Examining cholinergic function in the ventral tegmental area and dorsal hippocampus

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Abstract

Midbrain dopamine (DA) neurons project from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and ventral striatum (NAc and OT) forming a key part of the well-defined mesocorticolimbic system, as well as the hippocampal-VTA loop. The mesocorticolimbic system, at least partly, mediates the rewarding effects of drugs of abuse, such as nicotine. The VTA receives cholinergic projections from the pedunculopontine tegmental and laterodorsal tegmental nuclei (PPTg, LDTg). Previous studies have shown that selective bilateral cholinergic lesions of the posterior PPTg or LDTg can be achieved by directly infusing diphtheria-urotensin II toxin (Dtx-UII) into either region. This thesis aimed to selectively destroy the cholinergic input from both mesopontine nuclei to the posterior VTA (pVTA) by injection of Dtx-UII here. Unilateral or bilateral infusion of Dtx-UII into the pVTA did not destroy cholinergic terminals at any of the time points analysed. There was no evidence of neurodegeneration (as measured by Fluoro-jade C) present in the pVTA, PPTg or LDTg. A non-lesion approach was also adopted to better understand the actions of cholinergics in the pVTA. The effects of nicotine self-administered into the VTA or (as a point of contrast in the circuitry of the VTA) the dorsal hippocampus (dHPC) were assessed by measuring lever pressing and quantifying the expression of immediate early gene (IEG) *c-fos* as a marker of neural activation. Fos expression was quantified in the VTA and in structures on which VTA activity has effect – the shell and core of the NAc, the dorsal striatum and the dHPC. Fos was measured under two conditions: at the end of the very first exposure to nicotine and (in other groups) after repeated exposure. These results demonstrated that rats will lever press to directly administer nicotine into the VTA or dHPC, but that nicotine-induced Fos expression is not correlated with lever pressing. In addition, intra-VTA, but not intra-dHPC nicotine, activated all regions of interest. This demonstrates that intra-VTA nicotine mediates its effects through regions of the mesocorticolimbic system, but that intra-dHPC nicotine must mediate its effects

through other brain regions and systems. These results raise questions about the nature of reward processing in the brain.

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List of abbreviations

$^{\Delta9}$THC	$^{\Delta9}$-tetrahydrocannabinol
5-HT	serotonin
6-OHDA	6-hydroxydopamine
aca	anterior part of the anterior commissure
ACh	acetylcholine
aCSF	artificial cerebral spinal fluid
ADS	antibody dilutant solution
AF64A	ethylcholine mustard aziridinium ion
AMG	amygdala
ANOVA	analysis of variance
aPPTg	anterior pedunculopontine tegmental nucleus
Arch	archaerhodopsin-3
Arc	activity-regulated cytoskeleton-associated protein
ATP	adenosine triphosphate
aVTA	anterior ventral tegmental area
bNOS	brain derived nitric oxide synthase
BNST	bed nucleus of the stria terminalis
CA	catecholamine
Ca^{2+}	calcium
cAMP	cyclic adenosine triphosphate
CeA	central amygdala
CCK	cholecystokinin
ChR2	channelrhodopsin-2
CLi	caudal or central linear nucleus
CNO	clozapine-N-oxide
CNS	central nervous system
cp	cerebral peduncle

CPP	conditioned place preference
CPu	caudate putamen
CREB	cAMP response element
CRF	continuous reinforcement
CS	conditioned stimulus
D1	dopamine receptor-1
D2	dopamine receptor-2
DA	dopamine
DAB	3, 3-diaminobenzidine tetrahydrochloride
DG	dentate gyrus
DHβE	dihydro-β-erythroidine
dHPC	dorsal hippocampus
DREADDs	Designer Receptors Activated by Designer Drugs
DRN	dorsal raphe nucleus
Dtx	diphtheria toxin
Dtx-UII	diphtheria-urotensin II toxin
DV	dorsoventral
EC	entorhinal cortex
EF-2	elongation factor 2
EMIT	electrolytic microinjection transducer
EtOH	ethanol
fMRI	functional magnetic resonance imaging
fr	fasciculus retroflexus
FR2	fixed ratio-2
GABA	γ-aminobutyric acid
GAD65	glutamic acid decarboxylase 65-kD
GPCRs	G-protein coupled receptors
hif	hippocampal fissure
HPC	hippocampus
i.p.	intraperitoneal

IAL	interaural line
IC-CPP	intracranial conditioned place preference
ICSA	intracranial self-administration
ICSS	intracranial self-stimulation
IEG	immediate early gene
IF	interfascicular nucleus
IPI	inter-press interval
IPN	interpeduncular nucleus
IPSPs	inhibitory postsynaptic potentials
ITCN	image-based tool for counting nuclei
IUE	*in utero* electroporation
IVSA	intravenous self-administration
LC	locus coeruleus
L-DOPA	L-3, 4-dihydroxyphenylalanine
LDTg	laterodorsal tegmental nucleus
LGN	lateral geniculate nucleus
LH	lateral hypothalamus
LHb	lateral habenula
LS	lateral septum
LTP	long-term potentiation
mAChRs	muscarinic acetylcholine receptors
METH	methamphetamine
mfb	medial fiber bundle
ml	medial lemniscus
MLA	methyllycaconitine
MM	medial mammillary nucleus
mPFC	medial prefrontal cortex
MRN	median raphe nucleus
mRNA	messenger RNA
MS/DB	medial septum diagonal band area

MSNs	medium spiny neurons
MT	medial terminal nucleus of the optic tract
NA	noradrenaline
NAc	nucleus accumbens
nAChRs	nicotinic acetylcholine receptors
NACWO	Named Animal Care and Welfare Officer
NeuN	neuron-specific nuclear protein
NMDA	N-methyl-D-aspartic acid receptor
NpHR	neuron-silencing cation channel transporter halorhodopsin
NST	nucleus of the solitary tract
NT	neurotensin
OT	olfactory tubercle
PAG	periaqueductal gray
PaS	parasubiculum
PB	phosphate buffer
PBP	parabrachial pigmented nucleus
PBS	phosphate buffered saline
PCP	phencyclidine
PD	Parkinson’s disease
PER	perirhinal cortex
PFA	paraformaldehyde
PFC	prefrontal cortex
PHR	parahippocampal region
PIF	parainterfascicular nucleus
PN	paranigral nucleus
POA	preoptic area
POR	postrhinal cortex
PPTg	pedunculopontine tegmental nucleus
pPPTg	posterior pedunculopontine tegmental nucleus
PrS	presubiculum

pVTA	posterior ventral tegmental area
RLi	rostral linear nucleus
RMTg	rostral medial tegmental nucleus
Rn	red nucleus
RRF	retrorubral field
RSC	retrosplenial cortex
rsFC	resting state functional connectivity
s.c.	subcutaneous
SC	superior colliculus
scp	superior cerebellar peduncle
SCPx	superior cerebellar peduncle decussation
SN	substantia nigra
SNc	substantia nigra pars compacta
SNl	substantia nigra pars lateralis
SNr	substantia nigra pars reticulata
SQRT	square root transformed
STN	subthalamic nucleus
Sub	subiculum
SuMM	supramammillary nucleus
SVZ	subventricular zone
TH	tyrosine hydroxylase
tth	trigeminothalamic tract
UII	urotensin-II
UII-R	urotensin-II receptor
URP	urotensin-II related peptide
US	unconditioned stimulus
VAChT	vesicular acetylcholine transporter
VDB	vertical limb of the diagonal band
VMAT2	vesicular monoamine transporter 2
vGluT2	vesicular glutamate transporter 2

vHPC	ventral hippocampus
VP	ventral pallidum
vSub	ventral subiculum
VTA	ventral tegmental area
VTAR	ventral tegmental area rostral
VTT	ventral tegmental tail

Chapter 1: General Introduction

1.1 General overview – the Ventral Tegmental Area

In 1925, Tsai was the first to recognise the "nucleus tegmenti ventralis" (or ventral tegmental nucleus) in the brain of the opossum, using Nissl and Golgi staining. This organised triangular shaped structure spanned from the cerebral peduncle (cp) to the ventral tip of the substantia nigra (SN) (Tsai, 1925). Earlier studies referred to it as part of the SN (Kosaka and Hiraiwa, 1915; Castaldi, 1923). However, Tsai characterised the "ventral tegmental nucleus" as a separate region based on its substantial connectivity with surrounding fiber bundles, which the SN lacked (Tsai, 1925). Following this, Papez (1932) identified this region in the armadillo where it was termed "nucleus of the mammillary peduncle" (Papez, 1932). Subsequent studies investigated this region in other species and adopted Tsai's term "ventral tegmental nucleus" (Rioch, 1929; Brown, 1943; Taber, 1961). In 1956, Nauta coined the phrase "ventral tegmental area of Tsai" (Nauta, 1956). The term "area" was deemed more appropriate because of the region's heterogeneous nature and unclear borders that are best defined by surrounding structures.

By 1964 Dahlström and Fuxe had described 12 catecholamine-fluorescing cell groups in the rat brain. The ventral midbrain dopamine (DA) groups were termed A8, A9 and A10 which occupied the retrorubral field (RRF), substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), respectively (Andén *et al.*, 1964, 1966; Dahlström and Fuxe, 1964; Andèn *et al.*, 1965; Smeets and González, 2000; Björklund and Dunnett, 2007). However, it was long debated whether the VTA existed as a separate region from the neighbouring SN, because it was particularly difficult to differentiate between the two (Dahlström and Fuxe, 1964; Bentivoglio and Morelli, 2005; Wise, 2009). Phillipson (1979) demonstrated through Nissl preparations that the dorsal portion of the SNc appeared to be continuous with the VTA (Phillipson, 1979b). However, Golgi and tyrosine hydroxylase (TH) preparations of the SNc and VTA highlighted morphological differences that aided the differentiation between the two regions. A9 and A10 groups were characterised based on the lateral-medial topographical

organisation of DA neurons (Dahlström and Fuxe, 1964). A9 DA neurons are densely packed in the SNc (Hökfelt *et al.*, 1976). Golgi preparations revealed that SNc DA neurons had long dendritic processes that projected to regions of the SN pars reticulata and pars lateralis (SNr, SNl) (Juraska *et al.*,1977; Phillipson, 1979a). Generally, SNc DA neurons generated dendrites that projected in a perpendicular orientation – a prominent feature of SNc DA cells (Cajal, 1911; Juraska *et al.*, 1977; Phillipson, 1979a). Furthermore, A9 DA cells were reported to generate collateral projections that synapse back onto themselves, also known as autapses (autaptic synapses) (Van der Loos and Glaser, 1972). A10 DA cells were reported to extend horizontal dendritic projections to midline structures (Phillipson, 1979a). A10 DA cells are lightly packed throughout the VTA compared to the densely packed population of SNc DA neurons (Halliday and Törk, 1986). In the mouse and the rat, A10 DA neurons are much smaller than DA cells of the A9 group (Hökfelt *et al.*, 1976; Nelson *et al.*, 1996). Nissl preparations demonstrated that VTA DA cells were interspersed with non-DA neurons, which resulted in a much lighter staining compared to the SNc (Domesick *et al.*, 1983). Although the term VTA has been used synonymously with Dahlström and Fuxe's A10 nomenclature, the VTA also contains neurons that release glutamate, γ-aminobutyric acid (GABA) and other neuropeptides (Dahlström and Fuxe, 1964; Fallon and Moore, 1978; Moore and Bloom, 1979).

Functionally, although the VTA is composed of DA, GABA and glutamate releasing neurons, the majority of research has primarily focused on DA neurons. These have been associated with a number of functions: - reward-related behaviours (natural or drug-induced), aversion, salience, novelty, uncertainty, motivation, approach behaviours, learning, and goal-directed behaviours (Wise, 1978, 2008; Schultz and Dickinson, 2000; Ungless, 2004; Fields *et al.*, 2007; Grace *et al.*, 2007; Bromberg-Martin *et al.*, 2010; Ungless *et al.*, 2010; Volman *et al.*, 2013; Creed *et al.*, 2014; Lammel *et al.*, 2014; Walsh and Han, 2014). Furthermore, midbrain DA neurons play an important role in pathological conditions including Parkinson’s disease, schizophrenia, depression and addiction (Hornykiewicz, 1962; Marinelli and White, 2000;

Nestler and Carlezon, 2006; Yadid and Friedman, 2008; Cao *et al.*, 2011; Valenti *et al.*, 2011; Ikemoto and Bonci, 2014; Nikulina *et al.*, 2014).

1.2 VTA location and structure

The heterogeneous nature of the VTA (Figure 1.1) is a product of the differing connectivity, cell types, receptor expression and membrane channels present in each region. The anterior, posterior and tail regions of the VTA are further divided into 5 subnuclei based on their distinct DA-neuron cytoarchitecture: - ventral tegmental area rostral (VTAR), parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), parainterfascicular nucleus (PIF) and ventral tegmental tail (VTT). The anterior region of the VTA (aVTA) is located dorsal to the medial mammillary nucleus (MM) and medial to the substantia nigra pars compacta (SNc) (Zhao-Shea *et al.*, 2011). The aVTA is composed of the ventral tegmental area rostral (VTAR) and parabrachial pigmented nucleus (PBP). The aVTA does not include the midline nuclei (interfascicular nucleus [IF] and rostral linear nucleus [RLi], Figure 1.1) (Hökfelt *et al.*, 1984; Zhao-Shea *et al.*, 2011). The posterior VTA (pVTA) is located dorsal to the interpeduncular nucleus (IPN), medial to the SNc and ventral to Rn (Zhao-Shea *et al.*, 2011). The pVTA is comprised of the PBP, paranigral nucleus (PN) and parainterfascicular nucleus (PIF) and does not include the 3 midline nuclei (IF, RLi and central linear nucleus [CLi], Figure 1.1) (Hökfelt *et al.*, 1984; Zhao-Shea *et al.*, 2011). The most caudal region of the VTA is known as the ventral tegmental tail (VTT) and is located dorsolateral to the IPN (Perrotti *et al.*, 2005; Ikemoto, 2007; Zhao-Shea *et al.*, 2011). The VTT gradually becomes more dorsal and lateral, becoming embedded in the superior cerebellar peduncle decussation (SCPx, Figure 1.1) (Kaufling *et al.*, 2009; Zhao-Shea *et al.*, 2011).

The VTA is composed of three distinct neuronal populations: - dopaminergic (DA releasing), glutamatergic (glutamate releasing) and GABAergic (GABA releasing) (Van Bockstaele and Pickel, 1995; Steffensen *et al.*, 1998; Yamaguchi *et al.*, 2007; Nair-Roberts *et al.*, 2008).

Although widely thought of as a dopaminergic (DAergic) area only ~ 65% of neurons in the VTA release DA compared to the adjacent SNc where ~ 85% of neurons are DAergic (Yamaguchi *et al.*, 2007, 2015; Nair-Roberts *et al.*, 2008; Margolis *et al.*, 2006). Approximately 30% of the VTA neural population release GABA and ~ 5% release glutamate (Yamaguchi *et al.*, 2007; Nair-Roberts *et al.*, 2008). The distribution of DA, GABA and glutamate neural populations vary across VTA subregions. The pVTA has a higher percentage of DA neurons, particularly in PBP and PN subregions, compared to the aVTA and VTT (Zhao-Shea *et al.*, 2011). The DA content is sparse in the caudal VTT (Perrotti et al. 2005; Olson and Nestler 2007). VTA glutamatergic neurons are relatively few and primarily exist in the rostromedial PBP and VTAR (Yamaguchi *et al.*, 2007, 2011; Nair-Roberts *et al.*, 2008). GABAergic neurons are widely spread throughout the VTA, and are densest in the rostral and dorsolateral regions of the aVTA and VTT (Olson and Nestler, 2007; Kaufling *et al.*, 2010). The VTT is primarily composed of GABAergic neurons (Olson and Nestler, 2007). A proportion of VTT GABAergic neurons project to the aVTA and pVTA forming local microcircuits that can inhibit a/pVTA DA neurons (Kaufling *et al.*, 2010).

The rostromedial tegmental nucleus (RMTg) is positioned caudal to the VTA, lateral to the median raphe nucleus (MRN) and extends to the rostral edge of the pedunculopontine tegmental nucleus (PPTg). Previous studies have referred to the VTT and RMTg as part of the same structure (Kaufling *et al.*, 2009; Jhou *et al.*, 2009; Bourdy and Barrot, 2012; Sanchez-Catalan *et al.*, 2014, 2016; Yetnikoff *et al.*, 2014; Pistillo *et al.*, 2015). It has been stated that VTT specifically relates to the first third of the structure that is wedged into the pVTA, and RMTg refers to the remainder of the structure that extends rostrally towards the PPTg (Bourdy and Barrot, 2012). Although both terms are valid, the RMTg extends beyond the standard posterior boundary of the VTA and is primarily composed of GABAergic neurons. RMTg neurons predominantly project to DA neurons of the VTA and SNc. When RMTg GABAergic neurons are stimulated ~ 90% of VTA DA neurons are inhibited (Hong *et al.*, 2011; Ikemoto

and Bonci, 2014). Many studies describe the VTT and RMTg as separate entities and for the purpose of this thesis the VTT and RMTg will be referred to as 2 separate regions (Ikemoto, 2007; Volman *et al.*, 2013; Fowler and Kenny, 2014; Hong and Hikosaka, 2014; Ikemoto and Bonci, 2014; de Kloet *et al.*, 2015).

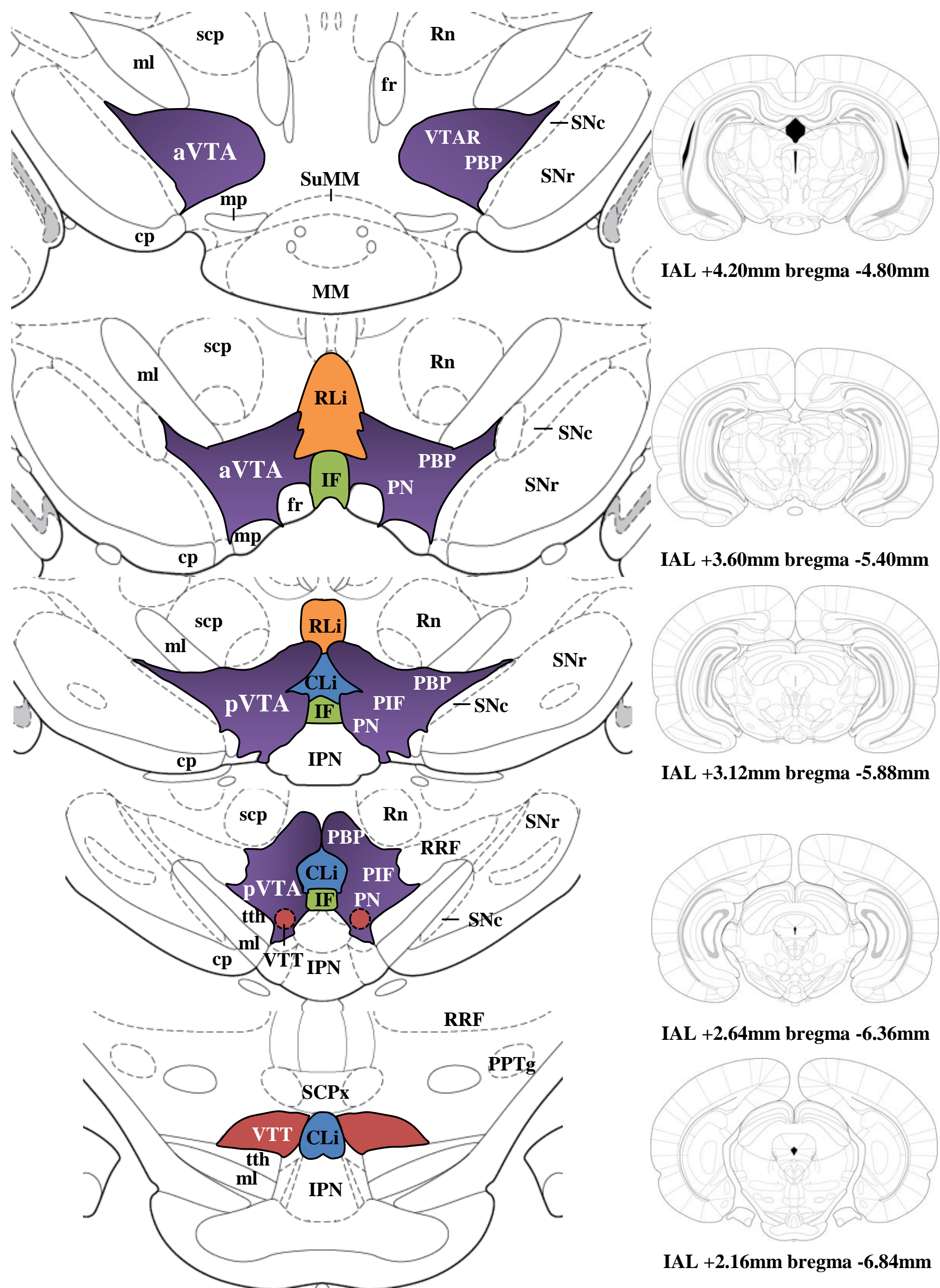


Figure 1.1 – Schematic diagram of the organisation of the rat VTA. The 5 subdivisions of the VTA: - ventral tegmental area rostral (VTAR), parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), parainterfascicular nucleus (PIF) and ventral tegmental tail (VTT) depicted in modified images from the rat brain atlas (Paxinos and Watson, 2007). The anteroposterior distance from interaural line (IAL) and bregma are indicated below corresponding coronal schematics. The border between aVTA and pVTA occurs at +3.48 mm from IAL (-5.5 mm from bregma) – the level at which the interpeduncular nucleus (IPN) appears. *Abbreviations:* - anterior VTA (aVTA), caudal linear nucleus (CLi), cerebral peduncle (cp), fasciculus retroflexus (fr), interfascicular nucleus (IF), medial lemniscus (ml), medial mammillary nucleus (MM), medial supramammillary nucleus (SuMM), posterior VTA (pVTA), red nucleus (Rn), rostral linear nucleus (RLi), retrorubral field (RRF), substantia nigra pars compacta (SNc), substantia nigra pars reticulata (SNr), superior cerebellar peduncle decussation (SCPx), trigeminothalamic tract (tth) and ventral tegmental tail (VTT). Image based on Sanchez-Catalan *et al.* (Figure 3, 2014).

1.3 VTA connections

1.3.1 VTA afferents

The VTA receives excitatory and inhibitory projections from many regions of the CNS (Figure 1.2) (Geisler and Zahm, 2005). In general, VTA afferents are topographically organised – projections to lateral portions of the VTA originate from more lateral regions of the forebrain and brainstem (Geisler and Zahm, 2005; Geisler *et al.*, 2007; Ikemoto, 2007). The VTA receives robust input from the prefrontal cortex (PFC), ventral striatum (nucleus accumbens [NAc] and olfactory tubercle [OT]), bed nucleus of the stria terminalis (BNST), amygdala (AMG), lateral septum (LS), medial septum diagonal band complex, ventral pallidum (VP), medial and lateral preoptic areas (POA), anterior hypothalamus, paraventricular nucleus of the hypothalamus, perifornical hypothalamus, lateral and dorsal hypothalamus, lateral habenula (LHb), SNc, mesopontine tegmental nuclei and dorsal raphe nucleus (DRN) (Wise, 2002; Geisler and Zahm, 2005; Bromberg-Martin *et al.*, 2010; Yetnikoff *et al.*, 2014).

Glutamatergic afferents

Glutamatergic afferents synapse onto VTA DA neurons regulating activity and DA release in target regions (Overton and Clark, 1997). One of the main glutamatergic inputs to the VTA is provided by the medial PFC (mPFC), in particular the infralimbic and prelimbic regions (Christie *et al.*, 1985; Sesack and Pickel, 1992; Lu *et al.*, 1997). The excitation or inhibition of the PFC triggers a respective increase or decrease in VTA activity (Gariano and Groves, 1988; Svensson and Tung, 1989; Murase *et al.*, 1993; Tong *et al.*, 1996). PFC glutamatergic neurons selectively innervate VTA DA neurons that project back to the PFC or VTA GABAergic neurons that then project to the NAc (Sesack and Pickel, 1992; Carr and Sesack, 2000; Fields *et al.*, 2007). These projections form part of the mesocorticolimbic system that is believed to be essential for reward and aversion (discussed below). Substantial glutamatergic projections are also supplied by the lateral hypothalamus (LH), BNST and superior colliculus

(SC) (Georges and Aston-Jones, 2002; Rosin *et al.*, 2003; Geisler and Zahm, 2005; McHaffie *et al.*, 2006). Further glutamatergic projections are provided by the subthalamic nucleus (STN), parabrachial nucleus from the nucleus of the solitary tract (NTS or NST), LHb, pedunculopontine tegmental and laterodorsal tegmental nuclei (PPTg, LDTg) (Kalivas, 1993; Geisler *et al.*, 2007). Excitatory glutamatergic afferents from the LDTg selectively target aVTA DA neurons that then project to the NAc (Omelchenko and Sesack, 2005).

GABAergic afferents

The VTA receives inhibitory GABAergic projections from the PPTg, LDTg, LH, LS, periaqueductal gray (PAG), DRN, NST, shell of the NAc, VP, LHb, BNST and RMTg (Figure 1.2) (Oades and Halliday, 1987; Floresco *et al.*, 2003; Geisler and Zahm, 2005; Blacktop, 2014). The VTA receives extensive GABAergic projections from the VP that regulates the firing activity of VTA DA neurons (Zahm, 1989; Zahm and Heimer, 1990; Mogenson *et al.*, 1993; Wu *et al.*, 1996). Inhibitory inputs from the LDTg selectively target GABAergic neurons in VTA that then project to NAc (Fields *et al.*, 2007). The BNST provides both glutamatergic and GABAergic projections to the VTA – these projections are believed to play an important role in mediating aversion and reward-related behaviours (discussed below).

Cholinergic afferents

The mesopontine tegmental nuclei provide the VTA with significant cholinergic input (Semba and Fibiger, 1992). The PPTg and LDTg project from Ch5 and Ch6 cells, respectively (Mesulam *et al.*, 1983). These cholinergic projections synapse onto VTA DA cell bodies providing the VTA with the only known source of acetylcholine (ACh) (Oakman *et al.*, 1995). The posterior PPTg (pPPTg) directly projects to the VTA, whereas the anterior PPTg (aPPTg) projects to DA neurons of the SNc (Lavoie and Parent, 1994; Oakman *et al.*, 1995; Omelchenko and Sesack, 2005; Holmstrand and Sesack, 2011). Glutamatergic and cholinergic projections from the LDTg synapse onto VTA DA and GABAergic neurons, which in turn,

project to the lateral shell of the NAc (Oakman *et al.*, 1995; Lammel *et al.*, 2012). As discussed above, the pPPTg and LDTg also provide robust GABAergic and glutamatergic projections to the VTA (Cornwall *et al.*, 1990; Semba and Fibiger, 1992; Oakman *et al.*, 1995; Charara, Smith and Parent, 1996; Parent, Parent and Charara, 1999; Geisler *et al.*, 2007; Wang and Morales, 2009). In the VTA, ACh activates metabotropic muscarinic acetylcholine receptors (mAChRs) and ionotropic nicotinic acetylcholine receptors (nAChRs) located on GABA, glutamate and DA neurons (Picciotto, Higley and Mineur, 2012). The activation of mAChRs is relatively slow (milliseconds to seconds) compared to the rapid activation of nAChRs (microseconds to sub-microseconds) (Albuquerque *et al.*, 2009). Therefore, ACh can precisely regulate inhibitory and excitatory input to VTA DA neurons, as well as directly regulating the activity of DA neurons (discussed further in Chapter 4).

Other afferents

Although the VTA primarily receives DAergic, GABAergic and glutamatergic input, certain regions of the CNS provide the VTA with projections that release other neurotransmitters or neuropeptides. The DRN primarily provides the VTA with GABAergic and glutamatergic projections. However, DRN-VTA projecting neurons that release serotonin (5-HT, serotonergic) have been identified (Azmitia, 1978; Hervé *et al.*, 1987; Charara and Parent, 1998; Fremeau *et al.*, 2002; Gras *et al.*, 2002; Geisler and Zahm, 2005; Commons, 2009; Hioki *et al.*, 2010). Projections from the striatum arise from medium spiny neurons (MSNs) that provide the VTA with substance P, dynorphin and endogenous κ-opioid innervation (Fallon, Leslie and Cone, 1985; Lu, Ghasemzadeh and Kalivas, 1998). Noradrenaline releasing (NA, noradrenergic) projections are supplied to the VTA by the locus coeruleus (LC) (Phillipson, 1979b; Geisler and Zahm, 2005). Input from the LH to the VTA is primarily glutamatergic (Rosin *et al.*, 2003). However, LH-VTA projections containing orexin, α-melanocyte stimulating hormone or dynorphin have been identified (Semba and Fibiger, 1992; Chou *et al.*, 2001; Fadel and Deutch, 2002). Orexins, also known as hypocretins, are solely expressed

in the LH and adjacent perifornical area (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). It has been reported that orexins are crucial for the modulation of sleep and wakefulness (Chemelli *et al.*, 1999; Peyron *et al.*, 2000; Hara *et al.*, 2001). Previous studies reported that orexins can regulate DA activity through LH-VTA projections (Nakamura *et al.*, 2000). In quiet waking, LH orexin projections to VTA DA neurons are silent, but were activated by sensory stimulation. In addition, these neurons were silent during non-REM sleep, but display burst firing activity during periods of REM sleep, implicating VTA DA neurons in the control of sleep and wakefulness (Mileykovskiy, Kiyashchenko and Siegel, 2005).

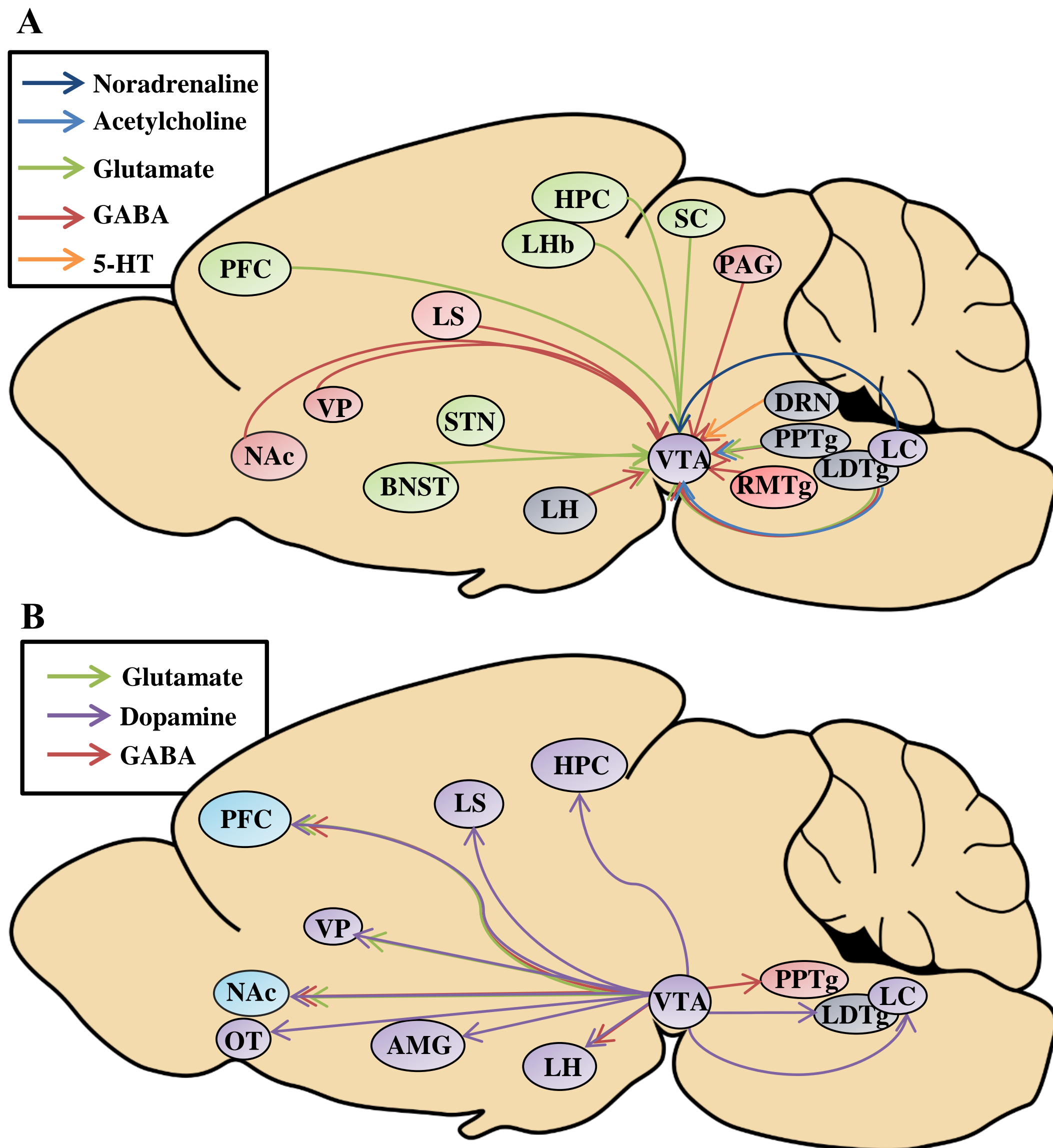


Figure 1.2 – Simplified diagrams of the afferent (A) and efferent (B) VTA connections. (A) Noradrenaline (dark blue), acetylcholine (blue), glutamate (green), GABA (red) and serotonin (5-HT, orange) releasing neurons project to the VTA from the bed nucleus of the stria terminalis (BNST), dorsal raphe nucleus (DRN), hippocampus (HPC), lateral habenula (LHb), lateral hypothalamus (LH), lateral septum (LS), laterodorsal tegmental nucleus (LDTg), locus coeruleus (LC), nucleus accumbens (NAc), pedunculopontine tegmental nucleus (PPTg), periaqueductal gray (PAG), prefrontal cortex (PFC), rostromedial tegmental nucleus (RMTg), subthalamic nuclei (STN), superior colliculus (SC) and ventral pallidum (VP). (B) VTA DA (purple), glutamate (green) and GABA (red) releasing neurons project to the amygdala (AMG), HPC, LC, LH, LS, NAc, olfactory tubercle (OT), PFC, PPTg and VP.

1.3.2 VTA efferents

The VTA and SN provide the only known source of DA to the striatum and limbic forebrain. VTA DA neurons densely, and mainly ipsilaterally, project to the mPFC and ventral striatum (NAc and OT) forming the well-defined mesocorticolimbic system. Lateral regions of the NAc (shell and core) and OT receive DA projections from the aVTA and lateral subregions of the VTA, predominantly the lateral PBP (Ikemoto, 2007). The medial NAc shell and medial OT (mOT) receive substantial input from the pVTA, primarily the medial PBP and PN (Fallon and Moore, 1978; Nauta *et al.*, 1978; Newman and Winans, 1980; Zhou, Furuta and Kaneko, 2003; Ikemoto, 2007, 2010). DA neurons of the PN appear to specifically project to the medial portion of the ventral striatum (Ikemoto, 2007). The VTT provides little, if any, projections to the ventral striatum and is not considered part of the mesocorticolimbic system (Ikemoto, 2007). This preferential mediolateral topography could partly explain the heterogeneous nature of the VTA. VTA DA neurons provide substantial input to other brain regions, such as the retrosplenial cortex (RSC), LC, VP, LS, AMG, LH, LDTg, subventricular zone (SVZ), entorhinal cortex (EC) and hippocampus (HPC) (Lindvall and Bjorklund, 1974; Lindvall *et al.*, 1974; Fallon and Moore, 1978; Beckstead, Domesick and Nauta, 1979; Swanson, 1982; McRae-Degueurce and Milon, 1983; Berger *et al.*, 1985; Ikemoto, Witkin and Morales, 2003). VTA DA neurons specifically project to the ventral subiculum (vSub), CA1 and CA3 regions of the HPC (Geisler and Zahm, 2005) (discussed further in Chapter 6).

Although the majority of VTA efferents are DAergic, VTA DA neurons are intermingled with GABA and glutamate neurons that also generate efferent projections. The lateral septal area receives a high proportion of VTA DA neurons (72%) whereas the AMG (53%), EC (46%) and HPC (6 – 18%) receive substantially less DA innervation from the VTA (Swanson, 1982; Fallon *et al.*, 1984; Gasbarri *et al.*, 1994; Margolis *et al.*, 2006). VTA-derived GABAergic neurons provide inhibitory input to the mPFC, PPTg, LH and NAc (Pirot *et al.*, 1992; Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000; Dobi *et al.*, 2010). Cholinergic

interneurons of the NAc receive inhibitory GABAergic VTA projections (Brown *et al.*, 2012). All VTA subregions contain glutamatergic neurons that project to the NAc and mPFC (Yamaguchi *et al.*, 2007, 2011; Gorelova *et al.*, 2012; Hnasko *et al.*, 2012). The NAc receives little glutamatergic input from the VTA, but receives robust DAergic innervation (65 – 85% DA). The mPFC receives substantial glutamatergic projections compared to VTA DAergic input (30 – 40% DA) (Yamaguchi *et al.*, 2011; Gorelova *et al.*, 2012). VTA glutamatergic neurons substantially innervate the AMG, LHb and VP – in particular the rostral portion of the VP (Hnasko *et al.*, 2012). The differential distribution of glutamate and GABA neurons could be an important factor in the functional heterogeneity demonstrated by the aVTA and pVTA.

Within the VTA, the aVTA receives robust projections from the pVTA, but reciprocal aVTA-pVTA projections are weak (Ferreira *et al.*, 2008). VTA GABAergic interneurons form inhibitory connections to local DA and glutamate neurons (Grace and Bunney, 1985; Johnson and North, 1992; Mansvelder, Keath and McGehee, 2002). In addition, VTA glutamatergic neurons locally synapse on DA and GABAergic VTA neurons providing excitatory input (Dobi *et al.*, 2010). GABAergic interneurons of the VTT provide inhibitory projections to DA neurons of the aVTA and pVTA (Klitenick, DeWitte and Kalivas, 1992; Marinelli *et al.*, 2006; Dobi *et al.*, 2010; Kaufling *et al.*, 2010). The majority of GABAergic VTT neurons project to DA neurons of the VTA, SNc and to a lesser extent the RRF (Ferreira, Del-fava and Hasue, 2008; Jhou *et al.*, 2009a, 2009b; Kaufling *et al.*, 2010). The pVTA also provides substantial, widespread projections to the VTA, SN and RRF (Ferreira *et al.*, 2008).

Co-expression

There is mounting evidence that a subpopulation of VTA DA neurons co-express glutamate and/or GABA (Rodríguez and Gonzalez-Hernandez, 1999; Stuber *et al.*, 2010; Tecuapetla *et al.*, 2010; Tritsch, Ding and Sabatini, 2012). A proportion of VTA DA neurons are reported to

express GABA synthesizing enzyme glutamic acid decarboxylase 65-kD isoform (GAD65) (Tritsch, Ding and Sabatini, 2012). These DA neurons load GABA into vesicles through DA vesicular monoamine transporter 2 (VMAT2). When activated, DA and GABA are co-released at the same synapses (Tritsch, Ding and Sabatini, 2012). A subpopulation of medial VTA DA neurons express vesicular glutamate transporter 2 (vGluT2, DA$^{+vGluT2}$) (Dal Bo *et al.*, 2004; Kawano *et al.*, 2006; Bérubé-Carrière *et al.*, 2009; Hnasko *et al.*, 2010). When stimulated these neurons trigger glutamatergic postsynaptic currents in the NAc and PFC (Chuhma *et al.*, 2004; Lavin *et al.*, 2005; Gorelova *et al.*, 2012). DA$^{+vGluT2}$ neurons of the aVTA project to the NAc, while pVTA DA$^{+vGluT2}$ neurons project to the PFC (Pistillo *et al.*, 2015). Mendez *et al.* (2008) reported that DA and glutamate co-transmission peaked in young animals and declined as the animal aged. At birth, 25% of midbrain DA neurons were reported to co-express vGluT2, which decreased to 14% by 6 weeks of age (Mendez *et al.*, 2008). Moreover, vGluT2 expression was reported to disappear from DA terminals in the striatum of adult rats (Bérubé-Carrière *et al.*, 2009). Further evidence has demonstrated that a subpopulation of midbrain DA neurons co-express specific neuropeptides. A select group of VTA DA neurons that project to the NAc, OT and orbitofrontal cortex were found to co-express cholecystokinin (CCK) (Hökfelt *et al.*, 1980; Seroogy and Fallon, 1989). A separate group of VTA DA neurons that project to the NAc and mPFC were reported to co-express neurotensin (NT) (Hökfelt *et al.*, 1984; Kalivas and Miller, 1984; Studler *et al.*, 1988). In addition, a subset of VTA DA neurons were reported to express both CCK and NT (Seroogy, Mehta and Fallon, 1987; Seroogy *et al.*, 1988).

1.4 Methods of assessing VTA function

In order to determine the functional role of the VTA, extensive experiments have been carried out on different species of animal. The most preferred animal model for the majority of experimental laboratories are rodents. In particular, the rat is small enough to easily house and care for, but large enough to undergo precise experimental manipulations and has the ability to complete complex behavioural tasks. The majority of VTA rodent studies involve lesion formation, cannula implantation, or electrical stimulation to assess neuroanatomical, neurophysiological and/or behavioural effects. Many of these methods aim to cause physical damage (unintentional tract damage from the pipette, cannula tip or electrode can be dealt with by use of appropriate control procedures). The main laboratory methods of assessing VTA function are: -

(a) Lesion studies. Lesions of the whole VTA, or lesions restricted to the aVTA or pVTA are formed using toxins that trigger cell death in a given neural space. Excitotoxins, such as ibotenic acid, are defined as 'non-selective' because they do not discriminate between neurochemically identifiable populations of neurons. A great advantage to excitotoxins is that they spare fibers of passage (Winn, 1991). However, the majority of lesion studies analysing VTA function involve neurotoxins rather than excitotoxins. Neurotoxins target a specific neural population and can be directly infused into brain regions to form selective lesions. 6-hydroxydopamine (6-OHDA) is specifically toxic to catecholamine groups (DA and noradrenaline [NA] neurons). It can be made selective for DA neurons by either infusing it into areas that contain no NA neurons or terminals, or by administering a NA reuptake blocker prior to 6-OHDA. This prevents uptake of toxin into NA, but not DA neurons. Previous studies have used 6-OHDA to destroy DA neurons of the VTA or SNc allowing the loss of function to be assessed (Emborg, 2004; Pioli *et al.*, 2008; Duty and Jenner, 2011; Ouachikh *et al.*, 2013). In addition, 6-OHDA can be infused directly into midbrain DA terminal regions or projections through the medial forebrain bundle (mfb) specifically destroying DA input to

selected regions, preventing DA release and reuptake (Yuan *et al.*, 2005; Sellings *et al.*, 2008). Previous studies investigated the functional and behavioural effect of 6-OHDA lesions combined with drug intravenous self-administration (IVSA). 6-OHDA lesions of the VTA or NAc DA terminals severely disrupted nicotine, amphetamine and cocaine IVSA (Lyness, Friedle and Moore, 1979; Roberts *et al.*, 1980; Roberts and Koob, 1982; Pettit *et al.*, 1984; Corrigall *et al.*, 1992; McGregor and Roberts, 1993; Caine and Koob, 1994). 6-OHDA DA terminal lesions of the NAc shell, but not the core, abolished IVSA of nicotine (Sellings *et al.*, 2008). In fact, DA lesions of the NAc core appeared to increase the rewarding effect of nicotine (Sellings *et al.*, 2008). These behavioural effects are not observed in the NAc unless > 90% of DA terminals are destroyed (Ikemoto and Panksepp, 1999). Although 6-OHDA can be directly infused into the VTA to selectively damage A10 neurons, it holds the potential to diffuse to A8 and/or A9 areas forming DA lesions. A number of studies have attempted to selectively destroy the afferent projections to VTA DA neurons. As previously discussed, VTA DA neurons receive cholinergic projections from the mesopontine tegmental nuclei. In order to investigate the role of mesopontine cholinergic input to VTA DA neurons, a selectively cholinergic toxin is required. Previous studies have attempted to develop a toxin that can selectively destroy cholinergic neurons. IgG 192-saporin can selectively lesion cholinergic neurons of the basal forebrain, but this toxin had no effect on the cholinergic neurons of the mesopontine tegmentum (Wiley, Oeltmann and Lappi, 1991; Book, Wiley and Schweitzer, 1994). The fusion toxin IgG 192-saporin selectively binds to low-affinity nerve growth factor receptors, which are absent in cholinergic neurons of the PPTg and LDTg (Cuello *et al.*, 1990; Suter *et al.*, 1992). It was thought that ethylcholine mustard aziridinium ion (AF64A) could selectively destroy PPTg cholinergic neurons – AF64A appeared to create selective lesion sites (Sandberg *et al.*, 1985; Lança *et al.*, 2000). However, it was found to form non-selective lesions damaging the site of infusion and (progressively) beyond (Rodríguez *et al.*, 1998). A fusion toxin diphtheria urotensin-II (Dtx-UII) has been developed which is highly selective for cholinergic neurons of the mesopontine tegmentum (Clark *et al.*, 2007) (discussed further

in Chapter 3). Dtx-UII binds to urotensin-II receptors (UII-Rs) on cholinergic neurons that activates the receptor, triggering a conformational change and receptor internalisation (Giebing *et al.*, 2005). The toxin is then engulfed by endosomes that facilitate the entry of Dtx-UII into the cytosol (Simpson *et al.*, 1998). Once Dtx-UII is released into the cytosol, protein synthesis is inhibited and cholinergic neurons are gradually destroyed over a period of days, leaving non-cholinergic neurons intact (Clark *et al.*, 2007; MacLaren, 2012).

(b) Histological analysis. Histological techniques can assess recent neuronal activation in post-mortem tissue. Staining for immediate early gene (IEG) activation, such as encoded transcription factor *c-fos*, can map recent neuronal activation in target brain regions (further discussed in Chapters 4, 5 and 6) (Duffield, Hastings and Ebling, 1998; Emmert and Herman, 1999; Novak, Smale and Nunez, 2000; Morrow, Elsworth and Roth, 2001; Pacák and Palkovits, 2001). IEG histology can be combined with selective staining for identifiable neuronal populations, such as tyrosine hydroxylase (TH) to analyse the recent activity of VTA DA neurons following drug administration or behavioural paradigms (Kiba and Jayaraman, 1994; Curran, Akit and Watson, 1996; Pich *et al.*, 1997; Lanca, Sanelli and Corrigall, 2000; Porter, 2008). Although this tool can be powerful, IEG expression is limited to use in post-mortem tissue within a specific timeframe. Therefore, this histological method is not ideal for all experimental designs.

(c) Electrical stimulation. In 1954, Olds and Milner discovered that direct electrical stimulation of discrete brain regions could be powerfully rewarding. Electrodes were surgically implanted into specific brain regions and rodents were subjected to behavioural testing. During testing, animals repeatedly returned to an area of the maze in which they had received intracranial electrical stimulation (Olds and Milner, 1954). This behaviour was supported by some electrode placements, but not others, which led to the interpretation that the brain had specialised “reward systems” (Wise, 1978). Olds and Milner mapped “pleasure centres” where electrode placements triggered activity that appeared to be rewarding. Olds and Milner

subsequently developed a method of electrical intracranial self-stimulation (ICSS). Animals were placed in an environment with free access to a lever. When pressed, the lever would deliver an electrical impulse to the tip of the implanted electrode, stimulating surrounding neurons. Animals worked to achieve direct electrical stimulation of discrete brain regions – in particular regions associated with the mesocorticolimbic system. The NAc, VTA or medial forebrain bundle (mfb, VTA neurons projecting to the NAc) have previously been targeted using ICSS (Phillips, Brooke and Fibiger, 1975; Phillips and Fibiger, 1978; Hunt and McGregor, 1998; Ishida *et al.*, 2001). Although ICSS can achieve some degree of anatomical specificity, there is no method of discriminating between neurochemical identities of neuron populations that are electrically stimulated. The implanted electrode can simultaneously stimulate multiple pathways implicated in reward. A further drawback of ICSS is that the electrode could stimulate fibers of passage rather than local cell bodies (Ranck, 1975). Following the powerful discovery that direct electrical stimulation of brain regions could be rewarding, a method of chemical self-administration was soon developed to identify neurochemicals involved in reward (discussed below).

