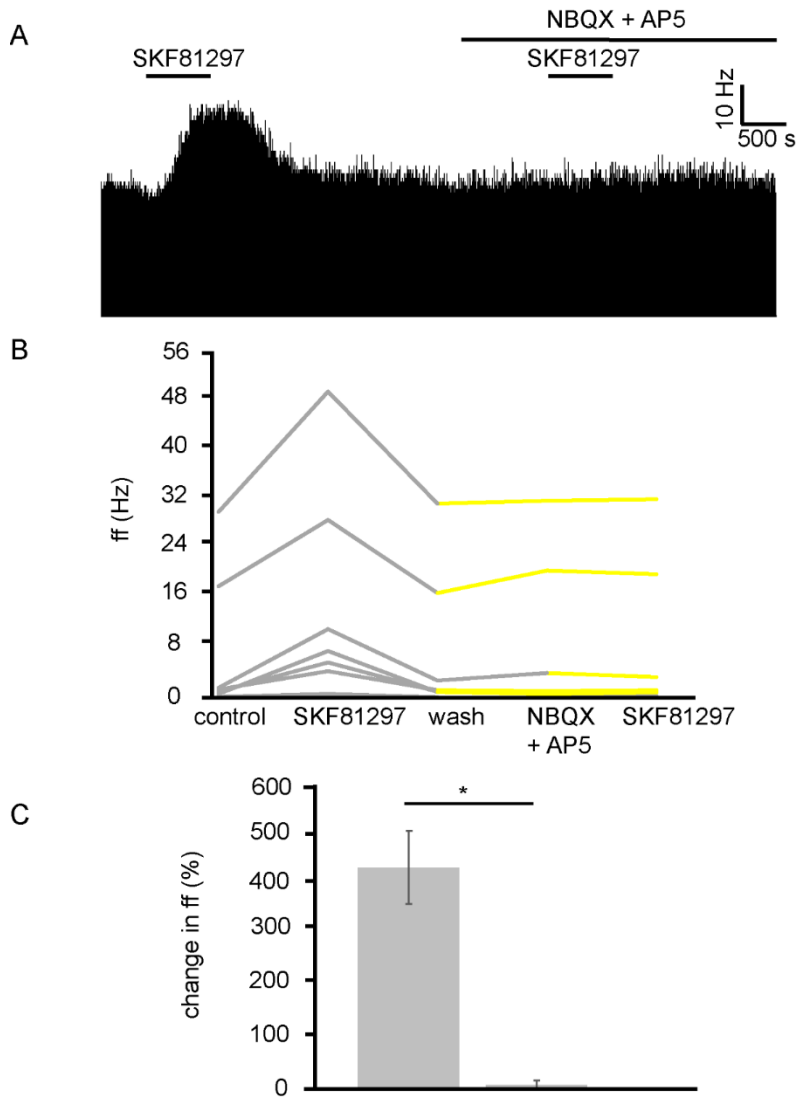


FIGURE 3.6: D1-like receptor mediated excitation requires ionotropic glutamate receptors. **(A)** Excitatory responses to SKF81297 application alone that are no longer present in response to SKF81297 in the presence of NBQX and AP5 **(B)** firing frequency for (ff) 7 VP neurons in response to SKF81297 application and SKF81297 application in the presence of NBQX and AP5, similar to that illustrated in panel A. Firing frequency was measured before the application of SKF81297, during the application of SKF81297, after a period of wash out, in response to the application of NBQX and AP5 and finally in response to SKF81297 in the presence of NBQX and AP5. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Significant ($P < 0.05$) differences in firing frequency (ff) to SKF81297 alone compared to the application of SKF81297 in the presence of NBQX and AP5.

3.3.7 Excitatory effects of D2-like receptor agonists require mGluRs

In order to establish the likely neurotransmitter, and receptor group that quinpirole was modulating to increase firing frequency in VP neurons, quinpirole was applied in the presence of MCPG, which is a non-selective mGluR antagonist. Because the current research has identified that quinpirole has its excitatory effects by modulation of presynaptic mechanisms (Figure 6) and D2-like receptors presynaptically are largely considered inhibitory (Mengual & Pickel, 2002; Vallone et al., 2000). Our hypothesis was that D2-like receptors might be modulating glutamate release (figure 1.1). Group 2 and 3 mGluRs are known to be inhibitory (Benarroch, 2008) and therefore provide a potential mechanism by which presynaptic inhibition of glutamate by D2-like receptors agonists might disinhibit neurons in the VP and therefore increase firing frequency in a number of VP neurons.

In 2 experiments 13 VP neurons were identified for analysis as they responded to quinpirole. All 13 neurons studied in the VP showed strong significant ($P < 0.05$) increases in firing frequency in response to quinpirole. After a period of wash out a further significant ($P < 0.05$) change was seen in 9/13 of those neurons studied in response to application of the non-selective mGluR antagonist MCPG. Subsequently only 4/13 of these neurons showed any significant ($P < 0.05$) increase in firing frequency in response to quinpirole in the presence of MCPG (Figure 3.7B). In the majority of cases the first application of quinpirole resulted in distinctly different responses to the second, as the firing frequencies measured during the first and the second application of quinpirole were significantly ($P < 0.05$) different for 12/13 neurons. The first application of quinpirole producing an average percentage increase in excitation of 588 ± 232 % compared to 41 ± 27 % for the second application of quinpirole, in the presence of MCPG (Figure 3.7C).

To further explore the role of mGluRs in the excitatory effects of D2-like receptor agonists we applied quinpirole alone and in the presence of MSPG, which is a selective antagonist for group 2 and 3 mGlu receptors, which are known to have inhibitory effects (Nicoletti et al., 2011) and be located postsynaptically (Petralia, et al., 1996).

From 3 experiments 11 neurons were identified for analysis as they responded to quinpirole application. All 11 neurons studied in the VP showed strong significant ($P < 0.05$) increase in firing frequency in response to quinpirole. After a period of wash out a further significant ($P < 0.05$) change was seen in 3/11 of those neurons studied in response to MSPG application. Subsequently only 2/11 of these neurons showed any significant ($P < 0.05$) change in firing frequency in response to quinpirole in the presence of MSPG (Figure 3.7E). In all cases the firing frequencies measured during the first (alone) and second (presence of MSPG) application of quinpirole were significantly ($P < 0.05$) different.

We therefore concluded that D2-like receptors inhibit the release of glutamate presynaptically, which in turn disinhibits VP neurons, which express inhibitory group 2/3 mGluRs, increasing their firing frequency.

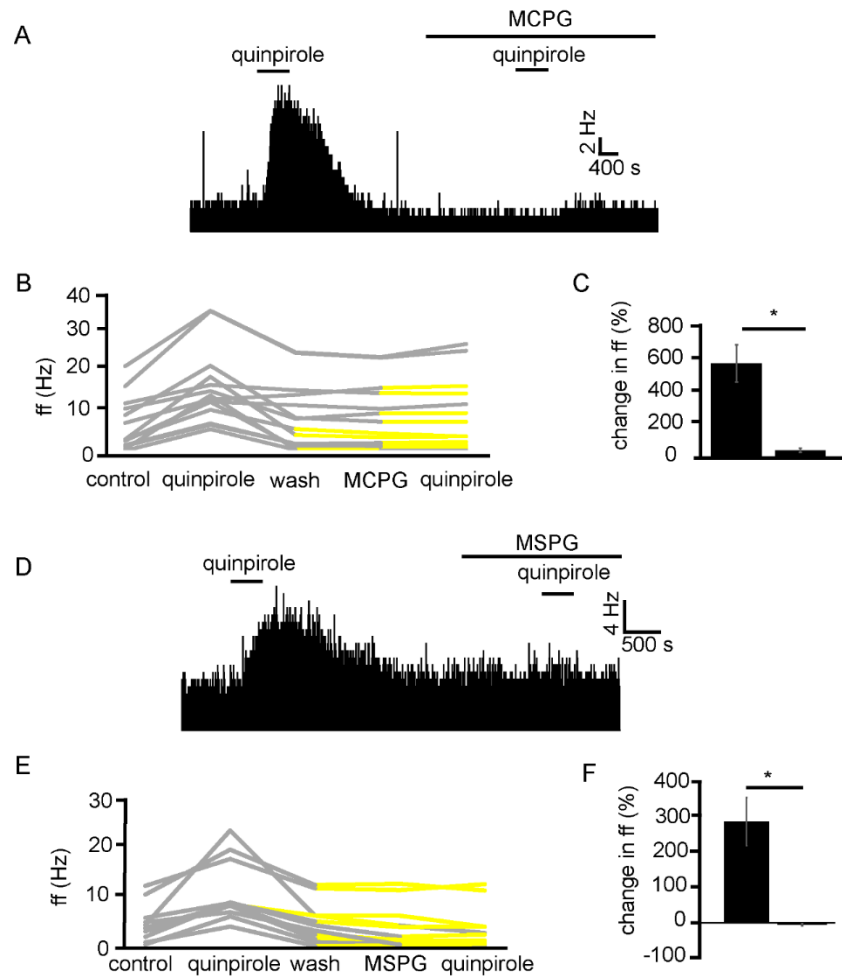


FIGURE 3.7: excitatory effects of D2-like receptor agonists require metabotropic glutamate receptors. **(A)** Excitatory responses to quinpirole that are not present for the same neuron in response to quinpirole in the presence of MCPG **(B)** Changes in firing frequency for 13 VP neurons to application of quinpirole and quinpirole in the presence of MCPG, similar to that illustrated in panel A. Firing frequency was measured before the application of quinpirole, during the application of quinpirole, after wash out of quinpirole, during application of MCPG and finally during the application of quinpirole in the presence of MCPG. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Significant ($*=P < 0.05$) differences in firing frequency in response to quinpirole compared to quinpirole in the presence of MCPG **(D)** Excitatory responses to quinpirole that are not present for the same neuron in response to quinpirole in the presence of MSPG **(E)** Changes in firing frequency for 11 VP neurons to the application of quinpirole and quinpirole in the presence of MSPG, similar to that illustrated in panel A. Firing frequency was measured; before the application of quinpirole, during the application of quinpirole, after wash out of quinpirole, during application of MSPG and finally during the application of quinpirole in the presence of MSPG. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(F)** Significant ($*=P < 0.05$) differences in firing frequency in response to quinpirole compared to quinpirole in the presence of MSPG.

3.3.8 Inhibitory effects of D2-like receptor agonists persevere in the presence of mGluR receptor antagonists

While excitation in VP neurons, as a result of quinpirole application, has been shown in the current study to be modulated by presynaptic mechanisms (Figure 3.5C, D). The current study suggests that inhibition of firing frequency in the minority of VP neurons is likely to be as a result of postsynaptic /direct inhibition (Figure 3.5E, F). This should mean that MCPG has no effect on the reduction in firing frequency seen in response to quinpirole.

In 3 experiments 5 VP neurons were identified for analysis as they responded to quinpirole application. 5/5 of these neurons measured in the VP displayed significant ($P < 0.01$) decreases in firing frequency in response to quinpirole. After a period of wash out MCPG application evoked significant changes in firing frequency in 2/5 neurons, subsequently application of quinpirole in the presence of MCPG evoked a further significant ($P < 0.05$) decrease in firing frequency in 5/5 of these VP neurons. In the majority of cases, the second application of quinpirole produced similar effects to the first one, as the firing frequencies measured during the first and the second application of quinpirole were not significantly different for 4/5 neurons (Figure 3.8B, C). The first application of quinpirole producing an average percentage inhibition of $-69 \pm 8 \%$ compared to $-66 \pm 9 \%$ for the second application of quinpirole, in the presence of MCPG (Figure 3.8C).

To further ensure that the inhibitory effect of D2-like receptor agonists were not mediated presynaptically or through mGlu receptors, we applied quinpirole in the presence of low Ca^{2+} aCSF and in the presence of low Ca^{2+} aCSF and MCPG.

From 1 experiment 3 neurons were identified for analysis. 3/3 displayed significant ($P < 0.05$) decreases in firing frequency in response to application of quinpirole. After a period of wash out 3/3 of the neurons responded with non-significant ($P > 0.05$) changes in response to MCPG application, subsequent application of the quinpirole in the presence of low Ca^{2+} and MCPG evoked further significant ($P < 0.05$) decreases in firing frequency in 3/3 of the neurons. In all cases, the second application of quinpirole (in the presence of MCPG and low Ca^{2+}) produced similar effects to the first one (Figure 3.8 E, F), as the firing frequencies measured during the first and the second application of quinpirole were not significantly different for 3/3 neurons.

We can conclude that mGluR are not involved in the inhibition seen in the VP to quinpirole application.

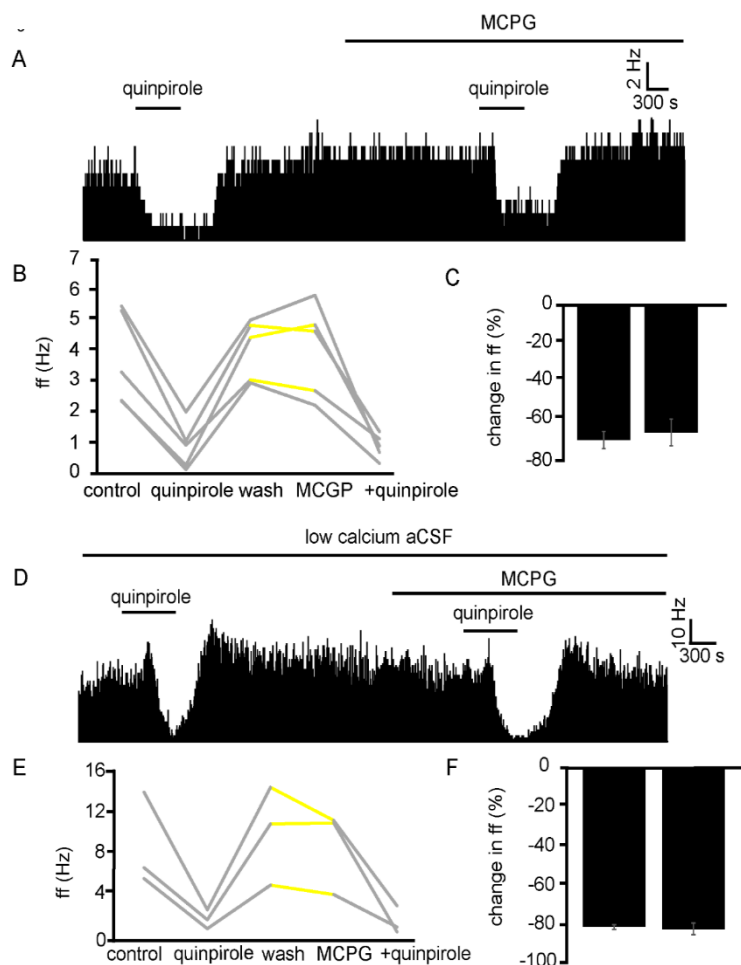


FIGURE 3.8: inhibitory effects of D2-like receptor agonists continue in the presence of mGluR receptor antagonists and low calcium aCSF. **(A)** Inhibitory responses to quinpirole application alone and in the presence of MCPG. **(B)** Changes in firing frequency for 5 VP neurons to application of quinpirole and quinpirole in the presence of MCPG, similar to that illustrated in panel A. Firing frequency was measured before the application of quinpirole, during the application of quinpirole, after wash out of quinpirole, during application of MCPG and finally during the application of quinpirole in the presence of MCPG. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Firing frequency in response to quinpirole compared to quinpirole in the presence of MCPG. **(D)** Inhibitory responses to quinpirole application in the presence of low calcium aCSF are repeated in the presence of low calcium aCSF and MCPG. **(E)** Changes in firing frequency for 3 VP neurons to application of quinpirole and quinpirole in the presence of MCPG, similar to that illustrated in panel A. Firing frequency was measured; before the application of quinpirole, during the application of quinpirole, after wash out of quinpirole, during application of MCPG and finally during the application of quinpirole in the presence of MCPG. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(F)** Firing frequency in response to quinpirole in low calcium aCSF compared to quinpirole in the presence of low calcium aCSF and MCPG.

3.3.9 Excitatory responses to D1-like receptor agonists are not effected by metabotropic glutamate receptor antagonists

We have already established that increases in firing frequency as a result of SKF81297 application are largely modulated by glutamate and ionotropic glutamate receptors. To ensure mGluRs do not interfere with this response, experiments were carried out with SKF81297 in the presence of MCPG. From 2 experiments 11 neurons were identified for analysis. 10/11 of these neurons were excited ($P < 0.05$) by SKF81297 application. After a period of wash out 4/11 neurons responded with significant ($P < 0.05$) changes in response to MCPG application, subsequent application of SKF81297, evoked a further significant increase in firing frequency in 11/11 of the VP neurons observed. In the majority of cases, the second application of SKF81297 (in the presence of MCPG) produced similar responses to the first one, as the firing frequencies measured during the first and the second application of SKF81297 were not significantly different for 10/11 neurons (Figure 3.9B, C). The first application of SKF81297 producing an average percentage excitation of 322 ± 105 % compared to 361 ± 153 % for the second application of SKF81297, in the presence of MCPG (Figure 3.9C).

We can therefore rule out the involvement of mGluRs in the excitation observed in response to SKF81297 in the VP.

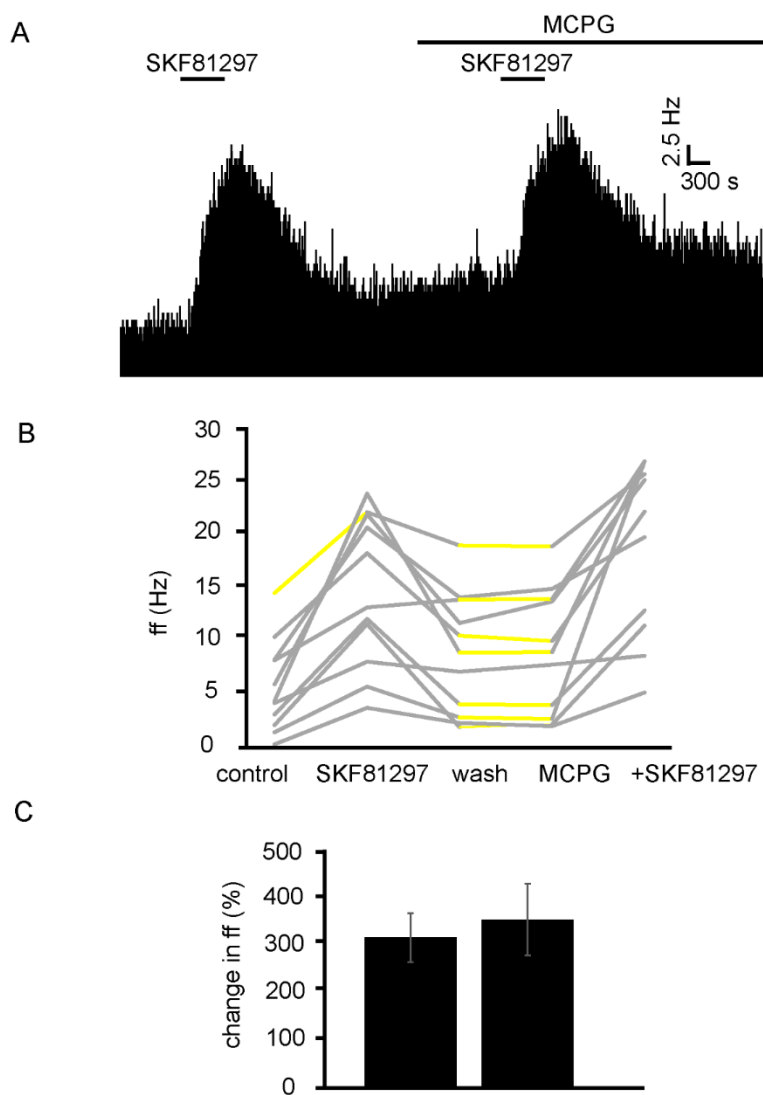


FIGURE 3.9: D1-like receptor agonists continue to excite VP neurons in the presence of MCPG. **(A)** Excitatory responses to SKF81297 application alone and in the presence of MCPG. **(B)** Changes in firing frequency for 11 VP neurons to application of SKF81297 and SKF81297 in the presence of MCPG, similar to that illustrated in panel A. Firing frequency was measured; before the application of SKF81297, during the application of SKF81297, after wash out of SKF81297, during application of MCPG and finally during the application of SKF81297 in the presence of MCPG. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Firing frequency in response to SKF81297 compared to SKF81297 in the presence of MCPG.