(d) Electrophysiology. This powerful technique determines the activity of a population of neurons in relation to discrete behavioural events. For example, if the initiation of an action is related to a specific neuron, a change in the spiking activity of the neuron must precede the onset of that action. On the other hand, if a neuron is thought to encode a specific function, such as the magnitude of a reward, a change in firing will follow the receipt of the reward. The current understanding of the functions of midbrain DA neurons has been strongly influenced by *in vitro* and *in vivo* single-unit electrophysiology. Many *in vivo* electrophysiological studies have been conducted aimed at the VTA of primates or rats (Schultz, 1998; Pan *et al.*, 2005). These studies reported that VTA DA neurons show transient changes in spike activity under a variety of conditions, such as: - exposure to novel or salient stimuli, unexpected rewards, reward predictive cues (visual or auditory), stress or noxious stimuli (Schultz, 1998; Hyland

et al., 2002; Ungless, 2004; Dommett *et al.*, 2005). For example, VTA DA neurons in monkeys respond with short-latency, phasic activity when the animal is presented with a primary food reward (Ljungberg, Apicella and Schultz, 1992; Schultz, Apicella and Ljungberg, 1993; Mirenowicz and Schultz, 1996). When the repeated presentation of food is reliably predicted by visual or auditory cues, DA activity is temporally advanced to become associated with the stimuli and the response to the reward stimulus is no longer present (Ljungberg, Apicella and Schultz, 1992; Mirenowicz and Schultz, 1996). However, if the reward is omitted, DA neuron activity is silenced at the time of the predicted reward (Schultz, Apicella and Ljungberg, 1993; Mirenowicz and Schultz, 1996; Schultz, 1997; Waelti, Dickinson and Schultz, 2001). Furthermore, if the animal receives an unpredicted reward, this elicits the activation of VTA DA neurons (Schultz, 1997; Bromberg-Martin, Matsumoto and Hikosaka, 2010). These studies strongly suggest that VTA DA neurons in animals play a central role in reward-mediated learning. However, it has been stated, that there is a need for improved criteria to reliably identify DA neurons (Margolis *et al.*, 2006). Subsets of DA neurons demonstrate different patterns of response (Kiyatkin and Rebec, 2001; Hyland *et al.*, 2002; Tobler, Dickinson and Schultz, 2003). The conclusion that these neurons are DAergic is critical to the hypotheses that the activity of DA neurons mediate motivational behaviours, reward and drug addiction.

(e) Optogenetics. This research method combines optics and genetics to specifically target discrete populations of neurons and their projections (Boyden *et al.*, 2005). Optogenetic techniques introduce the expression of microbial opsins in neurons of target brain regions. Opsins are light-activated ion-transporting membrane proteins or protein pumps, which allow the electrical activity of transfected neurons to be controlled by light. The most common opsins utilised for optical control are neuron-activating cationic channel protein channelrhodopsin-2 (ChR2), neuron-silencing cation channel transporter halorhodopsin (NpHR) or protein pump archaerhodopsin-3 (Arch) (Nagel *et al.*, 2003; Boyden *et al.*, 2005; Li *et al.*, 2005; Han and

Boyden, 2007; Zhang *et al.*, 2007; Chow *et al.*, 2010). Within milliseconds, a brief pulse of light can induce neural activation or inhibition with unprecedented specificity and temporal precision. Neural populations can be selectively inhibited or stimulated to determine functional circuitry of networks rather than neural connectivity (Ikemoto, Yang and Tan, 2015; Pistillo *et al.*, 2015). The majority of opsin genes are introduced using *in vivo* recombinant viral vectors or via *in utero* electroporation (IUE). Optogenetic techniques are primarily used in drosophila, *C. elegans* nematode, zebrafish and mouse models. Previous optogenetic studies targeted regions of the rodent mesocorticolimbic system. Combining optogenetics with conditioned place preference or self-simulation, the expression of ChR2 in the VTA confirmed that the VTA-striatal DA system is important for reward-related behaviours and mood-regulation (Tsai *et al.*, 2009; Witten *et al.*, 2011; Kim *et al.*, 2012; Ilango *et al.*, 2014a, 2014b). In addition, optogenetic stimulation of DA terminals in the NAc was found to be rewarding (Steinberg *et al.*, 2014). However, it took 2 days of high-frequency VTA DA optogenetic stimulation to establish conditioned place preference, while low-frequency stimulation had no effect (Tsai *et al.*, 2009). The inhibition of VTA DA neurons using photostimulation of NpHR induced aversion, demonstrated by place avoidance (Ilango *et al.*, 2014a). However, the reinforcing properties of ICSS were not reproducible through intracranial optogenetic self-stimulation of VTA DA neurons (Adamantidis *et al.*, 2011). This powerful technique provides the precise temporal control of neural activation or inhibition in a given circuitry. Although this stimulation is highly reproducible and produces stable responses, some disadvantages and conflicting reports have emerged (Tye *et al.*, 2012; Chaudhury *et al.*, 2013; Roeper, 2013). Roeper (2013) reports that optogenetics triggers behavioural patterns displayed after optical neural stimulation or inhibition in unnatural, spatial and temporal patterns that would otherwise never occur.

(f) DREADDs. Designer Receptors Activated by Designer Drugs (DREADDs) are modified G-protein coupled receptors (GPCRs) that are exclusively activated by synthetic ligands, but

are insensitive to their endogenous ligands (Armbruster and Roth, 2005; Armbruster *et al.*, 2007). This chemogenetic technique mediates transient control over the activation or inactivation of targeted brain regions. DREADD receptors can be introduced into the neural Systemic administration of a biologically inert synthetic ligand, such as clozapine-N-oxide (CNO), binds to DREADD receptors. tissue by methods of gene transfer, such as intracranial delivery of viral vectors containing the DREADD transgene (Rogan and Roth, 2011). For example, M4 muscarinic acetylcholine receptor is specifically engineered to selectively bind with CNO. The modified M4 receptor is known as hM4Di (Armbruster *et al.*, 2007; Rogan and Roth, 2011). DREADD activation leads to membrane hyperpolarisation, decreased cAMP signalling and increased activation of inward rectifying potassium channels (Armbruster *et al.*, 2007; Rogan and Roth, 2011). This results in a temporary suppression of neural activity similar to the action of the endogenous receptor. This relatively non-invasive technique can be combined with techniques such as lesion formation or drug administration, or even optogenetics. DREADDs have previously been used to target regions of the mesocorticolimbic system, such as the mPFC, NAc core and VTA (Bull *et al.*, 2014; Chang *et al.*, 2015; Scofield *et al.*, 2015; Yau and McNally, 2015). Projections from rostral and caudal subregions of the VP to VTA DA neurons were specifically targeted using modified hM4Di receptor expression and cocaine intravenous self-administration (Mahler *et al.*, 2014). DREADD expression demonstrated that the projections from the rostral VP to VTA DA neurons are critically involved in cocaine-seeking behaviour (Mahler *et al.*, 2014). DREADDs regulate the activity of discrete neuronal circuits to analyse the functionality of the targeted system and the behavioural response demonstrated by the animal. This technique possesses similar advantages to optogenetics, but without the temporal precision.

1.5 VTA function

Although the VTA is composed of neurons that release DA, GABA and glutamate, the majority of VTA research primarily focuses on VTA DA neurons. Midbrain DA neurons have been associated with a number of functions including reward-related behaviours (natural or drug-induced), aversion, salience, novelty, uncertainty, motivation, learning and goal-directed behaviours (Wise, 1978, 2008; Schultz and Dickinson, 2000; Ungless, 2004; Fields *et al.*, 2007; Grace *et al.*, 2007; Bromberg-Martin, Matsumoto and Hikosaka, 2010; Ungless, Argilli and Bonci, 2010; Volman *et al.*, 2013; Creed, Ntamati and Tan, 2014; Lammel, Lim and Malenka, 2014; Walsh and Han, 2014). Furthermore, midbrain DA neurons play an important role in pathologies such as Parkinson's disease, schizophrenia, depression, mood disorders, and addiction (Hornykiewicz, 1962; Marinelli and White, 2000; Nestler and Carlezon, 2006; Yadid and Friedman, 2008; Cao *et al.*, 2011; Valenti *et al.*, 2011; Ikemoto and Bonci, 2014; Nikulina *et al.*, 2014). The functional role of the VTA is a topic of extensive literature, and for the purpose of this thesis not all VTA functions shall be discussed – primarily reward-related behaviours and drug-induced reward shall be covered.

1.5.1 Reward

In behavioural neuroscience, the terms "reward" and "reinforcement" are often misused – especially in literature concerning drug addiction. Reward is defined as the positive incentive value that an animal assigns to a stimulus. For example, the reward value of a stimulus, such as food, fluctuates depending on the state of the animal. A hungry animal would find food more rewarding than a sated animal. Reinforcement is defined as the ability of a stimulus to support a learned behaviour. For example, if food is given following a lever press and the animal increases lever-pressing behaviour to acquire more food, then food is said to be acting as a reinforcer. A relationship exists between reward and reinforcement: a stimulus that is intrinsically rewarding supports reinforcement learning. However, these two terms have very

different definitions, and should not be used interchangeably. It should also be noted that not all rewards are "liked" in the sense of producing pleasure, but are critical for triggering goals to become "wanted" which leads to motivated actions to achieve them (Berridge and Robinson, 1998; Salamone *et al.*, 2007).

As demonstrated by Olds and Milner (1954), electrical stimulation of specific brain regions can be powerfully rewarding. Animals learn to lever-press repeatedly to achieve self-stimulation. This demonstrated that electrical stimulation of specific brain regions reinforced lever-pressing behaviour. In order to achieve ICSS, animals would run across aversive electrical shock grids or ignore warning signals of an aversive shock (Olds, 1958; Valenstein and Beer, 1962). This influential work demonstrated that ICSS was powerfully rewarding and led to numerous investigations to determine the neurotransmitters and neural systems that underlie this behaviour. Electrophysiological, pharmacological and behavioural studies identified DA as a crucial neurotransmitter contributing to reward-seeking and motivational behaviours (Wise and Bozarth, 1984; Wise, 2004, 2008). As previously mentioned, VTA DA neurons robustly project to the mPFC and ventral striatum (NAc and OT) forming the well-defined mesocorticolimbic system. This system is composed of two pathways that connect VTA DA neurons (in the mesencephalon: meso-) to the prefrontal cortex (-cortico: PFC) and the ventral striatum (-limbic: NAc and OT). Animals work to achieve direct electrical stimulation of brain regions associated with the mesocorticolimbic system, such as the PFC, NAc, VTA or medial forebrain bundle (mfb, fibers projecting from the VTA to the NAc) (Phillips, Brooke and Fibiger, 1975; Phillips and Fibiger, 1978; Hunt and McGregor, 1998; Ishida *et al.*, 2001). Previous studies reported that electrical self-stimulation of this system involves the activation of DA neurons (Fibiger and Phillips, 1986; Wise and Rompre, 1989). Selective DA 6-OHDA lesions of the NAc significantly reduced lever-pressing behaviour (Phillips and Fibiger, 1978; Fibiger *et al.*, 1987). Furthermore, electrical self-stimulation was attenuated when DA synthesis was inhibited, depolarisation of DA neurons was inactivated,

and when DA receptor antagonists were administered systemically or directly into the NAc (Fouriezos and Wise, 1976; Edmonds and Gallistel, 1977; Mogenson *et al.*, 1979; Rompré and Wise, 1989). ICSS studies show that DA projections from the VTA to the NAc are particularly vital for reward-related behaviours. Electrical self-stimulation of the mfb was reduced by microinfusion of DA antagonists into the ventral striatum, but not the dorsal striatum (Stellar, Kelley and Corbett, 1983; Stellar and Corbett, 1989) while amphetamine administration into the NAc decreased the frequency of ICSS (Colle and Wise, 1988). Electrical self-stimulation of the lateral hypothalamus (LH) was greatly reduced in animals with selective 6-OHDA lesions of the VTA (Koob *et al.*, 1978).

There is strong evidence that VTA DA neurons and target regions play an integral role in reward-related and approach behaviour (Wise and Rompré, 1989; Schultz, 1997, 2002, 2007; Wise, 2004). However, it should be noted that there is some controversy surrounding the precise role DA plays in reward, and if the mesocorticolimbic system actually mediates reward (Salamone, 1994; Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999). The majority of VTA DA neurons are inhibited by aversive stimuli, such as a foot shock or tail pinch (Mirenowicz and Schultz, 1996; Ungless, Magill and Bolam, 2004; Ungless, Argilli and Bonci, 2010). The population of DA neurons that were inhibited by the aversive stimulus were then activated once the foot shock had ended (Brischoux *et al.*, 2009). A relatively small population of DA neurons respond to aversive stimuli, such as an air puff to the hand of a non-human primate (Mirenowicz and Schultz, 1996). Zweifel *et al.* (2011) demonstrated that a tail pinch activated and inhibited roughly equal numbers of VTA DA neurons. It has been suggested that any acute increase in DA neuron activity could be due to the cessation of the aversive stimulus (Tanimoto, Heisenberg and Gerber, 2004).

Although ICSS achieves some degree of anatomical specificity, there is no control or method of discriminating between neurochemical identities of neuron populations that are electrically stimulated. Identifying the neurocircuitry and systems responsible for reward requires

experimental methods with precise control and manipulations of specific brain regions. A method of drug self-administration was developed to identify neurochemicals involved in reward. Pharmacological stimulation, rather than electrical stimulation, can selectively target receptors in discrete brain regions, such as regions of the mesocorticolimbic system which is heavily implicated in mediating the rewarding effects of drugs of abuse (Fibiger and Phillips, 1986; Wise and Bozarth, 1987; Koob, 1992; Robbins and Everitt, 1996; Bowers, Chen and Bonci, 2010). Because ICSS had so strongly implicated DA in reward, one of the original theories of drug-reward, was that all drugs of abuse act through DAergic systems (Wise, 1980). The VTA at least partly mediates the rewarding effects of drugs of abuse. There is mounting evidence that VTA DA projections to the NAc shell are particularly important in drug-induced reward (Fibiger and Phillips, 1986; Wise and Bozarth, 1987; Koob, 1992; Robbins and Everitt, 1996; Bowers, Chen and Bonci, 2010). The mesocorticolimbic system is considered essential for mediating the reinforcing and motivational properties of nicotine, cocaine, ethanol, cannabinoids, and opiates (Fibiger and Phillips, 1986; Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Koob, 1992; Robbins and Everitt, 1996; Bowers, Chen and Bonci, 2010). Mechanisms of drug reward are much more complex than first thought and DA is unlikely to solely mediate rewarding effects. The literature indicates that reward arises from the activation of distinct systems across multiple brain regions, which is experienced as rewarding (Ikemoto, 2010). To fully understand the large-scale circuitry that facilitates drug reward, it is necessary to identify the key regions involved, and to determine their relationship with the mesocorticolimbic system. The value of a reward cannot be measured or visualised: it must be quantified through behavioural reactions such as response times or choices. A number of behavioural paradigms have been developed to assess the role of the VTA in drug reward that demonstrate how hard an animal will work to receive a drug or neurochemically active compound.

1.5.2 Conditioned place preference

The motivational properties of drugs of abuse can be investigated through conditioned place preference (CPP) or intracranial CPP (IC-CPP) behavioural paradigms. During the conditioning phase animals are repeatedly administered a drug (stimulus) in a compartment of the place-conditioning chamber. In a second compartment, animals are administered a control stimulus an equivalent number of times. During testing no stimulus is present and the animals are allowed access to both compartments of the chamber. Typically, animals spend more time in the compartment paired with the rewarding stimuli. Therefore, if the time spent in the drug-paired environment is more than the vehicle-paired environment the drug is said to be reinforcing. Intra-NAc administration of amphetamine or DA receptor agonists were found to induce CPP (Carr and White, 1983, 1986; Hiroi and White, 1991). Intra-VTA administration of nicotine or nAChR agonists cysteine established CPP (Museo and Wise, 1994; Laviolette and van der Kooy, 2003). Microinjections of DA antagonists into the shell, but not the core of the NAc, attenuated CPP induced by systemic nicotine (Spina *et al.*, 2006). Furthermore, selective lesions of DA terminals in the medial OT disrupted CPP induced by intravenous administration of cocaine (Sellings, Mcquade and Clarke, 2006). CPP established by intravenous administration of cocaine or amphetamine is blocked by DA terminal lesions in the medial NAc shell, but not the core (Sellings and Clarke, 2003; Sellings, Mcquade and Clarke, 2006). In addition, cocaine injections into the NAc shell, but not the core, established CPP (Liao *et al.*, 2000). Carbachol administration into the pVTA, but not the aVTA, induced CPP (Ikemoto and Wise, 2002). These results suggest that the VTA and NAc are functionally heterogeneous structures. Furthermore, drug-induced CPP paradigms demonstrate the importance of the mesocorticolimbic system in drug-reward and reinforcement. However, this method of assessing drug-reinforcement does not determine response rates, evaluate extinction behaviour or examine patterns of drug administration behaviour.

1.5.3 Intravenous self-administration

The development of intravenous self-administration (IVSA) replicates intravenous drug-taking behaviour found in humans. Rats are trained to intravenously self-administer drugs through lever-pressing behaviour. IVSA is a direct measure of drug reward, because it demonstrates how hard an animal will work to receive one. Regions of the mesocorticolimbic system have been implicated in the rewarding effects of IVSA of drugs. Animals with selective NAc DA lesions or postsynaptic lesions demonstrate decreased IVSA of cocaine and d-amphetamine (Lyness, Friedle and Moore, 1979; Roberts *et al.*, 1980). Selective lesions of VTA DA neurons that project to the ventral striatum attenuated nicotine IVSA (Corrigall *et al.*, 1992). Nicotine IVSA was also prevented by intra-VTA infusion of nicotinic acetylcholine receptor (nAChR) antagonist dihydro-β-erythroidine (DHβE) (Corrigall, Coen and Adamson, 1994). These results demonstrate that animals will readily administer nicotine through IVSA. However, nicotine is not normally administered via the intravenous route. It should be noted that there are pharmacokinetic differences between intravenous nicotine and nicotine acquired from inhalation. Although IVSA allows the assessment of reinforcing effects of a variety of substances, systemically administered drugs have multiple actions at a wide range of sites. Some of which could conceivably counteract other actions, not necessarily contributing to reward. Occasionally, drugs that are not rewarding when administered systemically are readily administered directly into specific brain regions (Ikemoto and Bonci, 2014). For example, systemic picrotoxin leads to anxiety and aversion, but is readily administered by rats into the posterior hypothalamic area (File, 1986; Ikemoto, 2005). Furthermore, systemically administered baclofen results in a sedative effect, yet it can induce psychostimulant and rewarding effects when directly administered into the midbrain raphe nuclei (Shin *et al.*, 2010; Vollrath-Smith, Shin and Ikemoto, 2012).

1.5.4 Intracranial self-administration of drugs

Unlike IVSA, intracranial self-administration (ICSA) does not mimic drug-taking or drug-seeking behaviour exhibited by humans. ICSA aids the identification of specific brain regions that are chemical trigger zones for the rewarding actions of drugs of abuse. Through ICSA, rats learn to self-administer precise volumes (50 – 100nl) of drugs or neurochemically active compounds into discrete regions of their own brain (Stein and Olds, 1977; Ikemoto and Wise, 2004). Intracranially administering small volumes of drugs reduces diffusion from the injection site, therefore, drugs can activate specific receptors in discrete brain regions that trigger rewarding or aversive effects, or no effect at all (McBride, Murphy and Ikemoto, 1999). Larger volumes can diffuse from the injection site to act at more distal regions (McBride, Murphy and Ikemoto, 1999). In addition, the injected drug can cause local non-specific damage at the site of injection (McBride, Murphy and Ikemoto, 1999). Therefore, careful pharmacological characterisation of the drug must be carried out prior to testing. The ICSA system must accurately deliver precise volumes of the drug with minimal delay to achieve and maintain self-administration behaviour. Furthermore, the behavioural paradigm must have appropriate experimental controls to separate non-specific motor effects from goal-directed behaviours (Wise and Hoffman, 1992).

Bozarth and Wise (1981) demonstrated that animals would self-administer morphine directly into the VTA. An electrolytic microinjection transducer (EMIT) system was used to deliver morphine (100nl) from a head-mounted reservoir (Criswell, 1977; Bozarth and Wise, 1980, 1981). An electrical current was applied to the reservoir that produced hydrogen gas, increasing the internal pressure and forcing the drug solution out of the cannula and into the brain. Although this method was successfully used in several ICSA studies, the system was difficult to calibrate and the production of hydrogen gas could interact with certain chemical species (Bozarth and Wise, 1980, 1981; Ikemoto *et al.*, 1997; Ikemoto, Murphy and McBride, 1997, 1998; Ikemoto and Wise, 2004). A further drawback of the EMIT system is that it was

difficult to obtain consistent infusion volumes (Ikemoto and Sharpe, 2001). Welzel (1989) developed a micropipette delivery system for ICSA that consisted of a glass micropipette connected to an air-pressure system. This pressure system could deliver volumes of drugs (0.5 – 100nl) (Welzl, Kuhn and Huston, 1989). Drugs can also be intracranially administered through iontophoresis or reverse dialysis – these methods are not widely used for self-administration studies (Aghajanian and Davis, 1975; Quan and Blatteis, 1989). A head-attached micropump-injection system for intracranial drug delivery was developed (Ikemoto and Sharpe, 2001). Mechanical pressure produced by a computer-controlled stepping motor drives intracranial drug infusion, reliably and repeatedly delivering consistent volumes of the drug (Ikemoto and Sharpe, 2001). This simple system can be precisely controlled, and eliminates any potential electrochemical interaction between electrical currents and drug of interest. This method has been successfully used in several ICSA studies (Ikemoto and Wise, 2002, 2004; Zangen *et al.*, 2002; Ikemoto, Witkin and Morales, 2003; Farquhar, Latimer and Winn, 2012).

The majority of ICSA studies are carried out in an operant chamber where an animal is required to correctly respond to a stimulus or cue in order to trigger drug delivery. Bozarth and Wise (1981) demonstrated that experimentally naïve rats would learn to press a single lever with a light cue to deliver morphine directly into the VTA (Bozarth and Wise, 1981). However, a single-lever operant chamber does not determine if a drug is reinforcing or generally increasing motor activity. Therefore, it is beneficial to adopt a two-lever within-subjects approach. Here, the animal is presented with two identical levers, an active and inactive lever. Depression of the active lever triggers drug administration, while the inactive lever does not. Adopting the two-lever approach, Hoebel *et al.* (1983) demonstrated that d-amphetamine was readily administered into the NAc. Rats maintained amphetamine responding when the active and inactive levers were switched (Hoebel *et al.*, 1983). Furthermore, rats would stop lever pressing when amphetamine was substituted for the

vehicle, and resumed responding when amphetamine was reinstated (Phillips, Robbins and Everitt, 1994). ICSA of cocaine (90 – 100 pmol) into the NAc and VTA did not support lever-pressing behaviour in a single-lever operant paradigm until after 12 – 15 testing sessions (Goeders and Smith, 1983; Carlezon, Devine and Wise, 1995). In a two-lever operant paradigm, higher doses of cocaine (800 pmol) self-administered into the NAc supported lever-pressing behaviour (McKinzie *et al.*, 1999). These animals discriminated between the active and inactive levers. When the vehicle was substituted for cocaine lever-pressing behaviour was not present, but animals resumed lever pressing when cocaine was reinstated (McKinzie *et al.*, 1999). An alternative approach to within-subjects ICSA is a between-subjects yoked control procedure. Here, two animals are placed in separate operant chambers. The "executive" animal is presented with an active and inactive lever, whereas the "yoked control" animal is never presented with an active lever. When the "executive" animal responds to the cue stimulus, both animals receive a drug infusion at the same time. An advantage of this method is that the lever-pressing behaviour of the yoked control animal is never reinforced and its behaviour is only attributed to drug-induced arousal. However, a disadvantage to this approach is that the injection sites of the executive and yoked animals will not be identical. In addition, this procedure requires more animals, more surgeries and more operant chambers, which could be why this method is used less frequently than the within-subjects two-lever approach.

A popular alternative to lever-pressing operant chambers is the nose-poke apparatus. Animals are presented with one or two face-sized holes in a wall of the operant chamber. Animals break a photocell beam at the back of the hole with their nose to trigger the administration of a drug. A nose-poke to the active hole triggered drug administration, while a nose-poke to inactive hole did not. A light was presented for each nose-poke to the active hole. Welzel (1989) reported that rats would readily self-administer morphine by nose-poking a hole in the wall (Welzl, Kuhn and Huston, 1989). Welzel (1989) observed that rats would readily administer

morphine into regions of the VTA on a continuous reinforcement (CRF) or fixed ratio-2 (FR2) schedule. CRF delivered morphine after every nose-poke, whereas FR2 only delivered morphine after every second correct nose-poke. An FR2 schedule demonstrated that reinforcement remained as the response requirements increased. However, as with single-lever operant paradigms, single hole nose-poke apparatus does not differentiate between drug-induced motor activity and reinforced behaviour. Self and Stein (1993) developed a method of ICSA in an operant chamber with two nose-poke holes, where the active hole was illuminated by a cue light. A nose-poke in the active hole triggered the infusion of morphine over 5 sec and a continuous tone sounded with the infusion (Self and Stein, 1993). Although this study provided an inactive nose-poke hole, the number of nose-pokes for the active and inactive holes were not compared (Self and Stein, 1993). An advantage to using the nose-poke method of ICSA is that less effort is required to train an animal to exhibit nose-poke behaviour. However, this advantage is also a substantial disadvantage for this procedure. Nose-poke operant rates are high, which makes it difficult to determine the rate of learning that has taken place.

These results demonstrate that the ideal ICSA paradigm should allow an animal to: (1) distinguish between an active and inactive lever/hole, (2) demonstrate extinction when a drug is switched for a vehicle, (3) maintain reinforcement when response requirements increase, and (4) demonstrate lever/hole reversal when the active and inactive lever/holes are switched. Furthermore, it is essential to include appropriate control conditions to conclude that the target region triggered rewarding or aversive effects, or no effect at all (Wise and Hoffman, 1992).

ICSA lever-pressing studies have refined our understanding of specific regions of the mesocorticolimbic system and their involvement in mediating the rewarding effects of drugs of abuse. Animals will self-administer drugs directly into regions of the mesocorticolimbic system, in particular the pVTA. In a single-lever operant chamber, phencyclidine (PCP) was self-administered directly into the mPFC and NAc shell, but not the NAc core (Carlezon and

Wise, 1996). Animals readily discriminated between two levers to self-administer the principle active component of cannabis, $^{\Delta9}$-tetrahydrocannabinol ($^{\Delta9}$THC), into the pVTA and medial shell of the NAc, but not the core (Zangen *et al.*, 2006). In addition, low doses of $^{\Delta9}$THC were self-administered into the caudal portion of the aVTA (Zangen *et al.*, 2006). Several two-lever within-subjects ICSA studies report that the NAc shell, in particular the medial shell, appears to be more important than the core for drug reward. Rats learn to self-administer cocaine, amphetamine or DA receptor agonists directly into the shell but not the core (Phillips, Robbins and Everitt, 1994; Ikemoto *et al.*, 1997; Rodd-Henricks *et al.*, 2002; Ikemoto, Witkin and Morales, 2003; Ikemoto, 2010). Rats willingly self-administer cocaine and amphetamine in to the medial NAc shell, but not the lateral shell (Ikemoto, Qin and Liu, 2005). Furthermore, cocaine and amphetamine are readily administered into the medial OT (mOT), compared to the lateral OT (Ikemoto, Witkin and Morales, 2003; Ikemoto, Qin and Liu, 2005). Animals continue to self-administer cocaine into the OT at much lower doses compared to the NAc shell (Ikemoto, Witkin and Morales, 2003). Combined, these results suggest that VTA DA neurons that project to the NAc shell and mOT are involved in drug reward – and that the ventral striatum demonstrates functional heterogeneity. Similarly, ICSA revealed the functional heterogeneous nature of the VTA. The pVTA mediates the rewarding effects of neurochemically active chemicals and drugs more readily than the aVTA. Cholinergic agents (carbachol, nicotine and neostigmine), opiates (endomorphin-1), cocaine, cannabinoids ($^{\Delta9}$THC), alcohol-related compounds (ethanol, ethanol metabolites acetaldehyde and salsolinol) and serotonin-3 receptor agonists are more readily administered into the pVTA than into the aVTA (Rodd-Henricks *et al.*, 2000; Ikemoto and Wise, 2002; Zangen *et al.*, 2002, 2006; Rodd *et al.*, 2004, 2005, 2007, 2008; Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). Ikemoto and Wise (2002) demonstrated that rats learn to self-administer non-selective cholinergic agonist carbachol into the pVTA. Furthermore, rats self-administer the acetylcholinesterase inhibitor neostigmine (which increases local endogenous ACh concentration) into the pVTA (Ikemoto and Wise, 2002). Rats learn to self-administer opiates

directly into the VTA (Bozarth and Wise, 1981; Welzl, Kuhn and Huston, 1989; David and Cazala, 1994; Devine and Wise, 1994). Endomorphin-1, an endogenous ligand for mu opiate receptors, was self-administered directly into the pVTA (Zangen *et al.*, 2002). As discussed above, initial studies reported that animals would not self-administer cocaine into the VTA (Goeders and Smith, 1993). However, it has now been demonstrated that rats learn to self-administer higher doses of cocaine directly into the pVTA in a two-lever operant paradigm (Rodd *et al.*, 2005). In addition, Wistar rats which do not readily drink ethanol solutions, readily self-administer ethanol into the VTA (Rodd-Henricks *et al.*, 2000, 2003; Rodd *et al.*, 2004). Ethanol and acetaldehyde are self-administered into the pVTA, but not the aVTA (Rodd-Henricks *et al.*, 2000, 2002).

1.6 Aims

The aim of this thesis is to investigate the role of mesopontine cholinergic input to midbrain dopamine (DA) neurons. As discussed above, the mesopontine nuclei provide the VTA with significant cholinergic projections that release endogenous acetylcholine (ACh) (Semba and Fibiger, 1992). These projections provide excitation of midbrain DA neurons that are believed to be important for reward functions. However, the precise function of cholinergic input to the VTA is not known. To further understand the role of cholinergic projections to midbrain DA neurons, it is important to understand where ACh comes from – namely the PPTg and LDTg.

In Chapter 3, the first strategy was to use Dtx-UII fusion toxin as a method of cholinergic denervation of the pVTA. Since the pVTA receives cholinergic projections from the PPTg and LDTg, selective cholinergic lesions of the PPTg would not destroy LDTg projections to target structures, which could compensate for damaged PPTg input. In order to determine the behavioural effect of Dtx-UII PPTg lesions accurately, complete mesopontine cholinergic lesions had to be developed. Instead of performing multiple surgeries to lesion the cholinergic cell bodies of the pPPTg and LDTg, Dtx-UII was infused directly into the pVTA to destroy

the mesopontine cholinergic input. To assess lesion progression and the functional effect of Dtx-UII in the pVTA, basic locomotor behaviour was measured. Selective cholinergic lesions of the pVTA would allow the development of a better understanding of the functions of cholinergic projections from the pPPTg and LDTg to the pVTA.

Following on from this, a non-lesion approach was adopted to better understand the actions of cholinergics in the pVTA. For this, the effects of nicotine self-administered directly into the pVTA were examined following previous studies from this laboratory (Farquhar, Latimer and Winn, 2012). In Chapter 4, the effects of systemic nicotine on neural activation was quantified in the VTA and in structures on which VTA activity has effect – the shell and core of the nucleus accumbens (NAc), the dorsal striatum and the dorsal hippocampus (dHPC). Effects on these regions of interest were measured by immediate early gene (IEG) expression under two conditions: at the end of the very first exposure to systemic nicotine and (in other groups) after repeated exposure.

In Chapter 5, the direct effect of intracranial self-administration (ICSA) of nicotine into the pVTA was assessed. Systemic nicotine acts simultaneously at a wide range of sites and some actions could conceivably counteract others. ICSA does not mimic nicotine obtained through smoking, but it allows the effects of nicotine on specific structures to be examined in the absence of effects elsewhere. Neural activity was measured in the same VTA target regions under two conditions: at the end of the very first ICSA session and (in other groups) at the end of the fifth ICSA session. The question was, would ICSA of nicotine in the pVTA have different effects on the regions of interest dependent on the degree of learning that had taken place? As discussed above, the VTA is reciprocally connected to the HPC, in particular the CA1 region of the dHPC. These projections are reported to play an important role in drug-related learning (discussed in Chapter 6). In Chapter 6, a further approach was taken to contrast intra-VTA self-administration of nicotine with ICSA in the dHPC. The issue here is whether or not cholinergic activation at different points within the same neural circuitry has similar or

different effects on the identified regions of interest. Finally, the results of all the experimental chapters are discussed in Chapter 7.

Chapter 2: General Methods

2.1 Subjects

All experiments were performed on experimentally naïve adult male Lister Hooded rats (Charles River Laboratories, Kent, UK). Rats were housed in a temperature and humidity controlled room on a 12 h light/dark cycle. All behavioural testing was carried out during the light phase. Rats were housed in cages measuring 36 cm x 56 cm x 26 cm, with a solid plastic base and wire mesh top. Where possible, rats were housed in pairs and cages were environmentally enriched with Perspex tubes and a disposable chew toy. Animals with cannula implant assemblies were single housed and food hoppers were blocked off by a metal sheet to avoid losing or damaging headcaps. Animals had access to food (standard lab chow pellets) and water *ad libitum* in homecages (details of food restriction given in Chapter 5 and 6). For each chapter of this thesis that describes experiments, group sizes and weight ranges vary. The target surgery weight for lesion experiments was 330g (Chapter 3) and for cannulae implantation 400g (Chapter 5 and 6). Actual weight ranges and group sizes are stated in each chapter. Heavier body weights were needed for cannulae implantation studies to aid headcap stability, because heavier rats have thicker skulls. Prior to surgeries or locomotor testing all rats were handled once daily for a week. All experiments were performed under license from the UK Home Office under the Animals (Scientific Procedures) Act of 1986, the European Communities Council Directive of 24/11/86 (86/609/EEC) and University of Strathclyde local ethical review polices (PPL no. 60/3932 or 60/4555 and PIL no. 60/13235). These studies were carried out in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (Kilkenny *et al.*, 2010).

2.2 Surgical procedures

2.2.1 Anaesthesia

Rats were anaesthetised using isoflurane (Abbot Laboratories Ltd, Maidenhead, UK). Animals were placed in an induction box, which was fed 4 L/min O_2. After 90 sec, general anaesthesia

was induced by adding 0.5% isoflurane which was increased to 1% after a further 90 sec. Isoflurane was then increased by 1%, at 90 sec intervals, until a final concentration of 5% was reached. The timings used were approximate, and were adjusted so that the total loss of righting reflex was observed before the anaesthetic concentration was increased beyond 3%. In order to confirm that the rat was completely anaesthetised the absence of reflexes was checked (by retracting the hind limb to test for absence of withdrawal reflex) and deep steady breathing was observed. The animal was then removed from the induction box and placed in a stereotaxic frame (David Kopf, Tujunga, CA, U.S.A) where anaesthesia was maintained via a facemask mounted on the incisor bar. During incision and scalp cleaning 3% isoflurane in 1.4 L/min O_2 was delivered through the facemask, and following this anaesthesia was maintained at 2.5% isoflurane in 1.4 L/min O_2; this was adjusted appropriately if reflexes or shallow breathing were observed. Death during surgery was uncommon (5% of surgeries).

2.2.2 Stereotaxic surgery

All stereotaxic surgery was performed in a clean surgical environment on Model 900 Kopf Stereotaxic frames (David Kopf, Tujunga, CA, U.S.A).

2.2.3 General surgical procedures

Once secured in the stereotaxic frame, the animal was subcutaneously administered carprofen (s.c., 0.05 ml/rat; ‘Rimadyl’, Pfizer Ltd, Kent, UK) for analgesia. The scalp was shaved using electric clippers and the shaved area cleaned with iodine (10% iodine; Henry Schein, Dumfries, UK). A midline incision was made using a sterile scalpel, the skull was cleared of tissue and the incision held open with artery clamps. To ensure a flat skull position, lambda and bregma were measured and the height of the incisor bar was altered until lambda and bregma were level. Craniotomies were carried out using a handheld dental drill (Steel Komet drill burs, size 8, Komet dental, Gebr. Brasseler GmbH & Co. KG, Germany) with the aid of a surgical microscope (Leica M60 Microscope, Leica Microsystems, Milton Keynes, UK).

2.2.4 Cannulae implantation

The coordinates for posterior ventral tegmental area (pVTA) cannula surgeries were as follows: anterior-posterior, +3.2 mm from interaural line (IAL); mediolateral, distance from midline was not measured – the midline sinus was exposed and guide cannula was inserted adjacent to it; dorsoventral, the cannulae were cut to sit 7.7 mm below skull surface. The midline sinus was used as guide to significantly reduce variability in VTA cannulae placements. For surgeries targeting CA1 of the dorsal hippocampus (dHPC), coordinates for craniotomies were as follows: anterior-posterior, +5.2 mm from the IAL; mediolateral ±2.5 mm from the midline; dorsoventral, the cannulae were cut to sit 2 mm below skull surface. Six stainless steel rounded-head machine screws (0-80 x 3/32, 0-80 x 1/8 or 0-80 x 1/16; Plastics One, Roanoke, VA, U.S.A) were fixed to the skull, 2 in front, 2 behind the craniotomy and 2 in front of lambda to stabilise the head cap. Each rat was implanted with a unilateral guide cannula (24 ga; Plastics One, Roanoke, VA, U.S.A) and a solid pedestal (Plastics One, Roanoke, VA, U.S.A) that were lowered into place using an electrode holder mounted on the stereotaxic frame. The cannulae and solid pedestal were fixed in place using dental acrylic methyl methacrylate (Simplex Rapid, Kemdent Works, Wiltshire, UK) that bound around the cannula pedestal and skull screws. Internal dummy cannulae (31 ga; Plastics One, Roanoke, VA, U.S.A) were inserted into the implanted cannulae (which protruded 0.8 mm from guide cannulae tip). The rat was then allowed to inhale pure O_2 (flow rate, 1.4 – 1.8 L/min) until a positive reflex test was observed. Once removed from the stereotaxic frame, Hartmann's solution (1 ml/h of anaesthesia; Baxter Healthcare Ltd, Norfolk, UK) was administered i.p. to replace lost fluids and aid recovery. The rats were then placed in a recovery cage with a heat pad below where they were observed until they had recovered from anaesthesia and could be returned to their homecage. For further analgesia, rats received carprofen 24 h and 48 h after surgery (s.c., 0.05 ml/rat; 'Rimadyl', Pfizer Ltd, Kent, UK). Dummy cannulae were changed daily with clean replacements to prevent infection and protect the brain when no infusions

were taking place. Animals were given a minimum of 10 days to recover before starting behavioural testing.

2.2.5 Toxin microinfusion

Diphtheria-urotensin II (Dtx-UII) was manufactured and supplied through collaboration with Dr Stewart Clark (Department of Pharmacology and Toxicology, University of Buffalo, NY, U.S.A). Dtx-UII is not commercially available. Microinfusions of Dtx-UII (200 nl, 3% Dtx-UII in sterile phosphate buffer [PB]), ibotenic acid (180 nl, 0.06M solution in PB; final pH adjusted to pH 7.0 using 2M NaOH; Sigma-Aldrich, Gillingham, Dorset, UK) or sterile PB (0.1M, for sham controls) were performed using glass micropipettes.

Pipette preparation

Glass borosilicate capillary tubes (1.16 – 1.19 mm o.d.; 0.49 mm i.d.) were pulled into micropipettes using Model 720 Needle Pipette Puller (David Kopf, Tujunga, CA, U.S.A). The diameter of the pipette tips were adjusted to 40 – 50 µm under a light microscope and a scale (Dtx-UII; 10 mm, ibotenic acid; 0.9 mm) attached to each pipette.

Microinfusion surgery

The coordinates for pVTA microinfusion surgery were as follows: anterior-posterior, +3.2 mm from IAL; mediolateral, distance from midline was not measured – the midline sinus was exposed, and the filled glass pipette was inserted adjacent to it; dorsoventral (DV), to ensure a flat skull position, lambda and bregma were measured and the height of the incisor bar was altered until lambda and bregma were equal, pipette was lowered -7 mm from the brain surface once a craniotomy had been performed. Bilateral craniotomies were carried out unless stated in individual experimental chapters. A fine-tipped pipette was back-filled with the appropriate solution and secured in an electrode holder mounted on the stereotaxic frame. With surgical microscope (Leica M60 Microscope, Leica Microsystems, Milton Keynes, UK) guidance, the

IAL was measured once more to allow the pipette to be positioned correctly. The pipette was gently lowered into the brain and the solution was manually infused using a plastic 10 ml syringe and polyethylene tubing (both air-filled) that was attached to the pipette. This pressure system was carefully controlled by hand using a hole drilled into the side of the syringe, which was covered until the required volume of toxin had been injected (200 nl, indicated by the scale attached to the pipette). The pipette was left *in situ* for 5 min, which allowed the solution to diffuse away from the pipette tip; after this the pipette was carefully removed. Once all infusions had been carried out, the skull surface was cleaned thoroughly using 0.9% saline solution (TPS Healthcare, Cumbernauld, UK) and the incision was closed using Michel wound clips (Henry Schein Animal Health, Dumfries, UK). The rat was then allowed to inhale pure O_2 (flow rate, 1.6 – 1.8 L/min) until a positive reflex test was observed. Once removed from the stereotaxic frame, Hartmann's solution (1 ml/h of anaesthesia; Baxter Healthcare Ltd, Norfolk, UK) was administered i.p. to replace lost fluids and aid recovery. The rats were then placed in a heated recovery cage where they were observed until they had recovered from anaesthesia and could be returned to their homecage. For further analgesia rats received carprofen 24 h after surgery (s.c., 0.05 ml/rat; 'Rimadyl', Pfizer Ltd, Kent, UK).

2.3 Behavioural procedures

2.3.1 Self-administration

Self-administration studies were conducted in operant chambers (Med-Associates, St Albans, VT, U.S.A) that were individually housed in ventilated, sound and light attenuated cubicles. The dimensions of the chamber were 30.5 cm x 24.1 cm x 21 cm. During testing, 1 wall was illuminated by a house light (1.5 W, 28 v) and on the opposite wall 2 stimulus lights (1.5 W, 28 v) were positioned above (7 cm) 2 standard retractable levers (5 cm wide) that were spaced apart (11 cm). The stimulus light was programmed to illuminate when the active lever was pressed. Each testing session lasted 90 min and infusions were limited to a maximum of 60.

Animals either received 1 or 5 testing sessions. To minimise non-specific cell damage at injection site, the latter group (5 sessions) were tested every second day so that the injector cannula was only placed into the VTA a maximum of 5 times at 48 h intervals. The rat's injector cannula (31 ga; Plastics One, Roanoke, VA, U.S.A) was connected to a head-attached micropump by polyethylene tubing (PE50 thin wall, Plastics One, Roanoke, VA, U.S.A) and connected to an electrical swivel to prevent cable tangling and allow the rat to move freely in the chamber. A response on the active lever resulted in a 75 nl infusion of drug. The stimulus light illuminated above the lever and both levers retracted for 20 sec. The pump reliably and repeatedly delivers a set volume directly into the targeted brain region. A response on the inactive lever does not trigger drug infusion, light illumination or lever retraction. The allocation of active or inactive lever to left and right lever was randomly distributed across animals. The behavioural measurements were taken by the MED-PC software (Med-Associates, St Albans, VT, U.S.A) to determine the number of responses on the active and inactive levers and total number of infusions. Each testing session was video recorded using a standard VHS video recorder.

2.3.2 Locomotor testing

Locomotor monitoring was carried out under dull red-light illumination in four Perspex cages measuring 24.1 cm x 45.7 cm x 18 cm positioned in SmartFrame™ Cage Rack Stations (Kinder Scientific, San Diego, U.S.A). The rack stations contained a 7 x 15 infrared PhotoBeam grid positioned at the height of the rat's body and an additional 0 x 7 PhotoBeam frame that could only be reached when the rat reared vertically. All locomotor stations were connected to a computer with MotorMonitor software for data collection (Hamilton Kinder LLC, Poway, CA, U.S.A) which recorded sequential beam breaks as locomotion. This system measured parameters such as basic movements (total number of beam breaks made at the level of the rat's body), fine movements (number of beam breaks caused by smaller movements

such as forepaw grooming) and rearing (total beam breaks measured by the additional rearing frame). The duration of each testing session was 1 h.

2.4 Histological procedures

2.4.1 Transcardial perfusion

Animals were deeply anaesthetized for perfusion with a lethal dose of sodium pentobarbitone (0.6 – 0.8 ml/rat administered i.p., 200 mg/ml sodium pentobarbitone; Euthatal Merial Animal Health Ltd, Harlow, UK). Once rats were terminally anaesthetised, which was confirmed through negative blink and pedal reflex tests, they were immediately transcardially perfused using 0.1% phosphate buffered saline (PBS: 0.9% NaCl in 0.1 M PB [NaCl; Sigma-Aldrich, Gillingham, Dorset, UK]) followed by approximately 300 ml of PLP fixative (4% paraformaldehyde containing L-lysine acetate salt and sodium meta-periodate [Sigma-Aldrich, Gillingham, Dorset, UK] in 0.1 M PB; this fixative was used to prevent autofluorescence (McLean and Nakane, 1974)) (0.1 M PB: 2.2% Na_2HPO_4 [Fisher Scientific, Leicestershire, UK], 0.6% NaH_2PO_4 [Scientific Laboratory Supplies Limited, Newhouse, UK]). The completion of perfusion was confirmed through the inspection of the animal ensuring that their limbs and head had become hardened. Following perfusion, the brain was left *in situ* for 1 h to post-fix before it was removed and stored in sucrose solution (20% sucrose in 0.1 M PB) overnight at 4°C. In the event of incomplete fixation, the brain was incubated in PLP fixative overnight at 4°C and stored in sucrose solution (20% sucrose in 0.1 M PB) the subsequent night.

2.4.2 Tissue preparation and sectioning

In Chapters 4, 5 and 6, brains were embedded in PFA-fixed egg yolk prior to sectioning. Brains were incubated in egg yolk in plastic wells surrounded by PFA fixative (40% PFA in 0.1 M PB) for 72 h. Egg yolk embedding aided sectioning, mounting and analysis of delicate

cannulated tissue – especially sections containing the HPC. Coronal 30 µm sections were cut into 8 series using a freezing microtome (Sliding microtome Leica SM2010 R, Leica Microsystems Ltd, UK) and freezing stage (BFS-40MP, Linton Instrumentation, Norfolk, UK). The left hemisphere of the brain was marked as a reference using a needle to penetrate through an area of the brain considered not relevant to the study. In Chapters 5 and 6, 1:4 series were immediately mounted and processed for cresyl violet stain to confirm cannula placements. For storage, sections were transferred into 0.1 M PB, and stored in ethylene glycol and sucrose based cryoprotectant (ethylene glycol; Sigma-Aldrich, Gillingham, Dorset, UK) at -20ºC.

2.4.3 Immunohistochemical staining

Stains performed

Three different immunohistochemical stains were performed to analyse the regions of interest: (1) Neuron-specific nuclear protein (NeuN) and cresyl violet double stain: NeuN is a nuclear antigen which binds strongly to neuronal nuclei – and to a lesser extent the cytoplasm – that is commonly used as a non-selective marker to determine the presence and density of neurons. Here NeuN was used to determine the selectivity of mesopontine cholinergic neuron lesions (contrasting it with specific markers of cholinergic neurons) and to assess the extent of ibotenic acid lesions in the VTA. Cresyl violet counter stain marks Nissl bodies and the presence of gliosis (a CNS response to injury), which is frequently observed following excitotoxic lesions. However, it has been reported that this is rarely seen after Dtx-UII infusion. Glass pipettes occasionally cause small traces of gliosis and so this counter stain was used to analyse pipette tip location. (2) Tyrosine hydroxylase (TH): TH is the rate-limiting enzyme in catecholamine (CA) synthesis. It is responsible for the conversion of L-tyrosine to L-DOPA – the precursor for dopamine (DA) – so this staining can be used to determine the identity of DA neurons in different brain regions. Here TH staining was performed to determine the effect of Dtx-UII

infusion on DA cell bodies present in the VTA. (3) *c-fos*: Fos is the protein product of immediate early gene (IEG) *c-fos* which is expressed by many neurons upon activation. Expressed in the nucleus, Fos protein can be easily stained through immunohistochemistry, which has led to its frequent use as a marker of postsynaptic activation. Here, Fos expression was used to correlate neural activity in brain regions related to the rewarding effects of nicotine (regions of interest stated in each chapter).

Immunohistochemical protocol

All immunohistology was conducted at room temperature with sections incubated on a horizontal flatbed shaker (Advantage-Lab AL 05-05, Schilde, Belgium). The same protocol was used for NeuN, TH and *c-fos* staining; however, to avoid antibody cross-contamination, these processes were carried out separately. A 1:4 series of sections were used for immunohistochemical analysis. Sections were briefly washed in phosphate buffered saline (PBS; washed twice in PBS for 5 min) before being treated with blocking agent (blocking solution: 20% goat serum (Merck Millipore, Watford, UK), 0.1% Triton X-100 (Sigma-Aldrich, Gillingham, Dorset, UK), 79% PBS) for 1 h; *c-fos* was incubated for a slightly shorter time (45 min). Once blocked, sections were washed (as before) and incubated overnight in antibody dilutant solution (ADS; 1% goat serum, 0.1% Triton X-100, 98.9% PBS) that contained the primary antibody. Following overnight incubation, sections were washed with PBS (as before) and processed with Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK). Sections were incubated for 90 min with Biotinylated secondary antibody (IgG, 1:200 in ADS); sections were then washed (as before) and incubated for 45 min in avidin-biotin complex (ABC solution, 1:50 in ADS). After washing (as before), staining was revealed using 3, 3-diaminobenzidine tetrahydrochloride (DAB; Sigma Fast DAB solution, Sigma-Aldrich, Gillingham, Dorset, UK). Sections were washed in PBS for a final time. TH and *c-fos* stained sections were mounted on glass slides, cleared with xylene (Fisher Scientific, Leicestershire, UK) and coverslipped using DPX mounting medium (Sigma-Aldrich,

Gillingham, Dorset, UK) and glass coverslips. To prevent section loss during processing, NeuN stained sections were mounted onto gelatine coated slides (Southern Biotech, Birmingham, U.S.A) and prior to cresyl staining (see below), were placed overnight in a gas chamber containing paraformaldehyde (PFA).

Cresyl violet counterstain

NeuN stained sections were removed from the PFA chamber and soaked in xylene for 3 min. These sections were rehydrated through graded alcohol (100%, 50%, and 0% ethanol in water) before being rinsed thoroughly in running tap water. The slides were placed in cresyl violet stain for 60 sec and then rinsed in running tap water for 5 min. Sections were dehydrated (0%, 50% and 100% ethanol in water) before being soaked in xylene for a further 3 min and then coverslipped with glass coverslips using DPX mounting medium (Sigma-Aldrich, Gillingham, Dorset, UK).

Antibodies used

NeuN

For NeuN staining, normal goat serum was used for the blocking solution and ADS. The primary antibody was a mouse derived monoclonal antibody (NeuN; 1:20,000; Chemicon International Inc., CA, U.S.A). The mouse version of the Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK) was used for the secondary antibody and avidin-biotin complex. Sections were stained in Sigma Fast DAB solution (Sigma Fast DAB solution, Sigma-Aldrich, Gillingham, Dorset, UK) for 7 min.

TH

For TH staining, normal goat serum was used for the blocking solution and ADS. The primary antibody was a mouse-derived monoclonal antibody (TH; 1:25,000; Merck Millipore, Watford, UK). The mouse version of the Vector Labs Elite ABC kit (Vector Laboratories,

Peterborough, UK) was used for the secondary antibody and avidin-biotin complex. Sections were stained in Sigma Fast DAB solution (Sigma Fast DAB solution, Sigma-Aldrich, Gillingham, Dorset, UK) for 3 min.

c-fos

For *c-fos* staining, normal goat serum was used for the blocking solution and ADS. The primary antibody was a rabbit derived polyclonal antibody (*c-fos*; 1:10,000; Synaptic Systems, Gottingen, Germany). The rabbit version of the Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK) was used for the secondary antibody and avidin-biotin complex. Sections were stained in Sigma Fast DAB solution (Sigma Fast DAB solution, Sigma-Aldrich, Gillingham, Dorset, UK) for 10 min. To ensure against non-specific staining, control experiments were performed where anti-Fos primary antibody was omitted.

2.4.4 Fluorescent immunohistochemistry

Two different immunofluorescent stains were performed to analyse regions of interest: (1) vesicular acetylcholine transporter (VAChT; Synaptic Systems, Gottingen, Germany): anti-VAChT staining was used to identify midbrain cholinergic cell bodies and terminals. In order to analyse PPTg and LDTg projecting cholinergic neuronal populations in the VTA, sections that crossed through the VTA were processed to reveal VAChT immunofluorescence at cholinergic terminals. Sections that crossed through the PPTg and LDTg were processed with VAChT to determine the density of mesopontine cholinergic cell bodies after Dtx-UII infusion. (2) Fluoro-jade C (Merck Millipore, Watford, UK): Fluoro-jade dyes are fluorescent ligands which were found to detect and localise degenerating neurons, regardless of specific insult or mechanism of cell death. The mechanism in which the dyes bind to degenerating neurons is not fully understood. It has been hypothesised that Fluoro-jade dyes could be electrostatically attracted to polyamines that are produced during tissue degradation, such as cadaverine, spermidine and putrescine. Fluoro-jade C demonstrates the greatest affinity for

degenerating neurons, and provides the highest resolution and contrast to identify distal dendrites, axons and terminals. Fluoro-jade C was used here to determine whether intra-VTA infusion of Dtx-UII damaged VTA cholinergic terminals, or cell bodies of the PPTg and LDTg.

Vesicular acetylcholine transporter protocol

Sections were briefly washed in PBS (washed 3 times in PBS for 5 min) before being treated for 3 h with a blocking agent (blocking solution: 5% non-fat dry milk, 3% goat serum, 0.5% Triton X-100, 0.1% glycine and 0.1% L-lysine acetate). Once blocked, sections were incubated overnight with the primary antibody diluted in the same blocking solution (1:2000; Polyclonal Rabbit anti-VAChT, Synaptic Systems, Gottingen, Germany). Following overnight incubation, sections were washed in PBS (as before) and incubated for 90 min with the fluorescent-tagged secondary antibody diluted in blocking solution (1:500; Alexa Fluor® 488 Goat Anti-Rabbit IgG, Life Technologies, Renfrew, UK). Sections were washed thoroughly in PBS for a final time. Sections were then mounted on glass slides and coverslipped with hard setting mounting medium (VECTASHIELD® HardSet Mounting Medium with DAPI, Vector Laboratories, Peterborough, UK) and sealed using clear nail varnish. Slides were then stored at 4°C. During secondary antibody incubation and for all subsequent steps, sections were protected from the ambient light to prevent photobleaching.

Fluoro-jade C protocol

Prior to staining, sections were mounted on gelatine coated slides (Southern Biotech, Birmingham, U.S.A) and left to dry overnight in a gas chamber containing PFA. The following day, slides were immersed in a basic alcohol solution (1% sodium hydroxide [Fisher Scientific, Leicestershire, UK] in 80% ethanol) for 1 min. Slides were then rinsed for 1 min in 70% ethanol, then for 1 min in distilled water, and then incubated in a 0.06% potassium permanganate solution (Sigma-Aldrich, Gillingham, Dorset, UK) for 5 min. Slides were rinsed

in distilled water for 1 min before being transferred into a 0.0001% Fluoro-jade C solution dissolved in a 0.1% acetic acid (Sigma-Aldrich, Gillingham, Dorset, UK) vehicle for 15 min. Slides were rinsed in distilled water for 5 min and allowed to dry before being coverslipped using DPX mounting medium (Sigma-Aldrich, Gillingham, Dorset, UK). Coverslips were then sealed with clear nail varnish and slides were stored at 4°C.

2.4.5 Microscopy

Slides processed for NeuN/Cresyl, TH and *c-fos* were analysed through light microscopy using a Leica Microscope DM LB2 connected to a desktop computer system with a Leica DFC320 camera (both: Leica Microsystems, Milton Keynes, UK). Slides stained for VAChT and Fluoro-jade C were viewed using a Nikon Eclipse TE300 inverted epifluorescent microscope (Nikon Microscopy U, U.S.A) with Lambda SC Smart shutter controller or Nikon Eclipse E600 epifluorescent upright microscope LED (Nikon Microscopy U, U.S.A). Exposure and gain settings were adjusted to the same level for each sample and all files were saved as JPEG files.

2.4.6 Cell quantification

In order to quantify the number of Fos positive (Fos+) cells, software assisted manual counts were performed within specific nuclei (regions of interest stated in each chapter). Bilateral cell counts from the caudate putamen (CPu), dHPC, VTA, nucleus accumbens (NAc) shell and core were taken from at least 5 sections containing the area of interest from a 1:4 series. The distance between sections in a 1:4 series is approximately 120 µm; this lowered the risk of counting the same cell more than once. Sections stained with *c-fos* were photographed using the Leica Microscope DM LB2 (x20 magnification) connected to a desktop computer system with a Leica DFC320 camera. Images were uploaded to ImageJ analysis software (ImageJ, U.S National Institutes of Health, Bethesda, Maryland, U.S.A) with additional Cell Counter plugin installed. Once the image was loaded, every Fos+ cell within the region of interest was

manually selected by the operator and the program recorded the total number of cells. A spreadsheet of Fos+ cells along the anterior-posterior plane of each structure of interest was then created. Depending on the structure, neural activity was counted 1 of 4 ways: - (1) the boundaries of the VTA are not clearly defined by *c-fos*, therefore its boundaries were defined by neighbouring structures such as the SN or midline structures. The VTA is composed of VTAR, PBP, PN, PIF and VTT; the borders between these subregions are not clear, therefore they were counted as a whole. (2) The position of the NAc core was determined by the anterior part of the anterior commissure (*aca*), and NAc shell was determined by the midline and distance from NAc core (depending on the anterior-posterior position). (3) For the dHPC, 4 images containing CA1, CA2, CA3 and dentate gyrus (DG) were taken for both hemispheres. (4) For the CPu, the centre of the structure was estimated for each section.

This method of quantification is simply an estimation of the changes in cell counts, rather than a total count of neurons in each structure. It should be noted that during imaging and counting, the experimenter was blind to treatment conditions for each animal (i.e. the experimental group of each animal was withheld). However, there were obvious differences between nicotine and control groups in some sections. In sections containing the VTA, nicotine-induced substantial Fos expression in the medial terminal nucleus of the optic tract (MT), but saline had no effect. After counting, the MT could be used to determine if the animal successfully received nicotine and confirmed that there was no mix-up between experimental groups.

In order to quantify cholinergic terminals projecting to the VTA and mesopontine cholinergic cell density, software assisted counts of VAChT+ puncta and cell bodies were performed. Each VTA, PPTg and LDTg section stained with VAChT was photographed using a Nikon E600 epifluorescent upright microscope. Images were uploaded into ImageJ analysis software (ImageJ, U.S National Institutes of Health, Bethesda, Maryland, U.S.A) with the additional Image-based tool for counting nuclei (ITCN) plugin. This plugin allowed the minimum and maximum distance between puncta and puncta size to be set. Once the image was loaded,

every VAChT+ puncta within the region of interest was automatically selected by the program and the total number of puncta was recorded. For mesopontine cholinergic cell counts, the operator manually selected VAChT+ cells and the program recorded the total number of cells. The experimental groups were withheld from the experimenter; therefore, they were blind to which treatment each hemisphere or animal had received (treatments stated in each chapter). Furthermore, the experimenter conducted random a priori counts to check for reproducibility.

2.5 Statistics

2.5.1 Data analysis

Quantification of VAChT terminals (Chapter 3) and Fos positive cells (Fos+; Chapter 4, 5 and 6) was carried out using ImageJ analysis software (ImageJ, U. S. National Institutes of Health, U.S.A). Statistical analyses were performed in SSPS version 21 (SSPS Inc., Chicago, Illinois, U.S.A). The exact details of particular tests used are given in each chapter. Behavioural measures were also taken. Locomotor data (Chapter 3 and 4) were collated as square root transformed (SQRT) ± SEM in order to correct for positive skew in the data (identified by Shapiro-Wilk test). For intracranial self-administration data (Chapter 5 and 6) a range of ANOVAs were performed. Significant interactions were further investigated by post hoc tests or pairwise comparisons, where appropriate. Independent t-tests were used to compare smaller single data sets, with the exact details stated in each chapter. Results are expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. All effects were considered statistically significant when $p \leq 0.05$.

Chapter 3: Exploring the possibility of destroying cholinergic terminals in the rat ventral tegmental area

3.1 Chapter introduction

3.1.1 Diphtheria-urotensin II toxin

As previously discussed in the General Introduction, diphtheria urotensin-II toxin (Dtx-UII) was developed to selectively destroy cholinergic neurons of the mesopontine tegmentum, leaving non-cholinergic neurons intact (Clark *et al.*, 2007). The specificity of diphtheria toxin (Dtx) has previously been exploited because the naturally occurring targeting domain can be switched with the ligand of another receptor, producing a selective toxin for the new receptor (Fisher *et al.*, 1996; Benoliel *et al.*, 1999; Frankel, Kreitman and Sausville, 2000). The Dtx-UII fusion toxin was formed by replacing the targeting domain with urotensin II (UII) – a neuropeptide originally isolated from the spinal neurosecretory gland (urophysis) of teleost fish (Pearson *et al.*, 1980). The UII receptor (UII-R or UT receptor) is expressed in various mammalian tissues such as vasculature, heart, kidney and brain. However, UII-Rs are of particular interest because in the mesopontine tegmentum they are solely expressed on cholinergic neurons (Figure 3.1) (Clark *et al.*, 2001). In brain regions to which the mesopontine nuclei project, such as the VTA, UII binding sites were identified (Figure 3.1) (Clark *et al.*, 2001). When Dtx-UII binds to a UII-R, the receptor is activated which triggers conformational change and receptor internalisation (Giebing *et al.*, 2005). The toxin is engulfed by endosomes that facilitate the entry of Dtx-UII into the cytosol (Simpson *et al.*, 1998). Dtx inhibits protein synthesis through the ADP-ribosylation of elongation factor 2 (EF-2). EF-2 is responsible for peptide chain formation during the elongation stage of translation. The addition of ADP-ribose, triggered by Dtx, deactivates EF-2, which halts elongation, inhibits protein synthesis and leads to the death of the cell (Robinson, Henriksen and Maxwell, 1974). When Dtx is fused with UII, Dtx-UII only binds to UII-Rs, selectively destroying cholinergic neurons and leaving non-cholinergic neurons intact. Once Dtx-UII is released into the cytosol, cell death occurs gradually over a period of days.

3.1.2 Current lesion method

Clark *et al.* (2007) first reported that unilateral microinjections of Dtx-UII into the pedunculopontine tegmental nucleus (PPTg) could form selective lesions of cholinergic neurons here leaving surrounding non-cholinergic neurons intact (Clark *et al.*, 2007). Unilateral Dtx-UII PPTg lesions developed between 4 and 21 days after infusion – ~ 85% loss of cholinergic neurons occurred 21 days after infusion with little damage to surrounding neurons (Clark *et al.*, 2007). However, unilateral (unlike bilateral) non-selective PPTg lesions are reported to produce very few behavioural changes (Olmstead *et al.*, 1998; Bortolanza *et al.*, 2010), which is not surprising because the PPTg has extensive afferent and efferent contralateral projections. Therefore, unilateral lesions will not leave target structures completely without contralateral PPTg innervation (Winn, 2006). Particular PPTg target structures, such as the VTA and thalamus, also receive cholinergic projections from the laterodorsal tegmental nucleus (LDTg) (Oakman *et al.*, 1995; Usunoff *et al.*, 1999). Unilateral PPTg lesions would not destroy LDTg projections to target structures, which could compensate for damaged PPTg input (compensatory mechanisms discussed below). In order to determine the behavioural effect of Dtx-UII PPTg lesions accurately, bilateral mesopontine cholinergic lesions had to be developed.

As discussed in the General Introduction, the posterior region of the PPTg (pPPTg) provides cholinergic innervation to the ventral tegmental area (VTA), while the anterior PPTg (aPPTg) innervates the substantia nigra (SN). The subregions of the PPTg are functionally separate (Alderson, Latimer and Winn, 2008). Rather than whole PPTg cholinergic lesions, selective pPPTg Dtx-UII lesions were developed to destroy specifically cholinergic input to the VTA. Unlike bilateral excitotoxic PPTg lesions, which are performed in two separate unilateral procedures to reduce mortality, both hemispheres can receive pPPTg Dtx-UII infusion during the same surgery. Dtx-UII gradually induces cell death by protein synthesis inhibition with

lesion formation gradually occurring over a number of days. During the surgical recovery period it is hard to distinguish between toxin and control animals.

Fourteen days after bilateral Dtx-UII pPPTg surgery, extensive cholinergic cell loss was observed (~ 90%) but there was no change in basic locomotion or nicotine-induced locomotion in pPPTg Dtx-UII lesioned animals compared to control animals (MacLaren, 2012). Steidl *et al.* (2014) noted that bilateral PPTg infusion of Dtx-UII resulted in significant cell loss 4, 7, 14 and 28 days after infusion (~ 96% loss). However, 14 days after infusion it was observed that significant GABAergic and glutamatergic cell loss had occurred; the loss of cholinergic cells was significantly greater than the loss of non-cholinergic cells (Steidl, Wang and Wise, 2014). It was also reported that PPTg cholinergic lesions had no effect on cocaine or heroin intravenous self-administration (IVSA), conditioned place preference (CPP), or cocaine induced locomotion (Steidl, Wang and Wise, 2014). Excitotoxic lesions of the PPTg were reported to alter IVSA and CPP behaviour to drugs of abuse. IVSA of heroin (Olmstead *et al.*, 1998) and D-amphetamine (Alderson *et al.*, 2004) was impaired by PPTg lesions made before operant training, but not when animals acquired the appropriate operant response through training before lesion surgery. The role of excitotoxic PPTg lesions in nicotine IVSA was reported to be more complex. In pre-trained animals, dorsal PPTg (Corrigall, Coen and Adamson, 1994) and aPPTg (Alderson, Latimer and Winn, 2006) lesions did not alter IVSA of nicotine. However, pre-trained rats bearing pPPTg lesions showed an increased response to nicotine IVSA (Alderson, Latimer and Winn, 2006) and furthermore, excitotoxic pPPTg lesions (but not aPPTg) altered nicotine-induced locomotion (Alderson, Latimer and Winn, 2008). Studies examining CPP to drugs of abuse in PPTg-lesioned animals reported mixed results. PPTg lesions were found to prevent acquisition of CPP to amphetamine but not cocaine (Bechara and van der Kooy, 1989; Parker and van der Kooy, 1995). Critically, in lesions of the PPTg, excitotoxins destroy both cholinergic and non-cholinergic neurons. Therefore, any differences in behavioural or neuronal results post-surgery cannot be interpreted in terms of

cholinergic or other cell loss. At the correct time point, volume and concentration, the functional outcome of bilateral Dtx-UII PPTg lesions can be interpreted in terms of PPTg cholinergic cell loss. However, in order to determine the true behavioural effect of cholinergic lesions accurately, total cholinergic denervation of target structures should be achieved.

As discussed in the General Introduction, the mesopontine nuclei provide the VTA with significant cholinergic projections that release endogenous acetylcholine (ACh) (Semba and Fibiger, 1992). These mesopontine projections provide excitation of midbrain dopamine (DA) neurons that are important for reward functions. To further understand the role of cholinergic input into midbrain DA neurons, it is important to understand where ACh comes from – namely the PPTg and LDTg. Steidl *et al.* (2015) successfully developed bilateral LDTg Dtx-UII lesions (~ 80% cholinergic neurons) (Steidl, Cardiff and Wise, 2015). Although the LDTg provides the largest cholinergic input to the VTA, LDTg cholinergic lesions did not alter the rewarding effects of cocaine and appeared to reduce responsiveness to cocaine-predictive stimuli (Oakman *et al.*, 1995, 1999; Steidl, Cardiff and Wise, 2015). However, any behavioural effect of bilateral PPTg or LDTg cholinergic lesions can only be interpreted as partial destruction of the cholinergic input to VTA DA neurons. In order to achieve complete cholinergic denervation of the VTA, both mesopontine nuclei should be targeted.

3.1.3 Compensatory mechanisms

Little is known about the compensatory mechanisms that occur when cholinergic neurons are damaged. As dopaminergic systems demonstrate, functional compensatory mechanisms due to partial lesions should be strongly considered. Due to its involvement in Parkinson’s disease (PD) the dopaminergic nigrostriatal pathway has been rigorously studied. Although PD is comprised of many pathologies it is mainly characterised by the progressive loss of SN DA neurons (Hornykiewicz, 1962). Strikingly, humans do not show symptoms until > 80% of DA cells have depleted and this was also reported in animal models of PD (Singh, Pillay and

Choonara, 2007). In rats, selective DA lesions are achieved by selective neurotoxin 6-hydroxydopamine (6-OHDA) infusion. These lesions cause a condition with similar symptoms to idiopathic PD when near total DA cell loss is achieved – lesions where DA neurons are damaged, but not enough to cause motor symptoms, result in cognitive dysfunction (Castaneda, Whishaw and Robinson, 1990; Perez *et al.*, 2008). If smaller lesions formed, animals demonstrated transient behavioural deficits and could recover most behavioural functions (Robinson *et al.*, 1994). Robinson *et al.* (1990) suggested that compensatory mechanisms depend on lesion size (Robinson, Castaneda and Whishaw, 1990). For small DA lesions where < 80% of cells are lost, normal DA levels can be achieved by an increased DA output from the remaining DA cells (Robinson, Castaneda and Whishaw, 1990). Lesions where 80 – 95% of DA cells were destroyed animals were found to recover some behavioural function, but there were some permanent behavioural impairments (Robinson, Castaneda and Whishaw, 1990). It was suggested that this functional recovery is achieved by an increased pre-synaptic DA output and increased post-synaptic sensitivity to DA and this would result in near normal levels of DA. Animals with lesions greater than 95% never regained normal behavioural function and DA levels were significantly lower than control animals. No level of increased pre/post-synaptic output could achieve successful compensation with lesions this size. Administration of dopamine precursor, L-3, 4-dihydroxyphenylalanine (L-DOPA or levodopa), is the most common and effective treatment for symptoms of early PD (Mercuri and Bernardi, 2005). Animals with DA lesions greater than 95% failed to respond to L-DOPA treatment (Papa *et al.*, 1994). After years of L-DOPA therapy, patients develop motor complications and antiparkinsonian actions wear off; this is followed by random on-off fluctuations of symptoms (Ahlskog and Muenter, 2001). This progressive decline in L-DOPA efficacy and response duration is believed to be caused by the continuing degradation of DA neurons, loss of pre-synaptic DA terminals and alterations in post-synaptic sensitivity (Mouradian *et al.*, 1987).

Although less is known about compensatory mechanisms of the cholinergic system, it should be considered a possible reason for the lack of behavioural effect demonstrated by bilateral Dtx-UII lesions. It could be that mesopontine cholinergic input is not essential for mediating IVSA and CPP behaviour to drugs of abuse or could an intact LDTg compensate for PPTg lesions (or vice versa)? Grace and Floresco (2007) reported that the mesopontine nuclei work together to regulate the activity of VTA DA neurons. It is believed that the LDTg provides a cholinergic-dependant permissive "gate" to regulate VTA DA neurons firing (Lodge and Grace, 2006). Glutamatergic input, presumably from the PPTg, can only initiate burst firing in VTA DA neurons when the LDTg is active (Grace *et al.*, 2007). If this co-stimulation does exist, it is unlikely that an intact LDTg or PPTg could compensate for the lesioned mesopontine nuclei or is it possible that the remaining 10% of pPPTg or 20% LDTg cholinergic neurons could support normal behaviour? Ibotenic acid lesions often leave 10% of neurons intact, which still results in a strong behavioural deficit. However, ibotenate lesions form within 72 hours rather than weeks (Köhler, Schwarcz and Fuxe, 1979; Garey and Hornung, 1980). The long period of Dtx-UII lesion progression and delay before testing could support the development of compensatory mechanisms (testing can start up to 21 days after surgery). MacLaren (2012) investigated the possibility of post-synaptic compensation following bilateral pPPTg Dtx-UII lesions (MacLaren, 2012). After non-selective PPTg lesions the upregulation of VTA nicotinic acetylcholine receptors (nAChRs) is believed to occur, which is thought to be responsible for the altered locomotor response to nicotine (Blaha *et al.*, 1996; Alderson and Latimer, 2008). Based on this, MacLaren (2012) investigated the effect of nicotine following selective pPPTg lesions to determine if VTA nAChRs were upregulated (MacLaren, 2012). However, there were no differences in locomotion or rate of sensitivity to nicotine in control or lesioned animals. This would suggest no substantial nAChR upregulation, and therefore no development of functional compensation. Perhaps nicotine acts at regions other than the VTA or cholinergic input from the intact mesopontine nuclei is able to compensate for cholinergic cell loss? The cholinergic neurons of both mesopontine nuclei

should be targeted to achieve complete cholinergic denervation of the VTA, to rule out the possibility of cholinergic compensatory mechanisms.

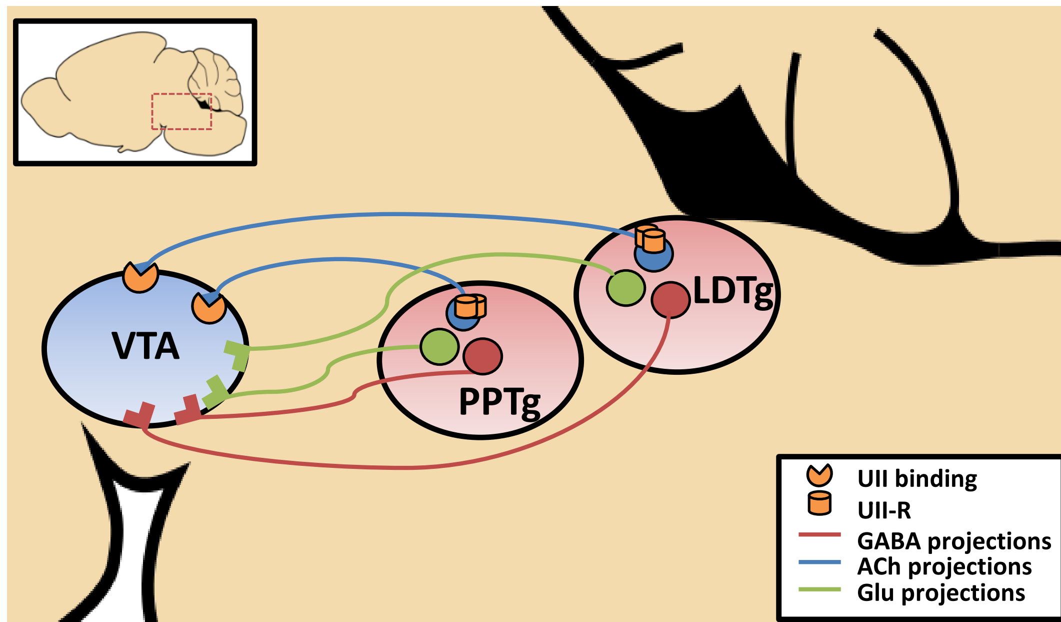


Figure 3.1 – Location of urotensin-II receptors (UII-Rs) or UII binding sites in the rat midbrain. UII-Rs are located on cholinergic cell bodies of the mesopontine tegmentum. Cholinergic neurons project from the pPPTg and LDTg which innervate the VTA. UII binding sites are present on cholinergic terminals in the VTA.

3.1.4 Current study

Previously, it has been demonstrated that Dtx-UII can destroy cholinergic cell bodies of the mesopontine tegmentum when infused directly. However, can this toxin destroy cholinergic terminals in the structures that receive cholinergic input from the PPTg/LDTg? The PPTg and LDTg express robust levels of UII-R; however, UII binding sites are present in brain regions that receive cholinergic projections from the mesopontine tegmentum (Figure 3.1) (Clark *et al.*, 2001). These UII binding sites are expressed at a lower level in brain regions such as parasubiculum (PaS), medial preoptic nucleus, medial habenular nuclei and VTA. It should be noted that not all PPTg/LDTg cholinergic terminals express UII binding sites; for example, cranial nerve nuclei and the pontine and medullary reticular formations, known PPTg/LDTg axonal termination sites, do not exhibit UII binding (Clark *et al.*, 2001), which potentially demonstrates the specific localisation of UII. If it were possible, it would be better to destroy

the mesopontine cholinergic input to the VTA rather than performing multiple surgeries to lesion the cholinergic cell bodies of the pPPTg and LDTg. Surgically targeting the LDTg is challenging and often unsuccessful due to its position to the fourth ventricle, lambdoid and midline sutures (Inglis and Semba, 1997; MacLaren, 2012; Steidl, Cardiff and Wise, 2015). As discussed in the General Introduction, the VTA is divided into three main subregions: - anterior (aVTA), posterior (pVTA) and tail (VTT). The aVTA and pVTA are structurally and functionally heterogeneous; the pVTA contains the highest percentage of DA neurons (Zhao-Shea *et al.*, 2011) and mediates the rewarding effects of many drugs of abuse. Intracranial self-administration (ICSA) studies demonstrated that rats would self-administer drugs directly into the pVTA more readily than the aVTA. Rats vigorously self-administer cholinergic drugs (nicotine, carbachol and neostigmine) directly into the pVTA (Ikemoto and Wise, 2002; Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). Other drugs, such as opioids (endomorphin-1), cannabinoids, cocaine, serotonin-3 receptor agonists, alcohol and alcohol related compounds (acetaldehyde and salsolinol) were also found to trigger rewarding effects when delivered directly into the pVTA (Bozarth and Wise, 1981; Zangen *et al.*, 2002, 2006, Rodd *et al.*, 2004, 2005, 2007, 2008). Based on these studies, it would be of particular interest to specifically target the mesopontine cholinergic input to the pVTA using Dtx-UII.

If Dtx-UII can form selectively cholinergic lesions in the pVTA, behavioural testing could be used to further understand the role of cholinergic projections from the pPPTg and LDTg. As previously mentioned, studies have utilised ICSA of nicotine directly into the pVTA (Farquhar, Latimer and Winn, 2012); these demonstrated that nicotine has reinforcement enhancing properties in the pVTA. Intra-pVTA ICSA of nicotine in pVTA Dtx-UII lesioned animals could potentially aid the understanding of the pVTA in addiction. This toxin could also assist in determining whether the results found in previous lesion studies of the PPTg are solely due to the loss of cholinergic neurons. Furthermore, the unique cholinergic mesopontine expression of UII-Rs in the PPTg and LDTg may be a useful tool for further understanding

the role of cholinergic input in reward related behaviours and the addictive properties of drugs of abuse. By furthering our knowledge of the structural organisation and functions of the VTA, PPTg and LDTg, our understanding of the implications of addiction will expand. It is important to study the specific connections between the nuclei of the brainstem, which will in turn advance our understanding of pathologies of various mental disorders and neurological diseases, as well as their role in addiction – possibly uncovering new potential targets for therapeutic intervention.

3.2 Chapter aim

The aim of this chapter was to determine whether complete cholinergic denervation of the pVTA could be achieved. The specific aim was to determine at what time point cholinergic lesions would form, if at all, using group sizes large enough to perform basic behavioural analysis. The following experiments were used to: - (1) confirm pipette location in the pVTA validating the stereotaxic coordinates used, (2) identify areas of neurodegeneration in the pVTA and PPTg/LDTg which could occur through retrograde degeneration, (3) determine the selective toxicity of Dtx-UII and (4) to analyse the effect of Dtx-UII on basic locomotion.

3.3 Toxin study 1: Dtx-UII and basic locomotion

3.3.1 Experimental overview

A fresh batch of freeze dried Dtx-UII was supplied by S.D. Clark (Department of Pharmacology and Toxicology, University of Buffalo, NY, U.S.A) and this was reconstituted and stored at -80°C as directed. The coordinates used for the pVTA had previously been tested and validated in this laboratory (Farquhar, Latimer and Winn, 2012). The volume and concentration of Dtx-UII (200nl; 3%) used for this study had been established for pPPTg and LDTg lesions (MacLaren, 2012). MacLaren (2012) studied a range of concentrations and volumes, which covered those developed by S.D. Clark, and found that a higher concentration

and/or volume caused non-specific damage in the mesopontine tegmentum (Clark *et al.*, 2007; MacLaren, 2012). Dtx-UII must be highly destructive to cholinergic neurons and leave non-cholinergic neurons intact. Multiple injections along the length of the PPTg were not only found to cause significant cholinergic cell loss but also substantial non-specific damage due to toxin accumulation (MacLaren, 2012). However, in this study one 200nl infusion (per hemisphere) ruled out the possibility of toxin accumulation in the pVTA.

In this study, rats received bilateral infusion of Dtx-UII into the pVTA. Cholinergic terminal density in the pVTA and cholinergic cell density in the mesopontine nuclei were assessed 9 days after Dtx-UII infusion – in the PPTg there was a 75% loss of cholinergic neurons 7 – 14 days after Dtx-UII injection (Clark *et al.*, 2007). During this period, basic locomotion was measured over 7 days to determine the behavioural effect of intra-pVTA Dtx-UII on locomotion. If cholinergic denervation of the pVTA could be achieved, baseline locomotor data would be useful as control data for Dtx-UII lesioned animals. This would allow assessment of Dtx-UII pVTA lesions on drug induced locomotor behaviour, or to investigate selective cholinergic lesions in other brain regions (providing UII-R or UII binding sites are present).

3.3.2 Materials and methods

Subjects

Twenty-four adult male Lister Hooded rats were used for this study. Rats were housed, anaesthetised and microinfusion surgery was performed as described in the General Methods. Animals weighed between 330 – 340g at the time of surgery. The coordinates for the pVTA were as stated in the General Methods.

Surgical procedure

Lesion surgery was performed as described in the General Methods. Rats in the toxin group (n = 14) received bilateral pVTA infusion of 3% Dtx-UII (200nl in 0.1M PB) – the concentration and volume optimal for pPPTg lesions. Control rats (n = 7) received bilateral pVTA infusion of sterile PB (200nl, 0.1M PB). The order of the first hemisphere infused (left or right) was alternated across rats – animals underwent surgery in order of increasing weight to ensure that their weights were approximately the same.

Locomotor testing

As described in the General Methods, Perspex cages positioned in SmartFrame™ Cage Rack Stations were used to monitor basic locomotion. Two days after surgery, locomotor monitoring was performed daily for a duration of 60 minutes. Animals were given 2 habituation sessions (data not shown) where they were placed in the cages but did not receive an injection. This was immediately followed by 7 locomotor sessions where the animals received an injection of saline (administered s.c.; 0.9%, 1ml/kg) directly before testing. All injections were performed in a procedures room opposite the locomotor test room. Each rat was individually taken to the procedures room, injected, and then placed in a locomotor cage for testing. Each rat was always injected by the same handler, in the same room and never injected in the presence of

another rat. All behavioural testing was carried out during the light phase under dull red-light illumination.

Histology

At the end of testing, animals were transcardially perfused and brains were stored in sucrose as described in the General Methods. Each brain was dissected, from the hippocampus to the anterior cerebellum and 30µm sections cut. All histological procedures were performed as stated in the General Methods. A 1:4 series of sections were processed for neuron-specific nuclear protein (NeuN) and cresyl double stain, tyrosine hydroxylase (TH) and vesicular acetylcholine transporter (VAChT) immunohistochemical analysis. All sections that crossed through the VTA were processed. Sections were mounted onto glass slides and viewed via light or fluorescent microscopy.

Data analysis

Quantification of VAChT+ terminals were carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Statistical analyses were performed in SPSS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). An independent-samples t-test was applied to determine if there were any differences in VAChT+ puncta between Dtx-UII and sterile PB infused tissue. Results are expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. A repeated measures ANOVA was applied to compare VAChT+ cells along the anterior-posterior plane of PPTg and LDTg with *groups* (Dtx-UII and sterile PB) and *level* (anterior-posterior plane) as factors. Locomotor results were collated as square root transformed (SQRT) ± SEM in order to correct for positive skew in the data (identified by Shapiro-Wilk test). A range of ANOVAs were performed to compare across *sessions* (1 – 7) and between *groups* (Dtx-UII and sterile PB) and the appropriate post hoc analysis were applied. Results were considered significant when $p \leq 0.05$.

Cohen's d was calculated to determine the effect size and to indicate the standardised difference between two means.

3.3.3 Results

Recovery

Animals recovered well from surgery (n = 21). Death during surgery (n = 2) or recovery (n = 1 – respiratory complications) occurred on three occasions. The general health of all rats was closely monitored for the duration of the experiment. Changes in body weight were closely monitored throughout the course of the experiment (Figure 3.2). When required, animals were given additional wet mash to help maintain a stable body weight. A repeated measures ANOVA found that there was no significant effect of *group* on body weight change ($F_{(1, 28)}$ = 1.757; p = 0.196). There was also no significant interaction between *group* x *day* ($F_{(6, 168)}$ = 1.214; p = 0.301) (Figure 3.2). As expected, there was a significant effect of *day* on body weight change over the course of the experiment as the animals gained weight ($F_{(1,168)}$ = 54.572; p = 0.000) (Figure 3.2).

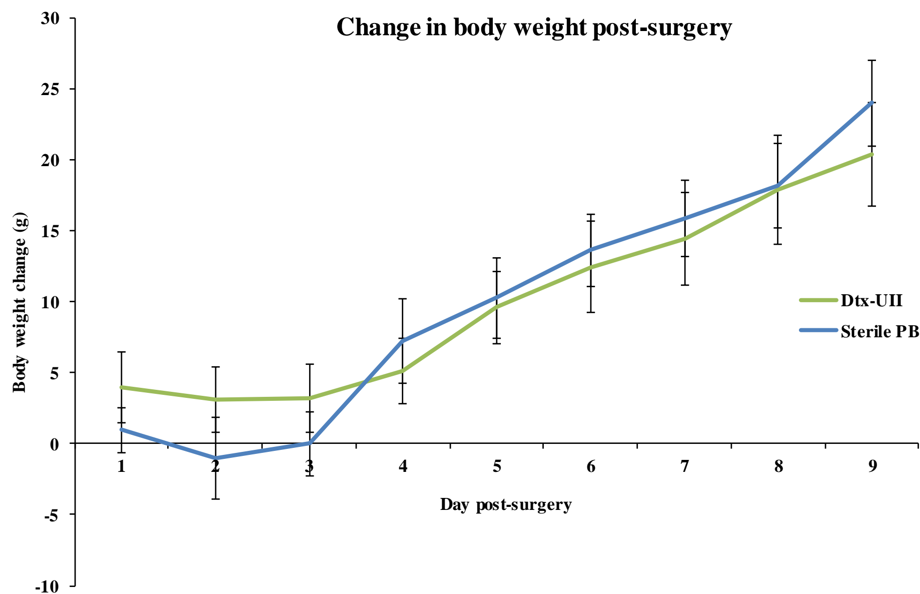


Figure 3.2 – Post-surgery body weight of Dtx-UII and sterile PB pVTA infused animals. Graph shows change in bodyweight during the 9-day period after surgery in Dtx-UII (green line; n = 14) and sterile PB (blue line; n = 7) animals. Graph shows group mean ± SEM.

Gross histological analysis

Fourteen rats received bilateral pVTA infusion of Dtx-UII aiming to destroy cholinergic terminals arriving in the pVTA from the pPPTg and LDTg. Toxin and control tissue was assessed through NeuN/cresyl double stain, TH staining and VAChT immunofluorescence. Representative pVTA tissue processed for NeuN/cresyl and TH are shown below (Figure 3.3). pVTA sections processed for TH were analysed for changes in DA cell morphology or neuron density following Dtx-UII infusion. Through visual inspection there were no obvious differences between toxin and control animals in pVTA DA cells (Figure 3.3A and B). NeuN/cresyl double stained sections were analysed for evidence of cell death, reactive gliosis, and changes in cell morphology or neuron density (Figure 3.3C and D). There was also no non-cholinergic cell death or damage in or around the pVTA following Dtx-UII infusion. There was no evidence of reactive gliosis – cell morphology and neuron density remained unchanged in the pVTA. NeuN/cresyl double stain did not reveal tract damage, at any level of VTA, from the glass micropipette in toxin or control tissue – which means pipette location could not be confirmed. Representative pPPTg tissue processed for NeuN/cresyl is shown below (Figure 3.4).

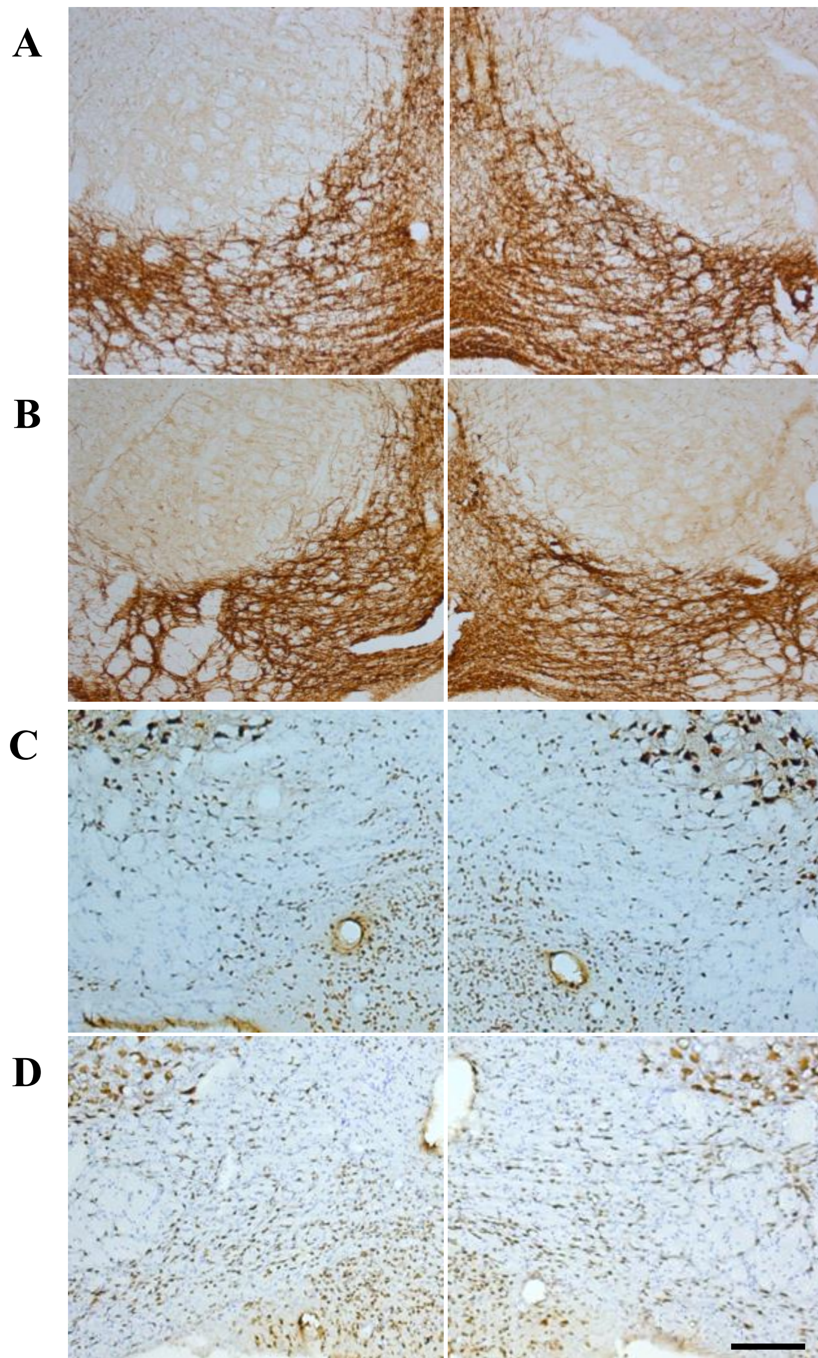


Figure 3.3 – Example pVTA sections of Dtx-UII and sterile PB infused rats. pVTA sections stained with TH after sterile PB (A) and Dtx-UII (B) infusion show no change in pVTA DA neuron density or morphology. Sections processed for NeuN/cresyl double stain (C and D) show no non-specific cellular damage following Dtx-UII (D) infusion when compared to sterile PB (C) infused tissue. NeuN/Cresyl staining did not reveal tract damage to indicate pipette location. Scale bar = 100μm.

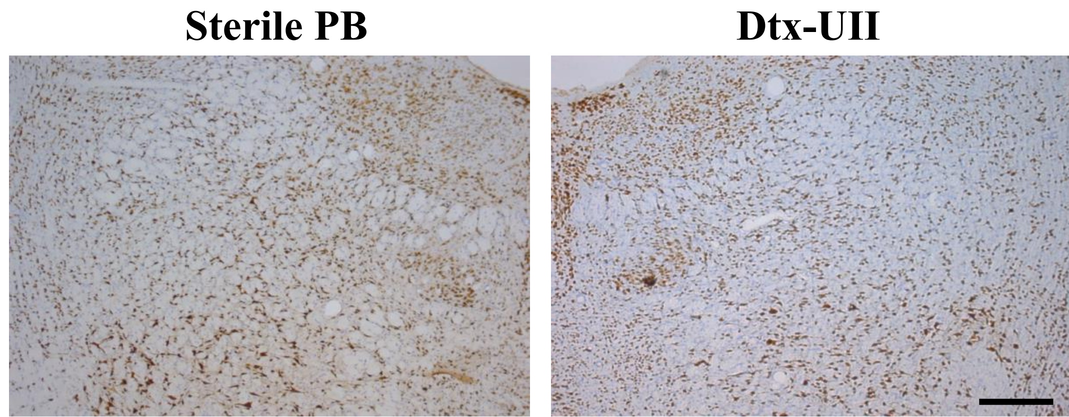


Figure 3.4 – Example PPTg sections of Dtx-UII and sterile PB pVTA infused tissue. PPTg sections stained with NeuN/cresyl after sterile PB and Dtx-UII pVTA infusion show no non-specific cellular damage. Sections appear to be denser in control PPTg sections, than toxin animals; however, this difference was observed throughout the section and was not localised to the PPTg. Scale bar = 100µm

Vesicular acetylcholine transporter

Using VAChT staining and fluorescent microscopy, cholinergic terminal density in the pVTA and cell density across the anterior-posterior plane of the PPTg and LDTg were analysed. Representative pVTA, pPPTg and LDTg tissue from Dtx-UII and control animals are shown below (Figure 3.5). These images were analysed using ImageJ software (ImageJ, U.S. National Institutes of Health, U.S.A) to calculate the number of individual cholinergic puncta or cells present (Figure 3.6 and 3.7).

There were no obvious differences in cholinergic terminal density in the pVTA of Dtx-UII and sterile PB infused animals (Figure 3.5 and 3.6). An independent-samples t-test was applied to determine if there were any differences in VAChT+ puncta between Dtx-UII and sterile PB infused tissue. There was no difference in cholinergic terminal density in Dtx-UII tissue (VAChT+ terminals = 1837 ± 328 [mean ± SEM]) compared to control tissue (VAChT+ terminals = 1806 ± 169 [mean ± SEM]) (Figure 3.5 and 3.6). The independent-samples t-test indicated that Dtx-UII had no effect on cholinergic terminal density ($t = 0.65$, df 30, $p = 0.948$). In all pVTA sections processed for VAChT, cholinergic terminals were arranged in a clear linear organisation (Figure 3.5). Further, Cohen's effect size value ($d = 0.3$) suggested a moderate practical significance.