3.4 Discussion

This study investigated the effects of dopamine on the tonic firing of VP neurons in vitro. Our data shows that dopamine has dual effects in the VP, producing increases and decreases in firing frequency in VP neurons. Two populations of neurons in the VP were identified based upon their spike half-width profile. Type I neurons displaying a markedly shorter spike half-width profile and being excited by both D1-like and D2-like receptor agonists (Figure 3.10). This resulted in a net excitatory effect in response to dopamine in type I neurons. Type II neurons however had a markedly longer spike half-width profile, were inhibited by D2-like agonists and excited by D1-like agonists (Figure 3.10). For type II neurons the net balance of inhibitory D2-like receptor activation and excitatory D1-like receptor activation, through application of dopamine, resulted in a net effect of increased firing rates in some type II neurons and decreased firing rates in others.

3.4.1 Presynaptic and postsynaptic effects of dopamine.

Previous studies of the dopaminergic modulation of VP neurons had been carried out in vivo. These studies showed that dopamine can exert both excitatory and inhibitory influences on VP neurons. Electrical stimulation of the VTA or SNc caused inhibitions or excitations of VP neurons, in a roughly 2:1 ratio (Mitrovic & Napier, 2002; Napier & Maslowski-Cobuzzi, 1994). Similar responses were observed with local microiontophoretic applications of dopamine (Mitrovic & Napier, 2002; Napier & Potter, 1989). Dopamine D1-like and D2-like receptors are thought to contribute to these responses, however it is unclear, which modulate excitations and which modulate inhibitions. Maslowski & Napier, (1991a) found D2-like receptor agonists reduced activity in 59 % of neurons studied and that D1-like receptor agonists excited 69 % of neurons. However, Napier & Maslowski-Cobuzzi's, (1994) data suggest that D1-like

receptor agonists largely produce inhibitions in VP neurons and D2-like receptor agonist largely produce excitation.

Both direct and indirect effects of dopamine are considered likely involved (Root et al., 2015) in modulation of VP neurons. However, a dissection of these effects and of the other neurotransmitters involved had not been possible in *in vivo* experiments. Our data clearly indicated that D1-like receptors acted at a presynaptic level, as a D1-like receptor agonist produced no significant effects in low Ca^{2+} (a condition in which synaptic transmission is blocked), even in neurons in which D1-like receptor agonist had previously elicited strong excitatory effects when applied in normal Ca^{2+} . Furthermore, we were able to show that D1-like receptors exerted their presynaptic effects by increasing ionotropic glutamate receptors activation, as antagonists of ionotropic glutamate receptors (NBQX and AP5) also consistently blocked the excitatory effects of D1-like receptor agonists. Thus, a facilitation of glutamate release from terminals impinging on VP neurons (figure 1.1) appears to underlie the excitatory effects of D1-like receptors in both groups of VP neurons.

On the other hand, D2-like receptors engaged both presynaptic and postsynaptic mechanisms. Low Ca^{2+} failed to block the decreases in firing frequency observed in the neurons of the second group in response to D2-like receptor agonist application. Thus, D2-like receptors caused their inhibitory effects acting postsynaptically on VP neurons of the second group. However, the excitatory effects of a D2-like receptor agonist were no longer observed (in neurons of the first group) in low Ca^{2+} aCSF, showing that these effects were mediated presynaptically. A study by Mengual and Pickel, (2002) showed that D2-like receptors are located at presynaptic locations on GABAergic terminals in the VP. However, the excitatory effects of D2-like receptor agonists persisted in the

presence of GABA_A and GABA_B receptor antagonists, showing that they did not result from inhibition of GABA release. On the other hand, a broad-spectrum mGluR antagonist completely prevented the excitatory effects of D2-like receptor agonists. Furthermore, a ligand that selectively blocks group 1 and group 2 mGluRs also prevented any excitatory action of D2-like receptor agonists. These observations suggested that the excitatory effects of D2-like receptors were caused by inhibition of glutamate release and reduced postsynaptic activation of inhibitory mGluRs. mGluRs are present in the VP (Testa, et al., 1994) and some mGluR groups are inhibitory (Anwyl, 1999). There is a paucity of data on mGluR action within the VP, but Ohishi, et al's., (1993) data suggests there is labelling for multiple mGluR in the VP, including the inhibitory mGluR2 group. They are also a likely candidate as Holmes, et al., (1996) found mGluR2 receptors to be the mediator of postsynaptic hyperpolarisations within the basal lateral amygdala.

The observation that NBQX and AP5 did not affect VP neurons firing but prevented the excitatory effects of D1 agonists, suggests that the glutamate terminals facilitated by D1 receptors were not spontaneously active in brain slices. We tentatively propose that a different set of glutamate terminals (figure 1.1) were responsible for the activation of postsynaptic inhibitory mGluRs in those VP neurons in which mGluR antagonists prevented inhibition by D2 agonists (as illustrated in Figure 3.10). These terminals are expected to release glutamate spontaneously, keeping postsynaptic inhibitory group 2 and/or 3 mGluRs in a state of basal activation. However, the lack of effects of mGluR antagonists on the firing frequency of these VP neurons was unexpected. It is possible that the excitatory influence caused by reduce activity of mGluRs was compensated by an inhibitory influence caused by reduced activity of other pre or postsynaptic mGluRs

affecting VP neurons through different mechanisms. Further experiments will be needed to test this hypothesis.

3.4.2 Identity of VP neurons modulated by dopamine

As mentioned above, VP neurons displayed two distinct responses to D1-like and D2-like receptor agonists. Type I neurons responded with increases in firing frequency to both specific D1-like and D2-like receptor agonists, while type II neurons were excited by D1-like receptor agonists and inhibited by D2-like receptor agonist (Figure 3.10). These responses can be attributed unambiguously to two distinct neuronal populations based on their spike half-width profile, with the type I neurons having a shorter spike half-width than type II neurons.

Further supporting this distinction, neurons with a shorter spike half-width displayed a decreased increase in firing frequency to dopamine in the presence of either D1-like or D2-like receptor antagonists, suggesting that both receptor classes contributed to increases in firing frequency for these neurons. Conversely, neurons with a longer spike half-width showed an increased excitatory response to dopamine in the presence of D2-like receptor antagonists and were inhibited by the application of dopamine in the presence of D1-like receptor antagonists, confirming that D2-like receptors inhibit these neurons.

Several studies have investigated spike half-width profiles in the VP and its dorsal extent, the GPe (Abdi et al., 2015; Bengtson, et al., 2004; Bengtson & Osborne, 2000; Hernández et al., 2015). VP neurons include prominent cholinergic and GABAergic populations (Duque, et al., 2007; Root et al., 2015; Zaborszky & Duque, 2000)).

Cholinergic cells have sparse axons and project to the cortex and the amygdala (Carlsen, et al., 2004; Zaborszky, et al., 1986), while GABAergic neurons can have dense axonal

arborisation and are thought to include both interneurons and projection neurons (Zaborszky, et al., 2012). The electrophysiological features of these neuronal types have not been completely identified. Bengtson and Osborne, (2000) found that only 2/13 cholinergic neurons in the VP had any tonic firing activity. Bugaysen et al, (2010) suggested that it is impossible to separate neuron populations from extracellular recordings in the rat GPe based on action potential half-width, although Becchetti et al, (2012) suggests that spike half-width maybe a viable way of distinguishing inhibitory from excitatory neurons in mature animals using MEA data.

Pang et al, (1998) identified “Type I” neurons in vivo, with features resembling those of noncholinergic neurons described by Bengtson and Osborne, (2000). Type B neurons of Lavín and Grace, (1996) also have features similar to these cells, and have short spike duration (approximately 1.3 ms). These neurons are probably GABAergic projection neurons (Root et al., 2015). Conversely, “Type II” neurons of Pang et al., (1998) and “Type A” neurons of Lavín and Grace, (1996) had relatively similar features, including slower spikes (approximately 2.8 ms). Pang et al. (1998) identified these neurons as noncholinergic (possibly GABAergic) interneurons. Based on this complex picture, we can tentatively classify our type I neurons as GABAergic projection neurons corresponding to type I of Pang et al., (1998) and type B of Lavin and Grace, (1996) and our type II neurons as GABAergic interneurons corresponding to type II of Pang et al., (1998) and type A of Lavin and Grace, (1996). Further investigation is needed to confirm the neurochemical and morphological identity of the two groups of neurons identified by this study based on their dopaminergic responses.

In some previous in vivo experiments dopamine inhibitions of VP neurons were more frequent than dopamine excitations (Napier & Maslowski-Cobuzzi, 1994), while we

found a prevalence of excitations. It is likely that this discrepancy results from the fact that in the intact brain the VP receives a number of external inputs (including glutamate) that are silent in brain slices and could be modulated presynaptically by dopamine.

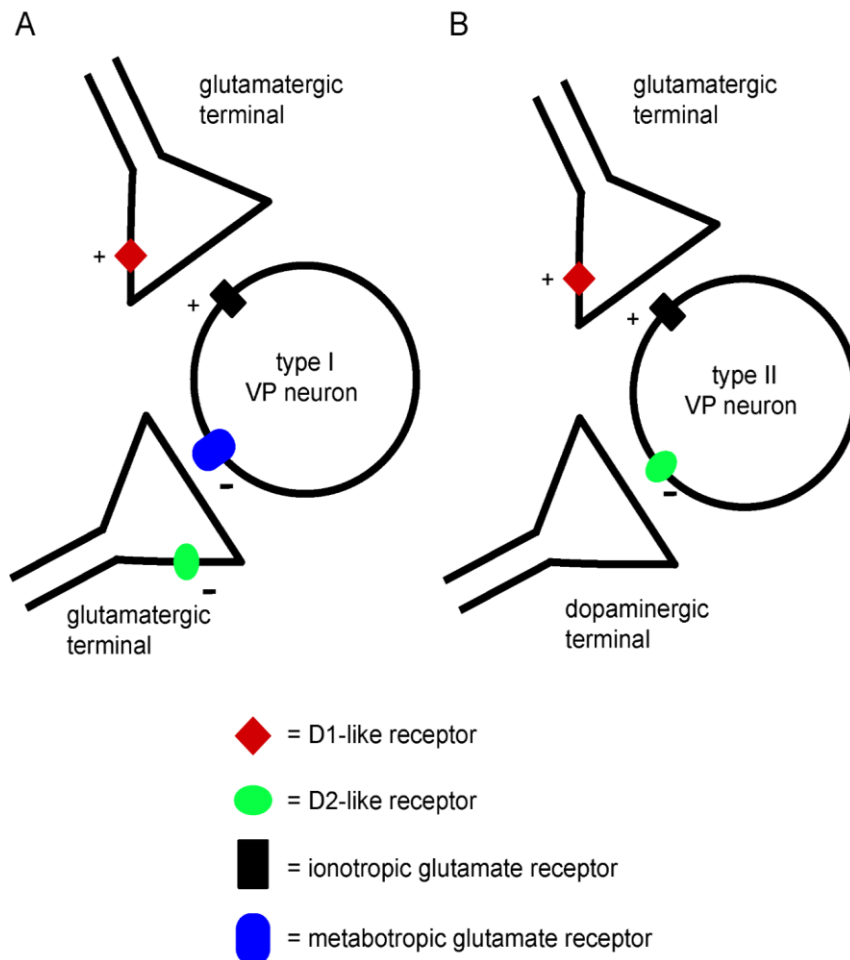


FIGURE 3.10: A minimal model explaining the differential effects of D1-like and D2-like receptor activation on type I and type II VP neurons in the VP.

(A) Presynaptic D1-like receptors facilitate glutamate release and excite type I VP neurons through increased activation of ionotropic glutamate receptors. Another glutamatergic input to type I neurons activates inhibitory metabotropic glutamate receptors. Presynaptic D2-like receptors have an inhibitory effect on this glutamate input, reducing glutamate release and disinhibiting type I neurons. Thus, both D1-like and D2-like receptor have excitatory influences on type I neurons.

(B) Presynaptic D1-like receptors facilitate glutamate release and excite type II VP neurons by increasing activation of ionotropic glutamate receptors. Postsynaptic D2-like receptors have direct inhibitory effects on type II VP neurons. The net influence of dopamine on type II neurons results from the balance of excitatory D1-like receptor effects and inhibitory D2-like receptor effects.

3.4.3 Functional implications:

The VP is crucially involved in reward processing (Smith et al., 2009) and drug-seeking behaviour (Kalivas & Volkow, 2005; Prasad & McNally, 2016). Dopamine levels rise dramatically within the VP as a result of sensitization to drugs of abuse (Stout et al., 2016) and dopaminergic mechanisms in the VP underlie morphine-induced conditioned place preferences (Gong, et al., 1996; Zarrindast, et al., 2007). The current study provides novel and clear insights into the cellular mechanisms by which dopamine modulates neuronal activity in the VP. Given the prominence of the VP in the reward circuits, understanding how dopamine works in this area is of primary theoretical value, and can also help developing rational pharmacological approaches to fight addiction and other dysfunctions of the reward system.

4 Chapter 4: Serotonergic modulation of VP neurons

4.1 Abstract:

Serotonergic afferents reach the VP from the dorsal raphe nuclei. Recent *in vitro* research suggests that serotonin produces dichotomous responses within the VP, however little else is known about the cellular effects of serotonin within the VP. The current study aimed to address this paucity of data using brain slices containing the VP and multi-electrode array (MEA) recordings. Our data supports the intracellular research of Bengtson et al., (2004). Serotonin affected firing in 100% of spontaneously active VP neurons. 72% of these neurons were excited and 28% were inhibited by serotonin application. The excitatory effects of serotonin were pre-synaptic in origin as blocking synaptic transmission with low- Ca^{2+} aCSF abolished these effects. Conversely, the inhibitory effects of serotonin persisted in low Ca^{2+} aCSF and therefore can be attributed to direct postsynaptic receptor activation. 5HT1a (WAY100635), 5HT5a (SB699551) and 5HT7 (SB269970) receptor antagonists modulated the excitatory effects of serotonin application, suggesting that they were all involved in the excitatory responses within the VP. In the presence of 5HT1a antagonists, 63% of neurons previously excited by serotonin showed no significant responses to serotonin application and 37% of neurons previously excited by serotonin showed significant inhibitory responses to serotonin application. In the presence of a 5HT7 antagonists, 72% of neurons previously excited by serotonin showed no significant responses to serotonin application and 28% of neurons previously excited by serotonin showed excitatory responses to serotonin application, but significantly reduced in magnitude compared to excitatory response to serotonin application alone. In the presence of a 5HT5a antagonists 83% of neurons, excited by serotonin application alone, continued to show significant excitation to serotonin application, however these neurons showed a significantly reduced magnitude of excitation to serotonin in the presence of 5HT5a antagonist compared to serotonin application alone.

Conversely, 5HT1a antagonists had no effect on the majority of inhibitory responses to serotonin application, with 75% showing similar significant decreases in firing frequency to serotonin application in the absence or presence of 5HT1a antagonist. The effect of 5HT7 and 5HT5a antagonists on inhibitory responses was inconclusive.

The inhibitory responses to serotonin application were found to be modulated by 5HT2c receptors, with 50% of neurons that had been inhibited by previous serotonin application showing no significant effects in response to serotonin application in the presence of 5HT2c antagonists (RS102221) and 33% responding with a significant increase in firing frequency to serotonin in the presence of 5HT2c antagonists. A 5HT2c antagonist had no effect on the excitatory responses to serotonin application, with 100% continuing to express significant increases in firing frequency to serotonin application in the presence of 5HT2c antagonists.

These data provide novel insights into the cellular mechanism by which serotonin modulates reward and affective responses and provides potential target receptors for pharmacological interventions.

4.2 Introduction:

Besides its strong dopaminergic projections, the VP also receives substantial serotonergic projections from the dorsal raphe nuclei (Vertes, 1991). Like dopamine, serotonin is heavily implicated in affective disorders, reward learning and responses to drugs of abuse (Der-Ghazarian et al., 2017; Kranz et al., 2010; Nakamura et al., 2008). Recent research suggests that the interaction between serotonin and dopamine are essential for reward and reward related behaviour such as drug addiction (Fischer & Ullsperger, 2017). The VP is a key site for research into drugs of abuse and reward (Creed et al., 2016; James & Aston-Jones, 2016; Root et al., 2015; Smith et al., 2009), it

therefore seems a likely site for the interaction between dopamine and serotonin to influence these behaviours.

Like the dual effects of dopamine, we have observed on VP neurons, Bengtson et al., (2004) have shown that serotonin has dual effects on neurons in the VP, with both increases and decrease in firing as a result of serotonin application. Bengtson et al., (2004) suggest that the dual effects of serotonin relate directly to two distinct populations of neurons in the VP, with serotonin decreasing firing in cholinergic neurons and increasing firing in non-cholinergic neurons of the VP. However the degree to which these distinctions maintain when serotonin is administered to both neuron types at once is questionable as cholinergic neurons in the VP are believed to innervate other populations of neurons within the VP (Duque et al., 2007). Collateral inhibition or excitation may therefore modulate the dichotomous response seen to serotonin in VP neurons by Bengtson et al., (2004). This can be investigated effectively using our MEA setup.

Our observations with dopamine suggest it too effects two distinct populations of neurons in the VP, with specific inhibitory effects being produced by D2-like agonists in neurons identified as having a larger spike half-width profile than those excited by D1-like and D2-like agonists. We could not however conclude if these differences in spike half-width profiles related to cholinergic or non-cholinergic neurons of the VP. In the current study we aimed to identify the spike half-width profile of those neurons that increase firing and those that decrease firing in response to serotonin application, so as to elucidate the potential modulatory interaction between serotonin and dopamine in the VP.

The VP is known to express multiple serotonin receptor subtypes, including: 5HT4 (Waeber et al., 1994), 5HT2 (Appel et al., 1990), 5HT1a (Drevets et al., 2007;

Heidenreich & Napier, 2000; Wright et al., 1995), 5HT1B(Murrough et al., 2011), 5HT7 (To et al., 1995) and 5HT5a (Oliver et al., 2000). These specific serotonin receptor subtypes, may well play specific roles in modulation of the VP circuitry. Indeed Heidenreich & Napier, (2000) have shown the specific 5HT1a receptor subtype, modulate the firing of VP neurons, with increases and decreases in firing potentials. Nishijo & Momiyama, (2016) have also suggested that 5HT1b receptors act as heteroreceptors on GABAergic terminals projecting into the VP, thus inhibiting the release of tonic GABA. Little else is known about how other specific serotonin receptor subtypes, known to be expressed in the VP, modulate the dichotomous responses identified by Bengtson et al., (2004). It is also not known whether serotonin's modulatory effects are pre/post synaptic, or indeed a combination of both. This is essential knowledge as the serotonin receptor subtypes, and their differential modulation of the VPs circuitry, may well play a key role in the modulation of affective disorders, responses to drugs of abuse and reward learning. Indeed Murrough et al., (2011) has recently suggested that there is a strong link between the specific Serotonin receptor; 5HT1b binding in the VP and major depressive disorder. We suggest that serotonin and its receptor subtypes that mediate its effects in the VP, are critical in terms of pharmacological interventions for major depressive disorder, responses to drugs of abuse and reward learning. In the current research we seek to elucidate the specific contribution several of these serotonin receptor subtypes play in modulating increases and decrease in firing observed in response to serotonin in the VP.

4.3 Results:

4.3.1 Serotonin application has dual effects on VP neurons.

To investigate the dichotomous responses seen by Bengtson et al., (2004) further we applied serotonin to a slice preparation, recording activity from multiple neurons across the VPs territories using a multielectrode array.