The number of cholinergic cell bodies in the PPTg and LDTg remained unchanged following toxin infusion (Figure 3.5 and 3.7). Distribution of VAChT+ cells along the anterior-posterior plane of PPTg and LDTg in Dtx-UII and control groups are shown below (Figure 3.7). A repeated measures ANOVA found that there was no significant effect of *group* on VAChT+ PPTg cells ($F_{(1, 2)} = 0.373$; $p = 0.604$). There was also no significant interaction between *group* x *level* ($F_{(10, 20)} = 1.365$; $p = 0.265$) (Figure 3.7). A repeated measures ANOVA found that there was no significant effect of *group* on VAChT+ LDTg cells ($F_{(1, 5)} = 0.049$; $p = 0.834$). There was also no significant interaction between *group* x *level* ($F_{(8, 40)} = 1.196$; $p = 0.326$) (Figure 3.7). Cohen's effect size value ($d = 0.56$) suggested a moderate to high practical significance.

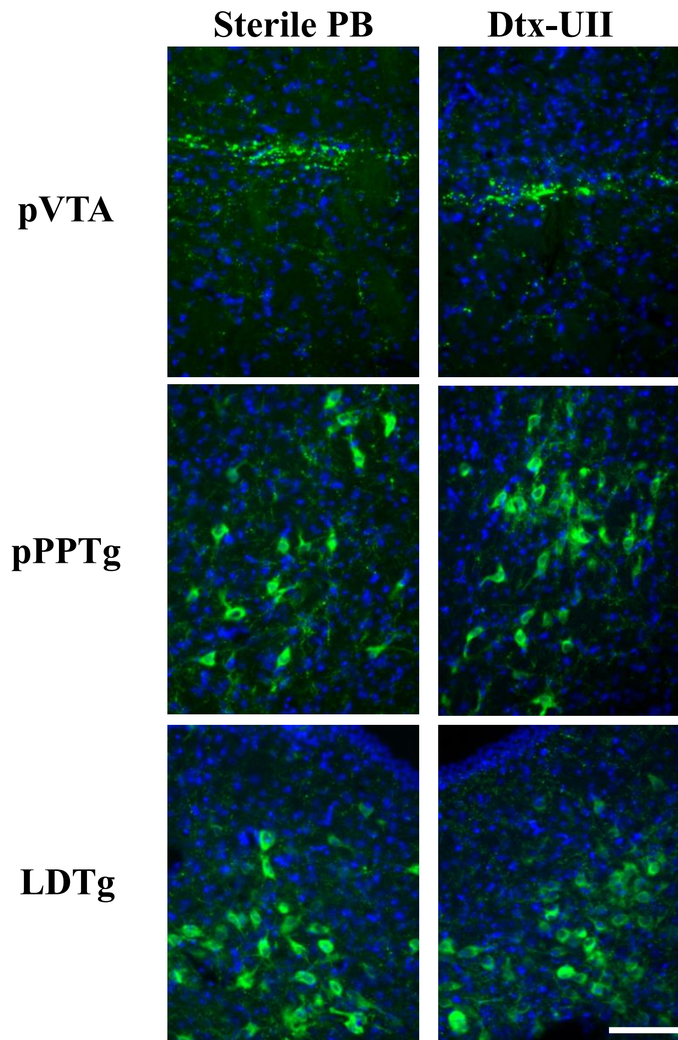


Figure 3.5 – The comparative distribution of cholinergic terminals and cell bodies (green) in the adult rat pVTA, pPPTg and LDTg after Dtx-UII pVTA infusion. Nine days after infusion no difference was observed between Dtx-UII and control hemispheres in the pVTA, pPPTg and LDTg. Nuclei were labelled with VECTASHIELD® Hard setting mountant with DAPI (blue). VAChT+ profiles were labelled with Alexa Fluor® 488 Goat Anti-Rabbit IgG. Scale bar = 100µm.

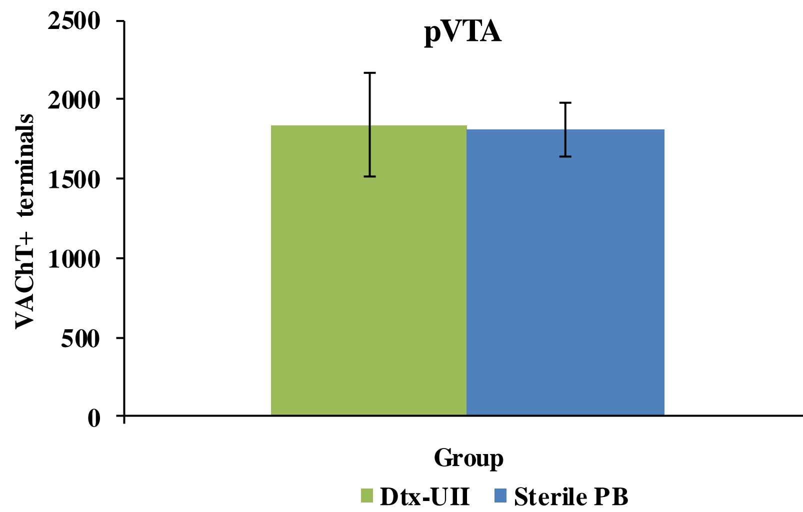
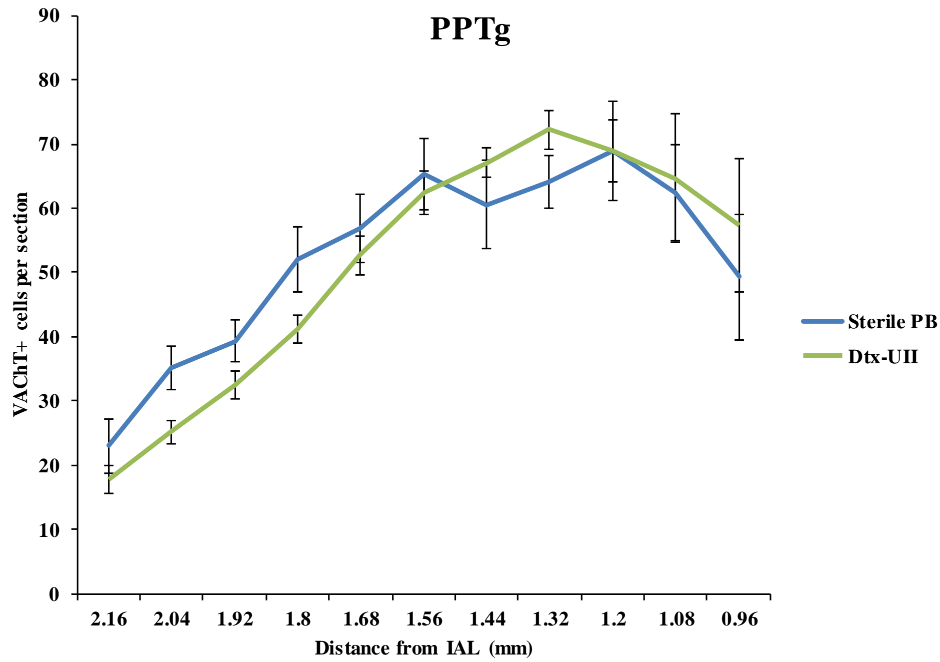


Figure 3.6 – Cholinergic puncta analysis after VAChT staining using ImageJ software. Cholinergic puncta of the pVTA remained unchanged after Dtx-UII infusion compared to control hemispheres 9 days after surgery. An independent-sample t-test was carried out at this time point ($p > 0.05$; $p = 0.948$). Dtx-UII (green bar; n = 14) and sterile PB (blue bar; n = 7) infused rats. Graph shows mean ± SEM.



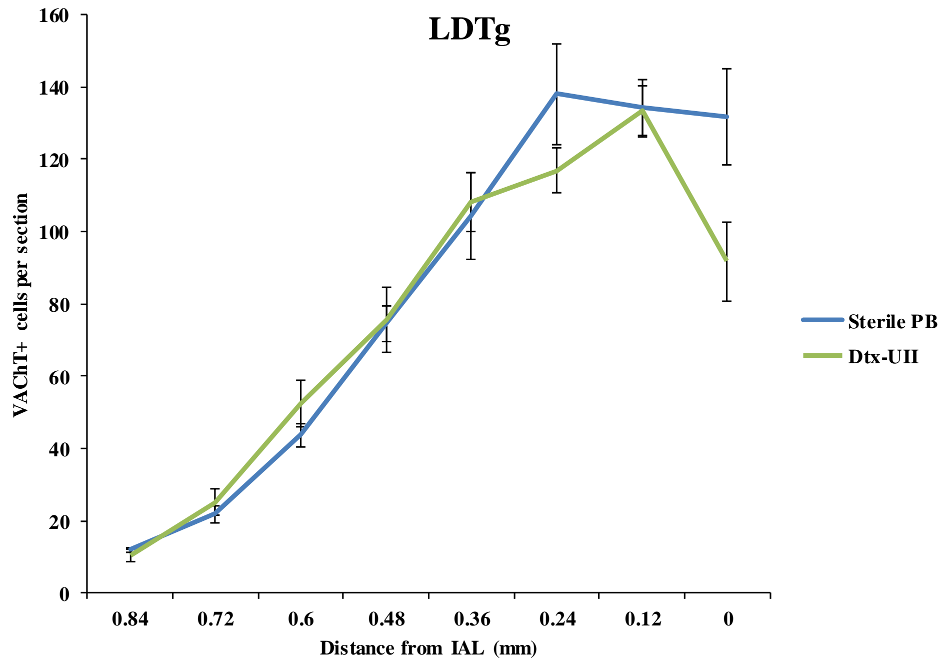


Figure 3.7 – Distribution of VAChT+ cells along the anterior-posterior plane of PPTg and LDTg in Dtx-UII and control groups. Dtx-UII (green line; n = 14) and sterile PB (blue line; n = 7) infused rats. Graphs show group mean ± SEM.

Behavioural analysis

The number of beam breaks (square root transformed: SQRT) for basic movements during 7 locomotor sessions are shown below (Figure 3.8A). Basic movements (the total number of beam breaks at the level of the animal's body) did not differ between toxin and control groups

as demonstrated through a repeated measures ANOVA ($F_{(1, 28)} = 0.082$; $p = 0.776$). There was also no effect of *session* ($F_{(6, 168)} = 1.051$; $p = 0.395$), and no *session* x *group* interaction ($F_{(6, 168)} = 0.583$; $p = 0.744$). There was a slight fluctuation in beam breaks by session 5 where toxin animals appeared to have an increased number of beam breaks but restricted pairwise comparisons failed to find any significant differences between sessions ($p > 0.05$).

The number of fine movements (such as forepaw grooming or head waves) measured as the number of beam breaks (SQRT) are shown below (Figure 3.8B). A repeated measures ANOVA for fine movement data found that there was no effect of *group*; Dtx-UII animals did not show a change in the number of fine movements compared to control animals ($F_{(1, 28)} = 0.134$; $p = 0.717$). It was further observed that there was no effect of *session* ($F_{(6, 168)} = 0.777$; $p = 0.589$), and no *session* x *group* interaction for fine movements ($F_{(6, 168)} = 0.572$; $p = 0.752$). As with basic movements, it was demonstrated that after session 5 there were slight differences in beam breaks but this was not of statistical significance, as restricted pairwise comparisons did not identify any significant differences between sessions ($p > 0.05$).

The number of rears completed by the animal (measured by the additional rearing frame) was expressed as the number of beam breaks SQRT (Figure 3.8C). Again, using the same methods of statistical analysis it was found that there were no significant differences between toxin and control *groups* ($F_{(1, 28)} = 0.554$; $p = 0.901$). It was also indicated that the number of rears were not affected by *session* ($F_{(6, 168)} = 1.556$; $p = 0.163$) and there was no interaction between *session* x *group* ($F_{(6, 168)} = 0.717$; $p = 0.636$). Restricted pairwise comparison failed to find any significant differences between sessions ($p > 0.05$). Together these results demonstrate that intra-pVTA infusion of Dtx-UII had no effect on locomotion or any other form of movement measured over a 7-day post-infusion period. These results also demonstrated that habituation to the environment and injections was achieved by both animal groups.

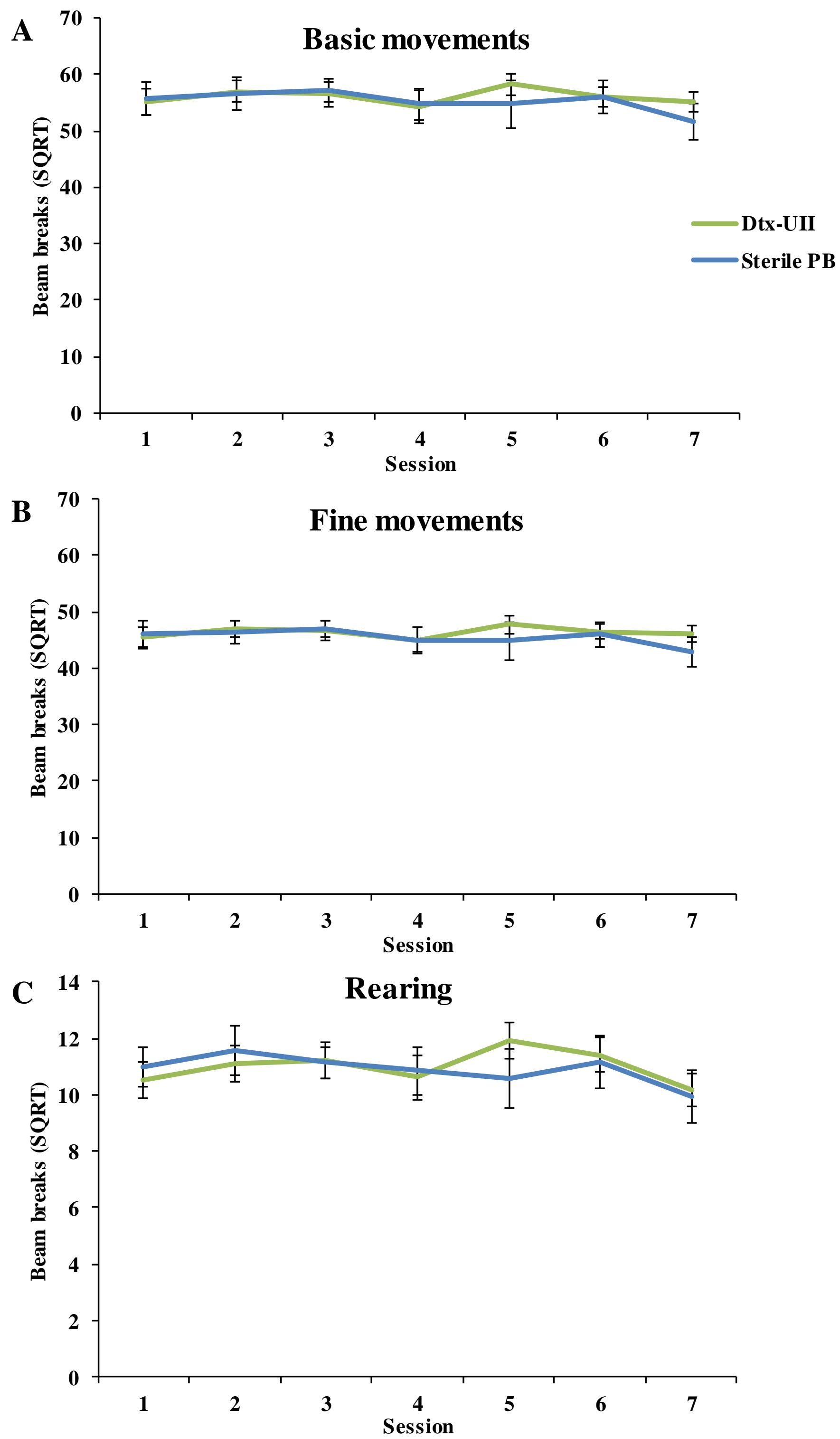


Figure 3.8 – Basic movements (A), fine movements (B) and rearing behaviour (C) (all SQRT transformed) made during locomotor testing sessions following saline injection. Graph shows mean beam breaks during 7 daily testing sessions by Dtx-UII (green line; n = 14) and sterile PB (blue line; n = 7) infused rats. Error bars = ± SEM.

3.3.4 Conclusions of toxin study 1

The main aim of this study was to determine the effect of bilateral pVTA Dtx-UII infusion. Nine days after surgery there was no evidence of non-selective damage in or around the pVTA. Dtx-UII had no visible effect on pVTA DA cells at this time point. There was no sign of tract damage to indicate pipette positioning and so pipette location and stereotaxic coordinates could not be confirmed. At this time point, through all pVTA sections examined, there were no detectable differences in cholinergic terminal density after Dtx-UII infusion. The number of cholinergic cell bodies across the PPTg and LDTg remained unchanged. Intra-pVTA Dtx-UII had no effect on basic movements, fine movements or rearing behaviour. The logical conclusions are that pVTA Dtx-UII infusion had no effect on cholinergic input from the pPPTg and LDTg after 9 days, or that the effect of Dtx-UII infusion can destroy cholinergic terminals but it does not manifest this early after toxin delivery because neurodegeneration would develop in a retrograde manner. A higher concentration or larger volume of Dtx-UII may be required to have a toxic effect on pVTA cholinergic terminals. However, as previous studies have shown, this can also cause damage to non-cholinergic neurons. The next aim was to confirm pipette placement to validate stereotaxic coordinates and then to further investigate post-surgery time points.

3.4 Toxin study 2: Dtx-UII time course experiment

3.4.1 Experiment overview

The principal aim of this study was to determine the time course and specificity of cholinergic terminal loss in the pVTA following Dtx-UII infusion. Rats received a unilateral infusion of Dtx-UII into the pVTA and sterile phosphate buffer (PB) into the contralateral hemisphere. As the vast majority of ascending and descending VTA projections are ipsilateral, each rat acted as its own control (Fields *et al.*, 2007). Three rats were euthanized at 5 different time points (2, 4, 6, 8 and 10 days post-surgery). In the PPTg there was 75% loss of cholinergic neurons

7 – 14 days after Dtx-UII injection (Clark *et al.*, 2007) and the brains were examined to assess cholinergic terminal density in the pVTA and cell body damage in the pPPTg and LDTg. This was achieved using immunofluorescent imaging for VAChT (as in toxin study 1) and Fluoro-jade C. As a control group, 3 rats received a unilateral infusion of ibotenic acid to confirm pVTA stereotaxic coordinates and to act as a positive control for a known source of neurodegeneration. Combined, these methods enabled assessment of the selective toxicity of Dtx-UII infusion in the pVTA.

3.4.2 Materials and methods

Subjects

Eighteen male Lister Hooded rats were used in this experiment. Rats were housed, anaesthetised and microinfusion surgery was performed as described in the General Methods. Animals weighed between 330 – 340g at the time of surgery. The coordinates for the pVTA were as stated in the General Methods.

Surgical procedure

All infusions were performed as stated in the General Methods. Rats for the time course study (n = 15) received a unilateral pVTA infusion of 3% Dtx-UII (200nl in 0.1M sterile PB) and an infusion of sterile PB (200nl, 0.1M PB) to the contralateral hemisphere. Excitotoxin rats (n = 3) received a unilateral pVTA infusion of 0.06M ibotenic acid (180nl in 0.1M sterile PB) but did not receive an infusion to the contralateral hemisphere to act as a control. Toxin infusion to the left or right hemisphere was alternated across rats – animals underwent surgery in order of increasing weight to ensure that their weights were approximately the same.

Histology

Three rats were euthanized and transcardially perfused (as described in the General Methods) at each of the 5 different time points (2, 4, 6, 8 and 10 days post-surgery). In the PPTg there is

loss of 75% of cholinergic neurons 7 – 14 days after Dtx-UII injection (Clark *et al.*, 2007). Excitotoxin-lesioned rats were perfused 72 h post-surgery. Each brain was dissected from the hippocampus to the anterior cerebellum, 30µm sections cut and a 1:4 series of sections were processed for histology (see General Methods). All sections that crossed through the VTA were processed. Tissue from excitotoxin-lesioned animals was processed with NeuN/cresyl double stain and Fluoro-jade C and tissue from the time course animals was processed for VAChT immunohistochemical analysis (see General Methods). Tissue from time course animals was examined through fluorescent microscopy to assess cholinergic terminal density in the pVTA and cell body density in the pPPTg and LDTg.

Data analysis

Quantification of VAChT+ terminals was carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Statistical analyses were performed in SPSS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). An independent-samples t-test was applied for each time point to determine if cholinergic terminal density altered after Dtx-UII infusion 2, 4, 6, 8 and 10 days after surgery. Results are expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. Results were considered significant when $p \leq 0.05$.

3.4.3 Results

Recovery

All animals recovered well from surgery (n = 18). Immediately after surgery, excitotoxin-lesioned rats were administered diazepam (i.p. 0.2ml) to avoid animals exhibiting seizures, barrel rolling, circling or Cheyne-Stokes breathing (all of which have been previously associated with ibotenic acid lesions). None of these were displayed. The body weight and general health of all rats were closely monitored throughout the course of the experiment and additional wet mash was provided when required to help maintain a stable body weight.

Pipette location

In order to confirm pipette position and stereotaxic coordinates, rats (n = 3) received a unilateral pVTA infusion of ibotenic acid. NeuN/cresyl double stain was used to verify the location of the glass pipette tip in the pVTA. Sections were analysed for evidence of cell death, neuron density, reactive gliosis and changes in cell morphology to identify the location of toxin infusion. In all pVTA sections there were clear differences between the hemisphere infused with ibotenic acid and control hemisphere. Representative tissue from pVTA ibotenic acid infusion is shown below (Figure 3.9). The area of cell loss clearly indicates pipette tip location and the site of ibotenic acid infusion at the level of the pVTA in the right hemisphere (Figure 3.9). There were no signs of track damage at any level of VTA, which can often be caused by glass pipettes. However, tracts are often hard to locate because of the narrow width of the pipette, differences in angle of pipette penetration and angle of brain blocking for sectioning.

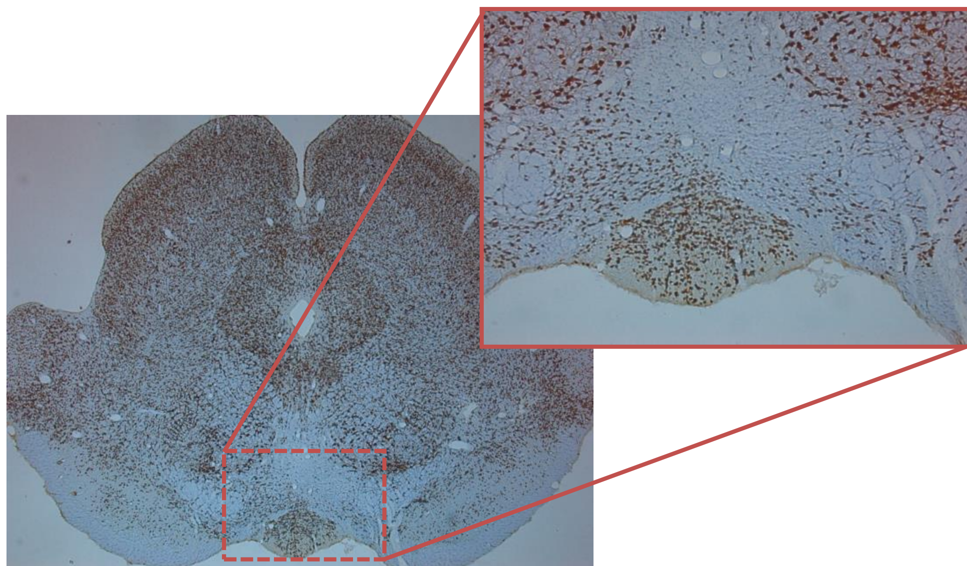


Figure 3.9 – Example section of pVTA unilateral ibotenic acid infusion. Cell death was clearly visible at the level of the pVTA in the right hemisphere following excitotoxin infusion. Sections were stained with NeuN/Cresyl double stain.

Time course analysis

Fifteen rats underwent unilateral Dtx-UII infusion aiming to destroy cholinergic terminals in the pVTA projecting from the pPPTg and LDTg. At each time point (2, 4, 6, 8 and 10 days after infusion) 3 animals were euthanized in order to determine whether a lesion had formed and, if a lesion had formed, to investigate lesion progression. Using VAChT staining and fluorescent microscopy, cholinergic terminal density in the pVTA after Dtx-UII infusion was analysed. Representative pVTA tissue from Dtx-UII and corresponding control hemispheres at the 5 time points are shown below (Figure 3.10A – J). All pVTA images were analysed using ImageJ software to calculate the number of individual cholinergic puncta (Table 3.1 and Figure 3.11A – E).

An independent-samples t-test was applied for each time point to determine if there were any differences in VAChT profiles between Dtx-UII and sterile PB infused hemispheres. At each time point after surgery there were no obvious differences between toxin and control hemispheres (Table 3.1, Figure 3.10A – J and 3.11A – E). An independent-samples t-test was carried out for each time point and each test indicated that Dtx-UII had no effect on cholinergic terminal density (Table 3.1). In all pVTA sections stained with VAChT, cholinergic terminals were arranged in a clear linear organisation (Figure 3.10 A – J).

VAChT+ terminals (mean ± SEM)			Independent t-test	Cohen's *d*
Day	**Dtx-UII hemisphere**	**Sterile PB hemisphere**		
2	276 ± 64	323 ± 47	*t* = - 0.591, df 4, p = 0.586	0.5
4	690 ± 91	702 ± 128	*t* = - 0.80, df 4, p = 0.940	0.1
6	264 ± 35	323 ± 57	*t* = - 0.875, df 4, p = 0.431	0.7
8	510 ± 100	447 ± 74	*t* = - 0.503, df 4, p = 0.642	0.4
10	658 ± 69	618 ± 116	*t* = - 0.297, df 4, p = 0.781	0.2

Table 3.1 – Quantification of VAChT+ terminals in the adult rat pVTA after Dtx-UII or sterile PB infusion using ImageJ software.

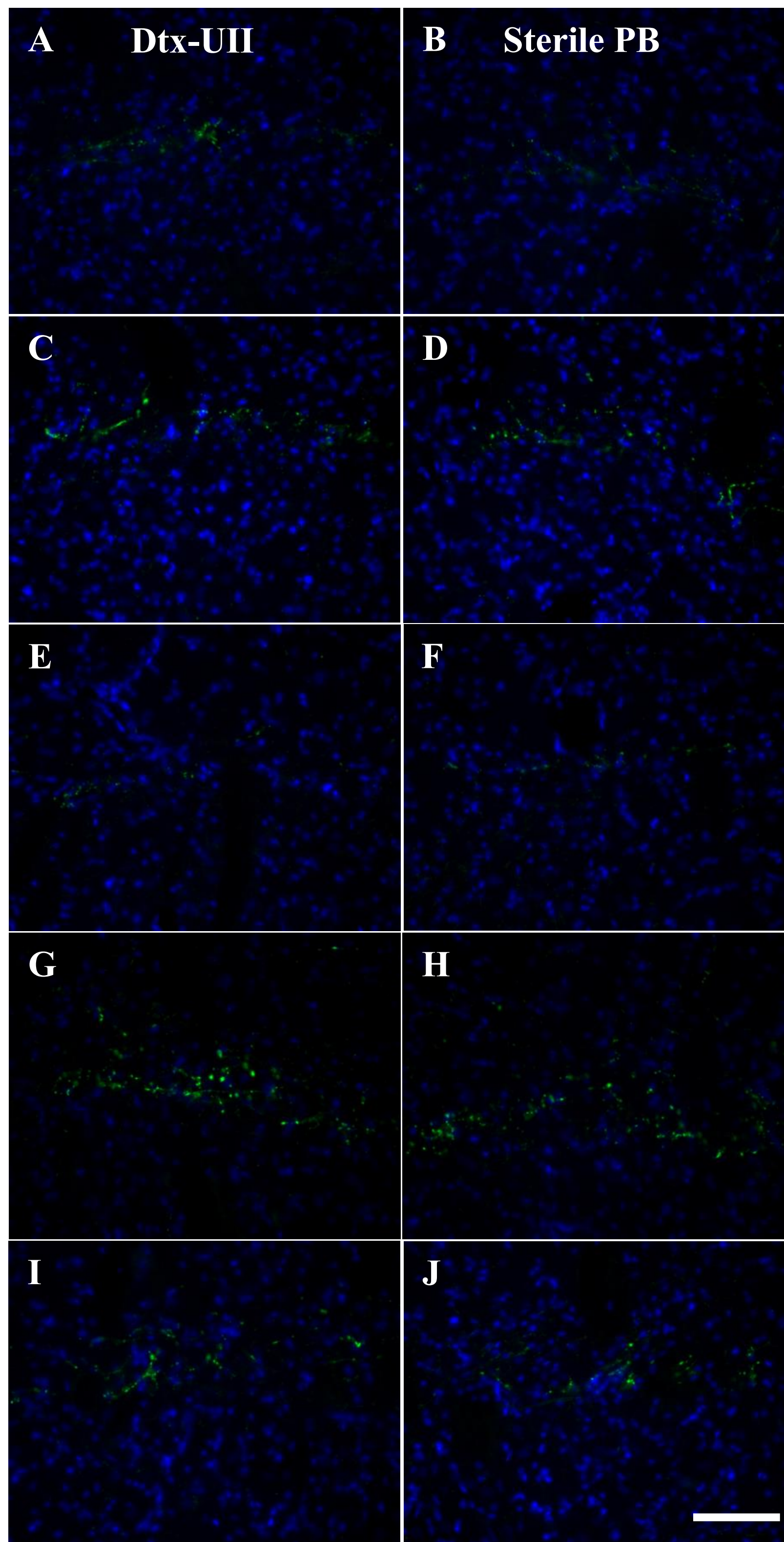


Figure 3.10 – The comparative distribution of cholinergic terminals (green) in the adult rat pVTA after Dtx-UII infusion. At 5 different time points it was observed that there were little differences between Dtx-UII and control hemispheres: - 2 (A and B), 4 (C and D), 6 (E and F), 8 (G and H) and 10 (I and J) days after surgery (n = 3 at each time point). Nuclei were labelled with VECTASHIELD® Hard setting mountant with DAPI (blue). VAChT+ profiles were labelled with Alexa Fluor® 488 Goat Anti-Rabbit IgG. Scale bar = 100µm.

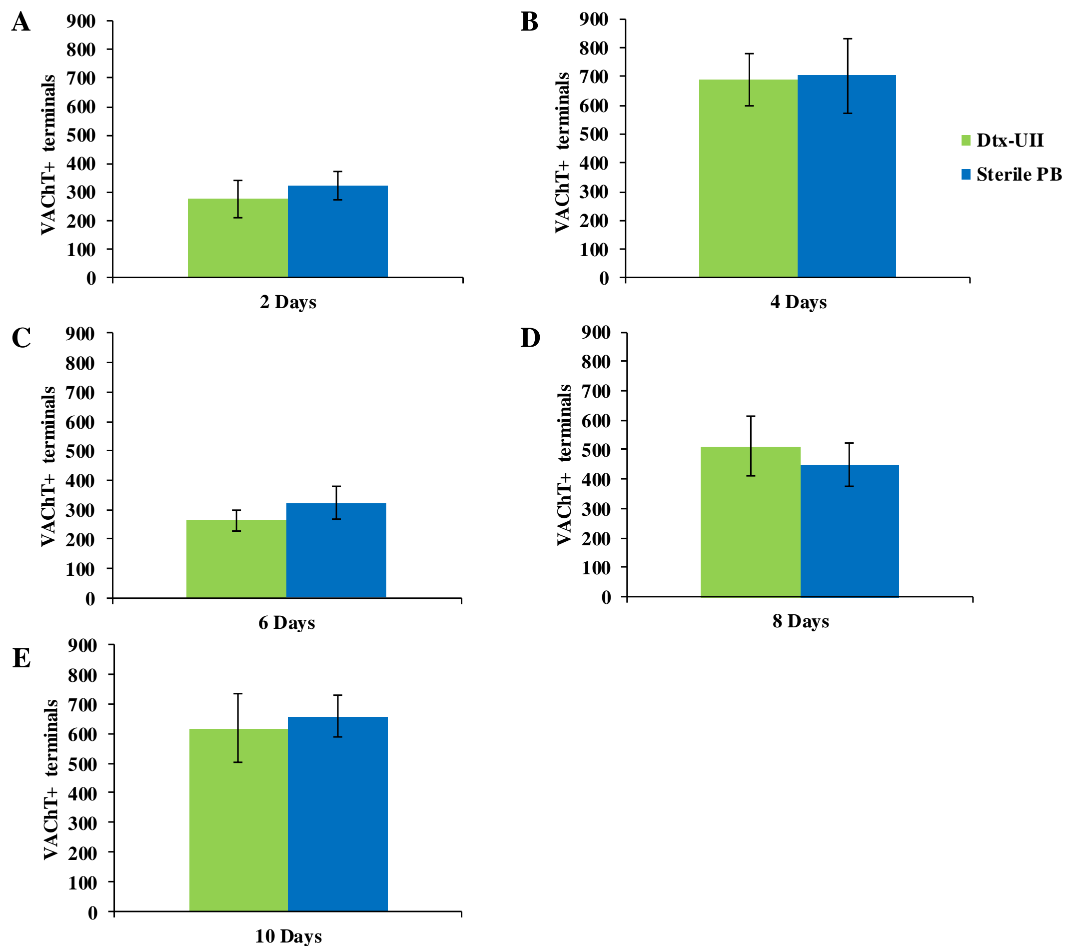


Figure 3.11 – Cholinergic puncta analysis after VAChT staining using ImageJ software. Cholinergic puncta of the pVTA remained unchanged after Dtx-UII infusion compared to control hemispheres; this was observed 2 (A), 4 (B), 6 (C), 8 (D) and 10 (E) days after surgery. An independent-samples t-test was carried out for each time point ($p > 0.05$; 2 days $p = 0.705$, 4 days $p = 0.687$, 6 days $p = 0.283$, 8 days $p = 0.479$ and 10 days $p = 0.459$). Dtx-UII (green bar; n = 15) and sterile PB (blue bar; n = 15) infused tissue. Graphs show mean ± SEM.

Neurodegeneration

In order to determine whether neurodegeneration occurred in the pVTA and pPPTg after Dtx-UII pVTA infusion, tissue sections were processed with Fluoro-jade C. Tissue from animals that received unilateral pVTA ibotenate infusion was used as a positive control because ibotenic acid is a known source of non-selective neurodegeneration (Figure 3.12A). Fluoro-jade C positive cells (Fluoro-jade C+) were observed in pVTA tissue infused with ibotenic acid whereas there were no positive cells visible in the contralateral hemisphere that received no infusion (Figure 3.12A and B); thus confirming that ibotenic acid is a suitable control for

examining neurodegeneration labelled by Fluoro-jade C. At each time point, no Fluoro-jade C+ cells were observed in either pVTA (infused with Dtx-UII or sterile PB). Representative tissue 2 days after infusion is shown below (Figure 3.12C and D). There were no Fluoro-jade C+ cells observed at any of the time points in corresponding pPPTg tissue (Figure 3.12E and F; 2 days after pVTA Dtx-UII infusion).

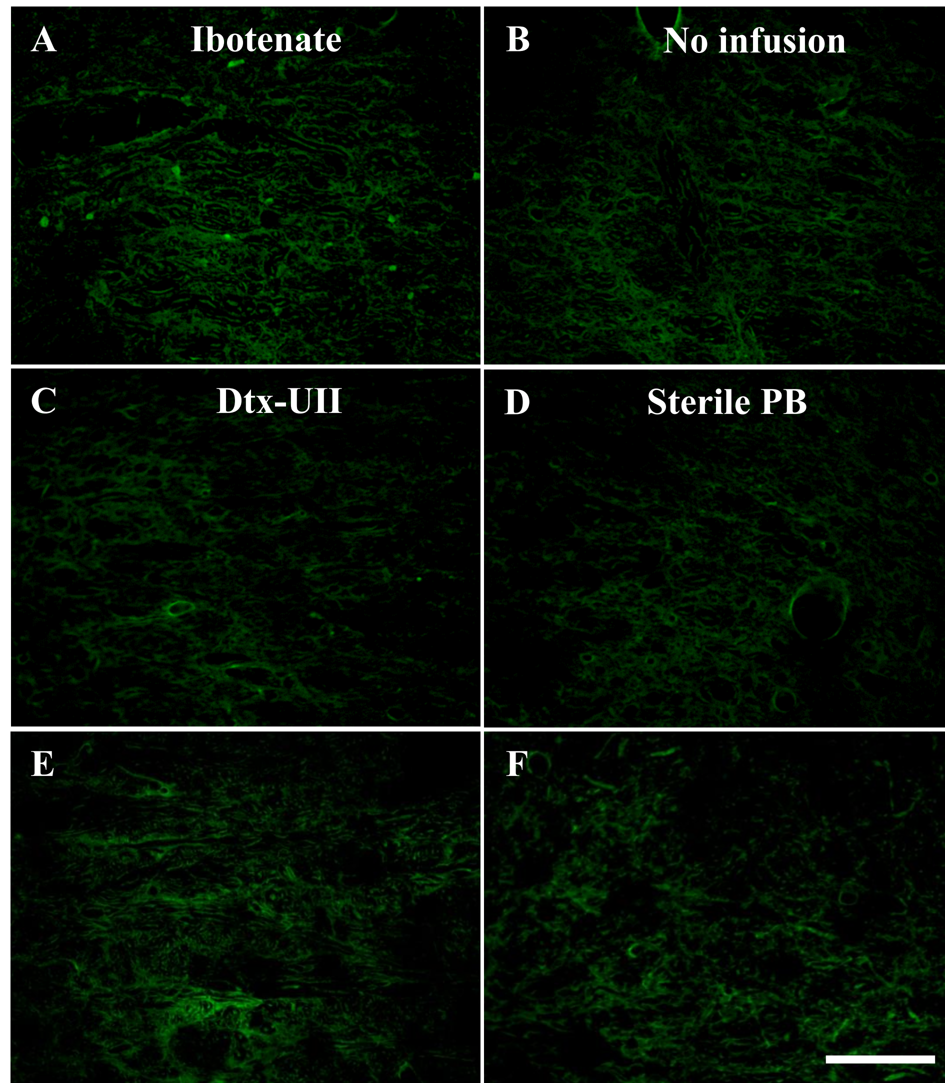


Figure 3.12 – Comparison of ibotenate induced neurodegeneration in pVTA vs. pVTA Dtx-UII infused tissue and corresponding pPPTg stained with Fluoro-jade C. Degenerating cells were labelled Fluoro-jade C+ after ibotenate infusion (A) but not in the control hemisphere (B). No cells in Dtx-UII hemispheres 2, 4, 6, 8 and 10 days post-surgery were stained with Fluoro-jade C, indicating that no neurodegeneration had occurred. Example tissue of pVTA (C and D) and pPPTg (E and F) Fluoro-jade C stained sections 2 days after surgery are shown. Scale bar = 100µm.

3.4.4 Conclusions of toxin study 2

This study revealed that Dtx-UII infusion into the pVTA had no visible effect on cholinergic terminals 2, 4, 6, 8 and 10 days after surgery. At each time point, through all VTA sections analysed, there were no detectable immunohistochemical differences observed in VAChT and Fluoro-jade C immunohistochemistry. This would indicate that cholinergic terminals remained present in the pVTA and that no neurodegeneration had occurred. In all VTA sections processed for VAChT, cholinergic terminals were arranged in a clear linear organisation – this was also observed in toxin study 1. Excitotoxin animals confirmed pipette position and stereotaxic pVTA coordinates. In addition, ibotenic acid reliably acted as a known source of neurodegeneration for Fluoro-jade C immunohistology. The logical conclusions are that pVTA Dtx-UII infusion had no effect on cholinergic input from the pPPTg and LDTg at any of the time points investigated or that the effect of Dtx-UII infusion can destroy cholinergic terminals but it does not manifest until a longer period of time as neurodegeneration would develop in a retrograde manner. The next aim was to look at a longer time point to see if retrograde degradation would occur 6 weeks after surgery.

3.5 Toxin study 3: Dtx-UII 6-week experiment

3.5.1 Experimental overview

Pilot data from S.D. Clark's laboratory indicated that cholinergic denervation of the pVTA using Dtx-UII was achieved 10 weeks after infusion. Sprague-Dawley rats received 6 bilateral infusions of 3% Dtx-UII (200nl x 6 per hemisphere) at graded dorsoventral (DV) coordinates with 60 seconds between each infusion. pVTA coordinates were: - anterior-posterior -6.2 mm from bregma (approximately +2.70 mm from interaural line [IAL]), midline ±3.2 mm (20º angle), and DV -8.3 mm from dura. Clark and his colleagues reported that the volume was considerably larger than the volume used for Lister Hooded rat PPTg surgeries. However, it was based on Dtx-UII PPTg surgeries in Sprague-Dawley rats that required a total volume of

toxin greater than 1µl (per hemisphere) for the formation of lesions equivalent to those made using 200 nl in Lister hooded rats. If direct PPTg and LDTg Dtx-UII lesions develop between 4 and 21 days after infusion, then retrograde cholinergic denervation of the pVTA must occur over a longer period of time. The principal aim of the current study was to determine the effect of Dtx-UII infusion into the pVTA 42 days (6 weeks) after surgery. This study used standard coordinates for the pVTA (+3.2 mm from IAL; used in toxin study 1 and 2) and coordinates slightly more posterior to the pVTA (+3 mm from IAL). Clark's pilot data indicated that pVTA cholinergic denervation could be achieved at more posterior coordinates (approximately +2.70 mm from IAL). VAChT immunohistology was used to assess pVTA cholinergic terminal density and cholinergic cell number in the pPPTg and LDTg. Fluoro-jade C immunohistology was used to investigate the presence of neurodegeneration in the pVTA.

3.5.2 Materials and methods

Subjects

Ten male Lister Hooded rats were used for this study. Rats were housed, anaesthetised and underwent microinfusion surgery as described in the General Methods. Animals weighed between 300 – 315g at the time of surgery. The coordinates for the pVTA were as stated in the General Methods and at a slightly more posterior location (+3 mm from the IAL).

Surgical procedure

As described in the General Methods, rats (n = 10) received a unilateral pVTA infusion of 3% Dtx-UII (200nl in 0.1M PB) while control hemispheres received an infusion of sterile PB (200nl, 0.1M PB). The order of the first hemisphere infused (left or right) was alternated across rats – surgery was performed on the animals in order of increasing weight. Animals received Dtx-UII infusion at the standard coordinates for pVTA (n = 5) or slightly more posterior (n = 5) following Clark's pilot data.

Histology

After 6 weeks, animals were transcardially perfused and brains were stored in sucrose (see General Methods). Each brain was dissected from the hippocampus to the anterior cerebellum, 30µm sections cut and a 1:4 series was processed for immunohistology. All sections that crossed through the VTA were processed. Tissue was stained for VAChT immunohistology and examined through fluorescent microscopy (see General Methods) to assess cholinergic terminal density in the pVTA and cholinergic cell body density in the pPPTg and LDTg. Fluoro-jade C immunofluorescence was used to determine whether neurodegeneration had occurred in the pVTA (see General Methods).

Data analysis

Quantification of VAChT+ terminals were carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Statistical analyses were performed in SSPS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). An independent-samples t-test was applied to determine if there were any differences in VAChT+ profiles between Dtx-UII and sterile PB infused tissue. Results are expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. Results were considered significant when $p \leq 0.05$.

3.5.3 Results

Recovery

Animals recovered well from surgery ($n = 9$). Death during surgery occurred on one occasion ($n = 1$). The body weight and general health of all rats were closely monitored throughout the course of the experiment. When required, animals were given additional wet mash to help maintain a stable body weight. As advised by the named veterinary surgeon, Michel wound clips were removed 2 weeks after surgery if they had not fallen out before this point. During

surgery, 2 animals received more than 200nl Dtx-UII (n = 2, ~ 500nl) and displayed circling behaviour during recovery. After a couple of hours animals began to behave normally and maintained a stable body weight for the course of the study. However, these animals were dropped from the study because large non-specific lesions were clearly visible during tissue sectioning and their tissue was processed for Fluoro-jade C to assess Dtx-UII non-selective neurodegeneration.

Histology results

Vesicular acetylcholine transporter

Seven rats underwent unilateral Dtx-UII infusion into the pVTA aiming to destroy cholinergic terminals projecting from the pPPTg and LDTg. Animals were euthanized 6 weeks after surgery to determine whether selective lesions had formed. Using VAChT immunohistochemistry and fluorescent microscopy, pVTA cholinergic terminal density and cholinergic cell number in the pPPTg and LDTg were analysed. There were no differences in tissue from animals infused at standard pVTA coordinates compared to slightly more posterior pVTA coordinates so the data were analysed together. Representative pVTA, pPPTg and LDTg tissue from Dtx-UII hemispheres and corresponding control hemispheres are shown below (Figure 3.13). These images were analysed using ImageJ software to calculate the number of individual cholinergic puncta and cells (Figure 3.14).

There were no obvious differences between Dtx-UII and sterile PB infused pVTA hemispheres 6 weeks after infusion (Figure 3.13A – B, and 3.14A). There was no difference in VAChT+ terminal density in Dtx-UII hemispheres (VAChT+ terminals = 1815 ± 150 [mean ± SEM] Figure 3.13A and 3.14A) compared to control hemispheres (VAChT+ terminals = 1657 ± 178 [mean ± SEM] Figure 3.13B and 3.14A). An independent-samples t-test was applied which indicated that Dtx-UII had no effect on cholinergic terminal density (t = - 0.678, df 10, p = 0.513). In all VTA sections stained with VAChT, cholinergic terminals were arranged in a

clear linear organisation (Figure 3.13A and B). Further, Cohen's effect size value ($d = 0.39$) suggested a moderate practical significance.

Representative pPPTg and LDTg tissue from intra-pVTA Dtx-UII hemispheres and corresponding control hemispheres are shown below (Figure 3.13C – F). These images were analysed using ImageJ software to calculate the number of cholinergic cells (Figure 3.14B and C). There was no change in VAChT+ cells in the pPPTg of Dtx-UII hemispheres (VAChT+ cells = 47 ± 5 [mean ± SEM] Figure 3.13C and 3.14B) compared to control hemispheres (VAChT+ cells = 46 ± 3 [mean ± SEM] Figure 3.13D and 3.14B). An independent-samples t-test was carried out which indicated that Dtx-UII had no effect on cholinergic pPPTg cell density ($t = -0.163$, df 10, p = 0.874). Cohen's effect size value ($d = 0.1$) suggested a low practical significance. There was no difference in VAChT+ cells in the LDTg of control hemispheres (VAChT+ cells = 67 ± 9 [mean ± SEM] Figure 3.13F and 3.14C) compared to toxin hemispheres (VAChT+ cells = 62 ± 7 [mean ± SEM] Figure 3.13E and 3.14C). An independent-samples t-test was carried out which indicated that Dtx-UII had no effect on cholinergic LDTg cell density ($t = 0.462$, df 10, p = 0.654). Cohen's effect size value ($d = 0.1$) suggested a low practical significance.