From 10 experiments, 76 neurons were analysed in the VP. 55/76 neurons displayed significant ($P < 0.05$) increases in firing frequency to the application of serotonin (Figure 4.1B and C). However, a minority 21/76 displayed significant ($P < 0.05$) decreases in firing frequency to serotonin application (Figure 4.1A, C). In the neurons that were excited by serotonin, the average increase in firing frequency was 88 ± 68 %. In the neurons that were inhibited by serotonin, the average decrease in firing frequency was -26 ± 2 %.

In order to ascertain if excitatory and inhibitory responses to serotonin were elicited in different types of neurons within the VP, baseline firing frequency, coefficient of variation and spike half-width were calculated for the two subpopulations. Baseline firing frequency and coefficient of variation (Figure 4.1D, E) were not significantly ($P > 0.05$) different for the two populations. There was also no significant differences in spike half-width (ms) between those neurons that were excited by serotonin application and those inhibited by serotonin application.

We can conclude that the dichotomous responses seen in the VP to serotonin by Bengtson et al., (2004) persist when serotonin is administered to the area as a whole. However there was no evidence to suggest that these responses were from two unique populations of neurons in the VP.

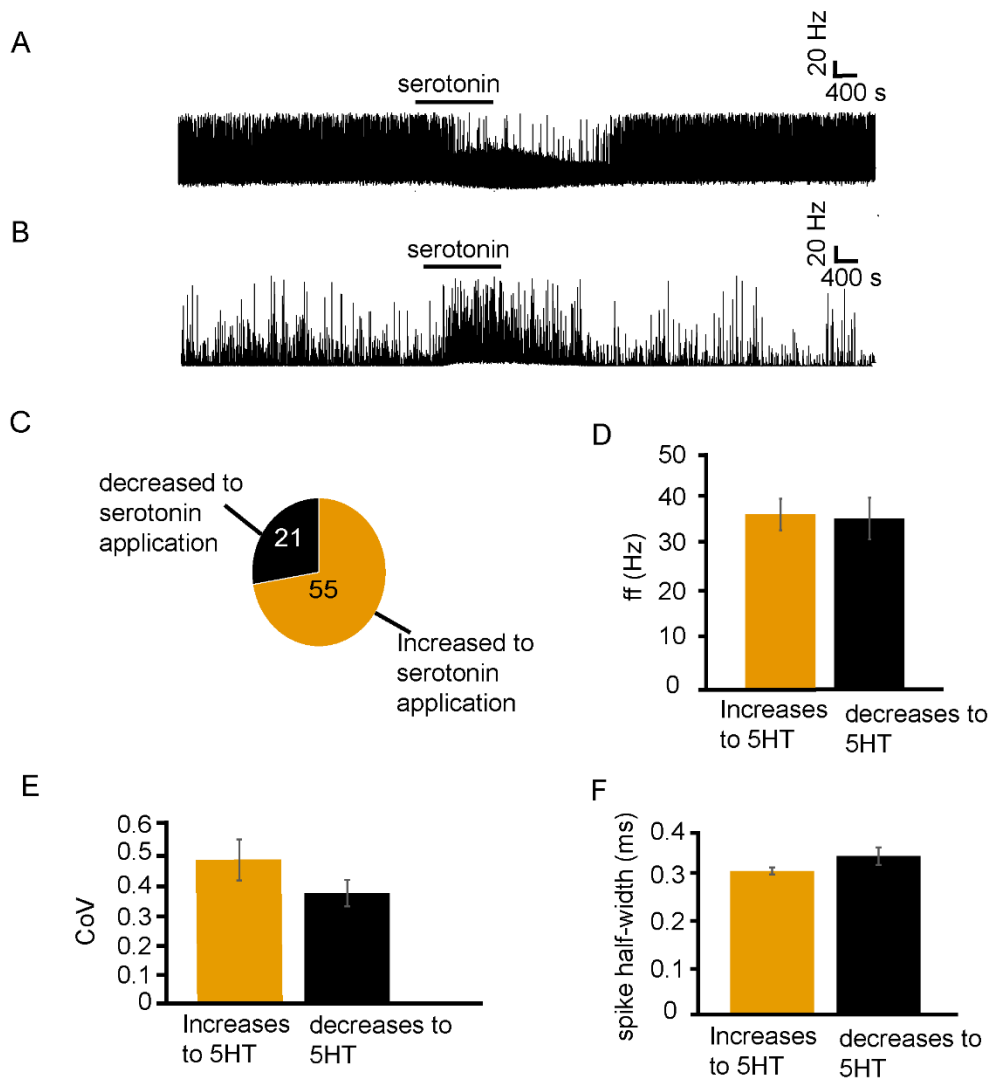


FIGURE 4.1: Serotonin application has dual effects on VP neurons. **(A)** Inhibitory response to serotonin application in a VP neuron, reversed after washout. **(B)** Excitatory response to serotonin application in a VP neuron, reversed after washout. **(C)** The majority of neurons analysed responded with increases in firing frequency in response to serotonin (orange), but a minority decreased their firing frequency in response to serotonin (black) **(D)** Firing frequency for those neurons excited by serotonin (orange) and those neurons that were inhibited by serotonin (black). **(E)** Coefficient of variation for those neurons excited by serotonin application (orange) and those inhibited by serotonin application (black). **(F)** Spike half-width (ms) for those neurons excited by serotonin application (orange) and those inhibited by serotonin application (black).

4.3.2 Repeated application of serotonin produces no clear sensitisation effects in VP neurons

Preliminary experiments showed that application of serotonin caused strong increases or decreases in firing frequency in VP neurons. As the following experiments involved pharmacological protocols with repeated applications of serotonin, in the presence of different specific serotonin receptor antagonists and in the presence of low Ca^{2+} aCSF, it was important to establish whether neuronal responses to subsequent short applications of serotonin were similar or whether significant sensitisation or desensitisations were observed.

Serotonin was applied twice (the second application was carried out after complete washout of the first). In 1 experiment, 10 VP neurons were analysed. In 7/10 of these neurons, serotonin significantly ($P < 0.05$) increased firing (Figure 4.2B), while in the other 3/10 serotonin significantly ($P < 0.05$) decreased firing (Figure 4.3B). In all cases, the second application of serotonin produced similar effects to the first one, as the firing frequencies measured during the first and the second application of serotonin were not significantly different for any of the 10 neurons (Figure 4.2C and 4.3C). We concluded that repeated exposure to serotonin elicited similar effects in VP neurons.

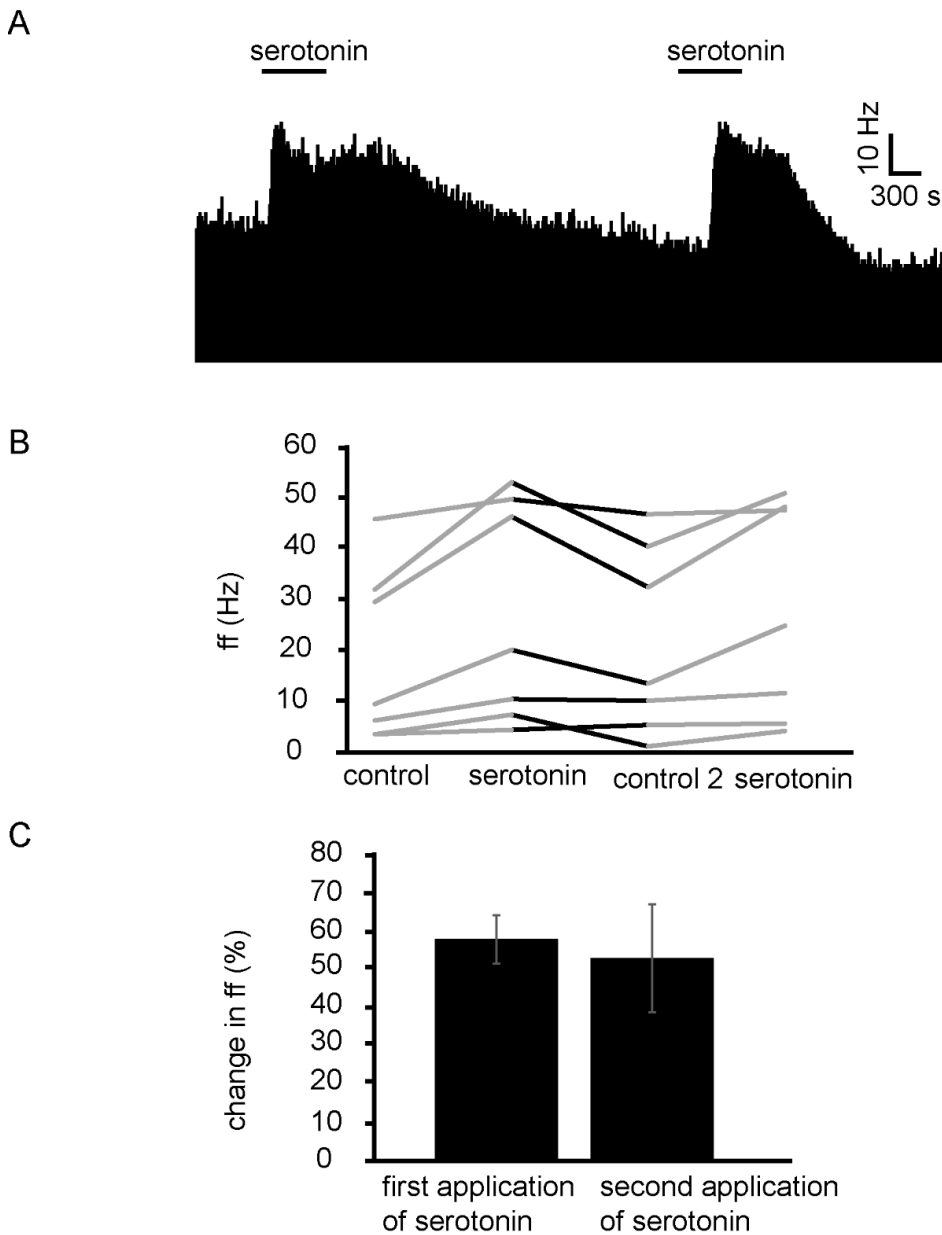


FIGURE 4.2: repeated application of serotonin produces no clear sensitisation affects in VP neurons excited by serotonin application. **(A)** Excitatory responses to two similar serotonin applications (separated by complete washout) in a VP neuron. **(B)** Changes in firing frequency for 7 VP neurons induced by repeated serotonin applications similar to that illustrated in panel A. Firing frequency was measured before the first application of serotonin, during the first application of serotonin, after serotonin wash out and finally during the second application of serotonin. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey. **(C)** Firing frequency during first and second serotonin application for neurons excited by serotonin.

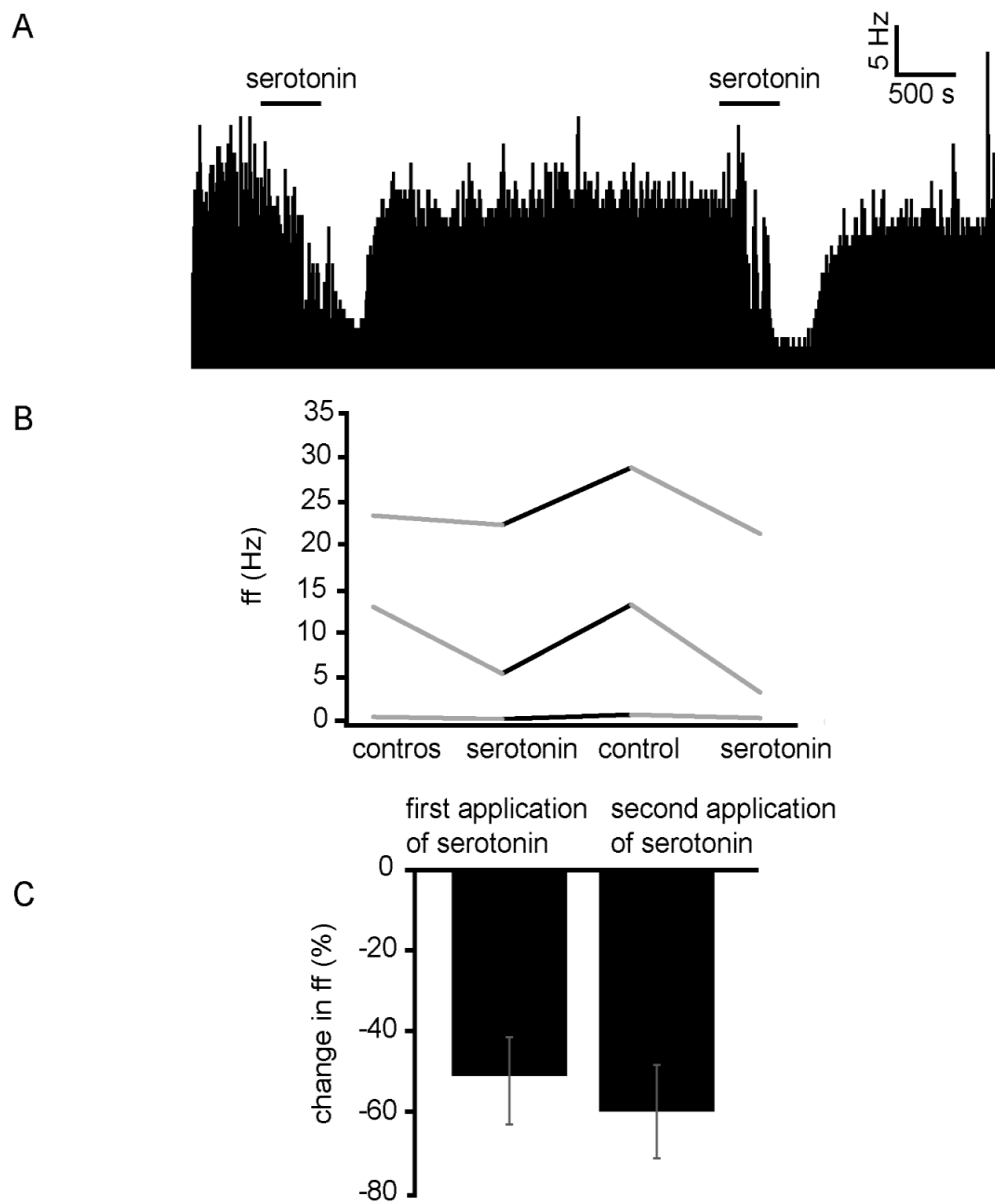


FIGURE 4.3: Repeated application of serotonin has no clear sensitisation effects in VP neurons inhibited by serotonin application. **(A)** Inhibitory responses to two similar serotonin applications (separated by complete washout) in a VP neuron. **(B)** Changes in firing frequency for 3 VP neurons induced by repeated serotonin applications similar to that illustrated in panel A. Firing frequency was measured before the first application of serotonin, during the first application of serotonin, after serotonin wash out and finally during the second application of serotonin. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey. **(C)** Firing frequency during first and second serotonin application for neurons inhibited by serotonin.

4.3.3 Serotonin produces excitatory effects on VP neurons through presynaptic modulation

To further explore the location of serotonin receptors and clarify the mechanism of action in the VP, the effects of serotonin in standard aCSF were compared to those in low Ca^{2+} aCSF. Low Ca^{2+} aCSF was used to block presynaptic transmission and reduce the impact of serotonin's potential presynaptic effects. Experiments were first carried out with serotonin in low Ca^{2+} aCSF followed by application of serotonin alone (Figure 4.4A). The treatment order was counterbalanced with serotonin alone first, followed by application of serotonin in low Ca^{2+} aCSF (Figure 4.4B) to ensure residual differences in Ca^{2+} levels were not affecting the response of VP neurons to serotonin alone.

From 3 experiments 9 neurons were analysed in the VP. Application of serotonin resulted in a significant ($P < 0.05$) increase in firing frequency in 7/9 neurons studied (figure 4.4A, B and C). Of these 7/7 showed no significant changes in firing frequency to serotonin application in the presence of low Ca^{2+} aCSF. In all cases the application of serotonin alone resulted in distinctly different responses to the application of serotonin in low Ca^{2+} aCSF, as the firing frequencies measured during serotonin alone and serotonin in low Ca^{2+} aCSF were significantly ($P < 0.05$) different for 7/7 neurons. The average percentage change in firing frequency for those neurons that increased firing to serotonin was 243 ± 91 % and 9 ± 3 % (Figure 4.4D) to the application of serotonin in low Ca^{2+} aCSF.

We therefore conclude that the increase in firing frequency is largely mediated by presynaptic processes.

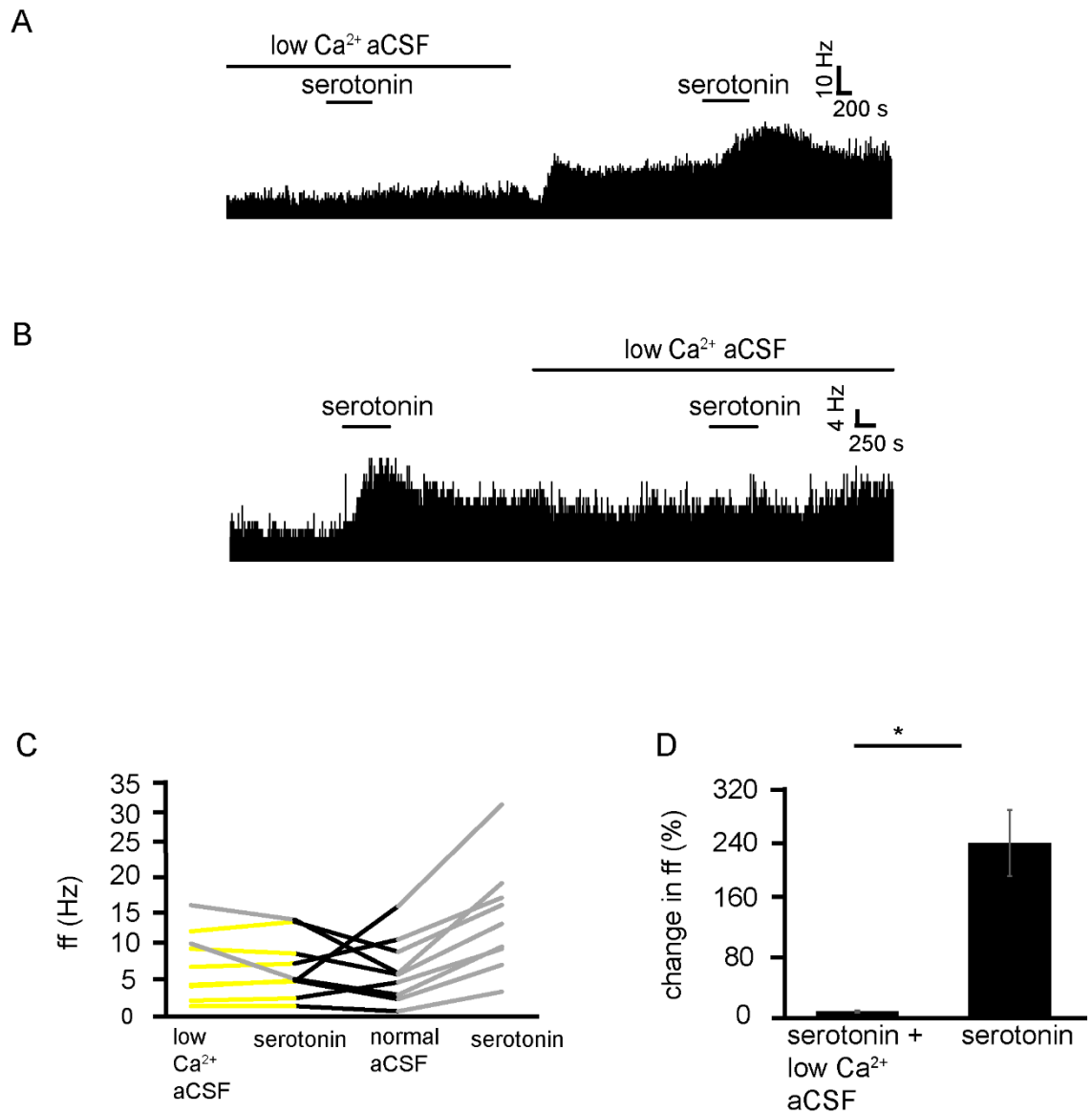


FIGURE 4.4: blocking synaptic transmission with low Ca^{2+} aCSF prevents the excitatory effect of serotonin application in the VP. **(A)** Excitatory responses to serotonin application alone were occluded in response to serotonin application in low Ca^{2+} aCSF **(B)** Another neuron in a different, but similar experiment to panel A, with the reverse order application of low Ca^{2+} aCSF. **(C)** Changes in firing frequency for 9 VP neurons in response to serotonin application in low Ca^{2+} aCSF and standard aCSF, similar to that illustrated in panel A. Firing frequency was measured; before the application of serotonin, during the application of serotonin in low Ca^{2+} aCSF, after the application of standard aCSF and finally during the second application of serotonin in standard aCSF. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(D)** Significant ($* = P < 0.05$) firing frequency changes to the application of serotonin in low Ca^{2+} aCSF compared to serotonin in standard aCSF.

4.3.4 Inhibitory responses to serotonin application persist in the presence of low Ca^{2+} aCSF

Those neurons that decreased firing frequency in response to serotonin application were also investigated in the presence of low Ca^{2+} aCSF to reduce the impact of presynaptic effects, and therefore to investigate their potential pre/postsynaptic modulation.

From 1 experiment 6 neurons were identified for analysis as they decreased in firing frequency in response to serotonin application. 6/6 neurons displayed a significant ($P < 0.05$) decrease in firing frequency to the application of serotonin. 6/6 of these neurons also showed a significant decrease in firing frequency to serotonin application in low Ca^{2+} aCSF (Figure 4.5B and C). In all cases the second application of serotonin (in low Ca^{2+} aCSF) produced similar effects to the first one (alone) as the firing frequencies measured during the first and second application of serotonin were not significantly different (Figure 4.5B and C).

We therefore conclude that the decrease in firing frequency in response to serotonin application is modulated postsynaptically and is therefore a direct effect on VP neurons.

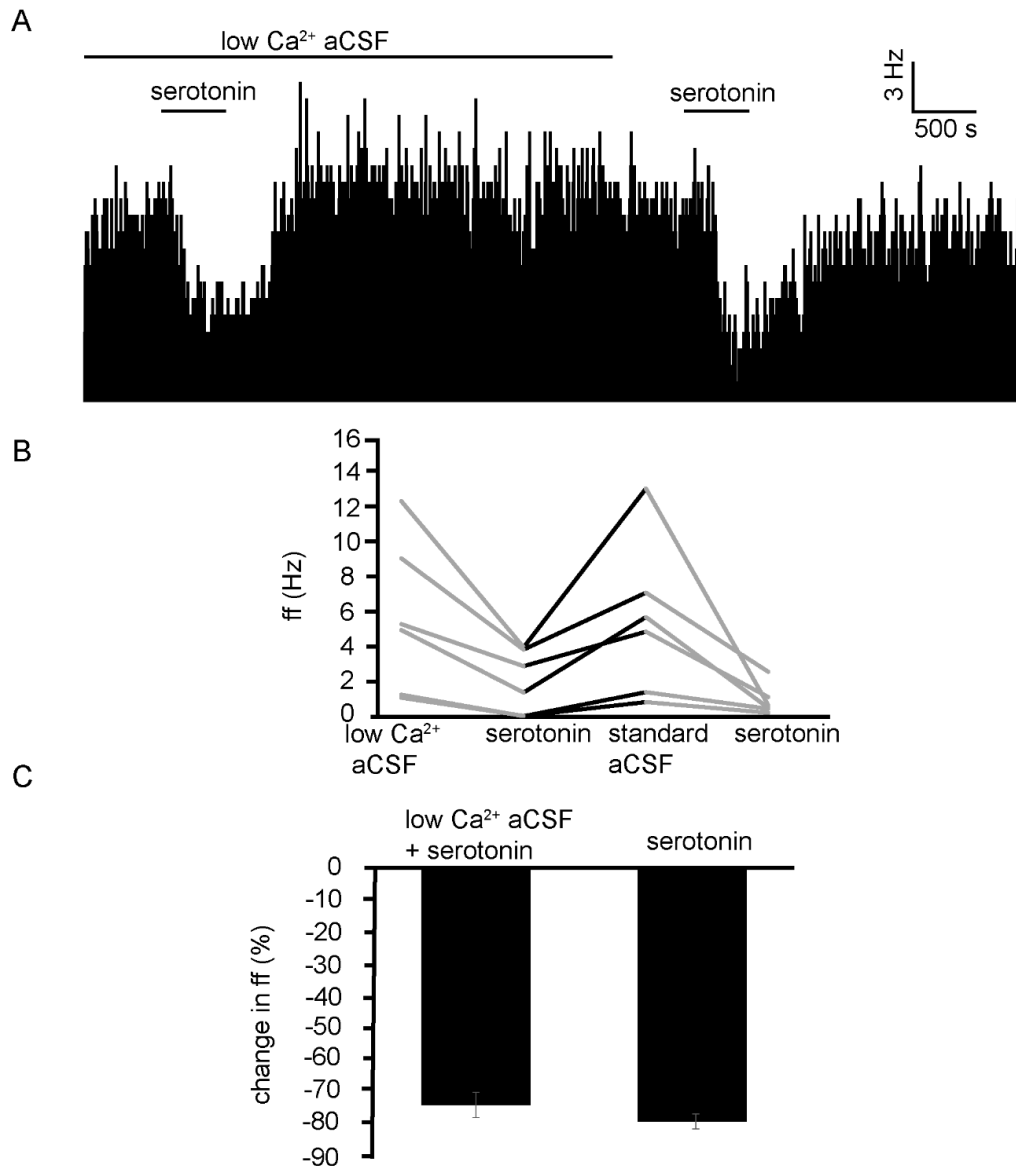


FIGURE 4.5: Inhibitory responses to serotonin application maintain in the presence of low Ca²⁺ aCSF. **(A)** Inhibitory responses to the application of serotonin occurred in the presence of low Ca²⁺ aCSF and standard aCSF. **(B)** Changes in firing frequency for 6 VP neurons in response to serotonin application in low Ca²⁺ aCSF and standard aCSF, similar to that illustrated in panel A. Firing frequency was measured; before the application of serotonin, during the application of serotonin in low calcium aCSF, after the application of standard aCSF and finally during the second application of serotonin in standard aCSF. Responses characterised by significant ($P < 0.05$) changes in firing frequency are in grey, the other ones in yellow. **(C)** Firing frequency changes to the application of serotonin in low Ca²⁺ aCSF and to serotonin in standard aCSF.

4.3.5 5HT1a receptors are required for the excitatory responses of VP neurons to serotonin

5HT1a receptors are known to be expressed in the VP (Wright et al., 1995) and have been shown to mediate the firing potential of neurons in the VP (Heidenreich & Napier, 2000). In other areas of the rat brain 5HT1a receptors are known to be located presynaptically acting as autoreceptors. One such area is the DRN (dorsal raphe nuclei) (Haj-Dahmane et al., 1991). 5HT1a receptors may therefore be a good candidate for the presynaptic increase in firing frequency that we identified in figure 4.4. To investigate their modulatory role in the VP, we applied serotonin in the presence of the 5HT1a antagonist WAY100635.

From 3 experiments 12 neurons were analysed in the VP. 8/12 VP neurons responded with a significant ($P < 0.05$) increase in firing frequency to the application of serotonin (Figure 4.6A and B) of these, 5/8 showed no significant change in firing frequency (Figure 4.6B) in response to serotonin application in the presence of WAY100635, and 3/8 showed a significant ($P < 0.05$) decrease in firing frequency to serotonin application in the presence of WAY100635. In all cases the firing frequencies measured during the first and the second application of serotonin were significantly ($P < 0.05$) different (Figure 4.6B). The first application of serotonin produced an average increase in excitation of $94 \pm 42\%$ compared to an average decrease in firing frequency of $-22 \pm 5\%$ for the second application of serotonin, in the presence of WAY100635.

From the same experiments, 4/12 neurons in the VP displayed a significant decrease in firing frequency to the application of serotonin (Figure 4.6C and D), of these 3/4 also displayed a significant ($P < 0.05$) decrease in firing frequency (Figure 4.6D) in response to serotonin application in the presence of WAY100635. In the majority of cases (3/4) the firing frequencies measured during the first and the second application of serotonin

were significantly ($P < 0.05$) different, with a larger decrease in firing frequency occurring to the application of serotonin in the presence of WAY100635 (Figure 4.6D). The first application of serotonin produced an average decrease in firing frequency of $-26 \pm 8 \%$ compared to $-39 \pm 9 \%$ for the second application of serotonin, in the presence of WAY100635.

We can therefore conclude that the increase in firing frequency seen in response to serotonin application is mediated by 5HT1a receptors, likely located presynaptically, and that the decrease in firing frequency seen in response to serotonin application does not depend on 5HT1a receptors. We also suggest that as 5HT1a receptors are known to have inhibitory influences, it is likely that they have their excitatory effects on VP neurons through presynaptic reduction of inhibitory inputs, perhaps through inhibition of the GABAergic inputs from the NAc. Further experiments will be needed to test this hypothesis. We can also suggest that there may be some interaction effects of 5HT1a receptors blockade on the inhibitory responses in the VP, as those VP neurons that were inhibited by serotonin application alone decreased to a greater extent (Figure 4.6D) in the presence of 5HT1a antagonists WAY100635, suggesting that antagonising 5HT1a receptors unmask the full direct effect of serotonin on VP neurons.

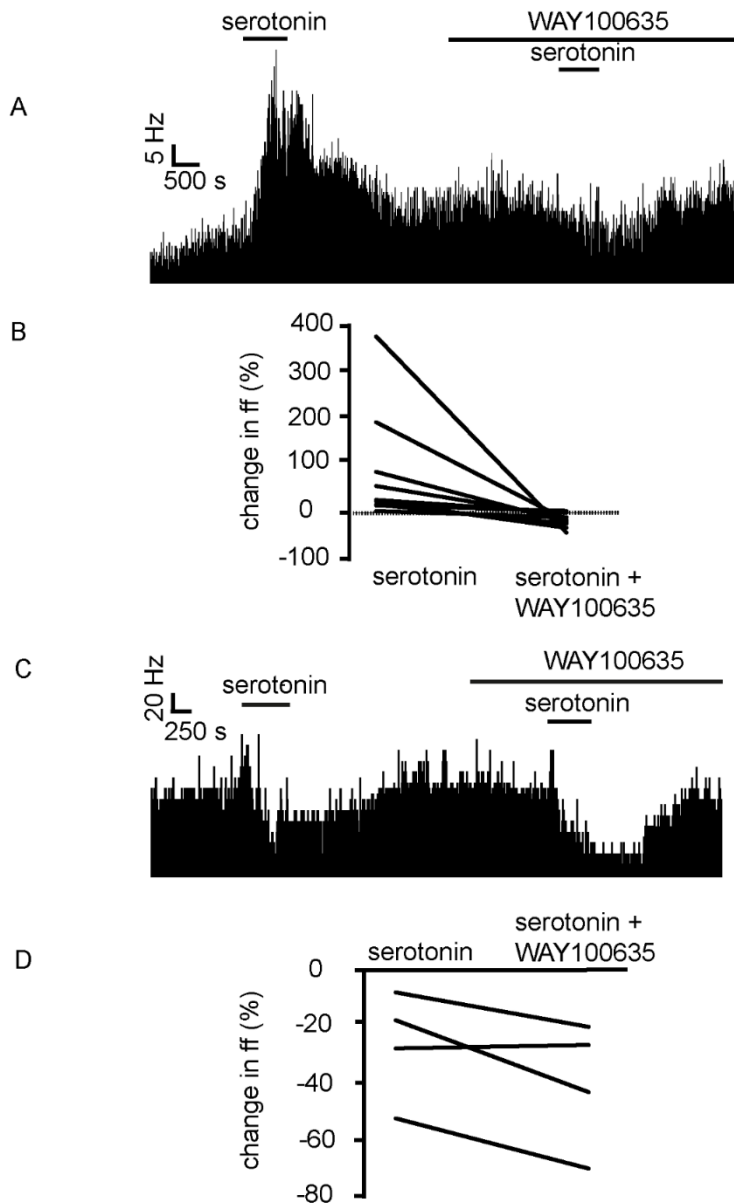


FIGURE 4.6: 5HT_{1a} antagonists block excitatory responses to serotonin application but do not block inhibitory responses to serotonin application in the VP. **(A)** Excitatory responses to serotonin application alone that are no longer present in response to serotonin in the presence of WAY100635. **(B)** Percentage change in firing frequency for 8 neurons in the VP in response to serotonin application and serotonin application in the presence of WAY100635 **(C)** Inhibitory responses to serotonin application alone and in response to serotonin in the presence of WAY100635. **(D)** Percentage change in firing frequency for 4 neurons in the VP in response to serotonin application and serotonin application in the presence of WAY100635.

4.3.6 5HT2c receptors have an inhibitory influence on VP neurons

5HT2 receptors are G_{q11} protein coupled and are considered to have a largely excitatory effect (Berger et al., 2009). A subpopulation of these receptors, 5HT2c receptors, are known to be expressed in the VP and are considered to be expressed in relatively high concentrations (Appel et al., 1990). We therefore applied serotonin in the presence of the 5HT2c antagonists RS102221 in order to investigate the possible involvement of this receptor in the dual effects of serotonin in the VP.

From 2 experiments 13 VP neurons were analysed. 7/13 responded with a significant (P<0.05) increase in firing frequency, while 6/13 responded with a significant (P<0.05) decrease in firing frequency to serotonin application.

Of the 7 that were excited by serotonin application 7/7 also showed a significant (P<0.05) increases in firing frequency to the application of serotonin in the presence of RS102221 (Figure 4.7A). In the majority of cases the firing frequencies measured during the first and the second application of serotonin were significantly (P<0.05) different, with a larger increase in firing frequency occurring to the application of serotonin in the presence of RS102221 in 6/7 (Figure 4.7B). The first application of serotonin produced an average percentage increase in firing frequency of 29 +/- 7 % compared to 46 ± 8 % for the second application of serotonin, in the presence of RS102221 (Figure 4.7B).

Of the 6 that were inhibited by serotonin application, 3/6 showed further significant changes in firing frequency to the application of serotonin in the presence of RS102221, however 2 of these were significant (P<0.05) increases in firing frequency and only one was a further significant (P<0.05) decrease in firing frequency. The remaining 3/6 showed no significant response to serotonin application in the presence of RS102221. In the majority of cases (5/6) the firing frequencies measured during the first (without

RS102221) and the second application of serotonin (in the presence of RS102221) were significantly ($P < 0.05$) different, with either a smaller decrease or an increase in firing frequency occurring serotonin was applied in the presence of RS102221 (Figure 4.7D). The first application of serotonin produced an average decrease in firing frequency of $-20 \pm 5 \%$ compared to an average increase of $17 \pm 12 \%$ for the second application of serotonin, in the presence of RS102221 (Figure 4.7D).

We can therefore conclude that activation of 5HT_{2c} receptors in the VP has an inhibitory influence on VP neurons firing frequency seen to the application of serotonin. Further experiments will be needed to ascertain whether this influence is due to the activation of pre- or postsynaptic mechanisms.

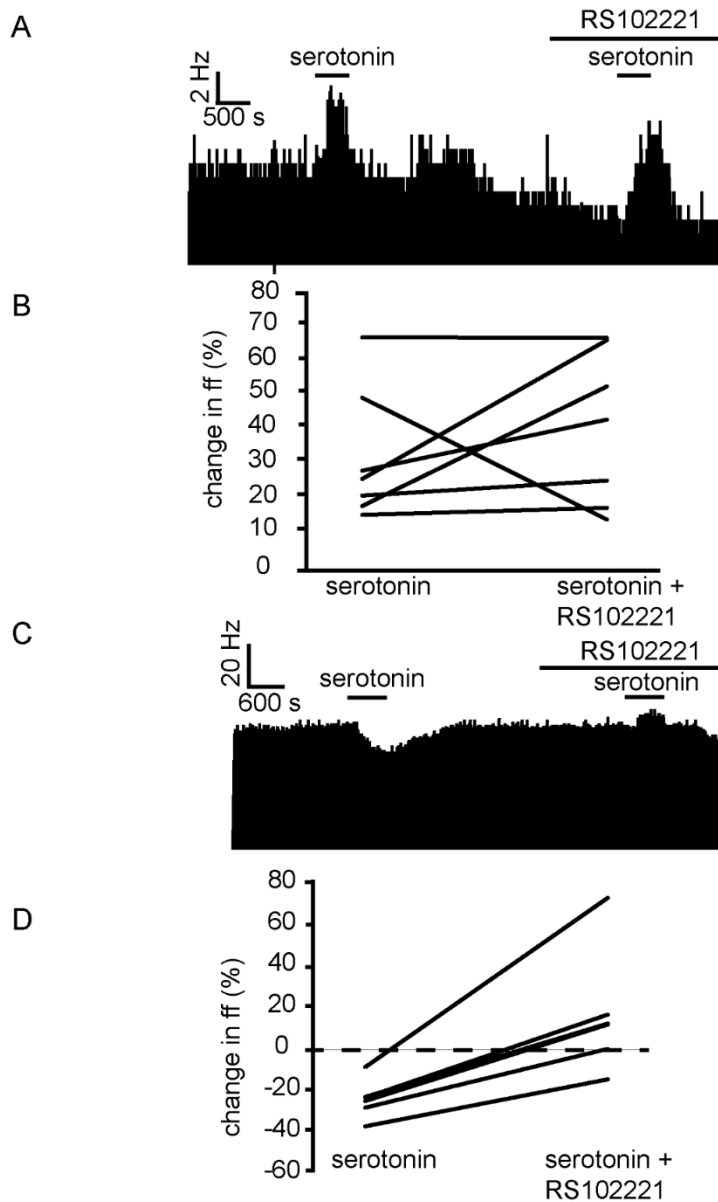


FIGURE 4.7: 5HT_{2c} antagonists modulate inhibitory responses to serotonin application, but do not block excitatory responses to serotonin application in the VP. **(A)** Excitatory responses to serotonin application alone and in the presence of RS102221. **(B)** Percentage change in firing frequency for 7 neurons in the VP in response to serotonin application and serotonin application in the presence of RS102221. **(C)** Inhibitory responses to serotonin application that are no longer present in response to serotonin application in the presence of RS102221. **(D)** Percentage change in firing frequency for 6 neurons in the VP in response to serotonin application and serotonin application in the presence of RS102221.

4.3.7 5HT5a receptors contribute to the excitatory effects of serotonin

Of the 7 subtypes of serotonin receptor only 5HT1 and 5HT5 are considered to be inhibitory (Berger et al., 2009), both by the reduction of intracellular cAMP levels (Thomas, 2006). Like 5HT1a, 5HT5a receptors are known to be expressed in the VP in moderate levels (Oliver et al., 2000). To investigate the role of these receptors, serotonin was therefore applied twice, first alone and then in the presence of the 5HT5a antagonists SB699551.

From 1 experiment 8 neurons were analysed. 6/8 neurons significantly ($P < 0.05$) increased their firing frequency in response to serotonin application, while 2/11 significantly ($P < 0.05$) decreased their firing frequency in response to serotonin application. Of those that were excited by serotonin application, 6/6 also showed significant changes in their firing frequency to the application of serotonin in the presence of SB699551, 5/6 maintaining a significant ($P < 0.05$) excitation, while 1/6 displayed a significant ($P < 0.05$) inhibition. In the majority of cases (5/6) the firing frequencies measured during the first and the second application of serotonin were significantly ($P < 0.05$) different with a smaller increase or a decrease in firing frequency to the application of serotonin in the presence of SB699551 (Figure 4.8B). The first application of serotonin produced an average increase in excitation of $57 \pm 12\%$ compared to $28 \pm 12\%$ for the second application of serotonin, in the presence of SB699551 (Figure 4.8B).