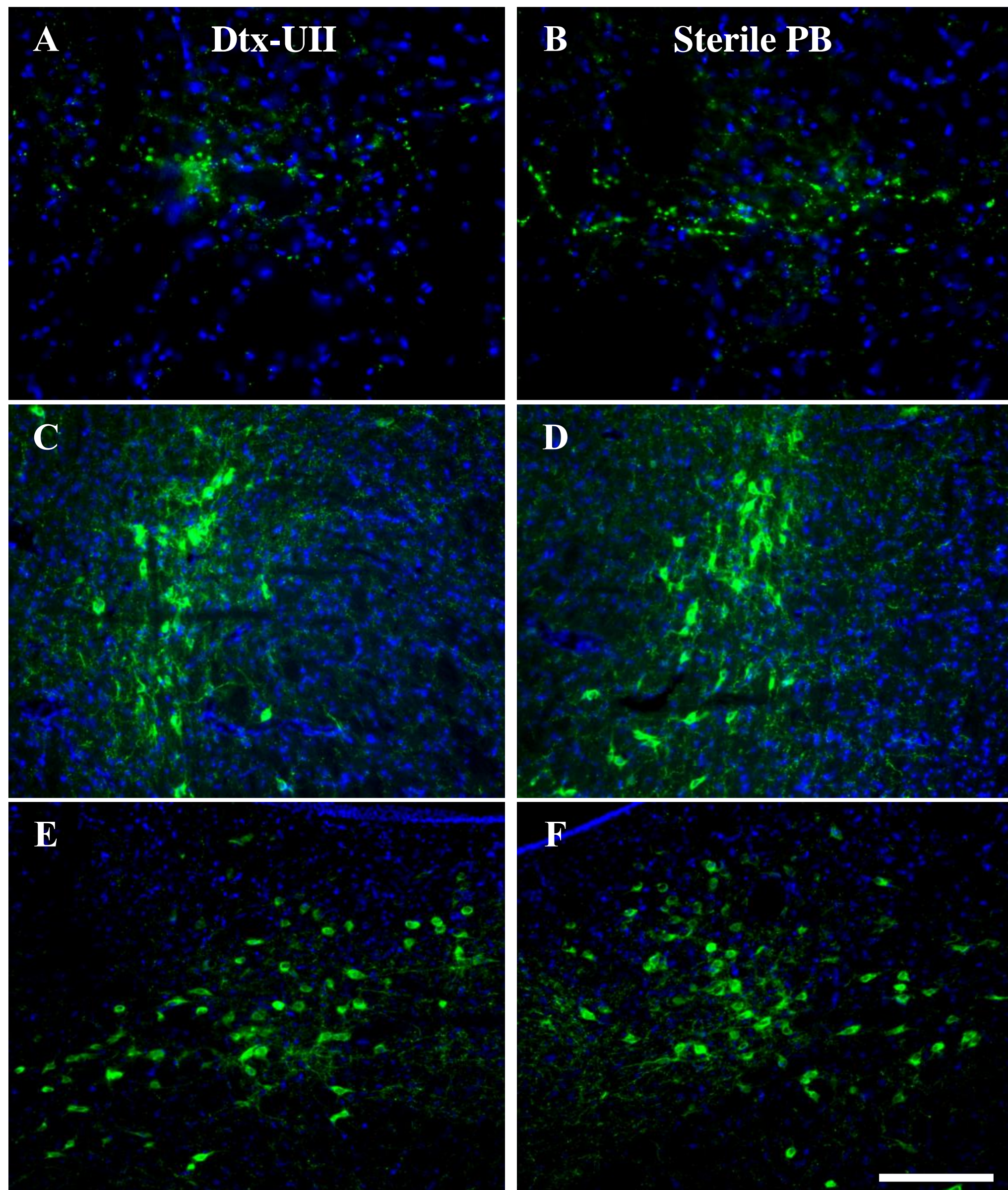


Figure 3.13 – The comparative distribution of cholinergic terminals and cell bodies (green) in the adult rat pVTA, pPPTg and LDTg after Dtx-UII infusion. After 6 weeks it was observed that there were no differences in cholinergic terminal density between pVTA Dtx-UII and control hemispheres (A and B). Cholinergic cell density in the pPPTg (C and D) and LDTg (E and F) remained unaffected 6 weeks after pVTA Dtx-UII infusion. Nuclei were labelled with VECTASHIELD® Hard setting mountant with DAPI (blue). VAChT+ profiles were labelled with Alexa Fluor® 488 Goat Anti-Rabbit IgG. Scale bar = 100µm.

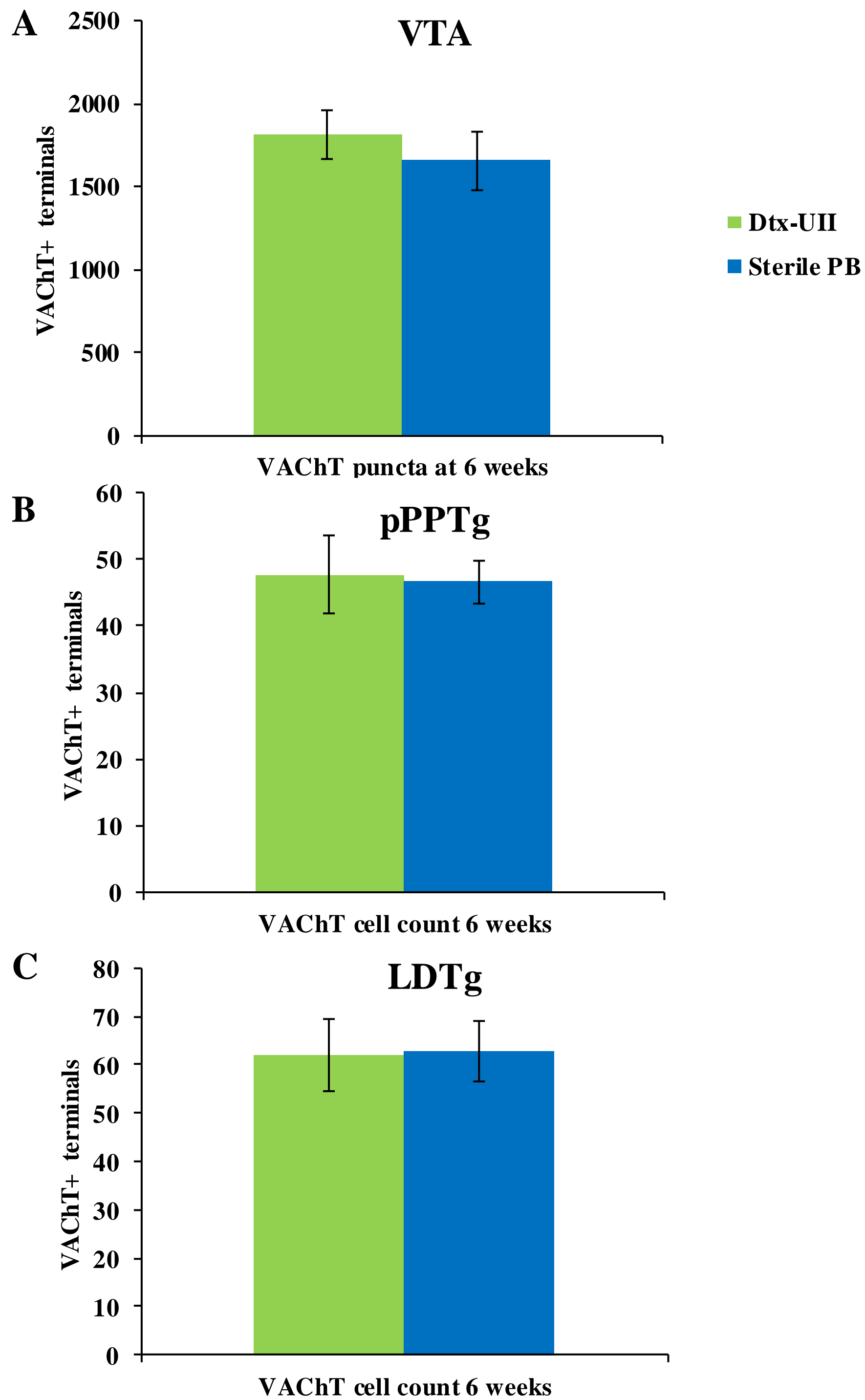


Figure 3.14 – Cholinergic puncta and cell analysis after VAChT staining using ImageJ software. Cholinergic pVTA puncta remained unchanged 6 weeks after Dtx-UII infusion when compared to control hemispheres (A). The density of pPPTg and LDTg cholinergic cells also remained unchanged 6 weeks after Dtx-UII pVTA infusion (B and C). Dtx-UII (green bar; n = 7) and sterile PB (blue bar; n = 7) infused tissue. Graphs show mean ± SEM.

Neurodegeneration

In order to determine whether neurodegeneration occurred in the pVTA 6 weeks after intra-pVTA Dtx-UII infusion, tissue sections were processed with Fluoro-jade C and visually analysed. Representative tissue processed with Fluoro-jade C from one of the animals that received a large infusion volume of toxin is shown below to visually assess non-selective neurodegeneration caused by Dtx-UII (Figure 3.15A). Fluoro-jade C+ cells were observed in all VTA sections from animals with a large non-specific lesion, whereas there were no positive cells visible in any VTA sections in animals that received the correct infusion volume of Dtx-UII (Figure 3.15A and B). This confirmed that a larger volume of Dtx-UII (~ 500µl) could cause non-selective damage in the pVTA (n = 2). No Fluoro-jade C+ cells were observed in any of the VTA hemispheres infused with sterile PB – representative tissue 6 weeks after infusion is shown below (Figure 3.15C).

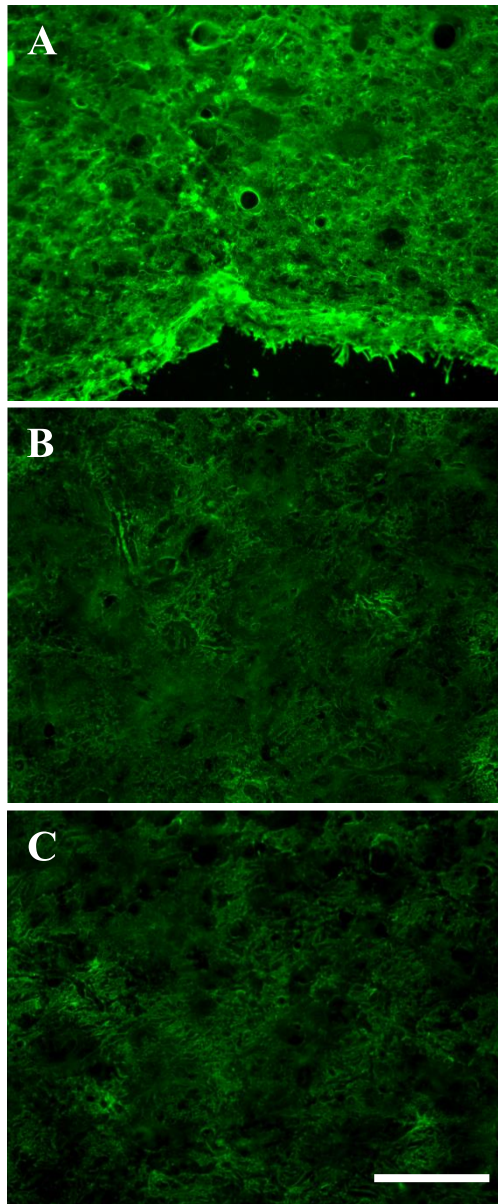


Figure 3.15 – Representative pVTA tissue processed with Fluoro-jade C 6 weeks after surgery. pVTA tissue from a non-specific Dtx-UII lesion caused by large infusion volume (~ 500nl) (A). The edge of the non-selective lesion is visible at the base of the image and Fluoro-jade C+ cells indicate the presence of neurodegeneration (A). No Fluoro-jade C+ cells were visible in any of the pVTA tissue 6 weeks after Dtx-UII (200nl) (B) or sterile PB infusion (C). Scale bar = 100µm.

3.5.4 Conclusions of toxin study 3

This study revealed that Dtx-UII infusion into the pVTA, at standard pVTA coordinates or slightly more posterior, had no visible effect on cholinergic terminals 6 weeks after surgery. Through all pVTA sections analysed there were no detectible immunohistochemical differences observed in VAChT and Fluoro-jade C immunohistochemistry. This would

indicate that cholinergic terminals remained present in the pVTA and that no neurodegeneration had occurred. In all VTA sections processed for VAChT, cholinergic terminals were arranged in a clear linear organisation – this was also observed in toxin study 1 and 2. VAChT immunohistochemistry also demonstrated that cholinergic cells in the pPPTg and LDTg were unaffected 6 weeks after intra-pVTA Dtx-UII infusion. The logical conclusions are that pVTA Dtx-UII infusion had no effect on cholinergic input from the pPPTg and LDTg 6 weeks after infusion or that the effect of Dtx-UII infusion can destroy cholinergic terminals but it does not manifest using this concentration or volume. However, as shown in this study, a larger volume of Dtx-UII can cause non-selective pVTA lesions. Fluoro-jade C reliably labelled non-selective Dtx-UII neurodegeneration in all pVTA sections from animals that received a large toxin infusion.

3.6 Chapter discussion

The main aim of this chapter was to determine the time course and specificity of cholinergic terminal loss in the pVTA following Dtx-UII infusion. Previous studies demonstrated that direct infusion of Dtx-UII into the mesopontine tegmentum achieved maximal cholinergic cell loss (~ 85%) 21 days after surgery, with ~ 75% loss between 7 and 14 days (Clark *et al.*, 2007). Further studies reported that bilateral pPPTg Dtx-UII infusion resulted in ~ 96% cholinergic cell loss 14 days after surgery (MacLaren, 2012). In the present study it was found that pVTA Dtx-UII infusion had no visible effect on cholinergic terminals 2, 4, 6, 8, 9, 10 and 42 days after surgery. At each time point, through all VTA sections analysed there were no detectible differences observed in VAChT and Fluoro-jade C immunohistochemistry. This would indicate that cholinergic terminals remained present in the pVTA and that no neurodegeneration had occurred.

Steidl *et al.* (2014) reported that bilateral PPTg Dtx-UII infusion in Long-Evans rats resulted in significant cholinergic cell loss 4, 7, 14 and 28 days after surgery (Steidl, Wang and Wise,

2014). Fourteen days after surgery it was observed that significant glutamatergic and GABAergic cell loss had occurred in addition to cholinergic cell loss, although relative cholinergic cell loss was greater than the loss of glutamatergic or GABAergic cells (Steidl, Wang and Wise, 2014). As Dtx-UII inhibits protein synthesis, pPPTg Dtx-UII lesions take considerably longer to form than ibotenic acid lesions. It has been suggested that compensatory mechanisms may develop to offset pPPTg cholinergic cell loss (MacLaren, 2012). Pilot studies in this laboratory had hinted that Dtx-UII initially caused terminal destruction followed by the re-sprouting of cholinergic terminals but this chapter demonstrates that no initial neurodegeneration and no increase in terminal density occurred between 2 and 10 days. If Dtx-UII pVTA lesion progression was longer than 6 weeks, or 10 weeks as Clark's pilot data indicated, this long period could support the development of compensatory mechanisms (behavioural testing would start at least 42 days after surgery). In all pVTA VAChT stained sections, cholinergic terminals appeared to be arranged in a linear formation (Figure 3.5, 3.10 and 3.13) as was previously observed in the pilot studies. The present chapter established that Dtx-UII infusion did not alter cholinergic terminal density in the pVTA at any of the time points analysed (Figure 3.6, 3.11 and 3.14A) and there were no visible areas of neurodegeneration observed in the pVTA or pPPTg (Figure 3.12 and 3.15). Dtx-UII infusion into the pVTA did not alter cholinergic cell density in the PPTg or LDTg (Figure 3.7 and 3.14).

Previous studies demonstrated that direct infusion of Dtx-UII into the mesopontine tegmentum destroyed cholinergic cell bodies – this toxin specifically targets cholinergic neurons expressing UII-Rs or UII binding sites (Clark *et al.*, 2007). The literature shows that UII binding sites are present in many areas of the central nervous system (CNS) including the VTA (Clark *et al.*, 2001). However, the expression of UII-Rs in the mesopontine tegmentum is much greater than the expression of UII binding sites in the VTA. This could potentially explain the results observed in the current study which showed that Dtx-UII could bind to UII sites in the pVTA but the concentration (3% Dtx-UII) was too low to have a toxic effect. Perhaps UII

binding sites do not trigger internalisation, which would mean Dtx-UII could not be released into the cytosol to inhibit protein synthesis. It could be the case that Dtx-UII works best in regions where UII-Rs are expressed highly such as the PPTg, LDTg or interpeduncular nucleus (IPN) (Clark *et al.*, 2001). UII binding sites are expressed at much lower levels in the VTA compared to other CNS regions such as lateral septal nuclei. Potentially, Dtx-UII could have a toxic effect in regions where UII binding sites are expressed at higher levels (Clark *et al.*, 2001).

The concentration of Dtx-UII used in the present study is optimal for pPPTg lesions (3% Dtx-UII) as higher concentrations were found to form non-selective lesions (MacLaren, 2012). MacLaren (2012) noted that the volume of Dtx-UII is also of great importance. A larger volume (600nl) of 3% Dtx-UII caused non-selective damage, whereas 200nl of 3% Dtx-UII was selectively toxic to mesopontine cholinergic neurons. The toxic effect of volume was observed in the current study and non-specific damage was visible 6 weeks after ~ 500nl 3% Dtx-UII (n = 2) (Figure 3.15). Steidl *et al*. (2014) demonstrated a slightly different method of Dtx-UII infusion. Instead of performing a singular toxin infusion of 200nl per hemisphere (as performed in the current study), 6 injections of 200nl per hemisphere were administered. This would explain the reason that non-specific damage was visible as early as 4 days post-surgery (Steidl, Wang and Wise, 2014).

Several studies have investigated the effect of UII infusions in regions of the CNS. Intra-VTA UII was found to modulate nucleus accumbens (NAc) DA efflux in a dose dependant manner; this result must be due to presynaptic UII binding site activation within the VTA, because VTA neurons do not express UII-R transcripts (Clark *et al.*, 2005). Mueller *et al.* (2014) investigated the effect of intra-VTA injections of UII and UII related peptide (URP) in cocaine-induced drug seeking behaviour (Mueller *et al.*, 2014); UII and URP possess equal EC50 binding properties for UII-R (Chatenet *et al.*, 2004). It was reported that high concentrations (1 nM) of UII would establish conditioned place preference (CPP) but

prevented cocaine induced CPP. Low doses of UII and URP did not induce CPP, however, when UII or URP were administered in combination with a sub-effectual dose of cocaine, CPP was formed. Mueller *et al.* (2014) acknowledged that the higher dose of UII was supra-physiological; therefore, any behavioural effect could be due to the activation of other receptors, such as somatostatin receptors, either in the VTA or in adjacent structures (SNc or IPN – receives cholinergic projections from medial habenula) (Uhl *et al.*, 1985; Martin *et al.*, 1991; Sato, Ota and Ogawa, 1991; Nothacker *et al.*, 1999). Furthermore, high doses of UII could diffuse and activate UII-Rs in neighbouring structures such as the IPN (Clark *et al.*, 2001). Mueller *et al.* (2014) also demonstrated that the effect of CPP induced by low dose UII was blocked by intra-VTA UII-R antagonist (SB657510) administration (Mueller *et al.*, 2014). In the present study the anterior-posterior coordinates for the pVTA were +3.2 mm from the IAL (approximately -5.8 mm from bregma); these coordinates are well defined as the location of the pVTA (Paxinos and Watson, 2007). The position of the pipette and location of peptide infusion were confirmed to be in the pVTA (Figure 3.9). However, the anterior-posterior pVTA coordinates used by Mueller *et al.* (2015) were -6.5 mm from bregma (approximately +2.5 mm from the IAL). It is possible that the behavioural effects of intra-VTA UII and URP are due to another mechanism more posterior than the pVTA. It would be interesting to carry out UII or URP infusions using the current coordinates for the pVTA. These anterior-posterior coordinates are similar to that used by Clark's laboratory for intra-pVTA Dtx-UII infusion (pilot data; -6.2 mm from bregma – approximately +2.70 mm from IAL), which could be triggering the same mechanism.

There are various methodological considerations that should be taken into account. For example, it is essential that the correct volume of toxin or sterile PB was infused via the glass pipette. On occasion, the glass pipette could become blocked as it entered the brain. If the air-filled syringe required more pressure than normal to eject, the glass pipette was carefully removed and replaced with a new toxin or sterile PB filled pipette. Although tissue was

carefully processed and sectioned, on occasion the angle of brain blocking for sectioning was slightly different and this could explain the different levels of VAChT+ puncta across the different time points in toxin study 2 (Figure 3.11). Prior to carrying out immunohistochemistry, all primary and secondary antibodies were tested to ensure that neurons were correctly labelled. As Fluoro-jade C also labels blood vessels, which can result in a high level of background stain, the optimum potassium permanganate incubation was determined – this can help reduce background staining. Fluoro-jade C was selected from the Fluoro-jade dyes as it has the greatest signal to background ratio and highest resolution compared to Fluoro-jade and Fluoro-jade B (Schmued *et al.*, 2005).

3.7 Future work

What we know from the current study is that Dtx-UII does not cause non-specific lesions; it does not affect cholinergic terminal density in the pVTA and it does not cause neurodegeneration in the pVTA or pPPTg at any of the time points examined. Moreover, larger volumes than normally used cause non-specific damage. The logical conclusion is that pVTA Dtx-UII infusion has no effect on cholinergic input from the pPPTg and LDTg and that Dtx-UII is not a viable tool for creating cholinergic denervation of the VTA.

Chapter 4: Fos expression after acute and chronic systemic nicotine administration

4.1 Chapter introduction

This thesis intended to investigate the role of mesopontine cholinergic input to midbrain DA neurons. The first strategy was to try to use the Dtx-UII toxin. If selective cholinergic lesions in the pVTA had been achieved using Dtx-UII, behavioural testing could have been performed, allowing for the development of a better understanding of the functions of cholinergic projections from the pPPTg and LDTg to the VTA (Chapter 3). For example, it would have been possible to investigate the effect of cholinergic drugs in rats with Dtx-UII pVTA lesions. Intra-pVTA intracranial self-administration (ICSA) of nicotine in rats with cholinergic denervation of the pVTA could have aided the understanding of the role of the pVTA in nicotine addiction and in reward-related behaviours more generally. This hypothesis was not unrealistic, but unfortunately Dtx-UII did not destroy cholinergic input to the pVTA from the pPPTg and LDTg at any of the time points investigated (Chapter 3). Given that the aim was to develop better understanding of cholinergic activity in the VTA an alternative approach was adopted. For this, the effects of nicotine self-administered directly into the pVTA were examined following previous studies from this laboratory (Farquhar, Latimer and Winn, 2012). Three approaches were taken: firstly, in the current chapter, examination of the effects of systemic nicotine on neural activity in the VTA and regions on which VTA activity has effect – the shell and core of the nucleus accumbens (NAc), the dorsal striatum and the dorsal hippocampus (dHPC). Effects on these regions of interest were measured by immediate early gene (IEG) expression in two conditions: following a single exposure to nicotine and (in different animals) after repeated nicotine exposure. Secondly, in the subsequent chapter, examination of the effects of intra-pVTA self-administration of nicotine on neural activity in the same regions of interest (Chapter 5). Effects on these regions were measured in two conditions: at the end of the very first ICSA session and (in other groups) at the end of the fifth ICSA session. The question was, would ICSA of nicotine in the pVTA have different effects on the regions of interest dependent on the degree of learning that had taken place? The third

approach taken was to contrast the effect of intra-pVTA self-administration of nicotine with ICSA in the dHPC (Chapter 6). The issue here is whether or not cholinergic activation at different points within the same neural circuitry has similar or different effects on the identified regions of interest.

4.1.1 Hippocampal-VTA loop

Midbrain DA neurons have been reported to display 3 patterns of activity *in vivo*: - (1) inactive and hyperpolarised, (2) slow, irregular single-spike firing (spontaneous tonic firing), or (3) brief pulse phasic firing (burst firing) (Grace and Bunney, 1983, 1984; Grace, 1991; Grace *et al.*, 2007). As previously discussed in Chapter 3, Grace and Floresco (2007) reported that the mesopontine nuclei work together to regulate the phasic activity of VTA DA neurons. It is believed that the LDTg provides a cholinergic-dependant permissive "gate" to regulate phasic firing of VTA DA neurons (Figure 4.1) (Lodge and Grace, 2006a). Glutamatergic input, which arrives from the PPTg as well as the prefrontal cortex (PFC) and bed nucleus stria terminalis (BNST), can only initiate burst firing to VTA DA neurons when the LDTg is active (Grace *et al.*, 2007). Spontaneous tonic firing of VTA DA neurons is regulated by γ-aminobutyric acid releasing (GABAergic) projections from the ventral pallidum (VP) (Zahm and Heimer, 1990; Wu, Hrycyshyn and Brudzynski, 1996). These projections hold VTA DA neurons in an inactive hyperpolarised state by constantly firing GABA-mediated inhibitory postsynaptic potentials (IPSPs). When the VP is inactivated it enables VTA DA neurons to display spontaneous firing (Floresco *et al.*, 2003; Lodge and Grace, 2006b). An important modulatory input to the VP arises from the ventral subiculum of the HPC (vSub) (Groenewegen *et al.*, 1987; Floresco, Todd and Grace, 2001). The vSub, an output region of the HPC, receives direct excitatory projections from the CA1 region of the HPC (Amaral, Dolorfo and Alvarez-Royo, 1991). The activation of the vSub leads to the spontaneous activation of VTA DA neurons. Excitatory glutamatergic input from the vSub triggers neuronal firing in the NAc which activates GABAergic neurons projecting to the VP (Figure 4.1). These projections inhibit the

VP and releases VTA DA neurons from constant inhibition (Floresco *et al.*, 2003). This polysynaptic pathway modulates baseline activity of VTA DA receptors (Grace, 1991). These afferent pathways regulate the activity of VTA DA neurons, because only neurons in a spontaneously firing state can be phasically activated by the PPTg/LDTg (Floresco *et al.*, 2003; Lodge and Grace, 2006). Together these regulatory systems modulate the dynamics of DA release in the NAc. Previous studies have shown that burst firing of VTA DA neurons occur when an animal is exposed to novel stimuli (Steinfels *et al.*, 1983; Ljungberg, Apicella and Schultz, 1992; Legault and Wise, 2001). Legault and Wise (2001) reported that this activation of VTA DA neurons was dependant on the vSub. Furthermore, when the vSub was chemically stimulated or when inhibition was blocked this led to VTA activation (Blaha *et al.*, 1997; Floresco, Todd and Grace, 2001; Floresco *et al.*, 2003). Lisman and Grace (2005) presented the hypothesis that the VTA and HPC form a functional loop designed to detect novelty. This was termed the hippocampal-VTA loop (HPC-VTA loop) (Lisman and Grace, 2005). It was suggested that VTA DA projections send signals to the HPC, "stamping" behaviourally relevant information for memory storage and identifying mismatches between incoming and previously stored information (Lisman and Grace, 2005; Bunzeck and Düzel, 2006). As previously mentioned, there is evidence of a polysynaptic pathway connecting the HPC to the VTA through the NAc and VP; this forms the downward arc of the HPC-VTA loop (Figure 4.1) (Legault, Rompré and Wise, 2000; Floresco, Todd and Grace, 2001). The downward arc is believed to carry novelty signals from the HPC to the VTA where it stimulates novelty-dependant firing of VTA DA neurons (Legault and Wise, 2001). The upward arc of the loop is formed by ascending DA projections from the VTA to the HPC (Scatton, Simon, Michel Le Moal, *et al.*, 1980; Gasbarri, Sulli and Packard, 1997). VTA DA neurons innervate the vSub, hilus and CA1 regions of the HPC (Gasbarri *et al.*, 1994; Gasbarri, Sulli and Packard, 1997). Previous studies have shown that long-term potentiation (LTP) in the CA1 region is dependent on VTA DA projections (Frey, Schroeder and Matthies, 1990; Frey *et al.*, 1991; Frey, Huang and Kandel, 1993; Bach *et al.*, 1999). LTP is defined as an activity-dependent

persistent increase in synaptic strength (Bliss and Collingridge, 1993). In the CA1 brief exposure to a novel environment mediates the induction of LTP-like synaptic plasticity (Li *et al.*, 2003). This synaptic strengthening is completely blocked by DA receptor-1 (D1) antagonists and in D1 knockout mice (Li *et al.*, 2003; Granado *et al.*, 2008). Furthermore, novelty-dependant LTP is enhanced by the activation of D1 receptors (Li *et al.*, 2003; Granado *et al.*, 2008). The upward arc of the HPC-VTA loop is reported to be responsible for inducing LTP-like synaptic plasticity in response to novel stimuli.

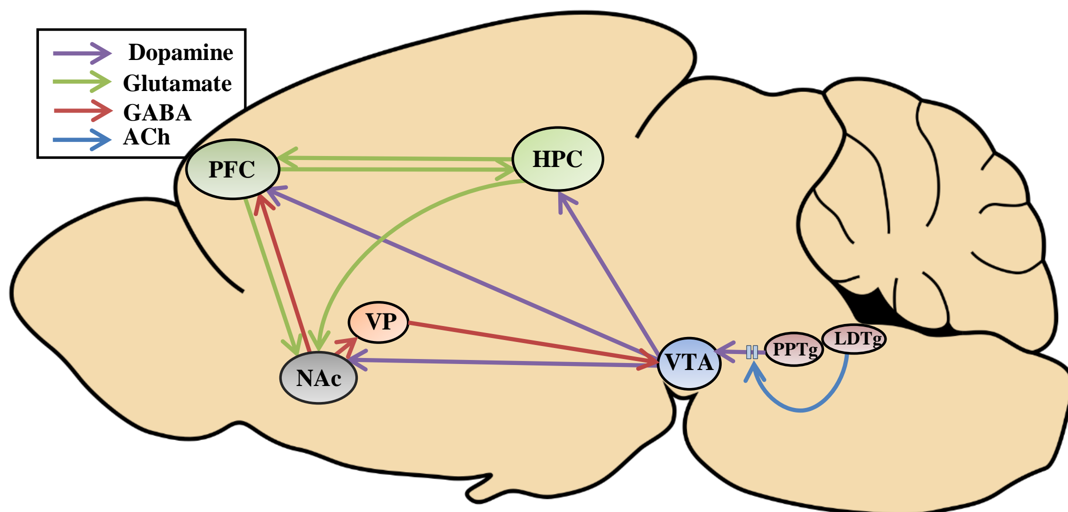


Figure 4.1 – A summary of VTA connectivity and the hippocampal-VTA loop. The downward arc of the loop carries information from the vSub of the HPC to the VTA via the NAc and VP. The upward arc is formed by DA neurons of the VTA projecting to the HPC. VTA DA neurons also project to the PFC and NAc. The PPTg provides glutamatergic innervation to the VTA this activity is "gated" by cholinergic neurons of the LDTg. Abbreviations: - hippocampus (HPC), nucleus accumbens (NAc), laterodorsal tegmental nuclei (LDTg), pedunculopontine tegmental nuclei (PPTg), prefrontal cortex (PFC), ventral pallidum (VP) and ventral tegmental area (VTA). Neurochemical identity of projections: - dopamine (DA; purple), glutamate (green), γ-aminobutyric acid (GABA; red) and cholinergic (ACh; blue).

HPC anatomy and function discussed further in Chapter 6. Briefly, the HPC is recognised as an important structure in learning and memory, but it is not typically considered part of the reward circuitry. However, an increasing number of studies report that the HPC is involved in mediating behaviours associated with drug reward (Davis and Gould, 2006; Gould, 2006; Franklin *et al.*, 2007; Tang and Dani, 2009; Rubinstein *et al.*, 2011; Ramirez, Tonegawa and Liu, 2013; Gould and Leach, 2014). The HPC is divided into 3 subregions: - the dentate gyrus

(DG), hippocampus proper and subiculum. The hippocampus proper is further divided into the areas of the Cornu Ammonis (Ammon's horn): - CA1, CA2 and CA3 regions. This structural organisation is repeated along the longitudinal axis of the HPC but the afferent and efferent connections differ along the dorsal to the ventral hippocampal pole (dHPC, vHPC) (Andersen, Bliss and Skrede, 1971; Moser and Moser, 1998). The dHPC is most prominently connected to the retrosplenial area, which is heavily implicated in reward-related behaviours (van Groen and Wyss, 1990, 2003; Ikemoto, Witkin and Morales, 2003; Jinno *et al.*, 2007; Miyashita and Rockland, 2007; Smith, Barredo and Mizumori, 2012). Conversely, the vHPC is strongly connected the rostral hypothalamus and amygdala nuclei – regions associated with emotionality and stress-related behaviours (Canteras and Swanson, 1992; Cenquizca and Swanson, 2007). The diverse projections of the dorsal and ventral HPC are likely to contribute to the functional differences between the poles. There is mounting evidence that the dHPC is critically involved in learning and memory paradigms that require spatial, temporal and working memory. Lesions or inactivation of the dHPC attenuate performance in tasks that required temporal memory, such as delay conditioning and memory of sequences (Compton, 1993; Czerniawski, Yoon and Otto, 2009; Esclassan *et al.*, 2009; Czerniawski *et al.*, 2012). Furthermore, the dHPC is critical for processing motor-related cognitive functions and spatial learning tasks (Anagnostaras, Maren and Fanselow, 1999; Czerniawski, Yoon and Otto, 2009). The vHPC is believed to mediate behaviours and process emotion. Lesions or inactivation of the vHPC result in the disruption of the expression of fear and anxiety behaviours (Henke, 1990; Trivedi and Coover, 2004; Czeniawski, Yoon and Otto, 2009; Czerniawski *et al.*, 2012). Furthermore, the heterogeneous nature of the HPC is demonstrated in response to cholinergic drugs. The administration of galanin, which modulates acetylcholine (ACh) release, increases ACh release in the dHPC and inhibits ACh release in the vHPC (Ögren *et al.*, 1998; Yoshitake *et al.*, 2011). Similarly, direct infusion of nicotine into the dHPC increased noradrenaline (NA) levels, whereas intra-vHPC nicotine decreased NA release (Shearman *et al.*, 2005).

Keleta and Martinez (2012) reported that the HPC-VTA loop is essential for the acquisition of methamphetamine (METH) intracranial conditioned place preference (IC-CPP). Based on the HPC-VTA loop, the order of sequential METH administration directly into the VTA, vHPC and NAc was investigated (Keleta and Martinez, 2012). The upward arc of the loop produced positive place preference (VTA-vHPC-NAc), whereas the downward arc (vHPC-NAc-VTA) attenuated METH-induced place preference and produced place aversion, disrupting place learning (Keleta and Martinez, 2012). Ricoy and Martinez (2009) reported that intra-dHPC METH induced drug seeking (place preference) and drug taking (lever pressing) behaviour. These responses were reliant on DA receptor activation in the dHPC (Zarrindast *et al.*, 2005; Ricoy and Martinez, 2009). Furthermore, dHPC lesions impair conditioned place preference (CPP), whereas vHPC lesions enhanced learning behaviour (Ferbinteanu and McDonald, 2001). VTA DA projections to the dHPC CA1 region are involved in reward-related learning (Rezayof *et al.*, 2003). Direct infusion of DA receptor antagonists into the dHPC CA1 region impaired morphine and ethanol (EtOH) CPP and drug seeking behaviour (Rezayof *et al.*, 2003, 2007). It has previously been reported that the DA content of the dHPC was found to be 4 fold to 10 fold-higher than the content vHPC (Ishikawa, Ott and McGaugh, 1982). These studies demonstrate that VTA DA projections to the HPC, in particular the dHPC CA1 region, play an important role in drug related learning. The hippocampal mechanisms for learning and memory may hold a central function in mediating the rewarding effects of drugs of abuse. The HPC-VTA loop hypothesis suggests that the HPC commits drug-associated contexts to memory once it has received DA signals from the VTA (Lisman and Grace, 2005). Although there is much to learn about the HPC-VTA loop, it has been implicated in the rewarding effects of METH, morphine and EtOH. This feedback loop may also play a role in mediating the effects of nicotine.

Following previous work from this laboratory (Farquhar, Latimer and Winn, 2012), it was decided to investigate the effect of systemic nicotine and self-administered nicotine directly

into the VTA and dHPC. Three approaches were taken: first, examination of the effects of systemic nicotine on neural activity in structures on which VTA activity has effect: - NAc shell and core, dorsal striatum and dHPC. Effects on these regions of interest were measured by immediate early gene (IEG) expression in two conditions: at the end of acute nicotine exposure and (in other groups) at the end of chronic nicotine exposure. The second approach was to examine the effects of intra-VTA self-administration of nicotine on neural activity in the same terminal regions of the VTA. Effects on these regions of interest were measured in 2 conditions: at the end of the very first ICSA session and (in other groups) at the end of the fifth ICSA session. The question was, would ICSA of nicotine in the VTA have different effects dependent on the degree of learning that had taken place? The third approach taken was to contrast the effect of intra-VTA self-administration of nicotine with ICSA in the dorsal hippocampus (dHPC). The issue here is whether or not cholinergic activation at different points within the same neural circuitry has similar of different effects on the identified regions of interest.

4.1.2 Nicotine

Smoking is still the leading cause of preventable mortality and disability, causing approximately 5 million premature deaths worldwide per year (Changeux, 2010; Centre for Disease Control and Prevention (CDC), 2010; Pistillo *et al.*, 2015). The main addictive agent in tobacco is nicotine which induces psychostimulation and reward, improving attention, reducing stress and anxiety (Stolerman and Jarvis, 1995; Benowitz, 2010). Although smoking cessation therapies are available cessation rates are poor and less than 3% of users who try to quit each year are successful (Centers for Disease Control and Prevention (CDC), 2005; Crane, 2007; Benowitz, 2010). Nicotine dependence is a chronic relapsing disorder with periods of compulsive smoking followed by years of abstinence that leads to withdrawal (Hughes, 2007). The development of nicotine dependence and avoidance of aversive withdrawal symptoms are the greatest reasons for frequent smoking and relapse (Hughes and Hatsukami, 1986).

Furthermore, nicotine-associated cues promote nicotine-seeking behaviour which encourages relapse (Markou and Paterson, 2009). Relapse can be triggered by stressful life events, environmental or sensory stimuli previously associated with smoking, or cigarette smoking itself (Ferguson and Shiffman, 2009). Repeated exposure to nicotine leads to cellular and synaptic alterations in the CNS.

4.1.3 Nicotine and the CNS

Nicotinic acetylcholine receptors (nAChRs) are activated by endogenous acetylcholine (ACh) or exogenous agonists, such as nicotine (Picciotto *et al.*, 2001; Gotti and Clementi, 2004; Dani and Bertrand, 2007; Albuquerque *et al.*, 2009). ACh can activate metabotropic muscarinic acetylcholine receptors (mAChRs) and ionotropic neuronal nAChRs while nicotine specifically activates ionotropic nAChRs (Picciotto, Higley and Mineur, 2012). nAChRs are ligand-gated cation channels composed of 5 transmembrane subunits (Dani and Balfour, 2011). These receptors exist as heteromers (combination of 5 α (2 – 10) and β (2 – 4) subunits) or homomers (5 repeated single α7, α8 or α9 subunits) (Heinemann *et al.*, 1990; Cooper, Couturier and Ballivet, 1991; Jones, Sudweeks and Yakel, 1999; Corringer, Sallette and Changeux, 2006; Gotti, Zoli and Clementi, 2006; Miwa, Freedman and Lester, 2011). The α8 subunit has only been isolated in avian tissue and has not been detected in mammalian tissue (Dani and Bertrand, 2007). Each specific subunit combination is functionally diverse – even the precise order and stoichiometry of the subunits effect physiological and pharmacological properties. For example, α4β2 exists as a major receptor subtype in the brain (Feduccia, Chatterjee and Bartlett, 2012). Expressed as $\alpha4_2\beta2_3$ the receptor exhibits nicotine sensitivity 10 fold-higher than $\alpha4_3\beta2_2$ (Moroni *et al.*, 2006; Miwa, Freedman and Lester, 2011). The latter is thought to possess an extra ligand binding site which results in a lowered sensitivity to nicotine (Fasoli and Gotti, 2015). Furthermore, the specific arrangement of nAChR subunits determines the receptors permeability to Ca^{+2}. For instance, α7 homomeric receptors have been reported to have the highest permeability to Ca^{+2}. This regulates intracellular Ca^{+2} levels and

can lead to the activation of downstream pathways involved in neural signalling (Fucile, 2004; Tapia, Kuryatov and Lindstrom, 2007). From the spinal cord to the olfactory bulb, nAChRs are widely spread throughout the CNS (Perry *et al.*, 2002; Gotti *et al.*, 2009). These receptors are located on dendrites, axons, cell somas, and at presynaptic and postsynaptic sites (Baddick and Marks, 2011). The majority of nAChRs exist at presynaptic or preterminal locations that enhance neurotransmitter release when activated (Dani and Balfour, 2011). A minority of nAChRs are present at somatodendritic postsynaptic locations that mediate neuron excitation (Dani and Bertrand, 2007; Albuquerque *et al.*, 2009; Millar and Gotti, 2009). The activation of mAChRs is relatively slow (milliseconds to seconds) compared to the rapid activation of nAChRs (micro- to sub-microseconds) (Albuquerque *et al.*, 2009). nAChRs typically exist in 3 states: - closed at rest, open pore or closed desensitised. Agonists, such as nicotine or ACh, bind to the receptor generating a rapid cellular response. The 5 subunits rearrange causing the pore to open for 1 – 2 milliseconds (Gotti *et al.*, 2009; Dani, 2015). This increases the permeability of cations (Na^+, K^+ and Ca^{2+}), inducing depolarisation and increasing the excitability of the neuron (McGehee and Role, 1995; Albuquerque *et al.*, 2009). Following agonist activation, the receptor enters a resting state or closed non-conducting state. This occurs through deactivation (dissociation of the agonist from the receptor) or desensitisation (agonist-bound conformational change). The rate and level of desensitisation depends on the specific subunit composition of the receptor and previous exposure to nicotine (Miwa, Freedman and Lester, 2011). For example, α7 homomeric receptors rapidly desensitise, whereas receptors that do not contain the α7 subunit (for example α4β2) desensitise at a steady rate. Brief exposure to a high concentration of nicotine synchronously activates nAChRs. However, prolonged exposure to a low concentration of nicotine (obtained through smoking) predominantly stabilises receptors in a deactivated state (Dani, Radcliffe and Pidoplichko, 2000). The chronic exposure to low nicotine concentration also leads to the selective increased expression (or upregulation) of specific nAChRs (Schwartz and Kellar, 1985; Benwell, Balfour and Anderson, 1988; Perry *et al.*, 1999). The level of nAChR upregulation depends

on the previous exposure to nicotine, receptor subunit composition, cell type and brain region. The highest levels of upregulation are believed to occur in α4β2 subtypes (Albuquerque *et al.*, 2009). Interestingly, some nAChRs have poor levels of upregulation (for example α4β4) and some are even downregulated (for example α6β3) (Lai *et al.*, 2005; Mugnaini *et al.*, 2006; Albuquerque *et al.*, 2009). Therefore, repeated exposure to nicotine does not simply cause nAChR desensitisation and upregulation, but initiates complex receptor alterations and neuroadaptations. The influence of nicotine is determined by a number of factors: - receptor subtype, location, density, functional properties, neurochemical identity of the neuron and brain region. Recent studies have suggested that the activation and subsequent desensitisation of nAChRs on DA, GABA, and glutamate neurons within the mesocorticolimbic system lead to the pleasant aspects of smoking (Mansvelder and McGehee, 2002; Laviolette, 2004; De Biasi and Dani, 2011; Picciotto and Kenny, 2013; Pistillo *et al.*, 2015). This promotes the onset of nicotine dependence and addiction. Therefore, the mesocorticolimbic system must be, at least partly, responsible for the symptoms of withdrawal (Mansvelder and McGehee, 2002; Pistillo *et al.*, 2015).

4.1.4 Nicotine and the VTA

As discussed in the General Introduction, the mesocorticolimbic system is considered essential for mediating the reinforcing and motivational properties of drugs of abuse, including nicotine (Fibiger and Phillips, 1986; Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Koob, 1992; Robbins and Everitt, 1996; Bowers, Chen and Bonci, 2010). DA is a key neurotransmitter in the mesocorticolimbic system, contributing to reward and aversion (Wise and Bozarth, 1987; Wise, 2004; Ungless, Argilli and Bonci, 2010). Previous studies reported that VTA DA neurons are involved in mediating the rewarding effects of drugs of abuse. Animals will learn to self-administer opiates, cocaine, cannabinoids, alcohol and nicotine directly into the VTA (Bozarth and Wise, 1981; Corrigall *et al.*, 1992; Gatto *et al.*, 1994; David *et al.*, 2004; Zangen *et al.*, 2006). These reinforcing effects are thought to be mediated through

VTA DA neurons. On the other hand, there is considerable evidence that aversive stimuli activate VTA DA neurons leading to DA release in target regions (Abercrombie *et al.*, 1989; Kalivas and Duffy, 1995; Lammel *et al.*, 2012). In particular, noxious stimuli have been reported to activate DA neurons in the ventral VTA (Brischoux *et al.*, 2009). However, this activation of VTA DA neurons may be due to avoidance or withdrawal of aversive stimuli. Learning to avoid harmful or aversive stimuli could be defined as rewarding. Avoidance behaviour can also be considered approach behaviour towards safety that has a rewarding effect (Ikemoto and Panksepp, 1999).

The neurochemical mechanisms that lead to nicotine dependence are somehow initiated by the activation of VTA DA neurons that ultimately lead to increased DA release in the NAc (Benwell and Balfour, 1992; Pontieri *et al.*, 1996; Balfour *et al.*, 2000; Balfour, 2004). As previously discussed, the HPC-VTA loop modulates VTA DA activity through a polysynaptic pathway from the HPC to the VTA through the NAc and VP. The VTA then provides DA innervation to the HPC; this feedback loop has been reported to commit drug-associated contexts to memory once the HPC has received DA signals from the VTA (Lisman and Grace, 2005). The activity of VTA DA neurons is mediated through complex interactions between ACh, GABA, glutamate and DA transmission in the VTA. Following nicotine exposure, the activation of VTA DA neurons is not fully understood. In the VTA, nicotine binds to nAChR subtypes located on DA, GABA and glutamate releasing neurons (Grillner and Svensson, 2000; Mansvelder, Keath and McGehee, 2002). VTA DA neurons possess nAChRs that are primarily composed of α3 – 7 subunits combined with β2 or β4 subunits (Wooltorton *et al.*, 2003). VTA DA projections mainly express α4, α6 and β2, whereas DA cell bodies express α4 – α7, β2 and β3 subunits (Klink *et al.*, 2001; Grady *et al.*, 2007). The α6 subunit appears to be selectively expressed in DA neurons (Le Novere, Zoli and Changeux, 1996; Drenan *et al.*, 2008; Gotti *et al.*, 2010). However, Yang *et al.* (2011) reported that GABA release onto VTA DA neurons was inhibited by a α6β2-selective antagonist (α-conotoxin MII). This would

suggest that α6 subunits are also present on GABAergic terminals (Yang *et al.*, 2011). Other studies reported that VTA GABAergic neurons express α4, α7 and β2 at the soma (Klink *et al.*, 2001; Dani and Bertrand, 2007). Less than half of VTA DA and GABA neurons express homomeric α7 nAChRs (Klink *et al.*, 2001). nAChRs possessing α7 subunits (α7* – where * denotes other potential subunits) has a low affinity for nicotine, relatively fast kinetics and does not desensitise at low agonist concentrations. Those that contain β2 (β2*) have a high affinity for nicotine, relatively slow kinetics and desensitise at low agonist concentrations (Paradiso and Steinbach, 2003). In the midbrain nicotine first acts at α4β2* and α7* nAChRs. Nicotine has a prolonged effect on presynaptic α7* nAChRs on glutamatergic afferents (Mansvelder and McGehee, 2002; Jones and Wonnacott, 2004). This stimulates presynaptic glutamate transmission onto VTA DA neurons and leads to an increased rate of burst firing (Kalivas, 1993; Grillner and Svensson, 2000; Mansvelder and McGehee, 2002; Jones and Wonnacott, 2004). Simultaneously, postsynaptic and somatic α4β2* nAChRs are activated on DA neurons. This activation triggers depolarisation that leads to action potential firing (Zhang *et al.*, 2009). VTA GABAergic local interneurons and terminals express α4β2* nAChR subtypes (Marubio *et al.*, 1999; Klink *et al.*, 2001; Changeux, 2010). Nicotine activation of inhibitory GABAergic neurons in the VTA is short and transient. At low nicotine concentrations α4β2* nAChRs on GABAergic neurons significantly desensitise within minutes and GABAergic inhibition of DA neurons decrease (Mansvelder and McGehee, 2002; Wooltorton *et al.*, 2003). Nicotine derived from smoking enhances excitation and decreases inhibition to DA neurons that result in increased DA neuronal firing. The DA neurons that project from the pVTA to the NAc commonly possess α6 and β3 subunits in combination with α4 and β2 (Ikemoto, 2007; Zhao-Shea *et al.*, 2011). At low nicotine concentrations obtained by smokers the presence of α6 subunits slows the rate of desensitisation observed in α4β2* subtypes (Liu *et al.*, 2012). Therefore, receptors containing α6 subunits remain persistently activated for minutes (in particular α6α4β2* nAChRs) and assist the prolonged release of DA in response to nicotine (Pidoplichko *et al.*, 2004; Leslie, Mojica and Reynaga, 2013).