Of the 2 that were inhibited by serotonin application, both also displayed a significant decrease in firing frequency to the application of serotonin in the presence of SB699551 (Figure 4.8C). In both cases the second application of serotonin (in SB699551) produced similar effects to the first one (alone) as the firing frequencies measured during the first and second application of serotonin were not significantly different (Figure 4.8D). The first application of serotonin produced an average decrease in

excitation of $-73 \pm 10 \%$ compared to $-75 \pm 0.5 \%$ for the second application of serotonin, in the presence of SB699551.

As serotonin produced a smaller increase in firing frequency when applied in the presence of SB699551, we conclude that $5HT_{5a}$ receptors contribute to the excitatory response of VP neurons to serotonin application, but that other serotonin receptor must be responsible for the residual excitatory effects.

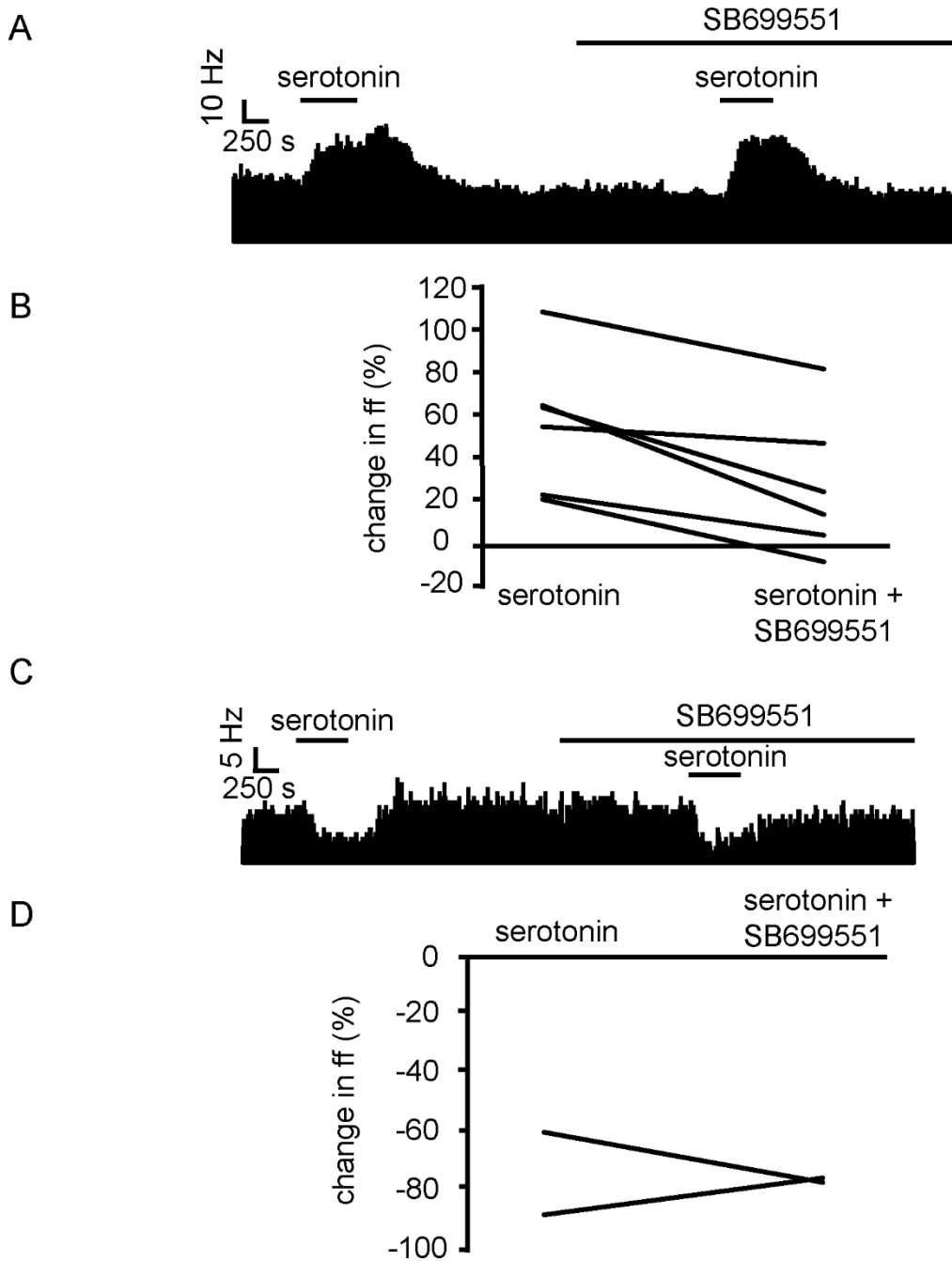


FIGURE 4.8: 5HT_{5a} receptors contribute to the excitatory responses of serotonin in the VP. **(A)** Excitatory responses to serotonin application alone and in the presence of SB699551. **(B)** Percentage change in firing frequency for 6 neurons in the VP in response to serotonin application alone and serotonin application in the presence of SB699551. **(C)** Inhibitory responses to serotonin application alone and in the presence of SB699551. **(D)** Percentage change in firing frequency for 2 neurons in the VP in response to serotonin application alone and serotonin application in the presence of SB699551.

4.3.8 5HT7 receptors contribute to VP excitatory response to serotonin

5HT7 receptors are the most recently identified members of the serotonin receptor extended family (Bard et al., 1993) and is a G_s protein coupled receptor that's mode of action is to increase intracellular levels of cAMP. 5HT7 receptors are known to be expressed in the VP (To et al., 1995). We therefore applied serotonin in the presence of 5HT7 antagonists SB269970 in order to investigate the possible involvement in mediating the dichotomous responses to serotonin seen in the VP.

From 2 experiment 9 neurons were analysed. 7/9 responded with significant ($P < 0.05$) increases in firing frequency to the application of serotonin and 2/9 responded with significant ($P < 0.05$) decreases in firing frequency to the application of serotonin (Figure 4.9A and C).

Of those neurons that were excited by serotonin application, only 2/7 showed a statistically significant ($P < 0.05$) increase in firing frequency to the application of serotonin in the presence of SB269970. In all cases the firing frequencies measured during the first and the second application of serotonin were significantly ($P < 0.05$) different with a smaller increase or a decrease in firing frequency to the application of serotonin in the presence of SB269970 (Figure 4.9B). The average percentage change in firing frequency was 51 ± 9 % to the application of serotonin alone, while it was 9 ± 4 % in response to the application of serotonin in the presence of SB269970 (Figure 4.9B).

Of those neurons that were inhibited by serotonin application, 1 showed a significant ($P < 0.05$) decrease in firing frequency to the application of serotonin in SB269970, while the other showed no significant response to serotonin in the presence of SB269970. In both cases the first application of serotonin resulted in a significantly ($P < 0.05$) different responses to the second (in the presence of SB269970), as one neuron

displayed a larger decrease in firing frequency and the other a smaller decrease in firing frequency to serotonin in the presence of SB269970 (Figure 4.9D).

We conclude that the 5HT7, along with 5HT1a and 5HT5a receptors, contribute to the increase in firing frequency observed in response to serotonin application in the VP.

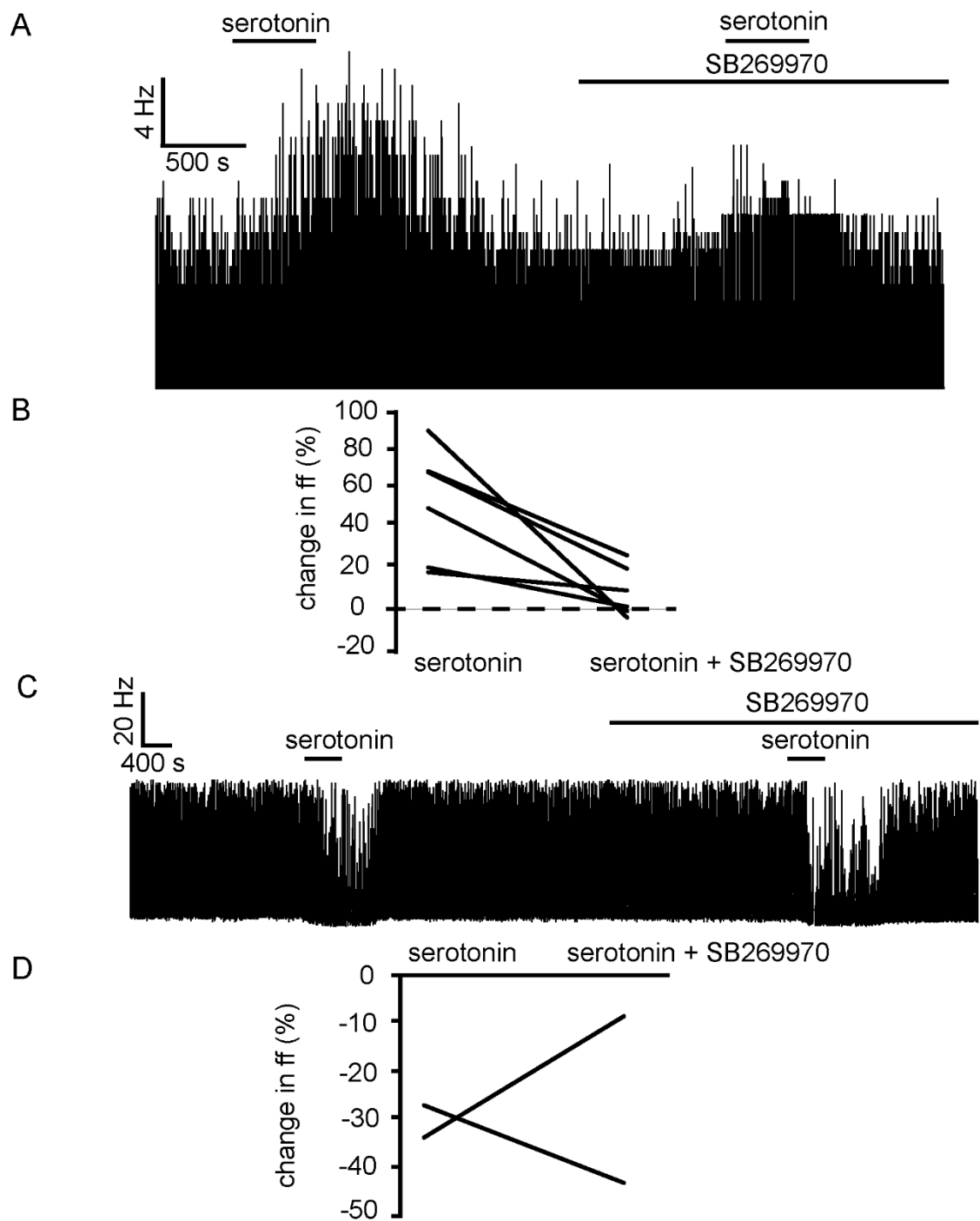


FIGURE 4.9: 5HT7 antagonist modulates the increase in firing frequency seen to serotonin application in the VP. **(A)** Excitatory responses to serotonin application alone that is no longer present in response to serotonin in the presence of SB699770. **(B)** Percentage change in firing frequency for 6 neurons in the VP in response to serotonin application alone and serotonin application in the presence of SB699770. **(C)** Inhibitory responses to serotonin application alone and in the presence of SB699770. **(D)** Percentage change in firing frequency for 2 neurons in the VP in response to serotonin application alone and serotonin application in the presence of SB699770.

4.4 Discussion:

Our study investigated the role serotonin and its receptor subtypes play in the modulation of VP neuronal circuitry. Our data indicates that the dichotomous response seen by Bengtson et al., (2004) to serotonin application persists when measured extracellularly and as population responses. We were however unable to find any consistent electrophysiological differences (spike half-width, firing frequency and coefficient of variation) between those neurons that increased firing frequency to serotonin application and those that decreased firing frequency. This means that the dual effects of serotonin observed in the current study were probably not exerted on the same neuronal subpopulation that were differentially affected by dopamine in the VP (as described in the previous chapter). We can also not support directly Bengtson et al., (2004) assertions that the responses correspond to cholinergic and non-cholinergic VP neuron populations.

4.4.1 Pre and postsynaptic effects of serotonin application

Our data suggest that the serotonin receptors responsible for the increases in firing frequency to serotonin application have an indirect effect through pre-synaptic modulation. We concluded this as neurons identified as increasing firing frequency to serotonin application in the VP, did not show any increase in firing when synaptic transmission was inhibited by a low Ca^{2+} aCSF solution. This supports Nishijo et al's., (2016) research, which suggests that serotonin receptors, acting as pre-synaptic heteroreceptors, inhibit GABA release onto the cholinergic neurons of the VP, thus disinhibiting these VP neurons. On the other hand those neurons that reduced their firing frequency in response to serotonin application continued to show significant reductions in firing frequency to serotonin application in the presence of low Ca^{2+}

aCSF. Our data therefore suggests that the serotonin receptors mediating the inhibitory effects of serotonin, are located postsynaptically on VP neurons.

4.4.2 Contribution of different serotonin receptors to serotonin responses in VP neurons

Our data reveals the contribution of several serotonin receptor subtypes to the dual response seen to serotonin application in the VP. We suggest that there are at least four distinct receptor subtypes responsible for the effects of serotonin. Our data suggests three of these mediate the increase in firing frequency (5HT1a, 5HT7 and 5HT5a) and one mediates the decrease (5HT2c) in firing frequency seen in response to serotonin application.

Our data are consistent with Nishijo and Momiyama's (2016) findings that excitatory responses to serotonin in the VP are a result of presynaptic modulation. However, Nishijo and Momiyama, (2016) suggest the 5HT1b receptors mediate this excitatory response to serotonin, while our data suggests that 5HT1a, 5HT5a and 5HT7 receptors also contribute to this net increase in excitation. This is because 5HT1a, 5HT5a and 5HT7 antagonists were capable of decreasing increases in firing frequency seen in response to serotonin application in the VP. Further research is undoubtedly required, however it is conceivable that 5HT1a receptors may act in a similar manner and at a similar location to 5HT1b receptors in the VP as 5HT1a receptors are largely considered to be inhibitory (Pytliak et al., 2011), therefore to modulate an increase in firing in the VP, 5HT1a receptors may, like 5HT1b receptors, be inhibiting GABAergic neurotransmission, therefore disinhibiting the target cells. Although 5HT1a receptors are most likely localised to GABAergic inputs in the VP, they could also act pre-synaptically at GA terminals, inhibiting GA release (figure 1.1), if the target neurons in the VP expressed one of the inhibitory mGlu receptors (Ohishi et al., 1993; Testa et al., 1994). Indeed this is the mechanism by which we have shown D2 agonists modulate

excitatory responses in the VP. This would be a potentially important factor for future research to clarify and could be a mechanism by which serotonin and dopamine interact at the cellular level in the VP.

As for 5HT7, our data suggests that 5HT7 receptors also play a role in the excitatory response to serotonin application in the VP. 5HT7 antagonists were able to partially block the increase in firing frequency seen in response to serotonin application in some neurons of the VP. Research has shown (Chen et al., 2008; Hashimoto & Kita, 2008) that 5HT7 receptors have a postsynaptic depolarising effect in the GPe, we therefore suggest that this may well be the case for some neurons in the ventral extents of the pallidum, although our data seems to largely suggest excitatory responses in the VP are modulated through pre-synaptic mechanisms, as excitatory responses did not occur in response to serotonin application in low Ca^{2+} aCSF. Further research with 5HT7 agonists within the VP would be useful to elucidate this.

Finally, our data suggests that 5HT5a receptors also contribute to the excitatory response to serotonin application in the VP. 5HT5a receptors are known to be located presynaptically and modulate inhibition, by inhibiting Ca^{2+} channels (Millan et al., 2008). Recent research also suggests that these receptors are found on both dopaminergic neurons and GABAergic interneurons in the VTA (Yamazaki et al., 2018). This provides an intriguing mechanism by which 5HT5a receptors may be interacting with dopamine transmission in the VP and could prove a fruitful target for future research, potentially elucidating, at the cellular level, interactions between serotonin and dopamine in the VP.

Our studies suggest that activation of 5HT1a, 5HT7 and 5HT5a receptors all contribute to the excitation of VP neurons in response to serotonin. Residual excitation in response to serotonin application in the presence of 5HT7 and 5HT5a antagonists supports the

contribution of multiple serotonin receptor subtypes; however the lack of residual excitation in response to serotonin in the presence of 5HT1a antagonist does not. A possible explanation for this lack of residual excitation, in the presence of 5HT1a antagonists, is a complex interaction between 5HT1a, 5HT5a and 5HT7 receptors, which means 5HT1a activation is required for the excitatory effects of 5HT7 and 5HT5a to be revealed. Research suggests that G-protein coupled receptors can form dimeric and oligomeric complexes (Devi, 2001). These dimeric and oligomeric complexes can result in changed receptor pharmacology, and result in different G-protein coupled responses to activation of one or all receptors in the complex (Franco et al., 2009; Renner et al., 2012). Recent research suggests that this could be the case for certain 5HT receptors. Renner et al., (2012) has shown that 5HT1a and 5HT7 receptors form functional heterodimers, and that this results in modulation of the 5HT1a receptors ability to activate G-protein gated potassium channels in hippocampal neurons. Functional crosstalk between these receptors in VP neurons is therefore possible, and provides a tentative explanation for the lack of residual excitation in response to serotonin in the presence of 5HT1a antagonists. It could be that 5HT5a receptors are also part of an oligomeric complex with 5HT1a and 5HT7 receptors. There is research which suggests an interaction between 5HT5a and 5HT1a receptors in cortical regions (Goodfellow et al., 2012). In this study depletion of 5HT5a receptors, in knockout mice, increased the inhibitory serotonin currents mediated by 5HT1a receptors in cortical regions. This may therefore provide tentative evidence for a heterodimeric relationship between 5HT1a and 5HT5a, providing a potential reason for the lack of residual excitation in response to serotonin in the presence of 5HT1a antagonists in VP neurons. Clearly, however further research is needed on this topic to elucidate these mechanisms in the VP.

4.4.3 Contribution of specific serotonin receptors to inhibitory serotonin responses in VP neurons

As for the neurons that decrease in firing frequency in response to serotonin application. Our data suggests that the most likely candidate for mediating this post-synaptic decrease in firing frequency is the 5HT_{2c} receptor. This is as inhibitory responses in VP neurons were blocked by 5HT_{2c} antagonists. The 5HT₂ group are largely considered excitatory (Berger et al., 2009), therefore it would seem unusual for these to be capable of inhibiting the VP neurons directly. However 5HT_{2c} receptors are known in the NAc to decrease the release of DA (De Deurwaerdère & Spampinato, 1999) and they have tonic inhibitory control over the mesolimbic dopamine pathway (Di Giovanni et al., 1999). Alex et al., (2005) have also shown that 5HT_{2c} receptors directly inhibit dopamine release in the striatum with increases in dopamine release produced by 5HT_{2c} antagonists. We, therefore, tentatively suggest that serotonin may produce inhibitory responses in a set of dopaminergic neurons within the VP. This provides an intriguing target for future research aimed at the interaction of dopamine and serotonin within the VP.

4.4.4 The 5HT_{1a} receptor antagonist WAY100635 is also a D₄ receptor agonists

A caveat of our data on the role of 5HT_{1a} receptors in the VP is that the antagonists used for these experiments: WAY100635, is also a D₄ agonist. This may be a problem as D₄ agonism may contribute to the lack of excitatory responses seen to serotonin application in the presence of WAY100635. We cannot therefore definitively conclude that 5HT_{1a} receptors modulate the excitatory responses to serotonin application in the VP. Indeed D₄ receptors are inhibitory (Neve et al., 2004) and are expressed in the VP (Noain et al., 2006). Our research has shown (chapter 3) that D₂-like (including D₂, D₃ and D₄) receptor agonists can produce inhibition of some VP neurons. This may therefore provide an explanation for the lack of excitation in the presence of

WAY100635, rather than it being a result of 5HT1a receptor inhibition. D4 receptors are also known to inhibit glutamatergic inputs from the BLA (basolateral amygdala) in the mPFC (medial pre-frontal cortex) (Laviolette et al., 2005). The VP has similar glutamatergic inputs from the BLA (figure 1.1). It could be that the lack of an excitatory response in VP neurons to serotonin, in the presence of WAY100635, is at least partly due to its agonism of D4 receptors, at pre-synaptic glutamate terminals, therefore inhibiting GA release from these terminals into the VP, resulting in a net response of no change in firing frequency.

4.4.5 Functional implications:

As previously mentioned in chapter 3, the VP is crucially involved in reward processing (Smith et al., 2009) and drug-seeking behaviour (Kalivas & Volkow, 2005; Prasad & McNally, 2016). Serotonin is also known to be involved in the modulation of these responses (Kranz et al., 2010), and serotonin levels are seen to increase in the VP when cocaine is self-administered (Sizemore et al., 2000).

The current study provides novel and clear insights into the serotonin receptor subtypes involved in the dichotomous responses to serotonin seen in the VP (Bengtson et al., 2004) and provides evidence for their pre and postsynaptic location of effect.

This information is of primary theoretical value and could also help to develop rational pharmacological approaches to fight addiction and other dysfunctions of the reward system, such as affective disorders.

**5 Chapter 5: Effects of electrical stimulation of NAc
afferents on VP neurons' firing**

5.1 Abstract:

Afferents from the NAc are considered to be a major source of input into the VP. Research reveals that these afferents are GABAergic, however stimulation of these afferents induces both excitatory and inhibitory responses within the VP. These are likely to be partially mediated by enkephalin and substance P, which are also released by these afferents, and are known to modulate VP neurons. However less is known about the potentially differential effects stimulation of these afferents has on subpopulations of neurons within the VP and the cellular mechanisms by which they exert their effects. The current study aimed to research this further using brain slices containing the VP, stimulation of the NAc afferents and multi-electrode array (MEA) recordings of their VP targets. Stimulation of the NAc afferents induced a pause in the tonic firing in 58 % of the neurons studied in the VP, while 42 % were not affected. Measures used to reveal electrophysiological difference between these groups found no significant differences in firing frequency, coefficient of variation and spike half-width. There was however significant differences in the pause duration between neurons in the dorsal and ventral VP, with stimulation of NAc afferents producing a significantly longer pause (0.48 ± 0.06 s) in tonic firing in dorsal VP neurons, compared to neurons in the ventral VP (0.21 ± 0.09 s). Pauses in the tonic firing of VP neurons, as a result of NAc afferent stimulation, were found to be largely mediated by GABA_A receptors, as application of picrotoxin significantly reduced their duration. Opioid agonists and antagonists were found to have no significant effects on the pause in tonic activity induced by NAc afferent stimulation. However, NK-1 receptor antagonists caused significant decreases in the pause duration, suggesting that substance P may contribute to the inhibitory effect of NAc afferent stimulation via activation of NK-1 receptors.

5.2 Introduction:

The VP is a major output structure of the striatum and is innervated by afferents from the NAc. Many consider these afferents to be the main input of the VP (Bolam et al., 1986; Haber et al., 1985). Activation of these afferents has been shown to be involved in pleasurable responses and sensitization to drugs of abuse (Creed et al., 2016; Smith & Berridge, 2005). These afferents affect VP neurons through release of associated neurotransmitters and peptides. The NAc afferents of the VP release GABA (Churchill et al., 1990b; Churchill & Kalivas, 1994; Kitamura et al., 2001; Mogenson et al., 1983; Reiner & Anderson, 1990; Walaas & Fonnum, 1979; Yang & Mogenson, 1985; Zaborszky & Cullinan, 1992). However research has shown that stimulation of these afferents produces both inhibition and excitation of VP neurons (Chrobak & Napier, 1993). Enkephalin and substance P, as well as GABA, are probably involved in these responses as both are known to be released by these afferents (Haber et al., 1985; Lu et al., 1997; Napier et al., 1995a). Enkephalin, has been shown to contribute (along with GABA) to the inhibition of VP neurons seen in response to stimulation of NAc afferents (Napier et al., 1992), and substance P is known to produce excitatory responses in the VP (Napier et al., 1995a), therefore substance P is a likely candidate to be involved in the excitation of VP neurons as a result of NAc afferent stimulation.

Questions remain as to what neurons the afferent connections of the NAc target in the VP. Research suggests they may directly target cholinergic interneurons in the VP (Grove et al., 1986; Zaborszky & Cullinan, 1992; Zaborszky et al., 1986), although recent research by Root et al., (2015) suggests NAc afferents also directly innervate GABAergic neurons of the VP. Further research needs to elucidate how stimulation of these afferents modulates the VP neurons they innervate. The question also remains as to what receptors are involved in the modulatory effects of NAc inputs to the VP. The research suggest a strong involvement of GABA_A receptors (Chrobak & Napier, 1993).

However NK-1 receptors (Chen et al., 2001; Maeno et al., 1993), δ -opioid receptors and μ -opioid receptors (Lahti, Mickelson, Jodelis, & McCall, 1989; Mitrovic & Napier, 1995) are also known to be present in the VP and neurons in the VP are responsive to their activation (Chrobak & Napier, 1993; Napier et al., 1995a). It can be hypothesised that substance P and enkephalin, released by NAc afferents to the VP (figure 1.1) target these receptors and modulate the effects of GABA on these neurons, however this has not been demonstrated.

It is important to understand further how activation of NAc inputs into the VP, and the neuromodulators released, impact on VP neurons. Unravelling the potentially divergent, modulatory effects these connections have on the interneurons and projection neurons of the VP may reveal important information relating to drugs of abuse, affective disorders and aspects of reward learning, as that these areas and their connections are heavily involved in these processes.

5.3 Results:

5.3.1 Stimulation of the NAc inhibits tonic firing in some VP neurons

In order to explore the effect that NAc afferents have on VP neurons, we stimulated the NAc every 10 seconds and measured the changes in tonic firing activity within the dorsal and ventral extents of the VP.

From 13 experiments 52 neurons were identified for analysis as they had a stable tonic firing rate after 1 hour of slice accommodation in the multielectrode array. Stimulation of the NAc induced a pause (referred to as responders) in firing in 30/52 of these neurons (Figure 5.1A and B), with 53 % being located in dorsal extents of the VP and 47% being located in ventral extents of the VP (Figure 5.1E). 22/52 were not affected by NAc stimulation (referred to as non-responders) with 41 % being located in the dorsal extent of the VP and 59 % being located in the ventral extent of the VP (Figure 5.1E). Pause duration was 0.29 ± 0.03 s for those classed as responders (Figure 5.1C).

In order to ascertain if pauses were elicited in different types of neurons within the VP, firing frequency, coefficient and variation and spike half-widths were calculated for responders and non-responders. There was no significant ($P > 0.05$) difference in the average baseline firing frequency rates between responders 18.16 ± 3.16 Hz and non-responders 15.27 ± 2.83 Hz (Figure 5.1F). There was no significant ($P > 0.05$) difference in the coefficient of variation between responders 27.07 ± 9.61 and non-responders 19.27 ± 2.51 (Figure 5.1G). Finally, there was no significant difference in spike half-width (ms) between responders 0.32 ± 0.02 ms and non-responders 0.35 ± 0.03 ms (Figure 5.1D).

We conclude that a significant populations of neurons in the VP are directly inhibited by afferents from the NAc, and that these neurons are fairly evenly dispersed between the dorsal and ventral extents of the VP. There is however a significant population that

were not directly inhibited by NAc stimulation. We could not find any marker that identified these neuron as separate types of neurons and it is possible that the NAc fibres impinging on these neurons were cut or absent in the brain slices used in these experiments.

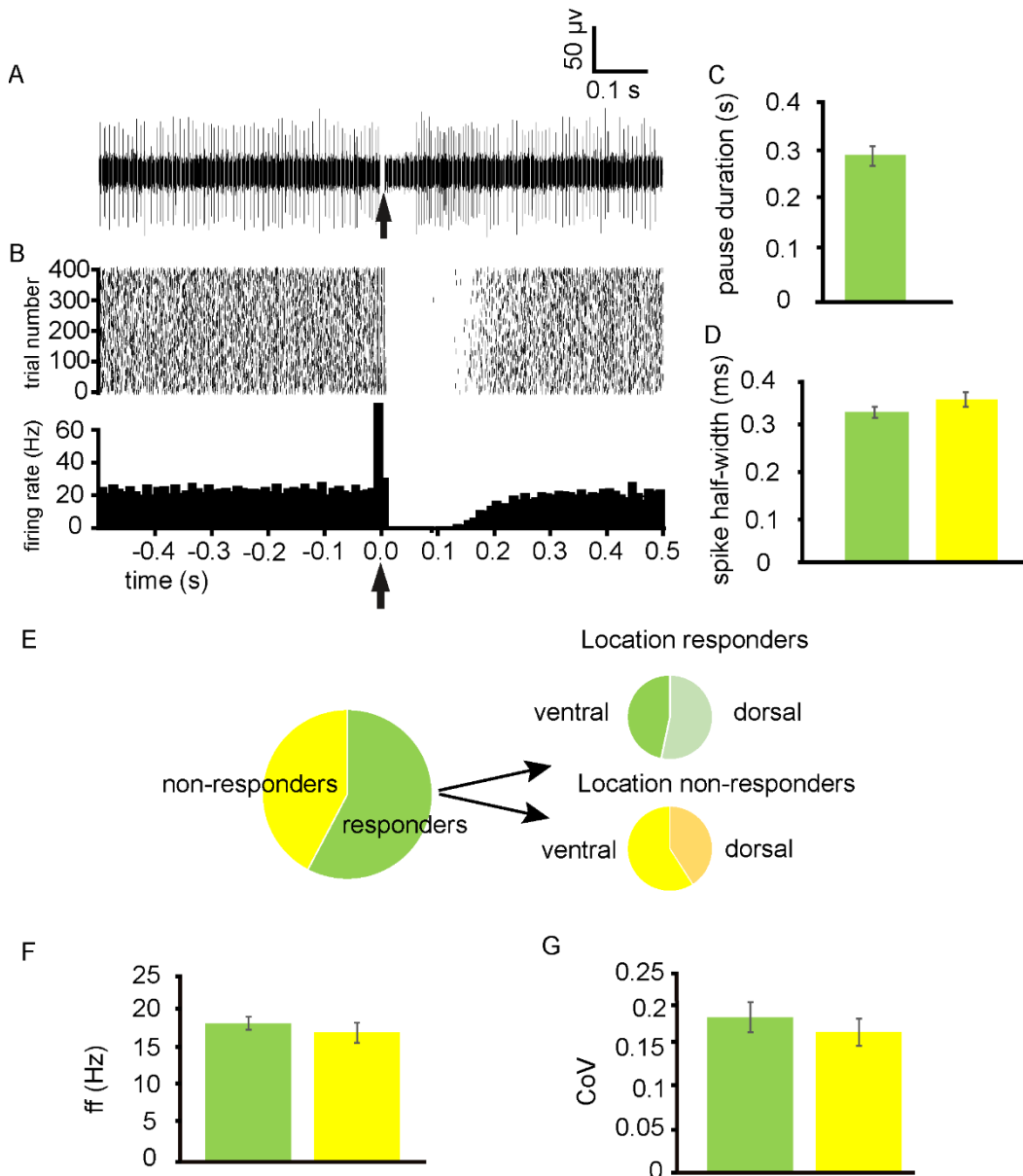


FIGURE 5.1: stimulation of the NAc results in inhibition of tonic firing in some VP neurons. **(A)** A typical example of a raw data trace from a neuron in the VP with a pause (responder) in tonic activity after stimulation of the NAc. **(B)** A typical example raster plot (above) and peri-stimulus time histogram (PTSH, below, in bins of 10 ms) of tonic firing activity in a VP neuron 0.5 s pre stimulation and 0.5 s post stimulation of the NAc. The raster plot exemplifies one neuron response during 400 applications of stimulation. **(C)** Bar chart representing average pause duration for those neurons that were responders to NAc stimulation **(D)** Bar chart representing non-significant difference in spike half-width (ms) for neurons that responded to NAc stimulation (green) and those that were non-responders (yellow) **(E)** Pie charts representing the proportion of neurons identified in the VP for analysis that responded to NAc stimulation, and the proportions that were located ventrally versus dorsally within the VP. **(F)** Bar chart representing non-significant differences in baseline firing frequency rates for neurons that responded to NAc stimulation (green) and those that were non-responders (yellow). **(G)** Bar chart representing non-significant difference in baseline coefficient of variation for those neurons that responded to NAc stimulation (green) and those that were non-responders (yellow).

5.3.2 NAc stimulation induces pauses of different durations dorsally compared to ventrally in the VP

In order to ascertain if there were any regional differences in pause duration between dorsal and ventral extents of the VP, the pause duration of neurons identified as in dorsal portions of the VP were compared to neurons in ventral portions of the VP.

From 10 experiments we identified 17 neurons in the VP for analysis as they responded to NAc stimulation. 7/17 of these were classified as being located dorsally (Figure 5.2A) in the VP and 10/17 of these were classified as located ventrally within the VP (Figure 5.2B). Ventrally located neurons paused for an average duration of 0.21 ± 0.09 s while dorsally located neurons paused for an average duration of 0.48 ± 0.06 s. This was significantly different ($P < 0.05$) (Figure 5.2C).

In order to ascertain if these differences represented different populations of neurons in the dorsal VP compared to the ventral VP we compared them based on the baseline firing frequency, coefficient of variation and spike half-width profiles. There was no significant difference in the average baseline firing frequency rates between those neurons located dorsally within the VP 17.13 ± 4.06 Hz and those located ventrally within the VP 16.13 ± 2.08 Hz (Figure 5.2D). There was no significant difference in the coefficient of variation between those neurons located dorsally 24.85 ± 11.06 and those located ventrally 22.05 ± 8.53 (Figure 5.2E). Finally, there was no significant difference in spike half-width (ms) between those located dorsally 0.29 ± 0.04 s and those located ventrally 0.36 ± 0.05 s (Figure 5.2F).

We tentatively conclude that inputs arriving from the NAc to dorsal regions in the VP have a greater inhibitory effect on the VP's tonic firing than those inputs from the NAc arriving in more ventral regions of the VP, but this does not appear to be related to the type of neuron they innervate within the VP

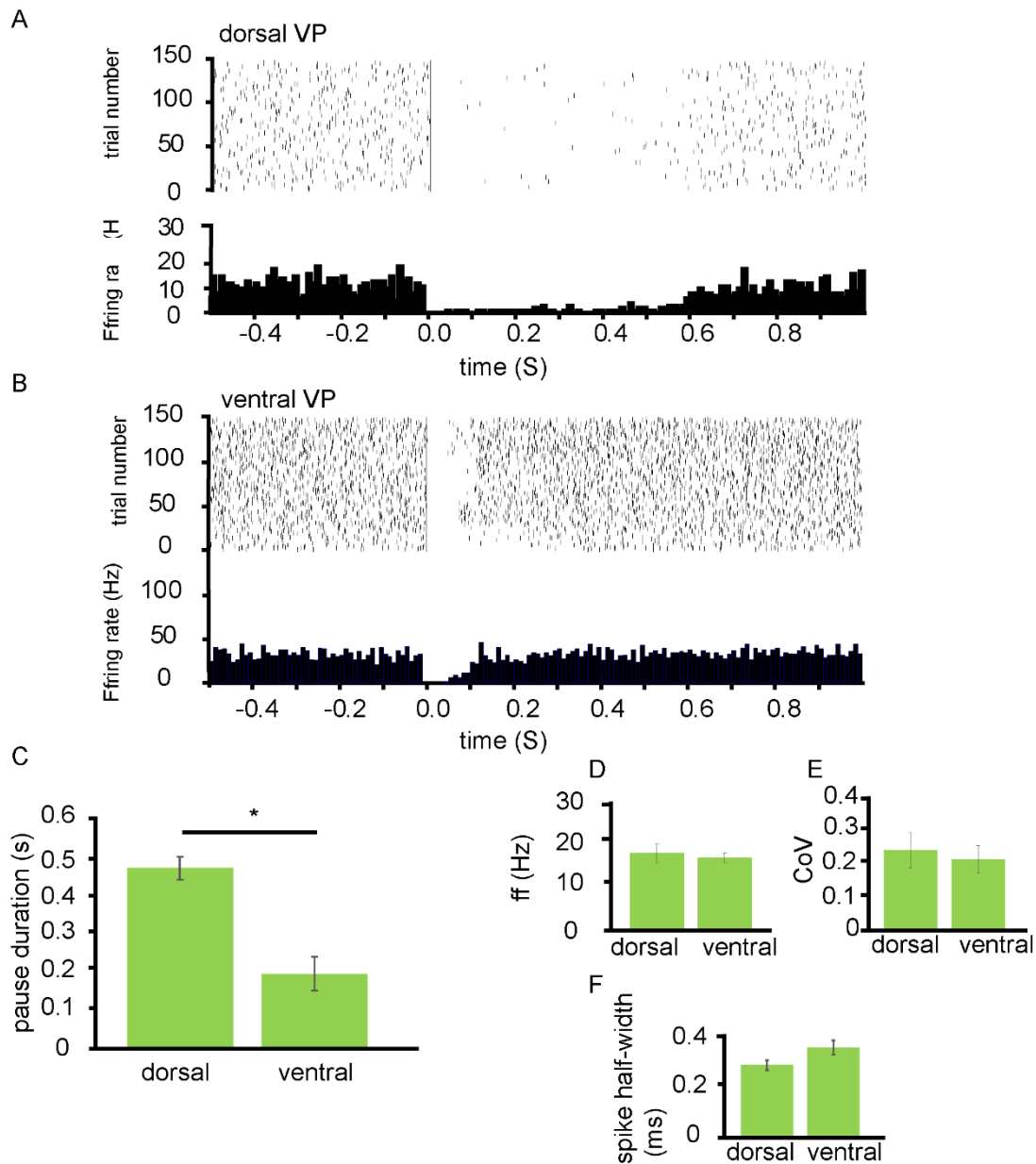


FIGURE 5.2: NAc stimulation induces a longer pause duration in dorsal VP neurons than ventral. (A) A typical example raster plot of tonic firing activity in a VP neuron 0.5 s pre stimulation and 1.0 s post stimulation of the NAc. The raster plot exemplifies one neurons response in the dorsal VP during 150 applications of stimulation. (B) A typical example raster plot of tonic firing activity in a VP neuron 0.5 s pre stimulation and 1.0 s post stimulation of the NAc. The raster plot exemplifies one neurons response in the Ventral VP during 150 applications of stimulation. (C) Bar chart representing significant differences ($= P < 0.05$) in pause duration between those neurons studied in the dorsal VP compared to those in the ventral VP. (D) Bar chart representing no-significant difference ($P > 0.05$) in firing frequency (Hz) for neurons that paused in response to NAc stimulation in the dorsal VP compared to those in the ventral VP. (E) Coefficient of variation for neurons that paused in response to NAc stimulation in the dorsal VP and those in the ventral VP. (F) Spike half-width (ms) for neurons that paused in response to NAc stimulation in the dorsal VP compared to those in the ventral VP.

5.3.3 Picrotoxin abolishes VP neurons inhibition by NAc stimulation.

Research suggests that afferent projections from the NAc innervating the VP are GABAergic. This should mean that application of GABA_a antagonists should largely ameliorate the inhibition in tonic firing seen in response to NAc stimulation. In order to explore the role of GABA in the inhibition of VP neurons, the GABA_a antagonist picrotoxin was applied during stimulation of the NAc.

From 12 experiments 26 neurons in the VP were identified for analysis that paused in response to NAc stimulation (Figure 5.3A and B). The average pause duration for these 26 neurons was 0.34 ± 0.04 s. For these 26 neurons, after the application of picrotoxin, the pause duration was 0.06 ± 0.01 s (Figure 5.3E). As the pause duration measured after the application of picrotoxin was significantly ($P < 0.001$) shorter than before the application of picrotoxin (Figure 5.3E) we can conclude that the inhibition induced in tonically active VP neurons by NAc stimulation is largely a result of GABA release and subsequently activation of GABA_a receptors.