The effect of nicotine in the VTA is more complex than simply activating DA releasing neurons that lead to increased DA transmission. Rather, many parallel events lead to a rapid and sustained increase in NAc DA levels. In order to further understand the mechanisms of nicotine addiction, it is necessary to determine the neuroadaptations and behavioural alterations that develop after a single exposure to nicotine, compared to subsequent changes after repeated exposure. Studies in laboratory animals have shown that nicotine reinforces self-administration, increases drug seeking behaviour, induces CPP, increases locomotor activity and enhances reward from ICSA (discussed further in Chapter 5) (Benwell and Balfour, 1992; Museo and Wise, 1994; Dani and Heinemann, 1996; Corrigall, 1999; Di Chiara, 2000; Laviolette and van der Kooy, 2003; David *et al.*, 2006; Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012).

4.1.5 Nicotine and locomotion

In rats, the effect of systemic nicotine on locomotor behaviour is well documented. After the first exposure to nicotine rats demonstrate decreased locomotion (hypolocomotion), which develops to increased locomotion (hyperlocomotion) following repeated administration of nicotine (concentrations ranging between 0.1 – 0.8 mg/kg) (Clarke and Kumar, 1983a; Ksir, Hakan and Kellar, 1987; Benwell and Balfour, 1992; Vezina, McGehee and Green, 2007). A single exposure to nicotine triggers profound synaptic alterations in specific brain regions (Mansvelder *et al.*, 2003). Repeated exposure to nicotine leads to locomotor sensitisation that many consider a vital component of addiction (Robinson and Berridge, 2008; Mao and McGehee, 2010). Sensitisation is defined as the enhanced effect a drug has after repeated exposure. Nicotine sensitisation can occur after as little as 5 days that leads to increased DA levels in the NAc (Benwell and Balfour, 1992; Nisell, Nomikos and Svensson, 1994; Panagis *et al.*, 1996; Balfour *et al.*, 1998). Locomotor sensitisation is believed to occur when nicotine binds to nAChRs in the VTA. The mesoaccumbens pathway is then activated and DA levels in the NAc increase – particularly in the NAc shell (Benwell and Balfour, 1992; Pontieri *et al.*, 1996; Balfour *et al.*, 1998). This hypothesis is strongly supported by a number of studies.

Panagis *et al.* (1996) demonstrated that direct infusion of nicotine into the VTA resulted in increased locomotion. This effect was blocked by pre-treating the animals with mecamylamine, a broad spectrum nicotinic receptor antagonist (Clarke and Kumar, 1983a; Panagis *et al.*, 1996). Nicotine-induced locomotion was blocked in animals with selective VTA DA lesions formed using 6-OHDA (discussed in the General Introduction) (Louis and Clarke, 1998). However, direct infusion of nicotine into the NAc had no effect on locomotion (Reavill and Stolerman, 1990). As discussed in the General Introduction, the VTA receives projections from the pPPTg and LDTg. Alderson *et al.* (2005) demonstrated that excitotoxic lesions of the LDTg altered the effects of nicotine-induced locomotion. Animals with LDTg lesions showed no difference in locomotor response to nicotine compared to saline (Alderson, Latimer and Winn, 2005). Furthermore, nicotine-induced locomotor behaviour was disrupted in rats with pPPTg lesions, but not anterior PPTg (aPPTg) lesions (Alderson and Latimer, 2008). Animals with pPPTg lesions did not demonstrate hypolocomotion following nicotine administration. Together, these results confirm that pPPTg and LDTg projections to the VTA are important in mediating the behavioural effects of nicotine – including sensitisation. Increased locomotor response and sensitisation following repeated nicotine exposure is believed to be partly caused by the upregulation of specific nAChRs in the VTA (Govind, Vezina and Green, 2009). Locomotion induced by systemic nicotine is not simply caused by activated VTA DA neurons that lead to increased NAc DA levels. It involves many parallel events across different brain regions that initiate complex receptor alterations and neuroadaptations in response to either acute or chronic nicotine exposure. It would be beneficial to visualise neural activity in specific brain regions to investigate the neural effects of acute and chronic nicotine. This would allow accurate detection of regional activity adaptations that occur from a single to repeated exposure to nicotine.

4.1.6 c-fos

In the late 1980s it was established that immediate early gene (IEG) encoded transcription factor *c-fos* was expressed in specific brain nuclei after pharmacological stimulation and physiological events (Morgan *et al.*, 1987; Saffen *et al.*, 1988; Sagar, Sharp and Curran, 1988) providing a novel marker of neural activity that could be easily and reliably measured. Nuclear phosphoprotein Fos, the product of *c-fos*, is expressed rapidly and transiently by many neurons upon activation and has been used extensively as a postsynaptic marker for recent neural activity (Duffield, Hastings and Ebling, 1998; Emmert and Herman, 1999; Novak, Smale and Nunez, 2000; Morrow, Elsworth and Roth, 2001; Pacák and Palkovits, 2001). Previous studies have used *c-fos* to determine the primary target areas for a variety of drugs of abuse (Kiba and Jayaraman, 1994; Curran, Akit and Watson, 1996; Pich *et al.*, 1997; Lanca, Sanelli and Corrigall, 2000; Porter, 2008). Once the particular drug binds to a receptor on the cell membrane, a secondary messenger cascade is triggered. G-proteins attached to the receptor activates adenylate cyclase which converts adenosine triphosphate (ATP) to cyclic adenosine triphosphate (cAMP). The role of cAMP is to activate several pathways to transfer the effect of the drug from the cell membrane into the nucleus. This is achieved by regulating intracellular levels of Ca^{+2} through ion channels on the cell membrane and activating protein kinases. These pathways trigger activation of Ca^{+2}/cAMP response element (CREB) located near the transcription start site of the *c-fos* gene (Sheng and Greenberg, 1990). This response element allows the transcription of many IEGs associated with different cell functions, including *c-fos* (Changelian *et al.*, 1989). Fos protein, produced by *c-fos* transcription, is essential for plastic modulation in the nervous system associated with physiological events such as activity-dependant synaptic plasticity (Jomphe, Lèvesque and Trudeau, 2003). Depending on the target regions of the drug, Fos levels peak after ~ 1 h and remains in the cell for ~ 3 – 4 h after transcription (Ren and Sagar, 1992; Herdegen and Leah, 1998). Fos protein can be successfully detected through immunohistochemistry using antibodies or *in situ*

hybridisation for *c-fos* messenger RNA (mRNA). One disadvantage in using Fos detection to map neural activation is that expression can be increased by a number of factors including novelty, stress, auditory and visual stimuli. Therefore, extra care must be taken to reduce excess IEG activation.

4.1.7 c-fos and nicotine

IEG expression mapping is a powerful tool used to visualise activated neural populations in select brain regions. Compared to other IEGs, Fos induction is relatively high and is best applied to behavioural regimes that have a strong cognitive effect (Okuno, 2011). Previous studies investigated the effect of nicotine on *c-fos* activation in restricted brain regions. Acute systemic nicotine was reported to induce Fos expression in many brain regions including: - midbrain DA neurons, interpeduncular nucleus (IPN), superior colliculus (SC), caudate (or central) linear nucleus (CLi), CPu, areas of the hypothalamus, NAc, medial PFC (mPFC), locus coeruleus (LC), PPTg, LDTg and areas of the visual pathways (Pang, Kiba and Jayaraman, 1993; Kiba and Jayaraman, 1994; Pich *et al.*, 1997; Mathieu-Kia, Pages and Besson, 1998; Ikemoto, Witkin and Morales, 2003; Shram *et al.*, 2007; Zhao-Shea *et al.*, 2011; Dehkordi *et al.*, 2015).

Lanca *et al.* (2000) reported that a single administration of nicotine resulted in increased PPTg Fos expression. Further analysis demonstrated that 95% of activated neurons were non-cholinergic – this represented the entire non-cholinergic population of the PPTg. Less than 5% cholinergic neurons were activated by systemic nicotine (Lanca, Sanelli and Corrigall, 2000). Based on this experimental protocol, Porter (2008) examined *c-fos* activation in mesopontine cholinergic neurons and midbrain DA neurons after acute and chronic systemic nicotine. This was achieved using double immunohistochemical stain for *c-fos* and brain derived nitric oxide synthase (bNOS) as a selective marker for cholinergic neurons of the mesopontine tegmentum (Porter, 2008). Midbrain DA neurons were visualised through double immunohistochemical

stain for *c-fos* and tyrosine hydroxylase (TH). Acute and chronic nicotine induced very low levels of *c-fos* and bNOS co-expression in the PPTg and LDTg. The majority of *c-fos* activation was in non-cholinergic neurons of the mesopontine tegmentum. There was minimal *c-fos* and TH co-expression in VTA DA neurons and the majority of Fos expression was in VTA non-DA neurons (Porter, 2008). Zhao-Shea *et al.* (2011) reported that nicotine activated DA neurons in distinct regions of the VTA. Acute systemic nicotine selectively activated subpopulations of pVTA neurons, but did not activate neurons in the aVTA or VTT (Zhao-Shea *et al.*, 2011). However, it has also been reported that there was no nicotine-induced Fos expression in the VTA after acute or chronic nicotine exposure (Pang, Kiba and Jayaraman, 1993; Mathieu-Kia, Pages and Besson, 1998). High levels of Fos expression were found in VTA terminal fields after acute and chronic nicotine administration (Mathieu-Kia, Pages and Besson, 1998). Substantial Fos expression was located in terminal regions such as: - NAc, central amygdala (CeA), lateral habenula (LHb), lateral septum (LS), cingulate, medial prefrontal cortices (orbital and piriform) (Mathieu-Kia, Pages and Besson, 1998). As previous studies have shown non-cholinergic neurons of the PPTg and LDTg, which project to the VTA, are activated by nicotine. Specific terminal regions of the VTA are also activated by nicotine. However, there appear to be conflicting results for the activation of VTA neurons. Previous studies report that VTA non-DA neurons were activated by nicotine; others report that nicotine triggered activation specifically in VTA DA neurons. Whereas, some studies report that there was no neural activation in the VTA at all.

4.2 Chapter aims

The aim of this chapter was to examine the mechanisms of acute and chronic systemic nicotine in the brain using IEG *c-fos* as a correlate of neural activity. Previous studies reported that nicotine increased *c-fos* transcription in many regions of the CNS. In the current study not all of these brain regions were examined. Fos activity was measured in key locations of the mesocorticolimbic system and the HPC-VTA loop – namely the VTA, dHPC and NAc. In the

ventral striatum (in which NAc is located) DA release has been linked to novelty and new stimuli (Legault and Wise, 2001). Whereas the dorsal striatum (in which CPu is located) is thought to mediate habit-based learning (Packard, Hirsh and White, 1989; Yin, Knowlton and Balleine, 2004; Packard, 2009a, 2009b). Fos activity in the CPu was quantified to determine if there was a shift in neural activation from session 1 to habit-based learning by session 5.

This chapter specifically set out to ask: - (1) what regions of the mesocorticolimbic system does systemic nicotine activate, (2) does nicotine mediate its effect through the HPC-VTA loop and (3) does acute and chronic nicotine exposure mediate its effects through the same neural mechanisms? Acute and chronic exposures were investigated in order to determine the effects after single exposure to nicotine, compared to subsequent changes after repeated exposure. Animals in the acute study were exposed to 1 day of nicotine testing, whereas the chronic study repeatedly exposed animals to nicotine over 5 days. Chronic exposure to nicotine investigated Fos expression in response to a final challenge by nicotine after sensitisation. Animals were injected with nicotine in a day-on-day-off testing regime due to the acidic nature of nicotine at the injection site. A moderate concentration of nicotine was chosen (0.8 mg/kg) that has previously been shown to induce Fos expression (Jorenby *et al.*, 1990; Matta *et al.*, 2007; Porter, 2008). Nicotine-induced locomotor behaviour and sensitisation was used to determine that nicotine administration had been achieved. Rats were habituated to the locomotor boxes and transport cages for 5 days prior to testing, because novelty of the environment can effect nicotine-induced locomotion (Clarke and Kumar, 1983b). Animals were habituated to the injection procedure for 3 days prior to testing, because injections can also trigger excess Fos expression (Dong *et al.*, 2010). On the final day of testing (day 1 or 5), animals were euthanized 1 h after nicotine administration when Fos levels are believed to peak.

It was hypothesised that acute nicotine exposure would lead to increased Fos activity in the VTA, and potentially the dHPC through the HPC-VTA loop. The repeated exposure to nicotine was expected to increase NAc Fos activity in the shell due to nicotine sensitisation.

The locomotor data were expected to show hypolocomotion after the first administration of nicotine, followed by hyperlocomotion after repeated nicotine exposure. The location of neural activity was examined by quantification of Fos+ cells in response to nicotine. The VTA, CPu, NAc (shell and core) and dHPC were examined for changes in Fos expression. Simultaneous quantification of nicotine-induced Fos activity in these specific regions will lead to a better understanding of interactions between nicotine and interrelated systems.

4.3 Materials and methods

4.3.1 Subjects

Eighteen adult male Lister Hooded rats were used for this study. Rats were handled and housed as described in the General Methods. Animals were either part of the acute (n = 10) or chronic (n = 8) nicotine study.[1] Animals weighed between 320 – 330g at the time of testing.

During the acute study, 3 rats had to be excluded from the experiment due to an unexpected adverse reaction to nicotine. Following nicotine administration, these 3 animals were placed in locomotor boxes as usual. They showed unusual whole body tremors and rapid breathing within a couple of minutes, followed by a lowered body posture and straub tail. Named Animal Care and Welfare Officer (NACWO) advice was sought and the animals were observed for a further 15 min to see if they would recover. There was no improvement so the animals were euthanized. The protocol and calculations were reviewed, and no calculation or mechanical error was found. The same concentration and batch of nicotine had recently been used on the same strain of rats with no unusual adverse effects. It was suspected that this particular batch of animals might have had an unusual sensitivity to the concentration of nicotine. The nicotine concentration was dropped to 0.6 mg/kg for the next animal, which was closely monitored. This animal exhibited the expected effects and continued onto locomotor testing. The

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[1] Behavioural data from the chronic nicotine study was collected with the help of Jordanna Magee, an Experimental Psychology Society vacation scholarship funded undergraduate student.

remaining animals also received the lower concentration of nicotine with no adverse effects (n = 4). Animals from the acute study were found to have further locomotor depression than the chronic nicotine animals that received a higher concentration of nicotine (Figure 4.2). This data would suggest that the batch of animals for the acute study were sensitive to nicotine concentration.

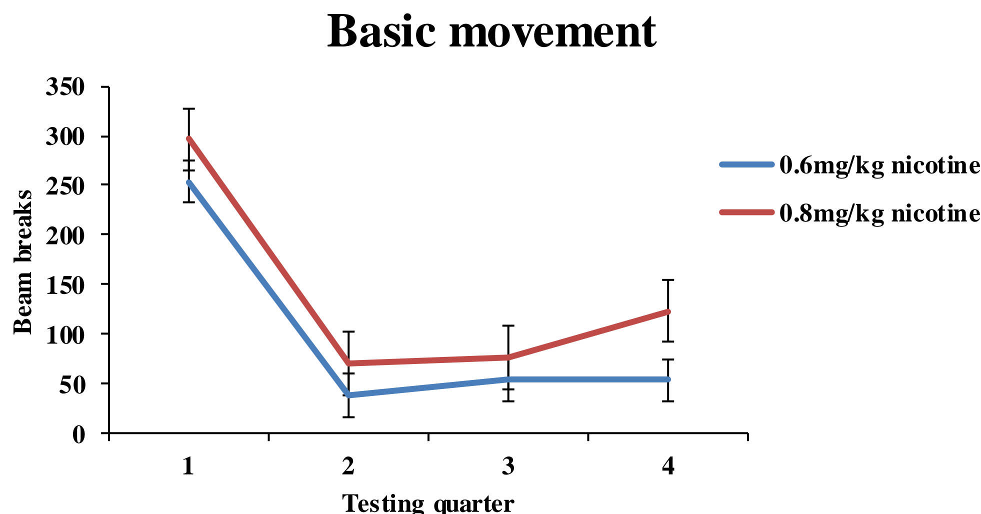

Figure 4.2 – Locomotor behaviour during nicotine session 1. Animals showed further hypolocomotion following administration of a lower concentration of nicotine (n = 4; 0.6 mg/kg) compared to chronic nicotine animals that received a higher nicotine concentration (n = 4; 0.8 mg/kg). Graph shows mean ± SEM.

4.3.2 Fos induction with locomotor testing

As described in the General Methods, Perspex cages positioned in SmartFrame™ Cage Rack Stations were used to monitor basic locomotion. Animals were habituated to locomotor boxes, handling procedures and transport cages for 5 days to reduce the effects of novelty on locomotion. Rats received an injection of saline on habituation days 3 – 5 to habituate animals to the testing procedure (administered s.c.; 0.9% saline 1 ml/kg). All injections were performed in a procedures room opposite the locomotor test room. Each rat was individually taken to the procedures room, injected, and then placed in a locomotor cage for testing. Each rat was always injected by the same handler, in the same room and never injected in the presence of another rat. Locomotor sessions were run daily for 1 h. After habituation and a 2-day break, rats started locomotor testing with nicotine (administered s.c.; 0.6 mg/kg or 0.8 mg/kg

dissolved in 0.9% saline; nicotine hydrogen tartrate salt, Sigma-Aldrich, UK; doses refer to salt). As previously mentioned due to nicotine sensitivity, animals in the acute nicotine study received a lower concentration of nicotine compared to the chronic nicotine animals (acute – 0.6 mg/kg and chronic – 0.8 mg/kg). For the chronic study, animals were injected with nicotine in a day-on-day-off testing regime. Control animals received injections of saline throughout (administered s.c.; 0.9% saline 1ml/kg). Animals were tested so that at least 1 nicotine and 1 control animal were in each testing group. Visual and audio cues were kept to a minimum to prevent excess IEG activation. All behavioural testing was carried out during the light phase under dull red-light illumination.

4.3.3 Histology

On the final day of testing (day 1 or day 5), animals were euthanized immediately after testing, transcardially perfused and brains were stored in sucrose as described in the General Methods. Each brain was dissected from the NAc to the VTA and 30µm sections cut. All histological procedures were performed as stated in the General Methods. A 1:4 series of sections were processed for *c-fos* immunohistochemical analysis. All sections that crossed through the NAc, CPu, dHPC and VTA were processed. Sections were mounted onto glass slides and viewed via light microscopy.

4.3.4 Data analysis

Statistical analyses were performed in SPSS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). Locomotor data were collated as square root transformed (SQRT) ± SEM in order to correct for positive skew in the data (identified by Shapiro-Wilk test). An independent-samples t-test was applied to determine if there were any differences in locomotor behaviour between acute nicotine and saline groups. Homogeneity of variances was assessed by Levene's test for equality of variances. Locomotor data from the chronic nicotine study were analysed through

a range of ANOVAs to compare across *sessions* (day 1, 3 and 5) and between *groups* (nicotine and saline). Mauchly's test of sphericity indicated sphericity was met.

Quantification of Fos+ cells was carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). A two-way ANOVA was performed to determine if there was an effect of *drug*, *brain region* or an interaction between *drug* x *brain region* on Fos expression following acute or chronic nicotine exposure. Results are expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. Results were considered significant when $p \leq 0.05$.

4.4 Results

4.4.1 Behavioural results

Acute nicotine study

Seven rats were habituated to the testing schedule for 5 days prior to 1 day of nicotine testing (habituation data not shown). On the day of testing, locomotor activity was recorded for 1 h immediately after nicotine (n = 4) or saline (n = 3) injection. Acute systemic nicotine administration supressed locomotor activity measured through basic movements, fine movements and rearing (Figure 4.3).

The number of beam breaks square root transformed (SQRT) for basic movements during testing is shown below (Figure 4.3A). An independent-samples t-test was applied to determine if there were any differences in basic movements between nicotine and saline animals. Basic movements (the total number of beam breaks at the level of the animal's body) were significantly different between nicotine and control groups (t = 5.966, df 5, $p = 0.002$) (Figure 4.3A).

The number of fine movements (such as forepaw grooming or head waves) measured as the number of beam breaks (SQRT) are shown below (Figure 4.3B). An independent-samples t-

test was applied to determine if there were any differences in fine movements between nicotine and saline animals. Fine movements were significantly different between nicotine and control groups (t = 18.971, df 5, p = 0.000) (Figure 4.3B).

The number of rears completed by the animal was also automatically measured by the apparatus; this was expressed as the number of beam breaks SQRT (Figure 4.3C). Again, using the same method of statistical analysis it was found that rearing was significantly different between nicotine and control groups (t = 5.442, df 5, p = 0.003) (Figure 4.3C). Together these results demonstrated that successful systemic nicotine administration was achieved. Following acute exposure to nicotine animals displayed nicotine-induced hypolocomotion (Figure 4.3).

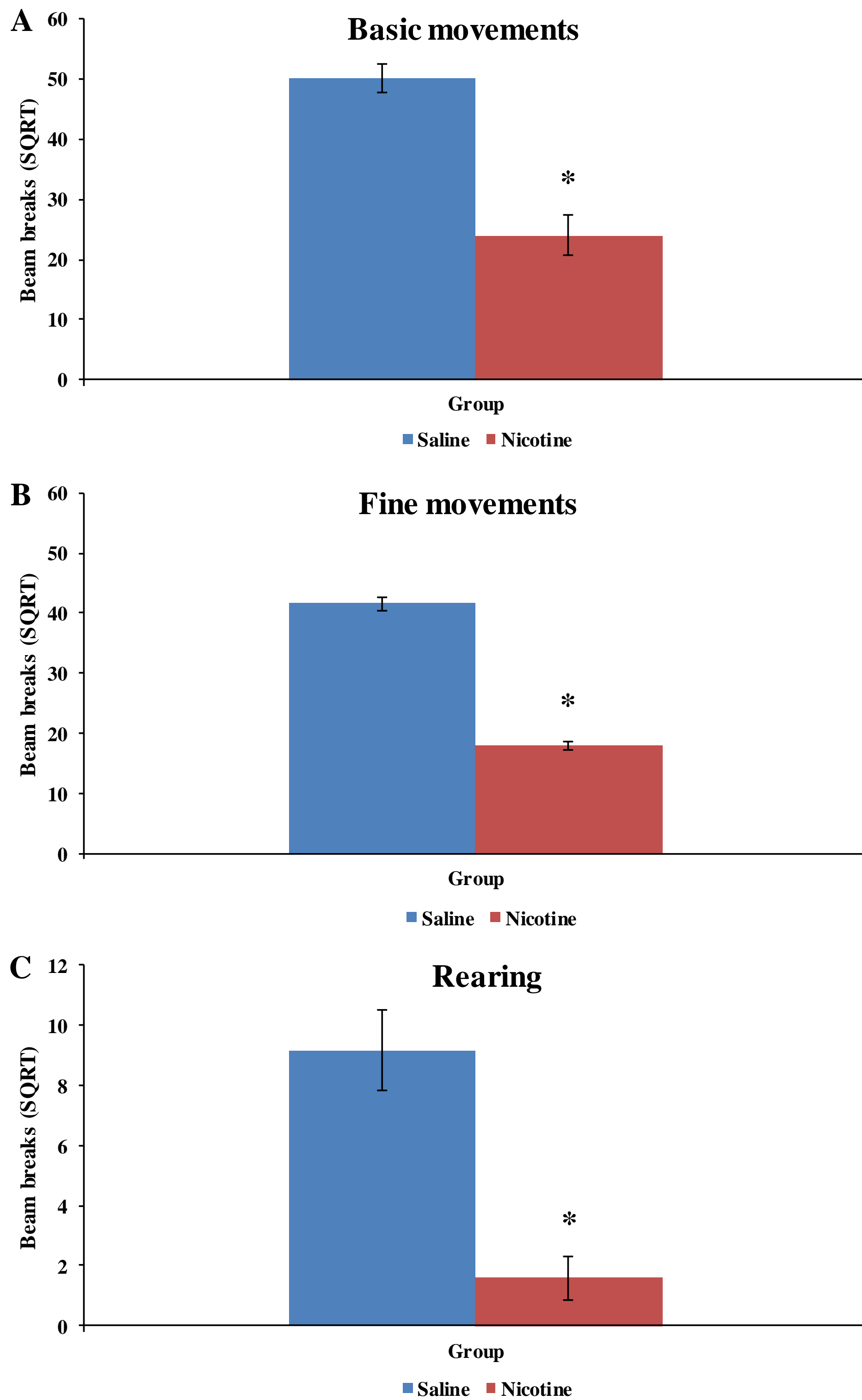

Figure 4.3 – Basic movements (A), fine movements (B) and rearing behaviour (C) (all SQRT transformed) made during locomotor testing following acute nicotine or saline injection. Graph shows mean beam breaks during day 1 test session by rats injected with nicotine (red bar; n = 4) or saline (blue bar; n = 3). Error bars = ± SEM. * indicates significant difference between saline and nicotine groups.

Chronic nicotine study

Eight rats were habituated to the testing schedule for 5 days prior to 5 days of testing (habituation data not shown). Rats underwent 5 days of locomotor testing with nicotine administration in a day-on-day-off design so that animals (n = 4) received nicotine injections on days 1, 3 and 5 while control animals (n = 4) always received saline. On days 2 and 4 all animals received saline and underwent testing (locomotor data not shown). Locomotor activity was measured through basic movements, fine movements and rearing for 1 h immediately after injection (Figure 4.4).

The number of beam breaks (SQRT) for basic movements during 3 nicotine testing sessions are shown below (Figure 4.4A). Basic movements were significantly different between nicotine and saline groups, demonstrated through repeated measures ANOVA ($F_{(1, 6)} = 13.012$; $p = 0.011$). There was no effect of *session* ($F_{(2, 12)} = 0.987$; $p = 0.401$). There was a significant interaction between *session* x *group* ($F_{(2, 12)} = 10.327$; $p = 0.002$).

The number of beam breaks (SQRT) for fine movements measured are shown below (Figure 4.4B). Repeated measures ANOVA for fine movement data found that there was a significant effect of *group* ($F_{(1, 6)} = 15.654$; $p = 0.007$). It was further observed that there was no effect of *session* ($F_{(2, 12)} = 1.541$; $p = 0.254$). There was a significant interaction between *session* x *group* for fine movements ($F_{(2, 12)} = 12.380$; $p = 0.001$).

The number of rears completed by the animal was expressed as the number of beam breaks (SQRT) (Figure 4.4C). Again, using the same methods of statistical analysis it was found that there was a significant difference between nicotine and saline *groups* ($F_{(1, 6)} = 10.312$; $p = 0.018$). The number of rears was not affected by *session* ($F_{(2, 12)} = 0.174$; $p = 0.843$). There was a significant interaction between *session* x *group* ($F_{(2, 12)} = 4.319$; $p = 0.039$).

Together these results demonstrated that successful nicotine administration had been achieved. Animals displayed nicotine-induced hypolocomotion on day 1, which tended towards

hyperlocomotion by day 3. On day 5, locomotor activity had increased further, demonstrating that nicotine-induced locomotor sensitisation had occurred following repeated nicotine exposure. These results also demonstrated that habituation to the environment and injections had been achieved by both animal groups.

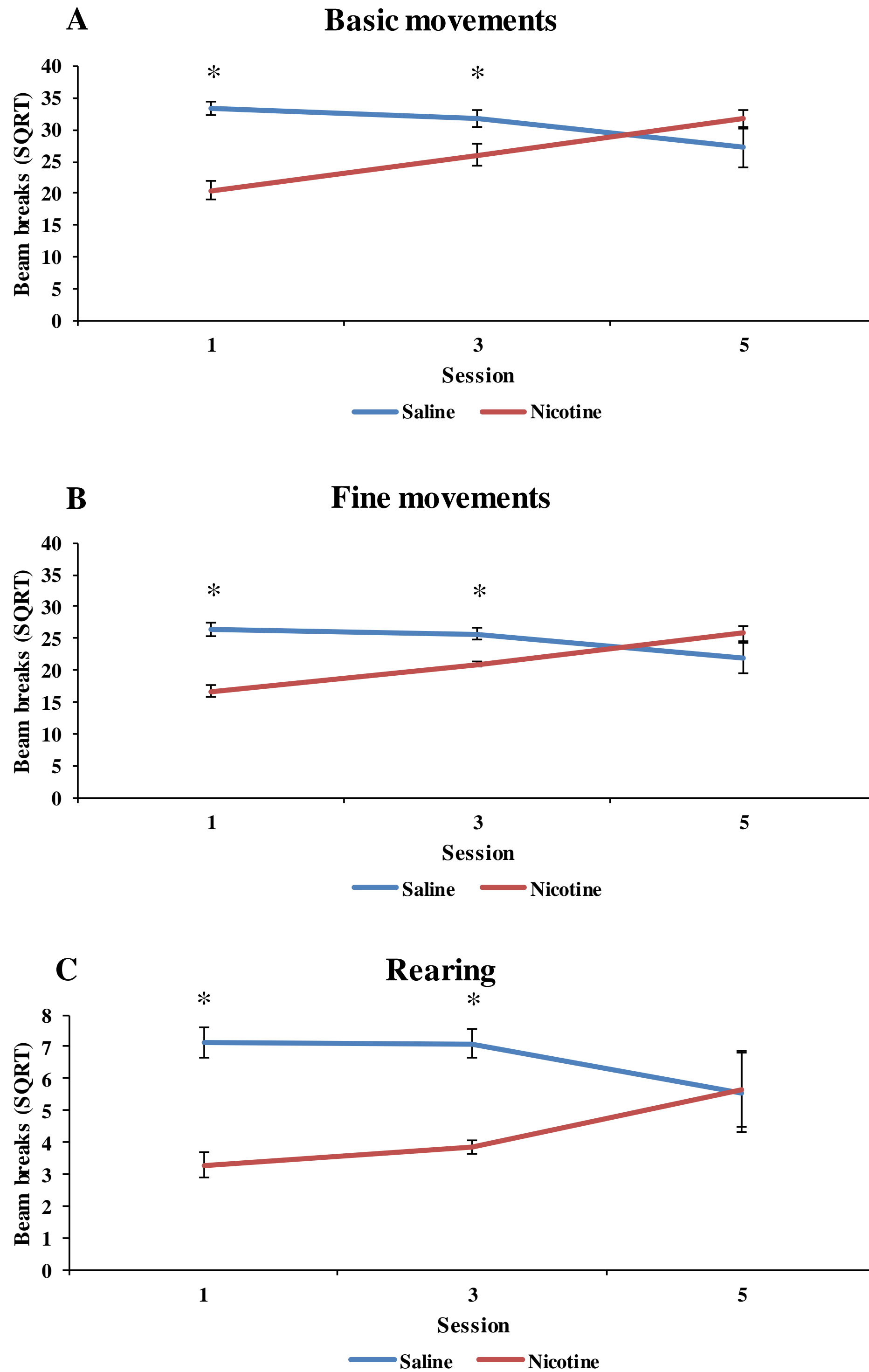

Figure 4.4 – Basic movements (A), fine movements (B) and rearing behaviour (C) (all SQRT transformed) made during locomotor testing sessions following chronic nicotine or saline injection (days 1, 3 and 5). Graph shows beam breaks (SQRT) during 3 nicotine testing sessions by rats injected with nicotine (red line; n = 4) or saline (blue line; n = 4). Error bars = ± SEM. * indicates significant difference between saline and nicotine groups in the corresponding session.

4.4.2 Histology

Animals were immediately perfused after the final session of locomotor testing (day 1 or 5). Tissue from nicotine and control groups was processed through *c-fos* immunohistology as described in the General Methods. All sections that crossed through the NAc, CPu, dHPC and VTA were processed. As stated in the General Methods Fos positive (Fos+) cells were quantified using ImageJ software (ImageJ, U.S. National Institutes of Health, U.S.A). Bilateral cell counts were taken from at least 5 sections containing the regions of interest from a 1:4 series. Representative tissue from the CPu, NAc shell and core, VTA and dHPC are shown below (Figures 4.5 – 4.7).

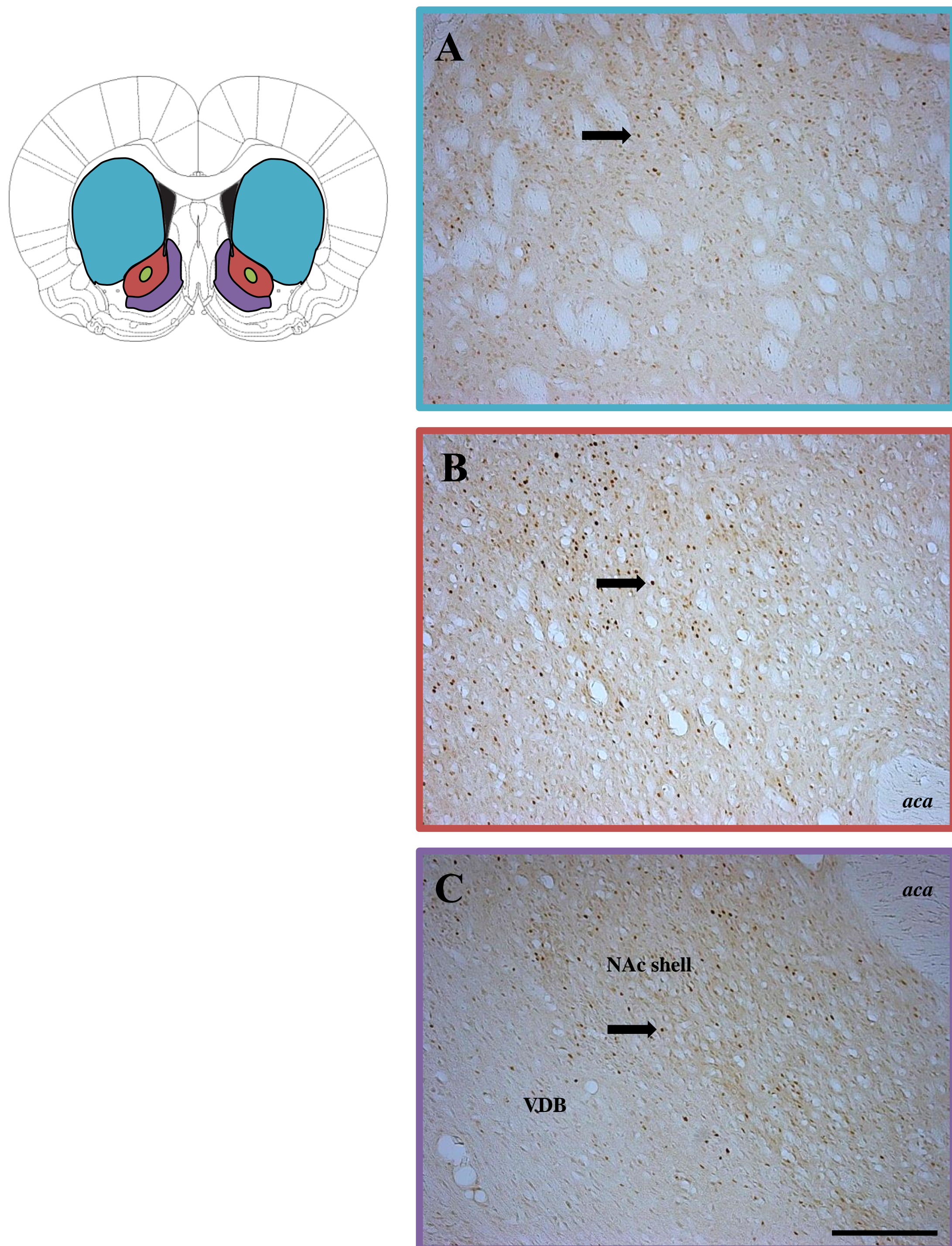

Figure 4.5 – Representative NAc and CPu tissue processed for *c-fos* activation after acute nicotine exposure. Example tissue from the CPu (A), NAc shell (B) and core (C) at +10.00 mm from the interaural line (IAL). All sections that crossed through the NAc and CPu were processed for *c-fos*. Cells were quantified using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Example Fos+ cells highlighted by black arrows. Schematic drawing highlights the CPu (blue), NAc shell (red) and NAc core (purple). The anterior part of the anterior commissure (aca; green) and the nucleus of the vertical limb of the diagonal band (VDB) were used to select the same region of NAc shell and core in each section. Schematic drawing adapted from Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007). Scale bar = 100µm.

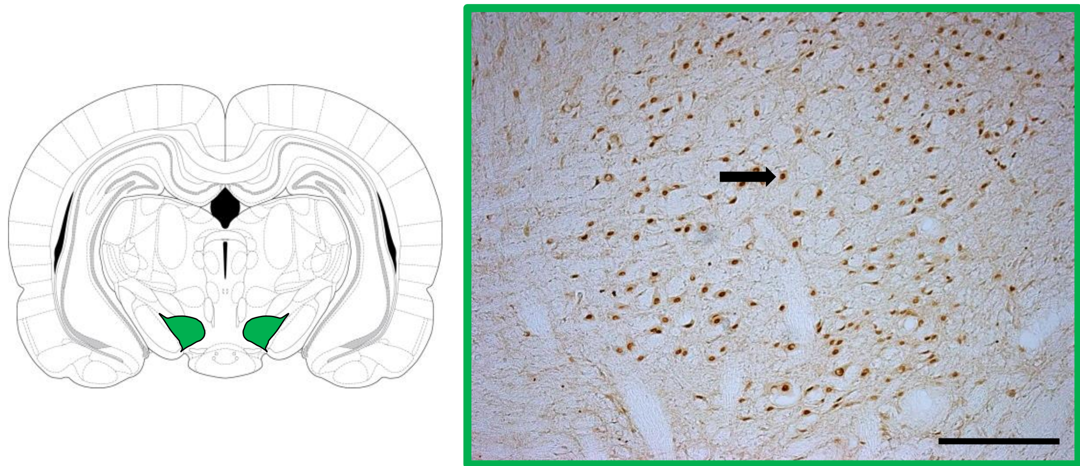

Figure 4.6 – Representative VTA tissue processed for *c-fos* activation after acute nicotine exposure. Example VTA tissue at +4.20 mm from IAL. All sections that crossed through the VTA were processed for *c-fos*. Cells were quantified using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Example Fos+ cell highlighted by black arrow. Schematic drawing highlights the VTA (green). Schematic drawing adapted from Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007). Scale bar = 100µm.

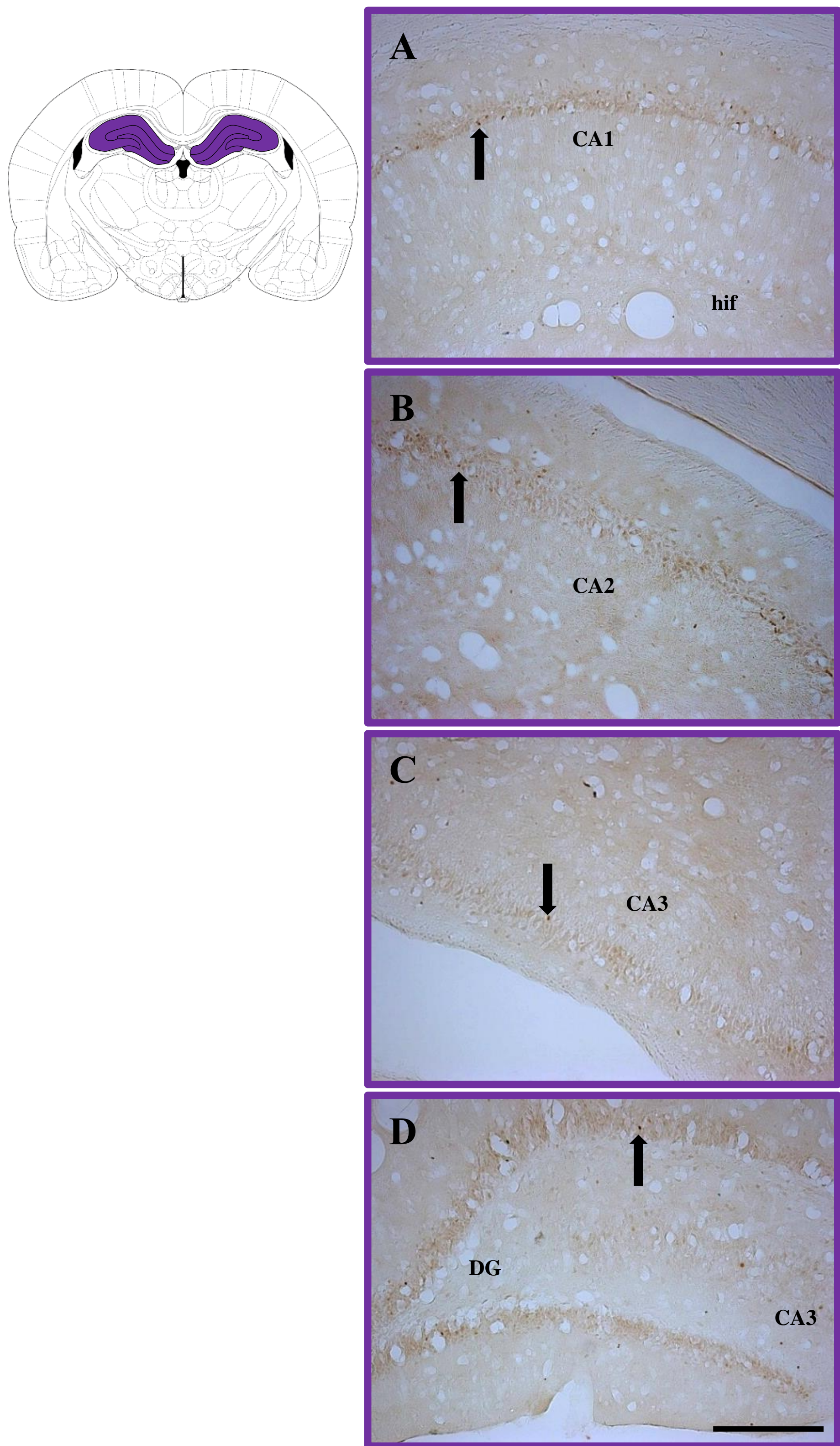

Figure 4.7 – Representative dHPC tissue processed for *c-fos* activation after acute nicotine exposure. Four images were taken per hippocampal section containing CA1 (A), CA2 (B), CA3 (C) and DG (D). Example images at +5.86 mm from IAL. All sections that crossed through the dHPC were processed for *c-fos*. The hippocampal fissure (hif) was used to select the same area of CA1 and CA2. Cells were quantified using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). Schematic drawing highlights the dHPC (purple). Example Fos+ cells highlighted by black arrows. Schematic drawing adapted from Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007). Scale bar = 100μm.

Acute nicotine study

The number of Fos+ cells in the CPu, NAc shell and core, VTA and dHPC from acute nicotine and saline tissue are shown below (Figure 4.8). A two-way ANOVA found that there was no significant effect of *drug* on Fos+ cells in these regions ($F_{(1, 25)} = 0.627$; $p = 0.436$). There was a significant effect of *brain region* ($F_{(4, 25)} = 9.251$; $p = 0.000$). There was also no significant interaction between *drug* x *brain region* ($F_{(4, 25)} = 0.135$; $p = 0.968$).

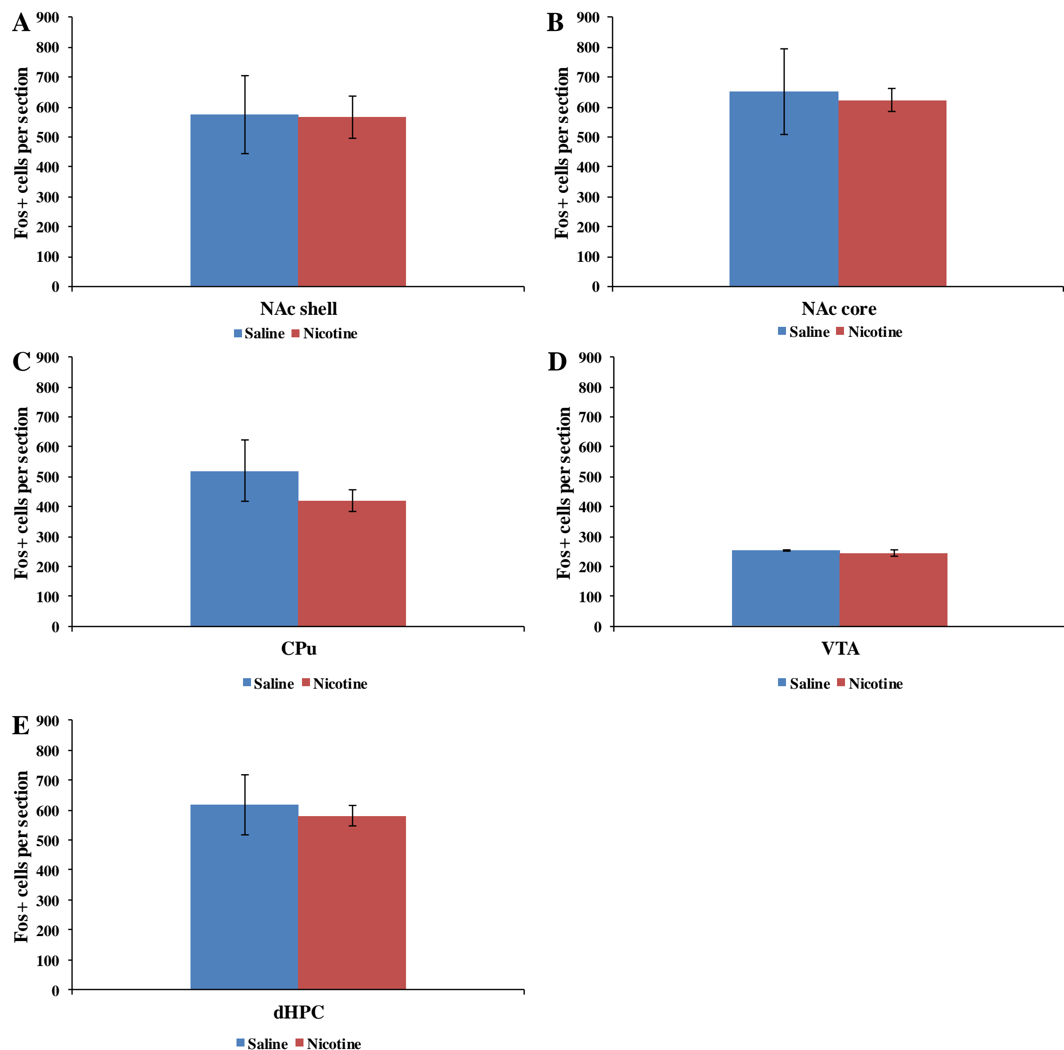

Figure 4.8 – Fos+ cells in the NAc shell (A), NAc core (B), CPu (C), VTA (D) and dHPC (E) after acute nicotine exposure. Animals were either administered with nicotine (n = 4; red bars) or saline (n = 3; blue bars). Error bars = ± SEM.

Chronic nicotine study

The number of Fos+ cells in the CPu, NAc shell and core, VTA and dHPC from chronic nicotine and saline tissue are shown below (Figure 4.9). A two-way ANOVA found that there was no significant effect of *drug* on Fos+ cells in these regions ($F_{(1, 28)} = 0.329$; $p = 0.571$). There was a significant effect of *brain region* ($F_{(4, 28)} = 11.511$; $p = 0.000$). There was also no significant interaction between *drug* x *brain region* ($F_{(4, 28)} = 0.573$; $p = 0.684$).