In order to investigate if the application of picrotoxin had any effect on electrophysiological characteristics of the VP neurons we measured baseline firing frequency rates and coefficient of variation for the VP neurons. The firing frequency (Hz) rates were not significantly ($P > 0.05$) different for any (pauses and non-pauses) VP neurons studied in the presence of picrotoxin 21.52 ± 0.49 Hz compared to control conditions 18.59 ± 1.97 Hz (Figure 5.3C). Coefficient of variation was also not significantly ($P > 0.05$) different for any (pauses and non-pauses) VP neurons studied in the presence of picrotoxin 29.75 ± 10.42 compared to control conditions 35.39 ± 4.34 (Figure 5.3D).

We can therefore conclude that application of picrotoxin has no significant ($P > 0.05$) effects on the electrophysiological characteristics of the VP neurons, but abolishes the inhibitory influence stimulation of NAc neurons has on VP neurons.

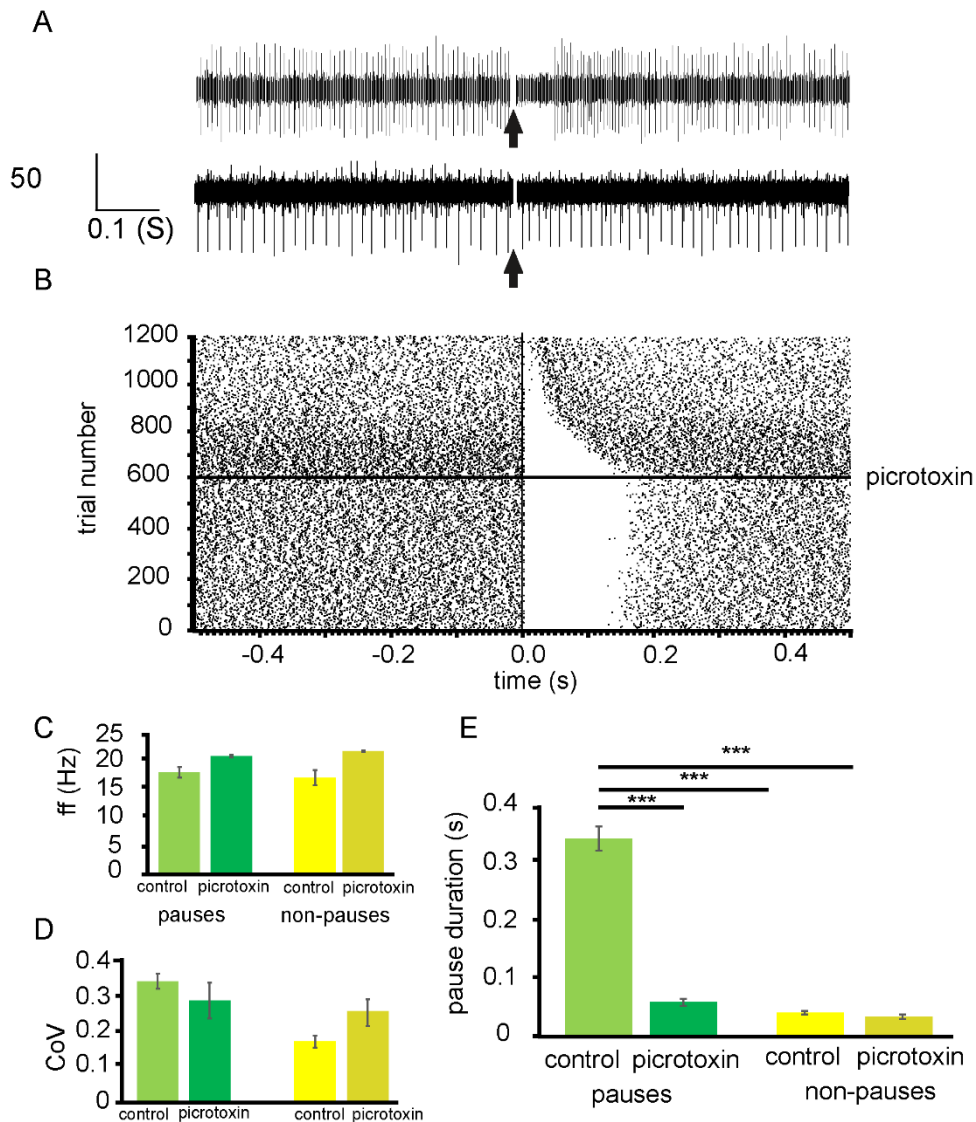


FIGURE 5.3: Picrotoxin substantially disinhibits VP neurons inhibited by NAc stimulation. **(A)** A typical example of a raw data trace from a neuron in the VP with a pause in tonic activity after stimulation of the NAc and a raw trace from the same neuron after the application of picrotoxin with no pause in tonic activity. **(B)** A typical example raster plot of tonic firing activity in a VP neuron from 0.5 s pre stimulation to 0.5 s post stimulation of the NAc. The raster plot exemplifies one neuron's response during 600 applications of stimulation in control conditions and 600 stimulations after the application of picrotoxin. **(C)** Bar chart representing no-significant difference in baseline firing frequency (Hz) for neurons that pause and those that didn't pause in response to NAc stimulation, in control conditions and in the presence of picrotoxin. **(D)** Bar chart representing no-significant differences in baseline coefficient of variation for neurons that pause and those that didn't pause in response to NAc stimulation, in control conditions and in the presence of picrotoxin. **(E)** Bar chart representing significant differences (***) in pause duration for those neurons that paused in response to NAc stimulation (green) compared to the same neurons in the presence of picrotoxin, and compared to those neurons that did not pause in response to stimulation (yellow) in control conditions and in the presence of picrotoxin.

5.3.4 Inhibition of VP neurons by NAc stimulation is not affected by Opioid antagonists

In order to investigate the role of opioids and opioid receptors in the inhibition of VP neurons by NAc stimulation, opioid agonists (DAMGO) and the non-specific opioid receptor antagonist, naloxone, were applied during stimulation of the NAc.

From 5 experiments 11 neurons were identified for analysis that paused in response to NAc stimulation (Figure 5.4A). In control conditions the average pause duration for these 11 neurons was 0.32 ± 0.06 s. For these 11 neurons after the application of naloxone the average pause duration was 0.31 ± 0.07 s. The pause duration after the application of naloxone was not significantly ($P > 0.05$) different to the pause duration before the application of naloxone (Figure 5.4C). However the pause duration (consistent with Figure 5.3) was significantly ($P < 0.05$) reduced, compared to control conditions and naloxone, after the application of picrotoxin 0.04 ± 0.07 s (Figure 5.4C). We therefore conclude that opioid receptors do not modulate the pause in firing seen in VP neurons, induced by stimulation of the NAc afferents.

In order to investigate if the application of naloxone had any effect on electrophysiological characteristics of the VP neurons we measured baseline firing frequency rates and coefficient of variation for the VP neurons (Figure 5.4E and F). The firing frequency (Hz) rates were not significantly ($P > 0.05$) different for the VP neurons studied in the presence of naloxone 19.35 ± 2.91 Hz compared to control conditions 18.00 ± 3.02 Hz and the presence of picrotoxin 19.82 ± 3.75 Hz. Coefficient of variation was also not significantly ($P > 0.05$) different for the VP neurons studied in the presence of naloxone 40.72 ± 16.79 compared to control conditions 27.07 ± 9.52 and picrotoxin 51.32 ± 22.93 .

To verify that opioids did not play a role in modulation of the pause duration induced by NAc stimulation, we also applied DAMGO during stimulation of the NAc.

From 3 experiments 5 neurons were identified for analysis that paused in response to NAc stimulation (Figure 5.4B). In control conditions the average pause duration for these 5 neurons was 0.40 ± 0.11 s. For these 5 neurons after the application of DAMGO the average pause duration was 0.31 ± 0.08 s (Figure 5.4D). The pause duration after the application of DAMGO was not significantly ($P > 0.05$) different to the pause duration before the application of DAMGO. The pause duration was, however significantly ($P < 0.05$) reduced, compared to control conditions and DAMGO, after the application of picrotoxin 0.04 ± 0.07 ms (Figure 5.4C).

To check if DAMGO affected the electrophysiological characteristics of VP neurons we measured baseline firing frequency rates and coefficient of variation for the VP neurons (Figure 5.4G and H). The firing frequency (Hz) rates were not significantly ($P > 0.05$) different for the VP neurons studied in the presence of DAMGO 14.01 ± 4.26 Hz compared to control conditions 15.89 ± 3.79 Hz. Coefficient of variation was also not significantly ($P > 0.05$) different for the VP neurons studied in the presence of DAMGO 60.89 ± 24.69 compared to control conditions 39.95 ± 8.63 .

We therefore conclude that opioid receptors appear to have no modulatory effect on the pause in firing seen in VP neurons, induced by stimulation of the NAc afferents.

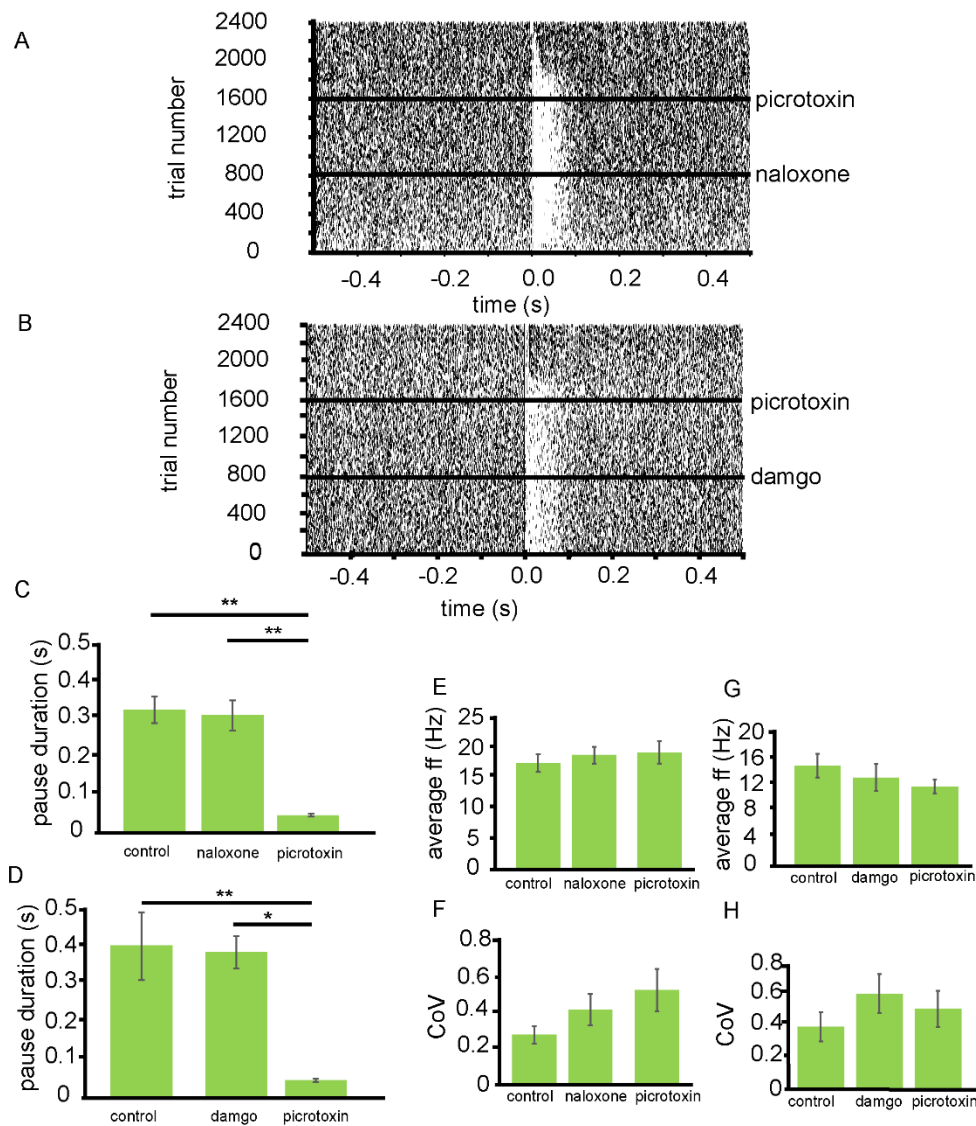


FIGURE 5.4: Inhibition of VP neurons by NAc stimulation is not effected by opioid agonists. **(A)** A typical example of raster plot of tonic firing activity in a VP neuron (from -0.5 s to 0.5 s post stimulation of the NAc). The raster plot shows a neuron's response during 800 applications of stimulation in control conditions, 800 stimulations after the application of naloxone and 800 stimulations after the application of picrotoxin. **(B)** A typical example raster plot of tonic firing activity in a VP neuron 0.5 s pre stimulation and 0.5 s post stimulation of the NAc. The raster plot exemplifies one neurons response during 800 applications of stimulation in control conditions, 800 stimulations after the application of DAMGO and 800 stimulations after the application of picrotoxin **(C)** Bar chart representing significant differences (** = $P < 0.01$) in pause duration for neurons in control conditions, the presence of naloxone and the presence of Picrotoxin. **(D)** Bar chart representing significant differences (** = $P < 0.01$ and * = $P < 0.05$) in pause duration for neurons in control conditions, the presence of DAMGO and the presence of Picrotoxin. **(E)** Bar chart representing non-significant ($P > 0.05$) differences in the average firing frequencies for neurons in control conditions, in the presence of naloxone and in the presence of picrotoxin. **(F)** Bar chart representing non-significant ($P > 0.05$) differences in the coefficient of variation for neurons in control conditions, in the presence of naloxone and in the presence of picrotoxin. **(G)** Bar chart representing non-significant ($P > 0.05$) differences in the average firing frequencies for neurons in control conditions, in the presence of DAMGO and in the presence of picrotoxin. **(H)** Bar chart representing non-significant ($P > 0.05$) differences in the coefficient of variation for neurons in control conditions, in the presence of DAMGO and in the presence of picrotoxin.

5.3.5 NK-1 receptors modulate the effect of NAc stimulation on VP tonic firing

In order to investigate the effects of substance P receptors on the inhibition of VP neurons by NAc stimulation, we applied L732,138, which is a NK-1 receptor antagonists, during stimulation of the NAc.

From 3 experiments 8 neurons were identified for analysis that paused in response to NAc stimulation (Figure 5.5A). In control conditions the average pause duration for these neurons was 0.39 ± 0.06 s, for these 8 neurons, after the application of L732,138 the average pause duration was 0.26 ± 0.06 s. The pause duration, after the application of L732.138 was significantly ($P < 0.05$) reduced compared to the pause duration in control conditions (Figure 5.5B). The pause duration was further significantly ($P < 0.001$) reduced, compared to control conditions, with the application of picrotoxin 0.07 ± 0.02 s (Figure 5.5B).

In order to investigate if the application of L732,138 had any effect on electrophysiological characteristics of the VP neurons we measured baseline firing frequency rates and coefficient of variation for the VP neurons. The firing frequency (Hz) rates were not significantly ($P > 0.05$) different for the VP neurons studied in the presence of L732,138 20.15 ± 3.67 Hz compared to control conditions 20.75 ± 1.69 Hz and the presence of picrotoxin 22.77 ± 3.15 Hz (Figure 5.5C). Coefficient of variation was also not significantly ($P > 0.05$) different for the VP neurons studied in the presence of L732,138 13.55 ± 1.57 compared to control conditions 12.90 ± 1.53 and picrotoxin 13.93 ± 2.9 (Figure 5.5D).

We conclude that substance P, released from NAc afferents into the VP, contribute to the pause duration produced as a result of NAc stimulation through activation of NK-1

receptors. This is unexpected as blocking a known excitatory influence was expected to prolong the pause duration.

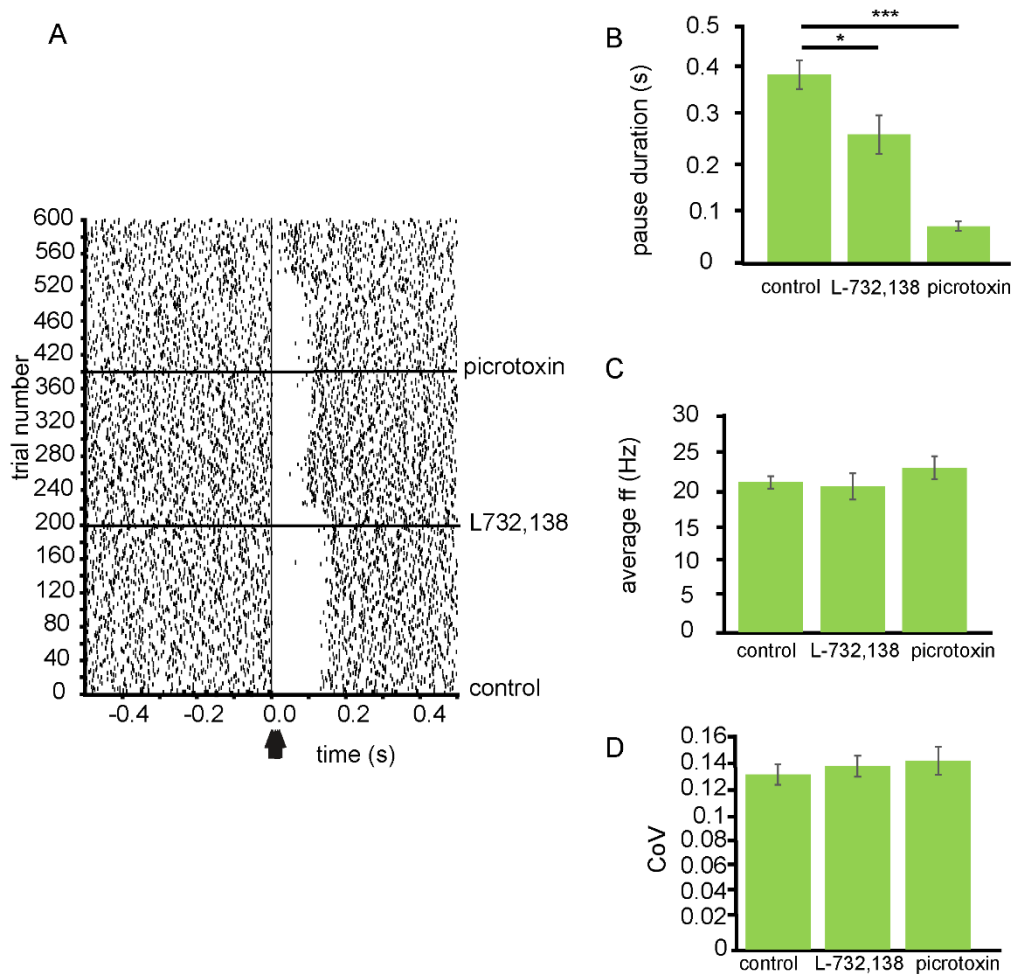


FIGURE 5.5: Inhibition of VP neurons by NAc stimulation is modulated by NK-1 receptor. **(A)** A typical example raster plot of tonic firing activity in a VP neuron from 0.5 s to 1.0 s post stimulation of the NAc. The raster plot exemplifies one neurons response during 200 applications of stimulation in control conditions, 200 stimulations after the application of L732,138 and 200 stimulations after the application of picrotoxin. **(B)** Bar chart representing significant (* = $P < 0.05$ *** = $P < 0.001$) differences in the average pause duration (ms) for neurons in the VP after NAc stimulation. Each bar represents the average pause duration in different pharmacological conditions: in control conditions, in the presence of L732,138 and in the presence of Picrotoxin. **(C)** Bar chart representing non-significant ($P > 0.05$) differences in the average firing frequencies for neurons in control conditions, in the presence of L732,138 and in the presence of picrotoxin. **(D)** Bar chart representing non-significant ($P > 0.05$) difference in the coefficient of variation for neurons in control conditions, in the presence of L732,138 and in the presence of picrotoxin.

5.4 Discussion:

The present research concludes that MEA setup can be used successfully to study the connections between the striatum and its output nuclei. In this case, specifically between the NAc and the VP (figure 1.1). We provide support, using a novel method, for previous research (Wang et al., 2014) suggesting that the NAc afferents of the VP have an inhibitory impact on VP neurons, as activation of the NAc afferents produced inhibition in tonic firing for a number of VP neurons. The current study also identified a number of, tonically active neurons, across all VP territories that were not inhibited by activation of afferents from the NAc.

We tentatively conclude that activation of NAc afferents does not inhibit all the neurons in the VP and this may relate to a type of neuron not directly targeted by NAc afferents. However, caution should be taken with these conclusions as brain slices are likely to only preserve a fraction of the connections between the NAc and VP. Therefore it is conceivable that those neurons that did not respond to NAc stimulation were simply inhibited by NAc fibres that were lost in the slice preparation.

Our findings, also revealed no distinct difference in any of our electrophysiological measures (firing frequency, coefficient of variation and spike half-width) for those neurons that responded to activation of NAc afferents and those that were non-responders. This may well support the fact that the difference between responders and non-responders was an artefact of the brain slice preparation, preserving some connections between the NAc and VP, while severing others.

Alternatively, the lack of electrophysiological differences between those neurons that responded to NAc stimulation and those that were non-responders may also be masked by within group variability. Research suggests NAc afferents target multiple different types of neurons in the VP, including GABAergic and cholinergic (Grove et al., 1986;

Root et al., 2015; Zaborszky, 1989), therefore the electrophysiological measures used may be distorted by this within group variability. Future research could use BAC transgenic animals with a GFP (green fluorescent protein) promoter for fluorescence in cholinergic neurons, so as to identify these in the slice.

5.4.1 Difference in pause duration dorsal to ventral within the VP:

The current study has also shown that the inhibitory effects of NAc inputs of the VP are evenly dispersed, with a similar number of neurons being inhibited in dorsal regions of the VP as were inhibited in more ventral regions of the VP. This even distribution across the VP is also the case for the neurons that were not inhibited by activation of NAc afferents. On the other hand there was, a significance difference in the duration of the inhibition between neurons in dorsal and ventral territories of the VP, with the dorsal regions exhibiting a significantly longer pause in firing in response to activation of the NAc afferents than did ventrally located neurons in the VP. This could relate to the differences in the functional roles of these territories or code an imbalance in projections from dorsal regions and from ventral regions to their prospective targets. Root et al., (2015) suggests that the connections between the NAcS and ventro-medial VP are involved in initiation of drug seeking (identifying the conditions for drug use) while NAcC to dorsolateral VP are involved in the continuation of drug seeking (addiction). We may therefore speculate that drugs of abuse alter this imbalance in pause duration between ventral and dorsal VP neurons resulting in a shift from initiation to continuation of drug seeking. Further research would be pertinent in slices from drug sensitised animals, to investigate if the difference in pause duration between dorsal and ventral VP territories, is altered by sensitization to drugs of abuse.

The fact that brain slice preparations are likely to only preserve a small proportion of the connections between brain regions, could again account for the differences in pause duration dorsally to ventrally within the VP. Therefore caution should be taken as these results could be due to more afferents to dorsal VP being intact in our brain slice preparations compared to the number of afferents to ventral VP. This is likely as the slice preparation technique used in the current study was based on that of Beurrier et al., (2006), whose aim was to preserve connections between more dorsal regions of the striatum and the GP. The current study investigated more ventral regions of the striatum (NAc) and the ventral pallidum, and therefore our findings, to some extent, may be an artefact of the preparation technique.

5.4.2 Neurotransmitters and neuropeptides modulating pause duration:

As for the pharmacology of these inhibitory effects on VP neurons, we found that the GABA_A antagonists (picrotoxin) largely removed the pause in tonic firing, seen in some VP neurons, induced by activation of the NAc afferents. We conclude that the inhibitory effect of NAc afferent activation on a subset of VP neurons is predominantly mediated by the release of GABA and the activation of GABA_A receptors on target VP neurons, thus resulting in a pause in tonic activity. This makes sense in the light of the dominant labelling/expression of GABA_A as opposed to GABA_B in the VP (Henderson, 1995; Zilles et al., 1991). This also supports the immunohistochemical research (Churchill & Kalivas, 1994) and the previous electrophysiological work of Chrobak and Napier, (1993), which suggests GABA antagonists disinhibit VP neurons.

Recent research suggests both direct and indirect pathway MSN's project into the VP (figure 1.1) (Creed et al., 2016; Kupchik & Kalivas, 2016). These release enkephalin (indirect) and substance P (direct) and therefore these neuropeptides would seem likely

candidates to contribute to the pause in tonic firing induced by activation of the NAc afferents. The current study found there to be no effect of opioid antagonists on the pause induced by stimulation of afferents from the NAc. We conclude that, although enkephalin may be released by these neurons (figure 1.1), it has no apparent modulatory effect on the neurons directly inhibited by activation of these afferents. Opioid antagonists were also not found to have any significant effect on the firing frequency and coefficient of variation for those neurons that paused. This is contrary to much of the research, which suggest enkephalin modulates VP neurons (Chrobak & Napier, 1993; Napier & Mitrovic, 1999). Indeed, caution is needed in the interpretation of our results. The stimulation technique used in our studies on opioids is known to favour the release of GABA/glutamate and not neuropeptides, such as enkephalin. Therefore, the lack of modulatory effect seen in response to opioid antagonists could simply be an artefact of the stimulation protocol not resulting in neuropeptide release. However Mitrovic and Napier, (1995) has shown that a significant proportion of VP neurons do not respond to any opioid agonist. It could therefore be that those neurons directly inhibited by NAc afferents are those that are not modulated by enkephalin.

5.4.3 Methodological issues with stimulation protocols:

The stimulation protocol used for the experiments involving opioid antagonists was probably biased against the release of neuropeptides, as it involved the use of a low frequency stimulation, which is known to favour the release of GABA and glutamate and not neuropeptides, such as enkephalin (Purves, 2001). Further to this we also used the non-selective opioid antagonist Naloxone. This meant that other opioid receptors (kappa and delta), which are known to be present in the VP (Mitrovic & Napier, 1995; Olive et al., 1997), would have been inhibited, thus potentially masking the effects of enkephalin by also modulating the effects of dynorphin. Future research should include

high frequency stimulation protocols, such as that used in our experiments with NK-1 antagonists, and selective enkephalin antagonists to avoid these issues.

5.4.4 Substance P modulation of pause duration:

The current study suggested, unexpectedly, that substance P contributes to the inhibitory effect of NAc input activation. Pause duration of VP neurons in response to NAc stimulation was significantly reduced by the application of NK-1 receptor antagonists, suggesting that substance P released from these NAc afferents contribute to the inhibitory effect of NAc afferent activation, via activation of NK-1 receptors. This contradicts previous research showing that substance P increases the firing rate of VP neurons and substance P antagonists block increases in firing rate seen in response to NAc stimulation (Mitrovic & Napier, 1998). However the research of Mitrovic and Napier, (1998) was carried out *in vivo*, therefore it plausible that the discrepancy results from the fact that in the intact brain the VP receives a number of other external inputs that are silent in brain slices and could be modulated presynaptically by substance P. Despite our data showing NK-1 receptor antagonists reduced the duration of the inhibition, they were not found to have any significant effect on the baseline firing frequency or coefficient of variation of the VP neurons, suggesting that there is little endogenous activation of these receptors in the VP in the absence of stimulation. Chen et al's., (2001) suggested that the majority of NK-1 receptors found in the VP are localised to cholinergic neurons. These cells could be involved in the effects reported here. Alternatively, substance P may have caused presynaptic facilitation of GABA release from NAc fibres, thus prolonging the GABA-induced pauses. Further experiments should be aimed at testing this intriguing hypothesis. Furthermore, it could be that substance P also has an effect at other tachykinin receptors in the VP, as NK-3

receptors are known to be found in the VP (Maeno et al., 1993; Shughrue et al., 1996) and this alters the effect seen as they were not blocked in the current study. Future research could target these receptors or repeat the current work with the application of NK-3 receptor antagonists.

5.4.5 Implications:

In the current study we have shown a novel method for investigation of the basal ganglia connections, which can be used to collect large amounts of data in relatively short periods of time, and also provides the ability to stimulate regions of interest and measure the effects in other afferent regions, providing insights into the modulatory connections and circuitry of brain regions.

We also provide evidence to show that NK-1 receptors modulate the inhibition in tonic firing as a result of NAc stimulation, which provides an intriguing mechanism, that could contribute to our understanding of how substance P application has reinforcing effects when applied to the VP (Nikolaus et al., 1999a). We tentatively suggests that this may be due to its facilitatory effect on inhibition produced by the NAc afferents of the VP, and may modulate reinforcement related signals between the NAc and VP, by increasing the duration of inhibition exerted by these inputs on the VP.

6 Chapter 6: General Discussion:

In this section the main overall findings of our studies will be summarised and links made to the potential implications of these results to our understanding of the VP and its role in both modulation of reward related responses and responses to drugs of abuse. Technical considerations will also be discussed and suggestions for future work outlined.

6.1.1 Dopaminergic modulation of VP neurons and their cellular mechanism

In chapter three of this thesis we examined the effect of dopamine and modulation of D1-like and D2-like dopamine receptors on the tonic firing of VP neurons. We revealed important cellular mechanisms underpinning their effects within the VP. These may provide insights into the cellular mechanism underpinning a range of functions dopamine has been associated with in the VP, such as avoidance learning (Lénárd et al., 2017; Péczely et al., 2014) and sensitization to drugs of abuse (Stout et al., 2016).

Further to this we were also able to consistently identify neuronal populations in the VP that had differential responses to D2-like receptor modulation based on their spike half-width profile. We found that neurons that decrease firing rates in response to D2-like agonist's application had a larger spike half-width profile (type II) than those that increased firing rates in response to D2 agonists (type I). This therefore provides a mechanism by which future research can aim to identify separate populations of neurons within the VP from extracellular recordings and via electrophysiological means. These data may also relate to those of Avila & Lin, (2014), whose findings suggests that there are two populations of neurons in the VP, each of which modulates different behavioural responses. They identified one populations with slow rates of firing and narrower spike waveforms coding motivational salience and one with faster rates of firing and more complex waveforms coding movement. These may have some relation

to the populations we have identified as type I and type II, that are modulated in different manners by D2-like receptor agonists and have shorter and longer spike half-width profiles respectively.

Other research has also identified two distinct populations of neurons within the VP that modulate separate symptoms of major depressive disorder. Knowland et al., (2017) identified that manipulation of either population resulted in social withdrawal or behavioural despair but not both. Investigation of these populations' spike half-width profiles and responses to D2-like agonists may prove insightful for future research.

Dopamine's effects in the VP have also been implicated in drug seeking behaviour. Stout et al (2016) has shown that upon exposure to cocaine, after sensitization, dopamine levels increase dramatically in the VP. Our data provides information on the likely cellular effects, in the VP, of this increased dopamine in response to cocaine, and therefore reveals likely cellular mechanisms for the role of dopamine in drug seeking behaviour in the VP. Our data reveals dopamine increases the activation of Type I neurons via presynaptic modulation of glutamatergic inputs and subsequent activation and deactivation of ionotropic and metabotropic glutamate receptors on type I neurons (Figure 3.10). Our data also reveals that dopamine results in both excitation and inhibition of type II neurons through presynaptic effects of D1-like receptors on glutamatergic terminals, facilitating the release of glutamate and excitation, via ionotropic glutamate receptors, and inhibitory effect via direct activation of D2-like receptors on type II VP neurons (Figure 3.10). These data may suggest fruitful and novel receptors to target in relation to pharmacological control of drug seeking behaviour.

Our data also suggests a possible cellular scenario for the cellular mechanism underpinning dopamine's role in avoidance learning. Lénárd et al., (2017) data suggest

that D2-like agonists injected into the VP directly increase memory and learning processes in avoidance learning tasks, therefore, according to our data, this may be modulated through the reduction in firing frequency of type II VP neurons, via direct effects and through increased activation of type I VP neurons via reduced activation of metabotropic glutamate receptors.

6.1.2 Serotonin receptor subtypes role in the dichotomous effects of serotonin on VP neurons

In chapter four of this thesis we examined the effect serotonin and several serotonin receptor subtypes have on the tonic firing of VP neurons. Understanding the role that these receptor subtypes play in the modulation of VP neuronal circuitry will shed light on potential pharmacological interventions for issues such as anhedonia and drug addiction that the VP is known to play a pivotal role in modulating. Our data suggests that the known excitatory responses to serotonin application in the VP (Bengtson et al., 2004) were largely modulated presynaptically by 5HT1a, 5HT5a and 5HT7 receptors, while inhibitory responses to serotonin application were largely modulated postsynaptically by 5HT2c receptors. Specific targeting of these serotonin receptor subtypes could therefore prove effective in the development of pharmacological interventions for anhedonia and drug addiction. Certainly 5HT2c antagonists (Goodwin et al., 2009) are known as affective antidepressants, however exploration of 5HT1a, 5HT5a and 5HT7 modulation may also prove fruitful.

5HT2c have also been implicated in the VP in drug sensitization (Napier & Istre, 2008). Napier and Istre (2008) found that 5HT2c receptors are upregulated in methamphetamine sensitized animals. Our data casts light onto the cellular mechanisms of this phenomenon, suggesting upregulation of 5HT2c receptors would result in an increase in neuronal inhibition within the VP, which is modulated at post-synaptic

locations. Our data may also provide insights into the cellular mechanisms underpinning 5HT2c known modulation of locomotor activity. Recent research has implicated the 5HT2c receptor in the VP in modulation of locomotor activity and rearing behaviour, with 5HT2c agonists attenuating both (Graves et al., 2013). Our data would suggest that 5HT2c activation reduced the activity of some VP neurons and this may well be the cellular mechanism for 5HT2c agonist's effects on locomotor activity.

6.1.3 Modulation of VP neurons tonic firing by stimulation of NAc afferents

In chapter five of this thesis we examined the effects of stimulation of the NAc afferents on the tonic firing of VP neurons. We provide evidence to support the fact that these afferents produce inhibitory responses in some VP neurons and that substance P contributes to this response by increasing the pause duration. We also provide evidence to show that some neurons in the VP are not modulated by NAc afferents and therefore may be involved in a separate circuit running through the VP which may be involved in the processing of different information.

We also identified that activation of NAc afferents to dorsal and ventral regions of the VP result in different pause durations. However, there is a major caveat of this conclusion as *in vitro* slice preparations, used in this study, only result in the preservation of a limited number of connections between regions, while severing others. It is therefore possible that the differences in pause duration between dorsal and ventral regions may simply be an artefact of the methodology employed in this study. On the other hand, we may also conceive, that if this finding is not simply an artefact of the method employed, it may link to evidence suggesting the dorsal and ventral VP regions are innervated by different inputs, with the medial NAcS innervating the ventromedial VP and the lateral NAcS and olfactory tubercle innervating the ventrolateral VP, while

the NAcC inputs innervate the dorsolateral VP subregion (Heimer et al., 1991; Zahm & Brog, 1992). The imbalance in pause duration seen between dorsal and ventral regions of the VP may therefore play some part in the role NAc inputs into the VP have in integration of firing.

6.1.4 Technical considerations

The main focus of the current research project was to investigate the cellular mechanism underpinning the effects of dopamine and serotonin within the VP and to elucidate the receptor types involved in these responses. We also wished to explore the effects of NAc afferent stimulation on VP neurons, and cast light on the modulatory role of various neuromodulators in the VP responses to activation of these inputs.

The main electrophysiological method used to answer these questions was in vitro multi-electrode array recordings with bath application of various pharmacological agents. We also employed the multi-electrode array as a method to stimulate the NAc afferents of the VP. While the multi-electrode arrays provide an opportunity to collect large amounts of data with a relatively high temporal and spatial precision, they do have some caveats. One issue stems from the fact multi-electrode arrays provide extracellular recordings of neuron populations, as such untangling the signal to reveal the unitary elements requires some skill and precision as well as a degree of judgement.

There are also some practical issues with this system. The MEA recordings required at least an hour for the slice to stabilise and to fix onto the electrode array. However, often part way through recording, some channels could become active, perhaps due to delayed fixation on the Multi-electrode array. It was impossible to include these channels in any analysis, therefore missing a potentially interesting population of neurons. Also despite waiting an hour for the slice to fix upon the multi-electrode array, some neurons showed

increases in spike amplitude over the course of an experiment, which often lasted several hours. This could have been a potential confound, as neurons that at the start of the study did not pass the threshold for spike detection, may have done so as the experiment progressed and the slice attached more to the MEA, moving closer to the electrodes and therefore having a higher amplitude. This therefore could have been misinterpreted as a result of the pharmacological agents being applied, rather than as a result of slice fixation. For this reason a significant number of neurons across all experiments had to be discounted from analysis.

Further to this several populations of neurons within the VP are known to be tonically silent (Bengtson & Osbourne, 2000). Due to the nature of the multi-electrode array, focus was on those neurons that were tonically active. This therefore excludes populations of neurons within the VP from my results. For example cholinergic neurons in the VP are considered to be tonically silent (Bengtson & Osborne, 2000), therefore it is unlikely that my data sheds any light on the role of these neuronal populations within the VP, despite evidence suggesting that they are effected by serotonin modulation (Bengtson et al., 2004).

Another restriction of in vitro techniques, such as the multi-electrode array is that it relies on healthy brain slices. There are multiple factors that can affect the viability and health of these slices, including PH, osmolarity, oxygenation levels. In vivo techniques can avoid some of these issues and allow you to study the brain in an intact state, however they have issues of their own, which results in difficulty to interpret the results of studies involving the mechanism of drug action. One major caveat to in vivo studies is the likely effects upstream and the effect of intact inputs into areas, such as the VP.

Another caveat of in vitro slice preparations is that many connections between regions are severed during slice preparation. This therefore undermines all studies carried out in

chapter 5 with stimulation of the NAc. Therefore all studies in chapter 5 and interpretation of these results must be treated with caution as the results and conclusions, at least to some extent, maybe an artefact of the preparation technique. It may be that, on balance, a more appropriate methodology for the type of studies in chapter 5 would involve in vivo techniques, where all connections and circuitry are intact.

6.1.5 Future work

6.1.5.1 Confirmation of neuronal populations identified by spike half-width profiles

In the current research we identified that two populations of neurons in the VP could be consistently identified based on the spike half-width profile and responses to D2-like receptor modulation. We speculated that these neurons are perhaps GABAergic projection neurons and GABAergic interneurons, however we were unable to provide conclusive evidence that this was the case. Future research will be needed to elucidate the biochemical identity of these subpopulations, probably by carrying out intracellular recordings of these two neuron populations and post-recording immunohistochemical labelling.

6.1.5.2 Interplay between serotonin and dopamine

Recent research has identified that focusing on the interaction between dopamine and serotonin in the VP may prove insightful to our understanding of reward related behaviour and responses to drugs of abuse (McDaid et al., 2007; Fischer & Ullsperger, 2017). The current research project has shown that both these neurotransmitters modulate neurons in the VP. An intriguing next step would be to study how dopamine

and serotonin agonists interact with each other at cellular level and whether there are differential interaction effects that relate to the two neuron population we identified in the VP (based on their spike half-width profile and response to D2-like receptor modulation).

6.1.5.3 Modulation of NAc inputs to the VP by serotonin and dopamine

In the current research project we managed to establish reliable techniques to stimulate the NAc and monitor the effects this has on tonic activity within the VP. However due to time restrictions we were unable to explore the effects that serotonergic and dopaminergic modulation had on these NAc inputs of the VP. Future research should focus on the role that serotonin and dopamine modulation has on these inputs from the VP, and perhaps look at the interplay between these two neurotransmitters in the modulation of NAc inputs of the VP.

6.1.6 Concluding remarks

Recent years have seen a surge in research focused on the VP and an identification of this area as crucially involved in reward-related behaviour, pleasure responses and addiction to drugs of abuse. However many of the cellular mechanism underpinning the VP's function are still unclear.

This thesis provides important novel insights into the cellular mechanisms responsible for dopamine's and serotonin's modulatory effects within the VP. It provides ideas on both future research directions and potential novel pharmacological targets for interventions, to treat dysfunction of affective responses and sensitization to drugs of abuse. As well as this, the current research project shows how multi-electrode arrays can be used to investigate the circuitry of the basal ganglia. It therefore provides a

demonstration of how this technique, is suitable to collect large quantities of data on tonic active brain areas, and can be used to study the responses of VP neurons to pharmacological intervention and modulation by activation of afferent inputs.

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