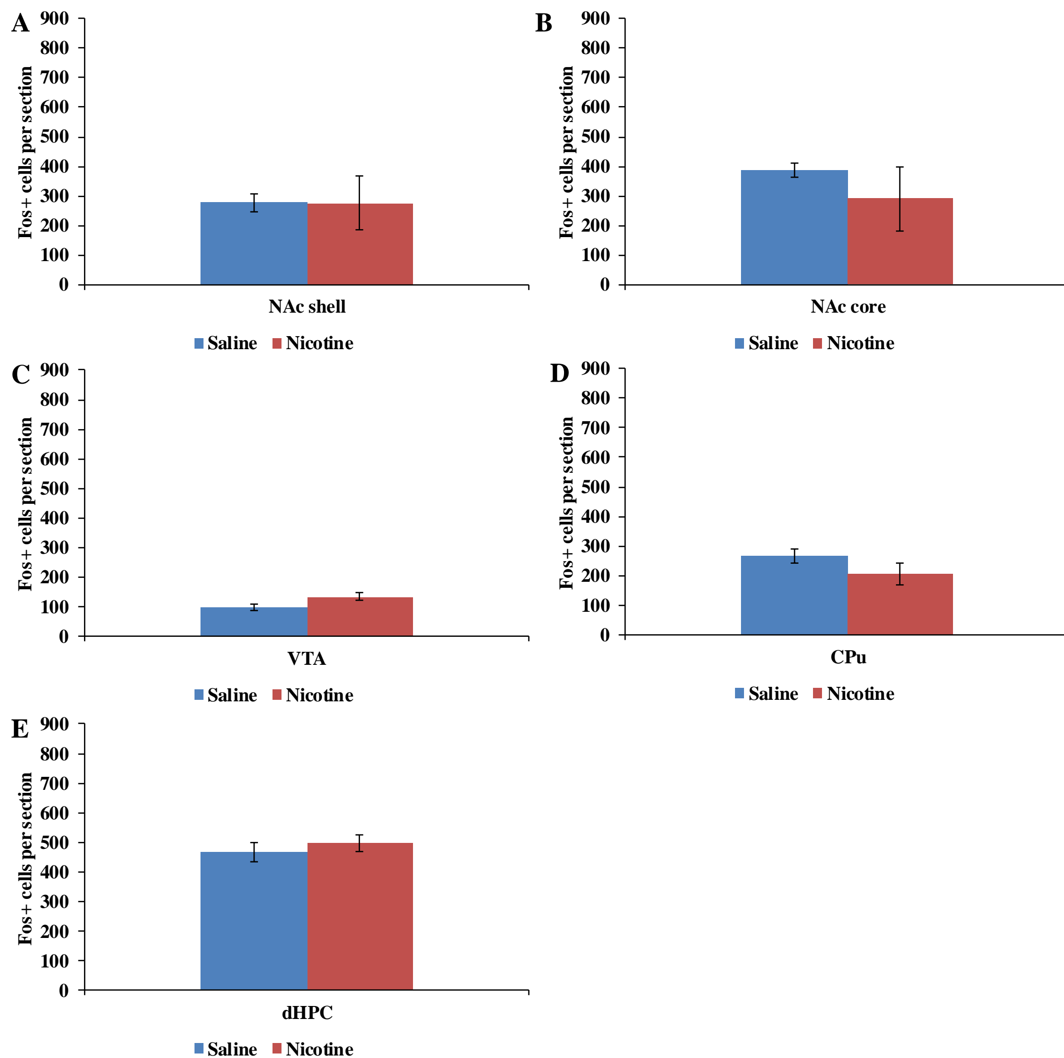

Figure 4.9 – Fos+ cells in the NAc shell (A), NAc core (B), CPu (C), VTA (D) and dHPC (E) after chronic nicotine exposure. Animals were administered with nicotine (n = 4; red bars) or saline (n = 4; blue bars). Error bars = ± SEM.

4.5 Chapter discussion

The main aim of this chapter was to quantify *c-fos* expression in selected brain regions after acute and chronic systemic nicotine. Fos quantification was carried out in the VTA, NAc shell and core, CPu and dHPC to investigate nicotine-induced activity in key regions of the mesocorticolimbic system and HPC-VTA loop. Investigating the effect of acute and chronic nicotine explored regional neural activation after a single administration of nicotine compared to repeated nicotine exposure. This would determine if acute and chronic nicotine mediates its effects through the same neural mechanisms.

In the current study it was found that acute or chronic nicotine had no visible effect on Fos expression in the VTA, NAc shell and core, CPu and dHPC (Figure 4.8 and 4.9). These results show that acute and chronic nicotine did not induce differential levels of Fos expression in key regions of the mesocorticolimbic system and HPC-VTA loop. Although there was no effect of *drug*, Fos expression was effected by *brain region* (Figure 4.8 and 4.9). The varying levels of Fos expression across brain regions could be due to neuron density. For example, in the VTA levels of Fos activity were low in saline and nicotine groups, but in the dHPC Fos levels were much higher for both experimental groups. This would suggest that there were fewer neurons in the VTA, therefore, fewer neurons to express Fos. In sections containing the VTA, nicotine induced substantial Fos expression in the medial terminal nucleus of the accessory optic tract (MT), but saline had no effect. Previous studies have used nicotine-induced Fos expression in the MT as a control to indicate successful nicotine administration and to confirm that there was no mix-up between experimental tissue (Porter, 2008). In the current study, after Fos quantification, the MT was used to confirm that all nicotine animals successfully received the drug. Nicotine administration was further confirmed by locomotor data (Figure 4.3 and 4.4). Animals exposed to acute nicotine demonstrated nicotine-induced hypolocomotion (Figure 4.3) and chronic nicotine animals demonstrated hypolocomotion, that tended towards hyperlocomotion by day 5 (Figure 4.4). Locomotor activity indicated that chronic nicotine

exposure achieved sensitisation (Figure 4.4). Locomotor sensitisation is reported to be mediated by midbrain DA neurons and with continued exposure to nicotine, sensitisation is thought to be regulated by the NAc (Kalivas and Duffy, 1995; Panagis *et al.*, 1996). Previous studies reported that nicotine sensitisation increased DA levels in the NAc, but not the dorsal striatum (Benwell and Balfour, 1992; Nisell, Nomikos and Svensson, 1994; Panagis *et al.*, 1996; Balfour *et al.*, 1998). It would be reasonable to assume that after acute nicotine exposure increased Fos expression in the VTA would be observed and that chronic nicotine would lead to increased Fos activity in the NAc. However, in the current study acute nicotine exposure did not increase VTA neural activity (Figure 4.8). Furthermore, chronic nicotine exposure did not increase Fos expression in the NAc shell or core (Figure 4.9).

Previous studies reported that acute systemic nicotine preferentially activated non-cholinergic neurons of the PPTg and LDTg (Lanca, Sanelli and Corrigall, 2000; Porter, 2008). In the VTA, acute nicotine induced Fos expression in non-DA neurons and only 7 – 15% of VTA DA neurons (Porter, 2008). Chronic exposure to nicotine also activated PPTg and LDTg non-cholinergic neurons, but had no effect on the expression of Fos in VTA DA neurons (Porter, 2008). Non-DA VTA neurons demonstrated increased Fos expression after chronic nicotine administration (Porter, 2008). Mathieu-Kia *et al.* (1998) also investigated Fos expression following acute and chronic nicotine exposure. Chronic nicotine induced high levels of Fos expression in VTA terminal fields: - SC, lateral habenula (LHb), medial terminal nucleus of the accessory optic tract (MT) and NAc, but there was no Fos activity in the VTA (Mathieu-Kia, Pages and Besson, 1998).

It has been suggested that *c-fos* may not be a sensitive marker for VTA DA neurons (Ren and Sagar, 1992). Perhaps the VTA stimulates neurotransmitter release that is not capable of stimulating *c-fos* gene transcription. Fos expression may be dependent on the dose or route of administration of nicotine. Different routes of administration would affect the time course of brain nicotine concentration (Ren and Sagar, 1992) – perhaps a longer time point is required

to induce *c-fos* transcription in VTA cells after subcutaneous nicotine administration. It has also been stated that no *c-fos* staining should not be taken as evidence of no neural activation (Kovács, 1998; Okuno, 2011).

4.6 Future work

What we know from the current study is that acute or chronic systemic nicotine does affect behaviour, that it does get into the brain but that it does not activate *c-fos* in the VTA, NAc shell and core, CPu and dHPC in adult rats. However, systemic nicotine has more-or-less simultaneous access to a wide variety of structures, and will likely have conflicting effects on them. Some effects might conceivably counteract other effects elsewhere. Following on from this study, it would therefore be interesting to investigate the effect of nicotine administration *directly* into the VTA, or into the dHPC as a different point in the circuitry where nicotine is known to have effects. This direct injection does not mimic naturally taken nicotine but does allow the effects of nicotine on specific structures to be examined in the absence of effects elsewhere. The specific questions being asked are: - (1) what is the action of intra-VTA nicotine, (2) does intra-dHPC nicotine mediate effects through the same neural mechanisms, (3) does acute nicotine activate the same regions as chronic nicotine, and (4) does intracranial nicotine modulate its effect through the key regions of the mesocorticolimbic system and/or the HPC-VTA loop?

Chapter 5: Fos expression after acute and chronic intracranial self-administration of nicotine into the posterior ventral tegmental area

5.1 Chapter introduction

As shown in the previous chapter, neither acute nor chronic systemic nicotine activated *c-fos* transcription in the VTA, NAc shell or core, CPu or dHPC in adult rats (Chapter 4). However, systemic nicotine acts simultaneously at a wide range of sites and some actions could conceivably counteract others. Therefore, it would be of interest to investigate the direct effect of nicotine in the pVTA. Intracranial self-administration (ICSA) does not mimic nicotine obtained through smoking, but it allows the effects of nicotine on specific structures to be examined in the absence of effects elsewhere.

5.1.1 Nicotine ICSA in the pVTA

As discussed in the General Introduction, rats willingly administer cholinergic agents carbachol, nicotine and neostigmine into the pVTA (Ikemoto and Wise, 2002; Ikemoto, Witkin and Morales, 2003; Farquhar, Latimer and Winn, 2012). In the aVTA, self-administration of carbachol is significantly weaker and nicotine self-administration does not occur (Ikemoto and Wise, 2002; Ikemoto, Qin and Liu, 2006). Intra-pVTA self-administration of carbachol is blocked by the muscarinic acetylcholine receptor (mAChR) antagonist scopolamine or nicotinic acetylcholine receptor (nAChR) antagonist dihydro-β-erythroidine (DHβE) (Corrigall, Coen and Adamson, 1994; Ikemoto and Wise, 2002). Nicotine self-administration in the pVTA appears to be mediated by nAChRs, because nicotine ICSA is blocked by co-administration of nAChR antagonist mecamylamine (Ikemoto, Qin and Liu, 2006). In addition, Ikemoto *et al.* (2006) demonstrated that the reinforcing effects of nicotine in the pVTA were blocked by quinpirole, dopamine-2 (D2) receptor agonist, as well as SCH 23390, dopamine-1 (D1) receptor antagonist. Intra-pVTA carbachol administration was reported to increase locomotion, induce CPP, and increase Fos expression in several brain regions (Ikemoto and Wise, 2002; Ikemoto, Witkin and Morales, 2003; Schifirnet, Bowen and

Borszcz, 2014). In the pVTA, cholinergic systems appear crucial for mediating nicotine-induced reinforcing and reward-related behaviours.

Previous studies have suggested that intra-pVTA nicotine may not be immediately recognised as a reward (Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). Rats willingly self-administer a range of nicotine concentrations into the pVTA (Ikemoto, Qin and Liu, 2006). However, first exposure to nicotine does not provide an incentive to lever press. Ikemoto *et al.* (2006) reported that lever pressing increased by ~ 50% between the first ICSA testing session with saline and the second session with nicotine. In addition, nicotine self-administration was not immediately reinforcing – nicotine administration did not encourage repetition of the action leading to its delivery (Ikemoto, Qin and Liu, 2006) whereas carbachol and neostigmine were self-administered right away (Ikemoto and Wise, 2002). On first exposure to carbachol self-administration, animals demonstrated high levels of lever pressing on both levers, with a preference for the active lever (Farquhar, Latimer and Winn, 2012). High frequency lever-pressing behaviour was maintained across 5 testing sessions. In contrast, on first exposure to nicotine animals demonstrated low rates of lever pressing (Farquhar, Latimer and Winn, 2012). Across 5 nicotine testing sessions, animals increased the proportion of reinforced lever presses. Animals reduced the number of inactive lever presses, rather than increasing active lever presses (Farquhar, Latimer and Winn, 2012). Together, these results suggest that pVTA cholinergic systems play a key role mediating the reinforcing and reward-related effects of nicotine in the pVTA. The question is, how does nicotine act on pVTA DA neurons to mediate reward and enhance reinforcement?

The transient activation of nAChRs on DA neurons does not fully explain what drives rats to continuously self-administer nicotine over a number of hours, or how systemic nicotine increases DA levels in the NAc for over an hour (Imperato, Mulas and Di Chiara, 1986; Pidoplichko *et al.*, 2004). Within seconds of being exposed to high, reward-mediating concentrations of nicotine these receptors desensitise (Pidoplichko *et al.*, 1997, 2004;

Wooltorton *et al.*, 2003). The activation of nAChRs on VTA DA neurons cannot be solely responsible for the maintenance of nicotine self-administration and prolonged DA release – other systems must be involved. Nicotine binds to nAChRs present on glutamatergic presynaptic terminals (Grillner and Svensson, 2000; Mansvelder and McGehee, 2002; Mansvelder, Keath and McGehee, 2002). Activation of glutamatergic nAChRs leads to increased glutamate transmission, triggering the activation of DA neurons (McGehee and Role, 1995; Ikemoto, 2007). The action of nicotine does not readily desensitise glutamatergic neurons and elevated glutamate transmission appears to persist (Mansvelder and McGehee, 2000, 2002; Pidoplichko *et al.*, 2004). Nicotine also activates VTA GABAergic neurons that inhibit local VTA DA neurons. However, GABAergic nAChRs desensitise rapidly and remain deactivated for long periods of time (Mansvelder, Keath and McGehee, 2002; Pidoplichko *et al.*, 2004). Due to the removal of GABA inhibition, VTA DA neurons are disinhibited which leads to the long-lasting excitation of DA neurons (Mansvelder, Keath and McGehee, 2002).

5.1.2 c-fos and VTA activation

There has been no quantification of *c-fos* activity investigating the mechanisms of intra-pVTA nicotine through ICSA. Previous studies have measured Fos expression after nicotine IVSA, ICSS or intra-pVTA administration of cholinergic agonists. Pagliusi *et al.* (1996) reported that IVSA of nicotine increased Fos expression in 21 brain regions compared to control animals that received saline IVSA (Pagliusi *et al.*, 1996). Substantial *c-fos* activity was reported in the superficial gray layer of the superior colliculus (SC), medial terminal nucleus of the accessory optic tract (MT) and the lateral geniculate nucleus (LGN). Fos levels were significantly increased in the mPFC, ventral shell of the NAc, piriform cortex and the cingulate cortex (Pagliusi *et al.*, 1996). Hunt and McGregor (1998) investigated Fos expression following ICSS of medial forebrain bundle (mfb) triggered by nose-poke response. Bilateral *c-fos* activity was identified in the core of the NAc, paraventricular nucleus of the hypothalamus, retrorubral fields and the locus coeruleus (LC). ICSS of the mfb did not trigger Fos expression in VTA

DA neurons, but *c-fos* activation was visible in noradrenaline releasing (NA, noradrenergic) neurons of the LC (Hunt and McGregor, 1998). Ikemoto *et al.* (2003) investigated *c-fos* activation following intra-pVTA infusion of carbachol (Ikemoto, Witkin and Morales, 2003). Little or no detectable Fos expression was observed in the NAc or VTA. Panagis *et al.* (1996) reported that bilateral intra-VTA infusion of nicotine increased Fos expression in the NAc but not the mPFC. In the NAc, nicotine-induced Fos activity was attenuated by pre-treatment of systemic mecamylamine, which further suggests that intra-VTA nicotine mediates its effects on local DA neurons (Panagis *et al.*, 1996).

5.2 Chapter aims

The aim of this chapter was to examine the mechanisms of acute and chronic ICSA of nicotine into the pVTA using IEG *c-fos* as a correlate of neural activity. Previous studies reported that direct electrical and chemical activation of the pVTA increased *c-fos* transcription in many regions of the CNS. In the current study not all of these brain regions were examined. Fos activity was measured in key locations of the mesocorticolimbic system and the HPC-VTA loop – namely the dHPC and NAc. In the ventral striatum (in which NAc is located) DA release has been linked to novelty and new stimuli (Legault and Wise, 2001) whereas the dorsal striatum (in which CPu is located) is thought to mediate habit-based learning (Packard, Hirsh and White, 1989; Yin, Knowlton and Balleine, 2004; Packard, 2009a, 2009b). Fos activity in the CPu was quantified to determine if there was a shift in neural activation from the novel events of session 1 to habit-based learning by session 5.

This chapter specifically aimed to ask: - (1) what regions of the mesocorticolimbic system does intra-pVTA nicotine activate, (2) does intra-pVTA nicotine mediate its effects through the HPC-VTA loop, and (3) does acute and chronic pVTA ICSA of nicotine mediate its effects through the same neural mechanisms? Acute and chronic exposures were investigated in order to determine the effects after single exposure to nicotine, compared to subsequent changes

after repeated exposure. Animals in the acute study were exposed to 1 session of ICSA nicotine testing, whereas the chronic study repeatedly exposed animals to nicotine over 5 sessions across 10 days. To minimise non-specific cell damage at injection site animals in the chronic study were tested every second day. The injector cannula was only placed into the pVTA a maximum of 5 times at 48 h intervals to prevent damage at injection site. Chronic exposure to nicotine investigated Fos expression in response to a final challenge by nicotine after sensitisation. On the final day of testing (session 1 or 5), animals were euthanized 1 h after ICSA testing when Fos levels are believed to peak.

It was hypothesised that acute intra-pVTA nicotine self-administration would lead to increased Fos activity in VTA terminal regions. Repeated exposure to intracranial nicotine was expected to increase Fos activity in the NAc shell due to nicotine sensitisation. It was further hypothesised that Fos expression in the CPu would increase by session 5 compared to session 1 through a shift in neural activation from the novelty of session 1 to habit-based learning by session 5. Based on the data of Farquhar *et al.* (2012), rats were expected to show relatively low levels of lever discrimination (nicotine vs. aCSF) on day 1 of ICSA testing but by session 5, after repeated nicotine exposure, increased lever pressing and discrimination between levers was expected.

5.3 Materials and methods

5.3.1 Subjects

Twenty-four adult male Lister Hooded rats with no previous operant training were used for this experiment. Rats were housed, anaesthetised and cannulae implantation surgery was performed as described in the General Methods. Animals weighed between 350 – 410g at the time of surgery. The coordinates for the pVTA were as stated in the General Methods. Animals

were either part of the acute (n = 12) or chronic (n = 12) nicotine ICSA study[2]. Following surgery animals were maintained on a food restriction regime to gain ~ 10 g per week. This was achieved by giving animals ~ 20 g food per day at the end of testing, or at the equivalent time on non-testing days. Animals had access to water *ad libitum* in homecages throughout the experiment.

5.3.2 Surgical procedure

Cannulae implantation surgery was performed as described in the General Methods. Unilateral guide cannulae (Plastics one, Roanoke, VA, U.S.A) aimed at the pVTA were implanted. The order of the hemisphere cannulated (left or right) was randomly alternated across rats. As before, animals underwent surgery in order of increasing weight to ensure that their weights were approximately the same. Dummy cannulae were changed daily with clean replacements. Rats were given a minimum of 10 days to recover from surgery before starting behavioural testing.

5.3.3 Behavioural testing

Self-administration was conducted in operant chambers as described in the General Methods. Animals were habituated to the testing procedure, operant chambers and head fixed micropump 1 day prior to testing. Self-administration testing began the next day. Each session lasted 90 min and infusions were set to a maximum of 60. Animals either received 1 or 5 testing sessions. To minimise non-specific cell damage at injection site the latter group (chronic study – 5 sessions) were tested every second day so that the injector cannula was only placed into the pVTA a maximum of 5 times at 48 h intervals. Rats were randomly assigned to the acute or chronic nicotine study, and randomly split into nicotine or control groups. Animals either received artificial cerebral spinal fluid (aCSF: consisting of 148 mM NaCl, 2.7

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[2] ICSA surgery was performed by Morag Farquhar, Philip Winn and Josie Fullerton. Behavioural testing was carried out by Morag Farquhar and immunohistology was performed by Josie Fullerton.

mM KCl, 1.2 mM $CaCl_2$ and 0.85 M $MgCl_2$, pH adjusted to 7.4 with NaOH) or 25 mM nicotine ((–)-Nicotine hydrogen tartrate salt dissolved in aCSF; nicotine Sigma-Aldrich, UK). The current study used the nicotine dose previously found to be the most rewarding in the pVTA (Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). The head-fixed micropump precisely and repeated delivered a volume of 75 nl when the appropriate response schedule was met. To minimise diffusion to neighbouring regions, a small volume of nicotine was infused. Animals were tested in groups of 3 so that at least 1 nicotine and 1 control animal were in each testing group. Visual and audio cues were kept to a minimum to prevent excess IEG activation. All behavioural testing was carried out during the light phase.

5.3.4 Histology

On the final day of testing (session 1 or session 5), animals were transferred in their homecage to a dark holding room after testing. Animals remained in this room for 1 h prior to sacrifice when peak levels of Fos expression are believed to occur. This carefully controlled environment had minimal auditory or visual stimuli to reduce excess Fos expression. Animals were euthanized 1 h after testing, transcardially perfused and brains were stored in sucrose as described in the General Methods. Each brain was dissected from the NAc to the VTA and 30µm sections cut. All histological procedures were performed as stated in the General Methods. 1:4 series of sections were processed for cresyl violet stain and *c-fos* immunohistochemical analysis. All sections that crossed through the NAc, CPu, and dHPC were processed for *c-fos*. Sections were mounted onto glass slides and viewed via light microscopy to quantify Fos expression. All sections that crossed through the VTA were processed for cresyl violet stain to determine cannula placement and tip location. This was confirmed using light microscopy to examine guide cannula location and evidence of injector track marks below. Only rats with cannulae tips located within the VTA were included in the subsequent analysis. The a/pVTA boarder is reported to be approximately -5.5 mm from

bregma (+3.40 mm from IAL) which corresponds to the position of the interpeduncular nucleus (IPN) ventral to the VTA (Olson, 2005; Sanchez-Catalan *et al.*, 2014).

5.3.5 Data analysis

Statistical analyses were performed in SPSS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). Following acute nicotine ICSA into the pVTA, a two-way ANOVA was performed to determine if there was an effect of *drug* or *lever*, or interaction between them on lever-pressing behaviour. Following chronic nicotine, a three-way ANOVA was performed to determine if there was an effect on *drug*, *session*, *lever*, or interactions between *drug* x *lever*, *session* x *lever*, *session* x *drug*, or three-way interaction between *drug* x *session* x *lever* on lever-pressing behaviour.

Quantification of Fos+ cells was carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). A three-way ANOVA was performed to determine if there was an effect of *drug, day, brain region* or an interaction between *drug* x *brain region*, *day* x *drug*, *day* x *brain region*, or three-way interaction between *day* x *drug* x *brain region* on Fos expression following acute or chronic nicotine ICSA into the pVTA. Data were normally distributed, as assessed by Shapiro-Wilk test.

All results were expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. Mauchly's test of sphericity indicated that sphericity was met. Significant interactions were further assessed with post hoc tests or pairwise comparisons, where appropriate. Results were considered significant when $p \leq 0.05$.

5.4 Results

5.4.1 Recovery

Animals recovered well from surgery (n = 22). Death during anaesthesia (n = 2) occurred on two occasions. The general health and body weight of all rats was closely monitored for the

duration of the experiment. When required, animals were given additional wet mash to help maintain a stable body weight.

5.4.2 Cannula placement

The location of the cannulae placements are illustrated below (Figure 5.1). One rat had to be excluded from the chronic nicotine study due to loss of head cap. Of the animals that completed the behavioural schedule, all cannulae were located within the VTA (n = 21). Six placements were located within the pVTA (IAL +3.20 mm). Nine placements were on the border between aVTA/pVTA (IAL + 3.40 mm) and 6 were in the aVTA (IAL +3.60 mm). The differences in placement were marginal (+3.20 to +3.60) and there was no obvious relationship between precise cannula location and behavioural outcome. As noted before, there was considerable variability in rats' lever-pressing behaviour (Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). One animal had to be discounted from the chronic nicotine study, because it did not demonstrate lever-pressing behaviour. On session 1, the animal carried out its highest number of lever presses across both levers (presses = 21), which was higher than its lever-pressing behaviour in sessions 2 – 5 (combined presses for sessions 2 – 5 = 25). All other animals (n = 9) increased lever-pressing behaviour across 5 ICSA sessions. Given that the point of the study was to look at Fos expression as rats learned to lever press this rat's data were not acceptable.

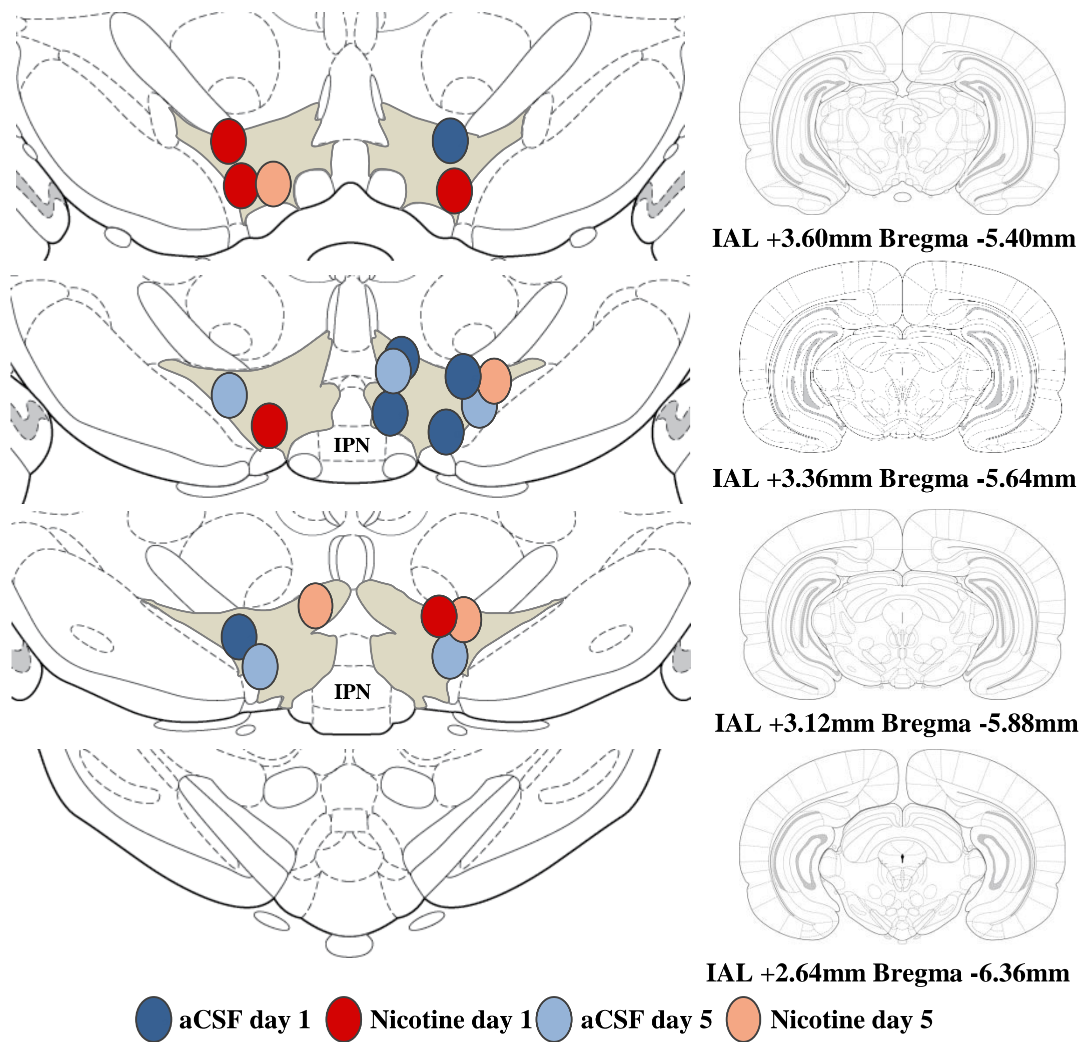

Figure 5.1 – Illustration of the location of unilateral cannula placements in the VTA. Coronal schematics adapted from the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 2007). The infusion site of acute nicotine (dark red circles; n = 5), acute aCSF (dark blue circles; n = 6), chronic nicotine (light red circles; n = 4) and chronic saline (light blue circles; n = 5) are shown. The interpeduncular nucleus (IPN) indicates the level of the pVTA.

5.4.3 Behavioural results

Acute nicotine ICSA

Eleven rats were randomly assigned to 1 of 2 groups to receive acute intra-VTA infusions of nicotine (n = 5) or aCSF (n = 6). Lever pressing on active and inactive levers is shown below (Figure 5.2). A two-way ANOVA was conducted to understand the effects of *drug* and *lever* on lever-pressing behaviour. There was no significant effect of *drug* ($F_{(1, 9)} = 0.480$; $p = 0.506$) or *lever* ($F_{(1, 9)} = 0.823$; $p = 0.388$). There was no *lever* x *drug* interaction ($F_{(1, 9)} = 0.29$; $p = 0.868$).

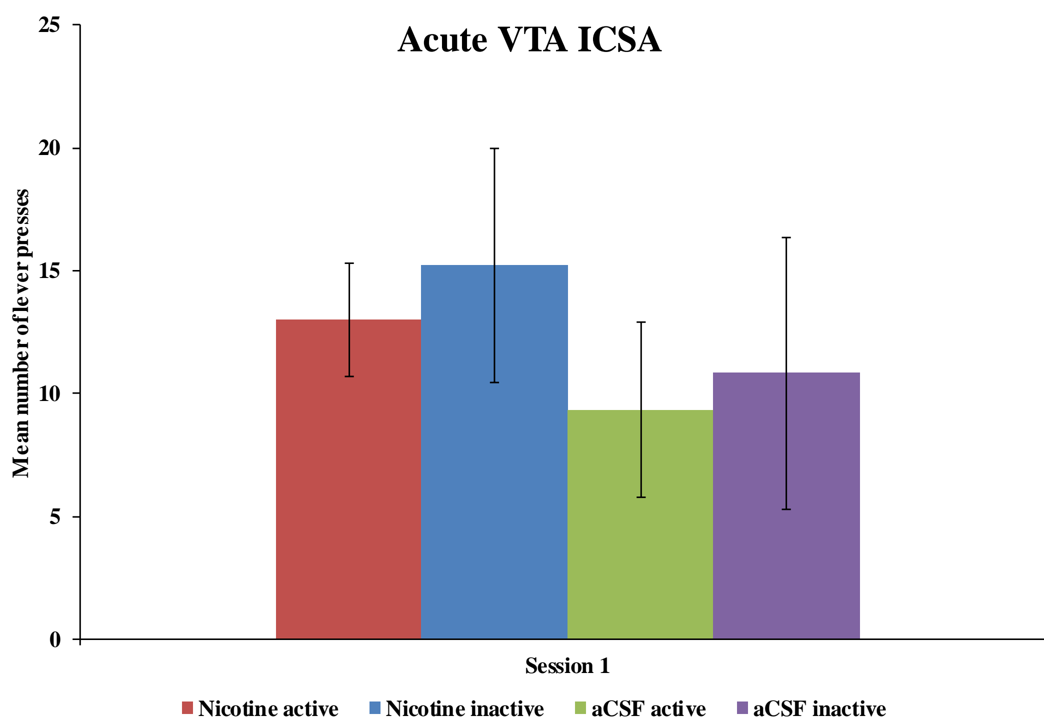

Figure 5.2 – Mean number of active and inactive lever presses during acute ICSA. Rats were given the opportunity to self-administer acute nicotine (n = 5) or aCSF (n = 6) directly into the VTA. Both groups were presented with an active lever (red bar: nicotine, green bar: aCSF) and an inactive lever (no drug: blue and purple bars). The number of lever presses on each were measured. Results presented mean ± SEM.

Chronic nicotine ICSA

Ten rats were randomly assigned to 1 of 2 groups to receive chronic intra-VTA infusions of nicotine (n = 4) or aCSF (n = 5) (n = 1 excluded). Lever pressing on active and inactive levers is shown across 5 sessions (Figure 5.3). A three-way ANOVA was conducted to understand the effects of *session*, *drug* and *lever* on lever-pressing behaviour. The three-way interaction was not statistically significant ($F_{(4, 32)} = 1.360$; $p = 0.270$). The two-way interactions between *session* x *drug* ($F_{(4, 32)} = 0.369$; $p = 0.829$), *session* x *lever* ($F_{(4, 32)} = 0.336$; $p = 0.852$) and *drug* x *lever* ($F_{(1, 8)} = 0.889$; $p = 0.373$) were not significant. There was a statistically significant effect of *lever* ($F_{(1, 8)} = 5.681$; $p = 0.044$), but there was no significant effect of *drug* ($F_{(1, 8)} = 0.727$; $p = 0.419$) or *session* ($F_{(4, 32)} = 1.749$; $p = 0.164$).

However, with small numbers of rats (nicotine n = 4, aCSF n = 5), it is possible that the relatively high variance in multiple conditions masks an effect. A one-way ANOVA was therefore also run to examine the effects of nicotine active / aCSF active / nicotine inactive / aCSF inactive as separate conditions (Figure 5.3). The one-way ANOVA shows that pressing on the active lever after nicotine increased over *session* while lever pressing in all other conditions did not change. For nicotine active, there was a significant effect of *session* ($F_{(4, 12)} = 4.001$; $p = 0.027$), but there was no effect of *session* for nicotine inactive ($F_{(4, 12)} = 0.140$; $p = 0.964$), aCSF active ($F_{(4, 12)} = 0.336$; $p = 0.849$) or aCSF inactive ($F_{(4, 12)} = 1.215$; $p = 0.343$).

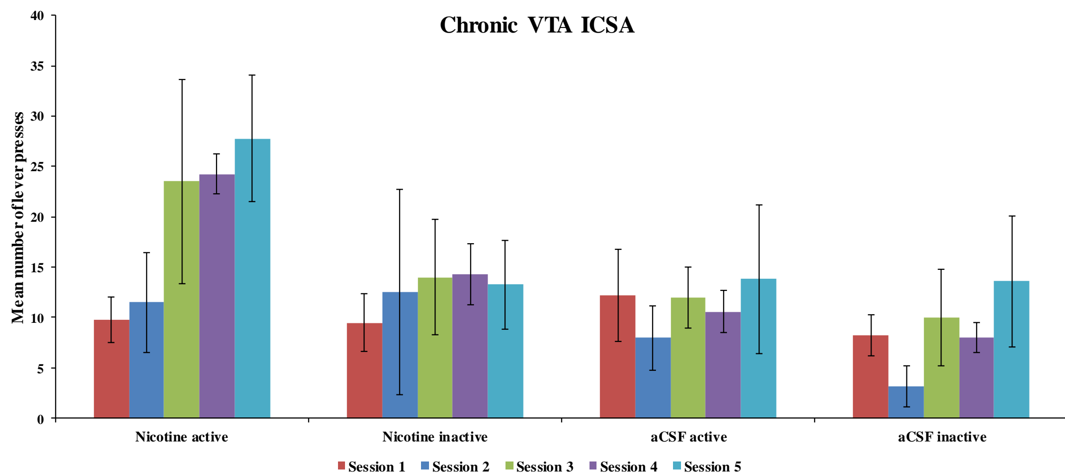

Figure 5.3 – Mean number of nicotine or aCSF active and inactive lever presses across chronic ICSA sessions 1 – 5. Rats were given the opportunity to self-administer nicotine (n = 4) or aCSF (n = 5) directly into the VTA. Both groups were presented with an active lever (nicotine or aCSF) and an inactive lever (no infusion). The number of lever presses on each were measured. Results presented mean ± SEM. For statistical significance, see text.

5.4.4 c-fos quantification

Once cannula placements were confirmed, sections from nicotine and aCSF groups were processed for *c-fos* immunohistology as described in the General Methods. All sections that crossed through the NAc, CPu and dHPC were processed. As stated in the General Methods, Fos positive (Fos+) cells were quantified using ImageJ software (ImageJ, U.S. National Institutes of Health, U.S.A). Bilateral cell counts were taken from at least 5 sections containing the regions of interest from a 1:4 series. The number of Fos+ cells in the NAc shell and core, CPu and dHPC from acute and chronic nicotine and aCSF groups are shown below (Figure 5.4).

A three-way ANOVA was conducted to understand the effects of *day*, *drug* and *brain region* on Fos expression. The three-way interaction between was not statistically significant ($F_{(3, 65)} = 0.449$; $p = 0.719$). The two-way interactions between *day* x *drug* ($F_{(1, 65)} = 0.466$; $p = 0.497$), *day* x *brain region* ($F_{(3, 65)} = 0.045$; $p = 0.987$) and *drug* x *brain region* ($F_{(3, 63)} = 0.246$; $p = 0.864$) were not statistically significant. There was a statistically significant effect of *drug* ($F_{(1, 65)} = 40.603$; $p = 0.000$) and *brain region* ($F_{(3, 65)} = 5.309$; $p = 0.002$), but there was no significant effect of *day* ($F_{(1, 65)} = 0.223$; $p = 0.638$).

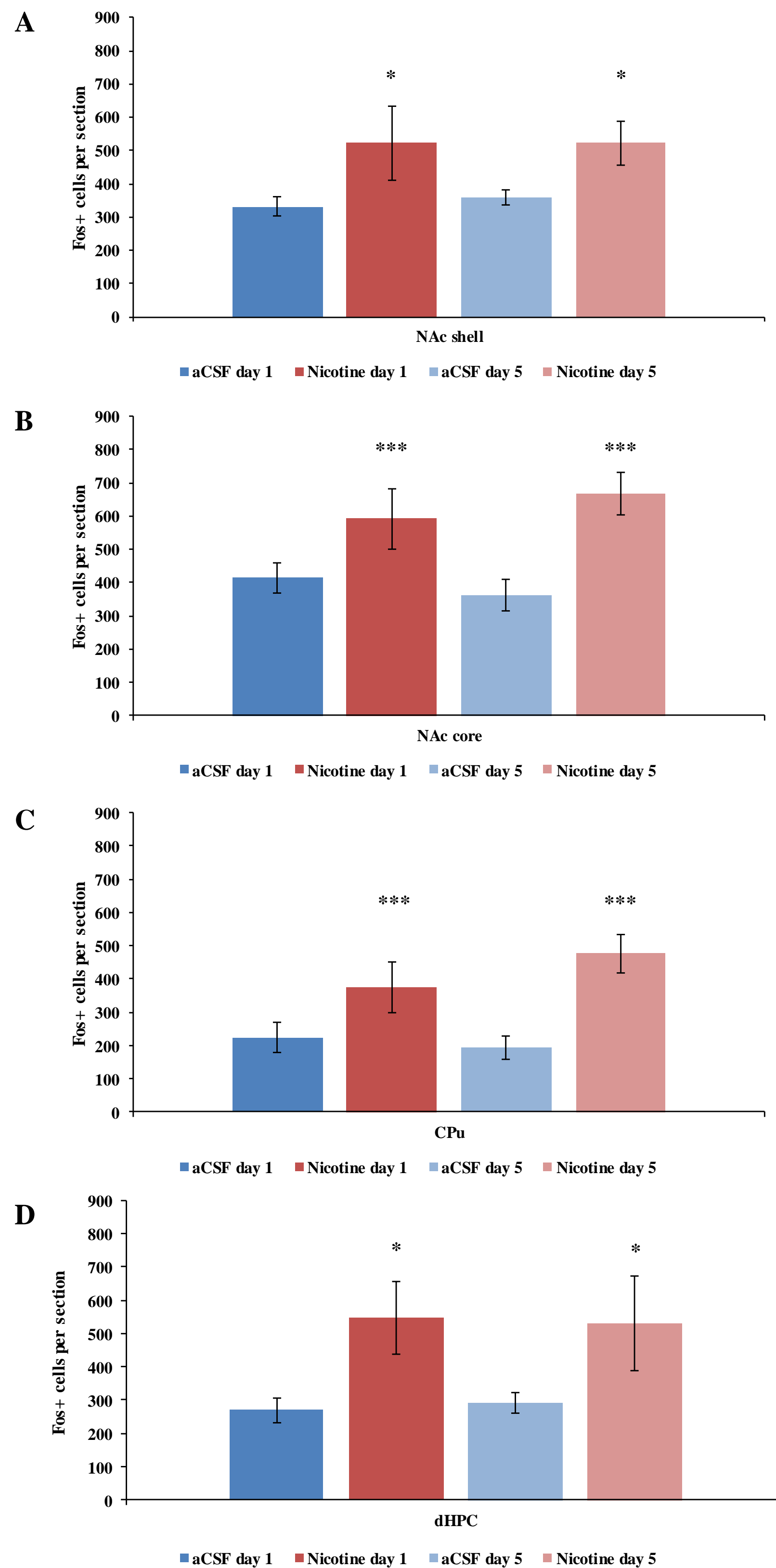

Figure 5.4 – Fos+ cells in the NAc shell (A), NAc core (B), CPu (C) and dHPC (D) after acute and chronic intra-VTA ICSA of nicotine. Animals were either administered with acute nicotine (n = 5; dark red bars), acute aCSF (n = 6; dark blue bars), chronic nicotine (n = 4; light red bars) or chronic aCSF (n = 5; light blue bars). Error bars = ± SEM. * indicates a significant difference between nicotine and saline groups $p \leq 0.001$; ** indicates $p \leq 0.01$; * indicates $p \leq 0.05$.**

5.5 Chapter discussion

The main aim of this chapter was to quantify *c-fos* activation in selected brain regions following acute and chronic ICSA of nicotine in the pVTA. Fos quantification was carried out in the NAc shell and core, CPu and dHPC to investigate nicotine-induced activity in key regions of the mesocorticolimbic system and HPC-VTA loop. Investigating the effect of acute and chronic nicotine explored regional neural activation after a single nicotine ICSA session compared to repeated ICSA sessions. This would determine if acute and chronic nicotine mediates its effects through the same neural mechanisms. In addition, Fos activity in the NAc and CPu were quantified to determine if there was a shift in neural activation from session 1 to habit-based learning by session 5.

In the current study, nicotine increased Fos expression in all regions of interest – the NAc shell and core, CPu and dHPC (Figure 5.4). There was as much nicotine-induced Fos activation on session 5 as there was on session 1, therefore there was no effect of session (Figure 5.4). As expected, animals appear to increase lever-pressing behaviour over 5 ICSA sessions only in the nicotine-active condition (Figure 5.3). Furthermore, the relationship between learning to lever press and Fos expression is not well correlated – nicotine increased Fos activity regardless of the level of lever-pressing behaviour (Figure 5.3 and 5.4).

Neural activation of the NAc shell was predicted, due to its reciprocal connections with the VTA that are strongly associated with drug reward (as discussed in the General Introduction). Although the NAc core also receives DA projections from the VTA, the core has not been implicated in drug reward. Therefore, nicotine-induced neural activation in the core was not predicted. The neural activation observed in the NAc core could be triggered by motivational behaviours. It has previously been reported that NAc core (but not the shell) is associated with motivational behaviours to overcome costs, such as physical effort, and for enabling reward-related behaviours to enhance motivation (Hall *et al.*, 2001; Floresco, 2006; Ghods-Sharifi and

Floresco, 2010). Perhaps the neural activity of the NAc core is associated with the physical effort required to perform lever pressing, or the motivational behaviour that leads to the execution lever-pressing behaviour. In addition, previous studies have shown that drug-induced sensitisation is associated with changes in DA responsiveness in the NAc shell and core (Cadoni and Di Chiara, 2000; Cadoni, Solinas and Di Chiara, 2000; Di Chiara, 2002). Nicotine sensitisation has been reported to reduce the responsiveness of the NAc shell to DA. On the other hand, nicotine sensitisation leads to increased DA transmission in the NAc core (Cadoni and Di Chiara, 2000; Cadoni, Solinas and Di Chiara, 2000; Di Chiara, 2002). In the current study, this could potentially explain the increased neural activation in the NAc core following chronic nicotine ICSA, but not acute nicotine exposure (Figure 5.4).

As discussed in the previous chapter, the HPC is connected to the VTA through the upward arc of the HPC-VTA loop. This functional loop is believed to detect and assess novelty (Lisman and Grace, 2005). In the current study, it was hypothesised that the novel experience of intra-VTA ICSA session 1 would activate the dHPC. The novelty of ICSA was expected to disappear or decrease by session 5, resulting in lower levels of dHPC neural activation. However, high levels of dHPC neural activation were maintained from session 1 to session 5 (Figure 5.4). The dHPC is heavily implicated in spatial and contextual learning (discussed further in Chapter 6), which could account for the neural activation observed following acute and chronic ICSA (Figure 5.4). Furthermore, there is mounting evidence that acute nicotine enhances HPC-dependent learning, which disappears following chronic nicotine exposure (discussed further in Chapter 6) (Gould and Wehner, 1999; Gould and Higgins, 2003; Davis *et al.*, 2005; Davis and Gould, 2006; Gould *et al.*, 2012). This could potentially explain the neural activation observed after acute nicotine ICSA. However, this would not account for the increased neural activity observed after ICSA session 5 (Figure 5.4).

In addition, it was hypothesised that neural activation would shift from the NAc in session 1 to the CPu by session 5. These results suggest that nicotine-induced Fos expression in the NAc

and CPu was not affected by the degree of learning that had taken place. It has previously been reported that increased Fos activity in the dorsal striatum was correlated with intra-VTA nicotine injections that were more laterally positioned (Panagis *et al.*, 1996). Panagis *et al.* (1996) recognised that this could be due to diffusion of nicotine to the neighbouring substantia nigra (SN), which provides substantial DA input to the dorsal striatum. In the current study, intracranial nicotine induced Fos expression in the CPu (located within the dorsal striatum, Figure 5.4). However, Fos activity in the CPu was not correlated to more medial or laterally placed VTA cannulae (CPu Fos quantification: - medial placements 478 ± 56 and lateral placements 428 ± 65 [mean ± SEM]). Therefore, it is unlikely that increased Fos expression in the CPu is due to nicotine diffusion to the SN. Increased neural activation in the CPu could be due to motor activation itself. During ICSA the animals are working to lever press, which could lead to increased Fos expression in the CPu. Panagis *et al.* (1996) raised an important methodological consideration. Intra-VTA cannula placement must be correctly positioned so that the injection does not diffuse into neighbouring brain regions.

As previously reported, rats demonstrated relatively low levels of lever discrimination (nicotine vs. aCSF) on session 1 of intra-VTA ICSA testing (Figure 5.2 and 5.3) (Farquhar, Latimer and Winn, 2012). By session 5, after repeated nicotine exposure, rats demonstrated increased lever pressing and discrimination between levers (Figure 5.3). Response levels on the inactive nicotine lever remained constant throughout sessions 1 – 5 (Figure 5.3). Low rates of lever-pressing behaviour were observed during session 1 compared to session 5, which could be due to noxious effects produced by the first exposure to intra-VTA nicotine. Many smokers report their first exposure to smoking as unpleasant, experiencing noxious symptoms such as nausea, coughing and dizziness (Kozlowski and Harford, 1976; Pomerleau, 1995; Laviolette and van der Kooy, 2004). Therefore, it should not be ruled out that the first exposure to intra-VTA nicotine could lead to unpleasant effects, explaining the low rates of lever

pressing. By ICSA session 5 animals demonstrated increased lever-pressing behaviour, with a clear preference for the active lever (Figure 5.3).

Although all cannula placements were located within the VTA, there was distinct variability in lever-pressing behaviour between animals (Figures 5.1 – 5.3). Due to small group numbers, it was difficult to relate the precise location of the cannula to the degree of lever-pressing behaviour, or Fos expression. In the chronic group, animals with placements more posteriorly located tended to demonstrate higher levels of lever-pressing behaviour (session 5; anterior placements = 21 presses, posterior placements = 58 presses). Furthermore, Fos activation appeared to be higher in all regions of interest with more posteriorly located cannulae (anterior: NAc shell = 323, NAc core = 502, CPu = 370, dHPC = 413; posterior: NAc shell = 643, NAc core = 809, CPu = 614, dHPC = 854). However, larger group numbers are required to confirm this effect. As previously noted, intra-VTA nicotine has a variable but modest effect in naïve rats, and despite consistent cannula placements in the VTA there was little consistency of lever-pressing behaviour (Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). All placements targeted the VTA, but each placement was slightly different, with varying sizes of infusion site.

5.6 Future work

What we know from the current study is that acute and chronic ICSA of nicotine into the VTA significantly activates *c-fos* in the NAc shell and core, CPu and dHPC in adult rats. The logical conclusions are that intra-VTA nicotine activates *c-fos* through regions of the mesocorticolimbic system and the HPC-VTA loop. Nicotine-induced Fos expression in the NAc and CPu was not affected by the degree of learning that had taken place. The current study demonstrated that acute and chronic intra-VTA nicotine, at this concentration, induced neuronal activation in the same brain regions. In addition, the current study confirmed that intra-VTA nicotine, at this concentration, results in low levels of lever pressing during ICSA

session 1. By session 5, after repeated nicotine exposure, rats demonstrated increased lever pressing and discrimination between levers.

Interestingly, intra-VTA nicotine triggered Fos expression in the dHPC. As discussed in the previous chapter, studies have identified VTA DA projections to the HPC, in particular the CA1 region of the dHPC, play an important role in drug-related learning. Following on from the current study, a second approach was taken to contrast intra-VTA self-administration of nicotine with ICSA in the dHPC. To determine whether or not cholinergic activation at different points within the same neural circuitry has similar or different effects on the identified regions of interest.

Chapter 6: Fos expression after acute and chronic intracranial self-administration of nicotine into the dorsal hippocampus

6.1 Chapter introduction

ICSA of nicotine into the VTA triggered neural activation in the NAc shell and core, CPu and dorsal hippocampus (dHPC) with no difference observed between acute (one session) and chronic (five sessions) administration (Chapter 5). Following this, nicotine ICSA into the dHPC was investigated to contrast with intra-VTA nicotine self-administration. This would determine whether nicotine mediates its effect through the same regions and circuitry as intra-VTA nicotine.

6.1.1 General overview – the Hippocampus

The hippocampus (HPC) is essential for many forms of learning and memory, such as episodic memory, spatial memory, contextual learning and working memory (Scoville and Milner, 1957; O'Keefe and Dostrovsky, 1971; Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Aggleton and Pearce, 2001). The HPC has been implicated in a multitude of functions ranging from spatial navigation to anxiety. It is becoming increasingly more apparent that the HPC is not just involved in memory formation. As well as learning and memory, the HPC is also critically involved in mediating emotionality and stress-related responses (Gray and McNaughton, 2003; Hasler *et al.*, 2007). In addition, the HPC is involved in transitive inference, arbitrary association and temporal ordering of events (O'Keefe and Dostrovsky, 1971; Kim and Fanselow, 1992; Phillips and LeDoux, 1992). The HPC works as a whole unit to produce these specialised functions, but the hippocampal poles and corresponding subregions play a larger role in some functions compared to others. Disorder of the HPC has been implicated in schizophrenia, Alzheimer's disease, epilepsy, depression and addiction (Nordberg and Winblad, 1986; Kellar and Wonnacott, 1990; Perry *et al.*, 1995; Durany *et al.*, 2000; Leonard *et al.*, 2000). It has been suggested that the HPC is involved in reward-related learning following drug administration (White, 1996). Drug addiction shares striking similarities with neural plasticity implicated by natural reward, learning and memory (Kelley,

2004). In fact, addiction, learning and memory share common brain regions, neural systems and cell signalling pathways (Abel *et al.*, 1997; Self *et al.*, 1998; Pierce *et al.*, 1998; Pierce, Pierce-Bancroft and Prasad, 1999; Adams and Sweatt, 2002; Sanna *et al.*, 2002; Cammarota *et al.*, 2002; Pittenger *et al.*, 2002; Hou *et al.*, 2004; Kelley, 2004; Lu *et al.*, 2005; Lynch and Taylor, 2005). The ability of drugs of abuse to interact with, and alter, neural machinery and substrates associated with learning and memory may contribute to their addictive properties. These alterations could exert strong, long-lasting influences on behaviour, leading to the development and maintenance of addiction (Gould and Leach, 2014). There is mounting evidence that the complex disorder of nicotine addiction, at least partly, mediates its effects through the HPC – particularly through the dorsal portion of the HPC. In fact, nicotine addiction has been termed a disorder of learning and memory (Gould, 2006; Ramirez, Tonegawa and Liu, 2013; Gould and Leach, 2014). Franklin *et al.* (2007) demonstrated that the dorsolateral prefrontal cortex (PFC) and HPC were activated by smoking-related cues in adult heavy smokers (Franklin *et al.*, 2007). A similar effect was also reported in adolescent light smokers (Rubinstein *et al.*, 2011). In addition, a group of nicotine-deprived smokers where shown smoking-related images, which increased neural activity in the amygdala (AMG) and HPC (Due *et al.*, 2002). Drugs of abuse, such as nicotine, may possess the ability to hijack brain regions associated with learning and memory, which could partially explain the addictive nature of drugs. To understand the effects of nicotine-induced learning and memory it is important to identify brain regions and circuitry involved in acute and chronic nicotine exposure. How does nicotine alter learning? How do these effects change with the duration of nicotine exposure? And can nicotine modulate its actions within specific hippocampal subregions, or across the entire structure?

6.1.2 Hippocampus – basic structure

The HPC extends from the septal nuclei of the basal forebrain, rostrodorsally over and behind the diencephalon, into the caudoventral portion of the hemisphere. The HPC is separated into

3 distinct subregions: - the dentate gyrus (DG), hippocampus proper and subiculum (Sub). The hippocampus proper consists of the areas of the Cornu Ammonis (Ammon's horn): - CA1, CA2 and CA3 regions. The parahippocampal region (PHR) lies adjacent to the HPC and is bordered by the Sub. The PHR consists of 5 subregions: - presubiculum (PrS), parasubiculum (PaS), entorhinal cortex (EC), perirhinal cortex (PER) and postrhinal cortex (POR) (Witter *et al.*, 1989). The main projections to the HPC from the PHR are supplied by the EC, via the perforant pathway. Together with the Sub, the EC also provides the major source of output for the HPC. Each hippocampal subregion has specific afferent and efferent projections, which form a circuitry that begins at the EC and ends in projections to the EC and Sub (Figure 6.1) (Andersen, Bland and Dudar, 1973; Bartesaghi, Gessi and Migliore, 1995).

Dentate gyrus

From layer II of the EC, the DG receives afferent projections via the perforant path – the primary output of the EC (Figure 6.1) (van Groen, Miettinen and Kadish, 2003). Through unmyelinated axons of granule cells, known as mossy fibers, the DG feeds information forward to the proximal dendrites of the CA3 region (Ribak, Seress and Amaral, 1985; Amaral, Scharfman and Lavenex, 2007).

CA3

CA3 receives afferent projections from the DG and layer II of the EC. It is possible that the indirect input from the EC to CA3 (via the DG) is strengthened by the direct input from the EC, which may facilitate changes in synaptic plasticity. CA3 reciprocally innervates the DG – particularly the hilus region (Li *et al.*, 1994; Vivar *et al.*, 2012). A number of CA3 pyramidal cells synapse onto local CA3 dendrites, and contralateral CA3 dendrites, to form an auto-associative network through recurrent collaterals (Ishizuka, Weber and Amaral, 1990; Bennett, Gibson and Robinson, 1994; Li *et al.*, 1994). The majority of CA3 axons ipsilaterally innervate CA1 through projections known as Schaffer collaterals (Figure 6.1) (Ishizuka, Weber and

Amaral, 1990; Li *et al.*, 1994; Ishizuka, Cowan and Amaral, 1995; Wittner *et al.*, 2007). Schaffer collaterals are important projections linked to neural plasticity associated with learning and memory (Bliss and Collingridge, 1993).

CA2

CA2 receives projections from CA3 Schaffer collaterals and layers II/III of the EC (Figure 6.1) (Ishizuka, Weber and Amaral, 1990; Chevaleyre and Siegelbaum, 2010). CA2 neurons project to CA1, distal CA3, and contralaterally to CA2. This region has been a matter of some controversy and has received much less attention than other hippocampal subregions. Compared to other subregions CA2 is structurally and functionally distinct (Chevaleyre and Siegelbaum, 2010; Mercer *et al.*, 2012; Piskorowski and Chevaleyre, 2012). This narrow zone of cells between CA1 and CA3 consists of large CA3-like cells, but unlike CA3 cells, do not receive input from DG mossy fibers (Ishizuka, Cowan and Amaral, 1995).

CA1

Schaffer collaterals from CA3 project to CA1 in a highly organised fashion (Li *et al.*, 1994; Ishizuka, Cowan and Amaral, 1995). Proximal CA3 cells primarily to project to dorsal CA1, whereas distal CA3 cells heavily project to ventral CA1. Layer III of the EC provides topographically organised projections to CA1, known as the temporoammonic pathway (Figure 6.1) (Empson and Heinemann, 1995). The medial EC, thought to primarily respond to spatial information, projects to the proximal portion of CA1 (Henriksen *et al.*, 2010; Deshmukh and Knierim, 2011), whereas the lateral EC, reported to respond to object/location information, projects to the distal portion of CA1 (Zhu, Brown and Aggleton, 1995; Henriksen *et al.*, 2010; Deshmukh and Knierim, 2011). Inhibitory CA1 interneurons innervate local CA1 cells, but also project to CA3 and the hilus of the DG (Sik *et al.*, 1994; Freund and Buzsáki, 1996). CA1 is a major source of hippocampal efferent output, projecting to subcortical structures through the Sub and to the cortex via the EC (discussed below) (Swanson and

Cowan, 1975; Witter and Groenewegen, 1990; Canteras and Swanson, 1992; Burwell and Amaral, 1998; Ishizuka, 2001; Agster and Burwell, 2009).

Subiculum

The Sub provides CA1 with sparse intrahippocampal projections from subicular pyramidal cells and interneurons (Harris and Stewart, 2001; Harris *et al.*, 2001; Commins, Aggleton and O'Mara, 2002; Seress *et al.*, 2002). Projections from CA1 and Sub to the EC provide the main source of hippocampal output to the PHR. Subicular pyramidal cells provide substantial input to other pyramidal cells within the Sub (Harris *et al.*, 2001). Layer III of the EC projects to the Sub and reciprocal projections from the Sub ipsilaterally innervate all regions of the EC – a small proportion of these projections are believed to be inhibitory (van Haeften, Jorritsma-Byham and Witter, 1995).

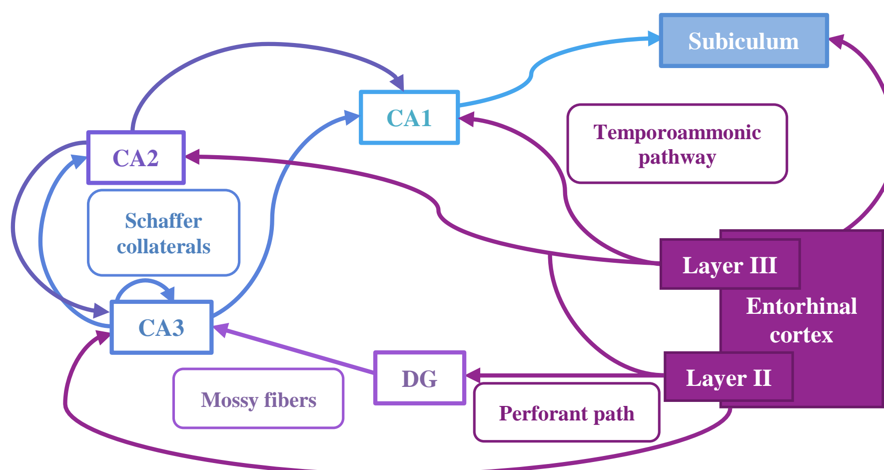

Figure 6.1 – Greatly simplified diagram of hippocampal connections. In summary, neural information flows from the entorhinal cortex (EC) to the dentate gyrus (DG) and CA3. The EC and DG feeds information to CA3. This information is either combined or compared before projecting to CA1 and CA2. The information that flows from CA3 to CA1 can then be combined or compared to the information supplied by direct EC-CA1 input. The hippocampal subregions project back to the EC and subiculum, generating the major output source of the HPC.

6.1.3 Dorsal and ventral HPC poles

As previously discussed, each hippocampal subregion has specific afferent and efferent projections, which form a circuitry that begins at the EC and ends in projections to the EC and Sub. This structural organisation repeats itself along the longitudinal axis of the HPC (Andersen, Bliss and Skrede, 1971; Moser and Moser, 1998). Despite the conserved intrinsic circuitry of the HPC, the afferent and efferent connectivity changes from the dorsal (septal) to the ventral (temporal) pole (Table 6.1 and 6.2). The dorsal HPC (dHPC) is defined as the first two-thirds of the hippocampal volume and the ventral (vHPC) is defined as the remaining one-third (Moser and Moser, 1998). There is mounting evidence that the hippocampal poles are not just structurally heterogeneous, but also functionally heterogeneous (Moser, Moser and Andersen, 1993; Moser *et al.*, 1995; Moser and Moser, 1998). The heterogeneous nature of the HPC is determined by differences in neurochemical function, associated behaviours and neural connections (Moser and Moser, 1998). The corresponding subregions of the dorsal and ventral hippocampi generate and receive diverse projections that are likely to contribute to the functional differences between the poles – including the differential response to nicotine administration. To understand how the hippocampal poles, and corresponding subregions, respond to acute and chronic nicotine administration it is important to identify target brain regions and circuitry that may be involved (Table 6.1 and 6.2).

	dHPC
Efferent projections	Retrosplenial area, anterior cingulate area, anterior thalamic nuclei medial and lateral mammillary nuclei, caudal region of the lateral septum, tenia tecta, medial septum, vertical and horizontal limbs of the diagonal band, supramammillary nucleus of the hypothalamus, rostrolateral NAc, rostral CPu (Raisman, Cowan and Powell, 1966; Swanson and Cowan, 1975; Vogt and Miller, 1983; Swanson and Köhler, 1986; Witter, Ostendorf and Groenewegen, 1990; Groenewegen, Wright and Beijer, 1996; Risold and Swanson, 1996; Risold, Thompson and Swanson, 1997; Naber and Witter, 1998; Kishi *et al.*, 2000; Ishizuka, 2001; van Groen and Wyss, 2003; Cenquizca and Swanson, 2006, 2007; Jinno *et al.*, 2007; Miyashita and Rockland, 2007)
Afferent projections	Retrosplenial area, dorsolateral band of the EC, vertical limb of the diagonal band, medial septum, locus coeruleus, VTA, medial and dorsal raphe nuclei, thalamic nuclei, supramammillary nucleus (Meibach and Siegel, 1977; Milner and Amaral, 1984; Haglund, Swanson and Köhler, 1984; Amaral and Kurz, 1985; Haring and Davis, 1985; Montone, Fass and Hamill, 1988; Canteras and Swanson, 1992; Halasy *et al.*, 1992; McQuade and Sharp, 1997; Risold, Thompson and Swanson, 1997; Vertes, Fortin and Crane, 1999; Pitkänen *et al.*, 2000; McKenna and Vertes, 2001; Van Der Werf, Witter and Groenewegen, 2002; Fyhn *et al.*, 2004; Vertes *et al.*, 2007; Fanselow and Dong, 2010; Broussard, 2012; Cui, Gerfen and Young, 2013; McNamara *et al.*, 2014; Strange *et al.*, 2014; Rosen, Cheung and Siegelbaum, 2015; Broussard *et al.*, 2016)

Table 6.1 – The efferent and afferent connectivity of the dorsal hippocampus.

	vHPC
Efferent Projections	Olfactory bulb, anterior olfactory nucleus, piriform cortex, endopiriform nucleus, septofimbrial nuclei, medial and ventromedial lateral septum, anterior thalamic nuclei, mammillary nuclei, contralateral and caudomedial NAc, amygdala nuclei (basolateral, posterior basomedial, posterior, posteromedial cortical nuclei and piriform-amygdala area), medial PFC (infralimbic and prelimbic areas), BNST, ventral regions of the lateral septum, supramammillary nucleus, ventrolateral region of the medial mammillary nucleus, hypothalamus, ventral pallidum (Raisman, Cowan and Powell, 1966; Swanson, 1981; Groenewegen *et al.*, 1987; Saunders, Rosene and Van Hoesen, 1988; Jay and Witter, 1991; Canteras and Swanson, 1992; Risold and Swanson, 1996; Groenewegen, Wright and Beijer, 1996; Naber and Witter, 1998; Pikkarainen *et al.*, 1999; Kishi *et al.*, 2000; Pitkänen *et al.*, 2000; Chiba, 2000; Thierry *et al.*, 2000; Petrovich, Canteras and Swanson, 2001; Dong *et al.*, 2001; Floresco, Todd and Grace, 2001; Kemppainen, Jalkkonen and Pitkänen, 2002; Witter and Amaral, 2004; Jones and Wilson, 2005; Herman *et al.*, 2005; Dong and Swanson, 2006; Cenquizca and Swanson, 2007; Roberts *et al.*, 2007; Hoover and Vertes, 2007)
Afferent projections	Ventromedial band of the EC, horizontal and vertical limbs of the diagonal band, intermediolateral septum, amygdala nuclei, medial PFC (infralimbic and prelimbic areas), BNST, supramammillary nucleus, locus coeruleus, VTA, dorsal raphe nucleus, thalamic nuclei (Meibach and Siegel, 1977; Scatton, Simon, M Le Moal, *et al.*, 1980; Gage and Thompson, 1980; Milner and Amaral, 1984; Haglund, Swanson and Köhler, 1984; Amaral and Kurz, 1985; Haring and Davis, 1985; Henke, 1990; Canteras and Swanson, 1992; Gasbarri *et al.*, 1994; Barbas and Blatt, 1995; Maren and Fanselow, 1995; Risold, Thompson and Swanson, 1997; Gasbarri, Sulli and Packard, 1997; Moser and Moser, 1998; Pikkarainen *et al.*, 1999; Pitkänen *et al.*, 2000; McKenna and Vertes, 2001; Kemppainen, Jalkkonen and Pitkänen, 2002; Van Der Werf, Witter and Groenewegen, 2002; Vertes *et al.*, 2007; Cenquizca and Swanson, 2007; Fanselow and Dong, 2010; Jennings *et al.*, 2013; Strange *et al.*, 2014)

Table 6.2 – The efferent and afferent connectivity of the ventral hippocampus.

Functional heterogeneity

There is strong behavioural evidence that the dorsal and ventral hippocampi support different functions – including the differential behavioural response following nicotine administration. Behavioural tests aimed at assessing spatial navigation, temporal and working memory, such as the Morris water maze or radial arm maze, have proved particularly useful at separating d/vHPC functions. During the water maze test, animals must swim to locate a hidden platform using landmarks outside the pool (Morris, 1981). Lesions of the dHPC impaired the animal's performance, while vHPC lesions had no effect on this behaviour (Moser *et al.*, 1995). Pothuizen *et al.* (2004) demonstrated that dHPC lesions caused a deficit in memory using the radial arm maze, while vHPC lesions did not. Returning to an arm previously associated with food was reduced in dHPC lesioned animals, but enhanced by vHPC lesions (Ferbinteanu and McDonald, 2001). Combined, these results show that lesions or inactivation of the dHPC severely disrupts the formation of spatial memory. Henke *et al.* (1990) reported that lesions of the vHPC altered stress responses and emotional processing, while dHPC lesions did not. Non-selective lesions of the vHPC, but not dHPC, aggravated gastric erosion after restraint stress (Henke, 1990). Using the elevated plus maze, Kjelstrup *et al.* (2002) demonstrated that animals with vHPC lesions failed to avoid the open (unprotected) arms of the maze – behaviour that is consistent with reduced anxiety (Kjelstrup *et al.*, 2002; Trivedi and Coover, 2004). Furthermore, vHPC-lesioned animals exhibited decreased neuroendocrine stress response during confinement to a brightly lit chamber (Kjelstrup *et al.*, 2002). Lesions of the vHPC produced behaviours that were consistent with anxiolytic effects. Lesion of the dHPC displayed little, if any, change in behaviour compared to sham controls. These results suggest that lesions or inactivation of the vHPC disrupts fear and anxiety related-behaviours (Trivedi and Coover, 2004; Czerniawski, Yoon and Otto, 2009; Czerniawski *et al.*, 2012).

Collectively these data demonstrate that the hippocampal poles exhibit functional dissociations that are consistent with their differential anatomical connections. As

demonstrated above, the hippocampal poles generate distinct afferent connections from the EC, efferent connections to cortical and subcortical areas (Table 6.1 and 6.2), and responses to behavioural or cognitive tasks. The dHPC is most prominently connected to the retrosplenial area, which is heavily implicated in reward-related behaviours. Conversely, the vHPC is strongly connected the rostral hypothalamus and amygdala nuclei – regions associated with emotionality and stress-related behaviours. The diverse projections of the dorsal and ventral HPC are likely to contribute to the functional differences between the poles – including the differential response to nicotine administration.

6.1.4 Nicotine in the d/vHPC

There is growing evidence that the dorsal and ventral hippocampi respond differently to nicotine and play diverse roles in nicotine-induced learning. Contextual fear conditioning paradigms have proved useful for defining the differential roles of the d/vHPC following acute and chronic nicotine administration. Infusion of acute nicotine directly into the dHPC or vHPC demonstrated differential effects on context and trace fear conditioning (Davis, Kenney and Gould, 2007; Davis and Gould, 2009; Raybuck and Gould, 2010; Kenney, Raybuck and Gould, 2012). In trace fear conditioning, a temporal delay is inserted between the conditioned and unconditioned stimulus (CS, US). This delay requires a memory trace of the CS to remain in the brain to form an association with the US. Trace conditioning is a similar process to working memory and is dependent on the HPC and frontal cortex (McEchron *et al.*, 1998; Gilmartin *et al.*, 2013). Infusion of nicotine into the dHPC enhanced context fear conditioning, whereas intra-vHPC nicotine disrupted this behaviour (Davis and Gould, 2006; Davis, Kenney and Gould, 2007; Raybuck and Gould, 2010; Kenney, Raybuck and Gould, 2012). Direct nicotine infusion into the dHPC prior to training enhanced trace fear conditioning. In addition, intra-dHPC administration of high-affinity nicotinic acetylcholine receptor (nAChR) antagonist dihydro-β-erythoidine (DHβE) blocked trace fear conditioning acquired by intra-dHPC nicotine, but not context fear conditioning (Davis and Gould, 2006; Raybuck and Gould,

2010). Administration of low-affinity nAChR antagonist methyllycaconitine (MLA) had no effect on trace or contextual learning (Davis and Gould, 2006; Raybuck and Gould, 2010). These data suggest that acquisition of trace fear conditioning following intra-dHPC nicotine may be mediated through high-affinity nAChRs. These data further suggest that nicotine-induced contextual and trace fear learning are mediated by different dHPC processes. In contrast, direct intra-vHPC nicotine produced deficits in acquisition and retrieval of trace and context fear conditioning – administration of DHβE or MLA had no effect on this response (Davis and Gould, 2006; Raybuck and Gould, 2010). Davis and Gould (2009) demonstrated that chronic exposure to intra-dHPC nicotine had no effect on context fear conditioning, but nicotine withdrawal produced deficits in contextual learning. Following chronic nicotine, intra-dHPC infusion of DHβE triggered deficits in contextual learning behaviour, suggesting that chronic intra-dHPC nicotine mediates its effects through high-affinity nAChRs (Davis and Gould, 2009).

As discussed above, acute nicotine enhanced dHPC-dependent learning, yet this effect disappeared following chronic nicotine exposure (Gould and Wehner, 1999; Gould and Higgins, 2003; Davis and Gould, 2009). This suggests that a tolerance to nicotine developed between acute and chronic exposure, which triggered neuroadaptations that alter brain function. When chronic nicotine exposure ceased, deficits in contextual learning were uncovered. As Gould *et al.* (2014) stated, the specific effects of nicotine withdrawal on dHPC-dependent learning suggest that chronic nicotine, and subsequent withdrawal, directly alter the HPC or hippocampal target regions (Davis and Gould, 2009; Gould and Leach, 2014). To understand the effects of dHPC-dependant nicotine-induced learning it is important to identify the hippocampal subregions and circuitry involved in acute and chronic nicotine exposure. Specifically asking, how does nicotine alter learning through the dHPC?

6.1.5 Learning, memory and nicotine addiction

There is a striking overlap between cellular and molecular substrates involved in nicotine addiction, learning and memory. Altered synaptic strength is likely to underlie changes in network dynamics that lead to memory acquisition (Hebb, 1949). Long-term potentiation (LTP) is a widely accepted model of synaptic plasticity that is thought to underlie learning and memory in the HPC (Bliss and Collingridge, 1993; Martin and Morris, 2002). LTP is defined as an activity-dependent persistent increase in synaptic strength (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). LTP is believed to occur through simultaneous depolarisation of N-methyl-D-aspartic acid (NMDA) receptors at pre- and postsynaptic terminals, leading to NMDA receptor-mediated calcium (Ca^{2+}) influx into the cell (Lynch *et al.*, 1990; Bliss and Collingridge, 1993; Nicoll and Malenka, 1999). Intracellular Ca^{2+} levels increase, which leads to altered protein activation, messenger RNA synthesis and protein translation (Frey *et al.*, 1988, 1996; Klann, Chen and Sweatt, 1991; Nguyen, Abel and Kandel, 1994). The activation of nAChRs also leads to increased intracellular Ca^{2+} levels (Karadsheh *et al.*, 2004; McKay, Placzek and Dani, 2007). A wide variety of nAChR subtypes, predominantly α7 (low-affinity) and α4β2 (high-affinity), are distributed throughout the HPC at pre- and postsynaptic locations (Séguéla *et al.*, 1993; Dominguez del Toro *et al.*, 1994; Radcliffe *et al.*, 1999; Fabian-Fine *et al.*, 2001; Perry *et al.*, 2002; Rush *et al.*, 2002; Dani and Bertrand, 2007). Both α7 and α4β2 receptors are Ca^{2+} permeable, which could directly influence Ca^{2+}-dependent cell signalling cascades (Kutlu and Gould, 2016). Therefore, NMDA receptors and nAChRs may mediate learning through the same cellular cascades. Nicotine has been reported to facilitate and induce LTP within the HPC, the intensity of this effect depends on hippocampal subregion and nAChR subtype (Tang and Dani, 2009). Nicotine-facilitated LTP has been reported at CA1 interneurons and pyramidal neurons, predominantly through high-affinity nAChRs (Ge and Dani, 2005; Yamazaki *et al.*, 2005; Jia *et al.*, 2009, 2010). In the dHPC there is a greater density of high-affinity nAChRs compared to the vHPC (Mugnaini *et al.*, 2002). The majority

of high-affinity nAChRs are located in the DG and CA1 regions (Perry *et al.*, 2002). Nakauchi *et al.* (2007) observed that nicotine simultaneously enhanced synaptic performance in Schaffer collaterals and attenuated synaptic performance of EC projections to CA1. This may disrupt the flow of information within the HPC, potentially modifying learning and memory formation. Nicotine has also been reported to enhance synaptic signalling in the DG (Welsby *et al.*, 2006; Zhang *et al.*, 2010). It has been hypothesised that nicotine mediates synaptic plasticity in the CA1 and DG, which supports the enhancement of behaviours such as spatial and contextual memory (Gould and Leach, 2014). Through nAChR activation, nicotine possesses the ability to alter cellular cascades associated with synaptic plasticity, which may lead to long-lasting behavioural changes (Gould, 2006).

As previously discussed, the dHPC is responsible for spatial and contextual learning. Context fear conditioning was enhanced by acute intra-dHPC nicotine administration. After chronic nicotine administration, this behaviour was no longer affected. In addition, nicotine can facilitate and induce LTP within CA1. Dorsal CA1 projects to regions associated with reward-related learning, such as: - NAc, retrosplenial area and tenia tecta. Furthermore, VTA DA neurons project to dorsal CA1 and vSub forming part of the HPC-VTA loop. In order to further understand the mechanisms of nicotine reinforcement and nicotine-induced learning, it would be helpful to determine the neuroadaptations that develop after a single exposure to intra-dHPC nicotine, compared to subsequent changes after repeated exposure. As nicotine exposure moves from acute to chronic exposure, how does nicotine induce changes in these cellular signalling cascades? Specifically asking, how does nicotine alter learning? How do these effects change with the duration of nicotine exposure? And where does this activation differ across brain regions? It would be beneficial to visualise neural activity in specific brain regions to investigate the neural effects of acute and chronic intra-dHPC nicotine exposure. Neural activation of regions associated with the CA1 of the dHPC can be mapped using IEG *c-fos* activation (discussed in Chapter 4 and 5).

6.2 Chapter aims

The aim of this chapter was to examine the mechanisms of nicotine ICSA directly into the dHPC using *c-fos* as a correlate of neural activity, specifically investigating the effect of acute and chronic nicotine self-administered directly into CA1 of the dHPC. Previous work from this laboratory demonstrated that animals willing self-administer nicotine into CA1 of the dHPC, but not the vHPC.

Based on the methodology of Chapter 5, this chapter specifically aimed to ask: - (1) what regions of the HPC-VTA loop does intra-dHPC nicotine activate, (2) does intra-dHPC nicotine mediate its effects through the same mechanisms as intra-VTA, and (3) does acute and chronic dHPC ICSA of nicotine mediate its effects through the same neural mechanisms? It was hypothesised that ICSA of nicotine into CA1 of the dHPC could mediate its effects through the HPC-VTA loop. This would lead to increased Fos expression in the VTA and NAc. However, if intra-dHPC nicotine mediates its effects through other systems, VTA and NAc *c-fos* activity would remain unchanged compared to aCSF controls. The behavioural data were expected to show high levels of lever-pressing behaviour after day 1 ICSA testing. By day 5 ICSA testing, after repeated nicotine exposure, increased lever-pressing behaviour and lever discrimination was expected.

The location of neural activity was examined through Fos quantification in response to nicotine. To determine the key regions activated by intra-dHPC nicotine Fos expression was quantified in the NAc (shell and core), CPu and VTA after acute and chronic nicotine. Simultaneous quantification of nicotine-induced Fos activity in these specific regions will lead to a better understanding of interactions between nicotine and interrelated systems.

6.3 Materials and methods

6.3.1 Subjects

Twenty-four adult male Lister Hooded rats with no previous operant training (naïve) were used for this experiment. Rats were housed, anaesthetised and cannulae implantation surgery was performed as described in the General Methods. Animals weighed between 350 – 410g at the time of surgery. The coordinates for CA1 of the dHPC were as stated in the General Methods. Animals were either part of the acute (n = 12) or chronic (n = 12) nicotine ICSA study[3]. Following surgery, animals were maintained on a food restriction regime to gain ~ 10 g per week. This was achieved by giving animals ~ 20 g food per day at the end of testing, or at the equivalent time on non-testing days. Animals had access to water *ad libitum* in homecages throughout the experiment.

6.3.2 Surgical procedure

Cannulae implantation surgery was performed as described in the General Methods. Unilateral guide cannulae (Plastics one, Roanoke, VA, U.S.A) aimed at CA1 of the dHPC were implanted. The order of the hemisphere cannulated (left or right) was alternated between surgeries. Dummy cannulae were changed daily with clean replacements. Rats were given a minimum of 10 days to recover from surgery before starting behavioural testing.

6.3.3 Behavioural testing

Self-administration was conducted in operant chambers as described in the General Methods and Chapter 5. Animals were habituated to the testing procedure, operant chambers and head fixed micropump as previously described. Rats were assigned to the acute or chronic intra-dHPC nicotine study, and randomly split into nicotine or control groups. As before, animals

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[3] ICSA surgery and behavioural testing were performed by Morag Farquhar. Sections were processed through immunohistology by Josie Fullerton.

either received aCSF or 25 mM nicotine ((–)-Nicotine hydrogen tartrate salt dissolved in aCSF; nicotine, Sigma-Aldrich, UK).

6.3.4 Histology

As described in Chapter 5, on the final day of testing (day 1 or day 5) animals were transferred to a dark holding room for 1 h. Animals were euthanized, transcardially perfused and brains were stored in sucrose as described in the General Methods. Each brain was dissected from the NAc to the VTA and 30μm sections cut. All histological procedures were performed as stated in the General Methods. As before, 1:4 series of sections were processed for cresyl violet stain and *c-fos* immunohistochemical analysis. All sections that crossed through the NAc, CPu, and VTA were processed for *c-fos*. Sections were mounted onto glass slides and viewed via light microscopy to quantify Fos expression. All sections that crossed through the dHPC were processed for cresyl violet stain to determine cannula placement and tip location. Only rats where cannulae tips were located within the dHPC were included in the subsequent analysis.

6.3.5 Data analysis

Statistical analyses were performed in SPSS version 21 (SPSS Inc., Chicago, Illinois, U.S.A). Following acute nicotine ICSA into the dHPC, a two-way ANOVA was performed to determine if there was an effect of *drug* or *lever*, or interaction between them on lever-pressing behaviour. Following chronic nicotine, a three-way ANOVA was performed to determine if there was an effect on *drug*, *session*, *lever*, or interactions between *drug* x *lever*, *session* x *lever*, *session* x *drug*, or three-way interaction between *drug* x *session* x *lever* on lever-pressing behaviour.

Quantification of Fos+ cells was carried out using ImageJ analysis software (ImageJ, U.S. National Institutes of Health, U.S.A). A three-way ANOVA was performed to determine if there was an effect of *drug, day, brain region* or an interaction between *drug* x *brain region*, *day* x *drug*, *day* x *brain region*, or three-way interaction between *day* x *drug* x *brain region*

on Fos expression following acute or chronic nicotine ICSA into the dHPC. Data were normally distributed, as assessed by Shapiro-Wilk test.

All results were expressed as mean ± SEM and homogeneity of variances was assessed by Levene's test for equality of variances. Mauchly's test of sphericity indicated that sphericity was met. Significant interactions were further assessed with post hoc tests or pairwise comparisons, where appropriate. Results were considered significant when $p \leq 0.05$.

6.4 Results

6.4.1 Recovery

All animals recovered well from surgery (n = 24). The general health and body weight of all rats were closely monitored for the duration of the experiment. When required animals were given additional wet mash to help maintain a stable body weight.

6.4.2 Cannula placement

Of the animals that completed the behavioural schedule 22 cannula tips were located within the dHPC, 16 cannula tips were located in CA1 and 6 were located in CA2. The location of the cannulae tips are shown below (Figure 6.2). Two animals were excluded from the study due to cannula placement outwith the HPC (n = 1) and perfusion pump failure (n = 1).

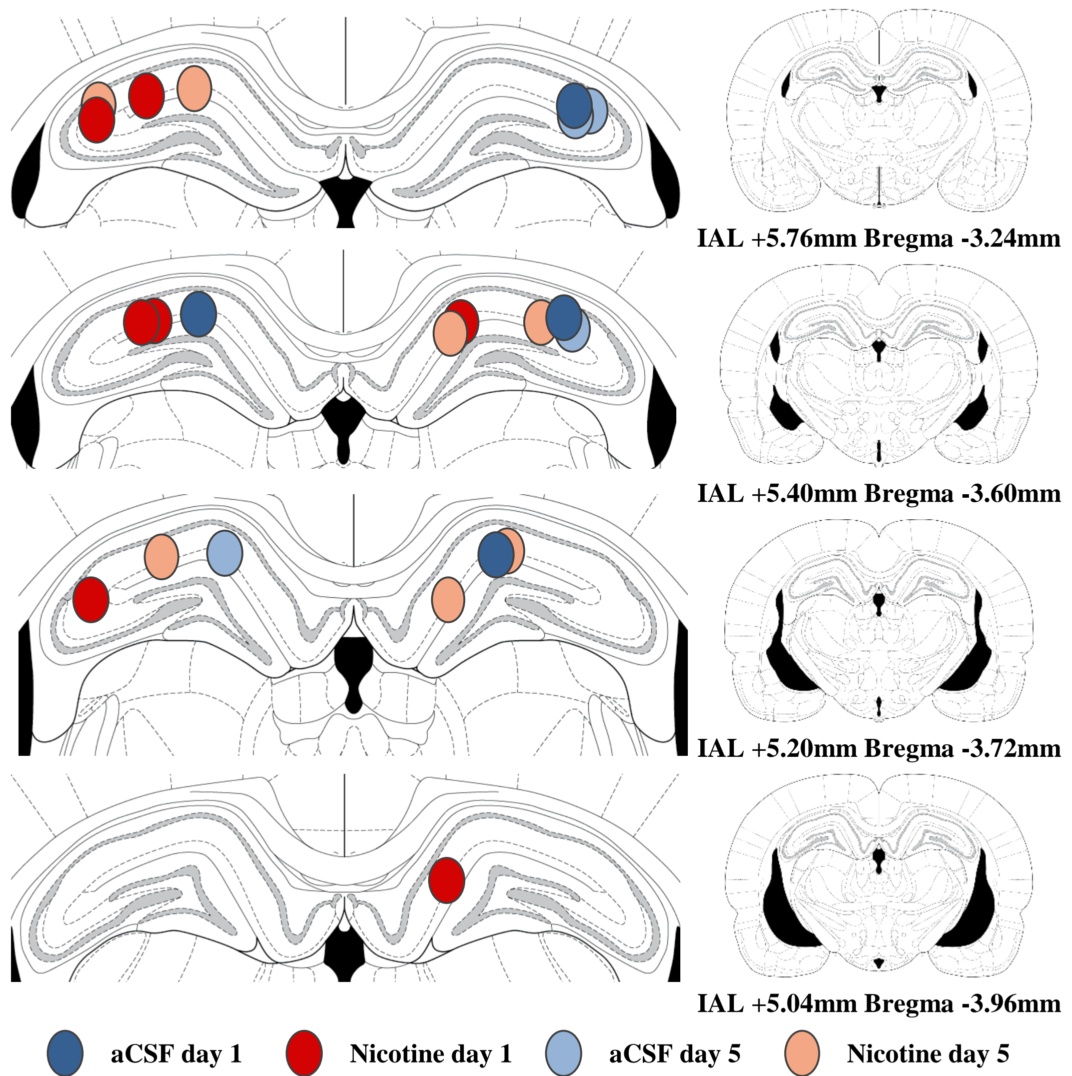

Figure 6.2 – Illustration of the location of unilateral cannula placements in CA1 of the dHPC. Coronal schematics adapted from the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 2007). The infusion site of acute nicotine (dark red circles; n = 7), acute aCSF (dark blue circles; n = 4), chronic nicotine (light red circles; n = 7) and chronic saline (light blue circles; n = 4) are shown.

6.4.3 Behavioural results

Acute nicotine ICSA

Eleven rats were randomly assigned to 1 of 2 groups to receive acute intra-dHPC infusions of nicotine (n = 7) or aCSF (n = 4). Lever pressing on active and inactive levers is shown below (Figure 6.3). There was a significant effect of *drug* ($F_{(1, 9)} = 6.625$; $p = 0.030$), but there was no significant effect of *lever* ($F_{(1, 9)} = 0.025$; $p = 0.878$). There was no *lever* x *drug* interaction ($F_{(1, 9)} = 0.107$; $p = 0.751$).

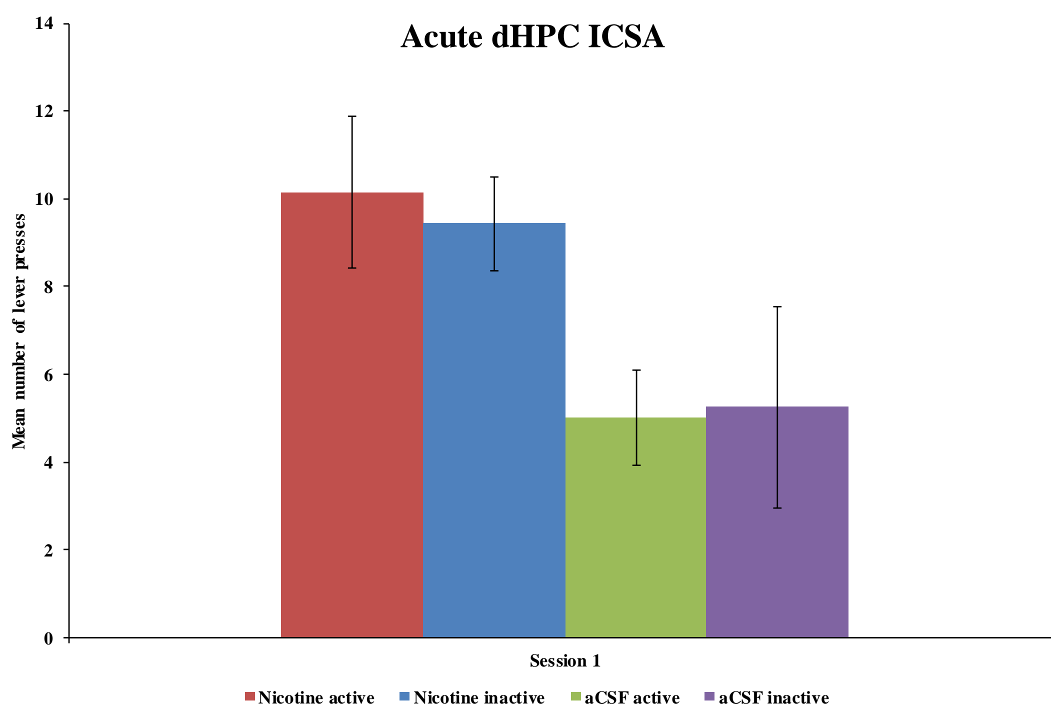

Figure 6.3 – Mean number of active and inactive lever presses during acute ICSA. Rats were given the opportunity to self-administer acute nicotine (n = 7) or aCSF (n = 4) directly into CA1 of the dHPC. Both groups were presented with an active lever (red bar: nicotine, green bar: aCSF) and an inactive lever (no stimulus: blue and purple bars). The number of lever presses on each were measured. Results presented mean ± SEM. For statistical significance, see text.

Chronic nicotine ICSA

Eleven rats were randomly assigned to 1 of 2 groups to receive chronic intra-dHPC infusions of nicotine (n = 7) or aCSF (n = 4). Lever pressing on active and inactive levers is shown across 5 sessions (Figure 6.4). A three-way ANOVA was conducted to understand the effects of *session*, *drug* and *lever* on lever-pressing behaviour. The three-way interaction was not statistically significant ($F_{(4, 36)} = 1.753$; $p = 0.160$). The two-way interactions between *session* x *drug* ($F_{(4, 36)} = 2.297$; $p = 0.078$) and *session* x *lever* ($F_{(4, 36)} = 1.075$; $p = 0.383$) were not statistically significant. There was a significant interaction between *drug* x *lever* ($F_{(1, 9)} = 6.813$; $p = 0.028$) and there was a statistically significant effect of *session* ($F_{(4, 36)} = 3.443$; $p = 0.018$), but there was no significant effect of *drug* ($F_{(1, 9)} = 0.806$; $p = 0.393$) or *lever* ($F_{(1, 9)} = 3.737$; $p = 0.085$).

In order to analyse the data further, each session was assessed independently. On the first session, there was a significant interaction between *lever* x *drug* ($F_{(1, 9)} = 5.657$; $p = 0.041$), a significant effect of *lever* ($F_{(1, 9)} = 5.657$; $p = 0.041$) and *drug* ($F_{(1, 9)} = 7.271$; $p = 0.025$). By session 2, there was no interaction between *lever* x *drug* ($F_{(1, 9)} = 1.710$; $p = 0.223$). There was no effect of *lever* ($F_{(1, 9)} = 1.459$; $p = 0.258$), but there was a significant effect of *drug* ($F_{(1, 9)} = 7.496$; $p = 0.023$). On session 3, there was no interaction between *lever* x *drug* ($F_{(1, 9)} = 2.301$; $p = 0.164$) and no significant effect of *lever* ($F_{(1, 9)} = 0.330$; $p = 0.580$) or *drug* ($F_{(1, 9)} = 0.002$; $p = 0.969$). On session 4, there was a significant interaction between *lever* x *drug* ($F_{(1, 9)} = 15.271$; $p = 0.004$) but there was no significant effect of *lever* ($F_{(1, 9)} = 0.503$; $p = 0.496$) or *drug* ($F_{(1, 9)} = 0.017$; $p = 0.900$). On the final session, there was no interaction between *lever* x *drug* ($F_{(1, 9)} = 0.458$; $p = 0.516$). There was a significant effect of *lever* ($F_{(1, 9)} = 5.333$; p = 0.0.046) but not *drug* ($F_{(1, 9)} = 0.004$; $p = 0.953$).

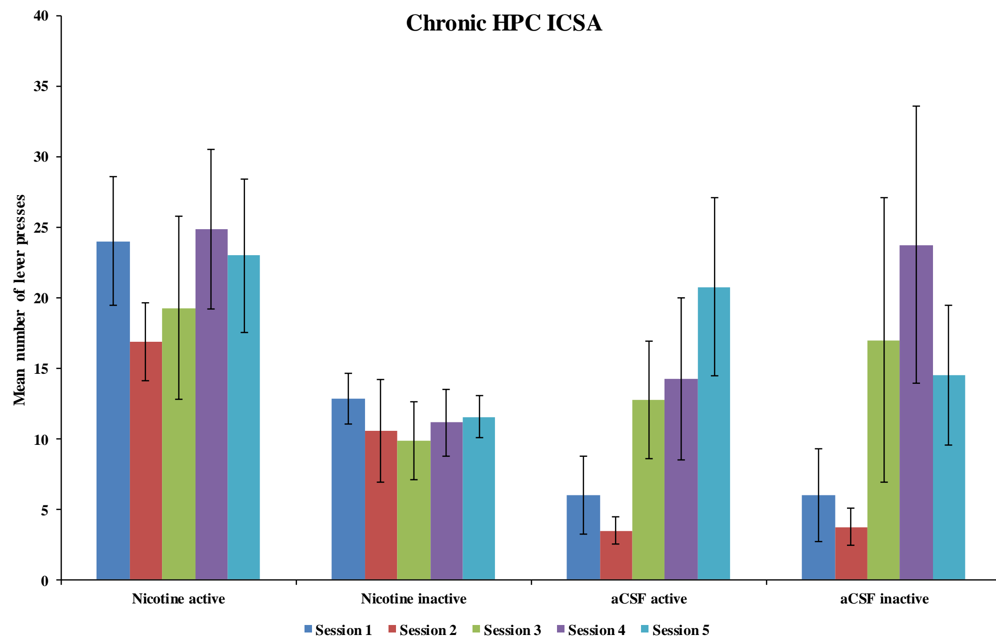

Figure 6.4 – Mean number of nicotine or aCSF active and inactive lever presses across chronic ICSA sessions 1 – 5. Rats were given the opportunity to self-administer nicotine (n = 7) or aCSF (n = 4) directly into CA1 of the dHPC. Both groups were presented with an active lever (nicotine or aCSF) and an inactive lever (no infusion). The number of lever presses on each were measured. Results presented mean ± SEM. For statistical significance, see text.

6.4.4 c-fos quantification

Once cannula placements were confirmed, sections from nicotine and control groups were processed for *c-fos* immunohistology as described in the General Methods. All sections that crossed through the NAc, CPu and VTA were processed. As stated in the General Methods Fos positive (Fos+) cells were quantified using ImageJ software (ImageJ, U.S. National Institutes of Health, U.S.A). Bilateral cell counts were taken from at least 5 sections containing the regions of interest from a 1:4 series. The number of Fos+ cells in the NAc shell and core, CPu and VTA from acute and chronic nicotine and aCSF groups are shown below (Figure 6.5).

A three-way ANOVA was conducted to understand the effects of *day*, *drug* and *brain region* on Fos expression. The three-way interaction was not statistically significant ($F_{(3, 72)} = 0.781$; $p = 0.508$). The two-way interactions between *day* x *brain region* ($F_{(3, 72)} = 0.108$; $p = 0.955$) and *drug* x *brain region* ($F_{(3, 72)} = 0.368$; $p = 0.508$) were not statistically significant. There

was a significant interaction between *day* x *drug* ($F_{(1, 72)} = 5.603$; $p = 0.021$). There was a significant effect of *brain region* ($F_{(3, 72)} = 62.985$; $p = 0.000$), but there was no significant effect of *drug* ($F_{(1, 72)} = 0.098$; $p = 0.755$) or *day* ($F_{(1, 72)} = 1.488$; $p = 0.227$).

In order to analyse the data further, nicotine and aCSF groups were assessed independently. For the aCSF group, the interaction between *day* x *brain region* was not significant ($F_{(3, 24)} = 0.491$; $p = 0.692$). There was a significant effect of *brain region* ($F_{(3, 24)} = 24.777$; $p = 0.000$) but not *day* ($F_{(1, 24)} = 0.501$; $p = 0.486$). For the nicotine group there was no interaction between *day* x *brain region* ($F_{(3, 48)} = 0.343$; $p = 0.795$). There was a significant effect of *day* ($F_{(1, 48)} = 8.987$; $p = 0.004$) and *brain region* ($F_{(3, 48)} = 43.063$; $p = 0.000$).

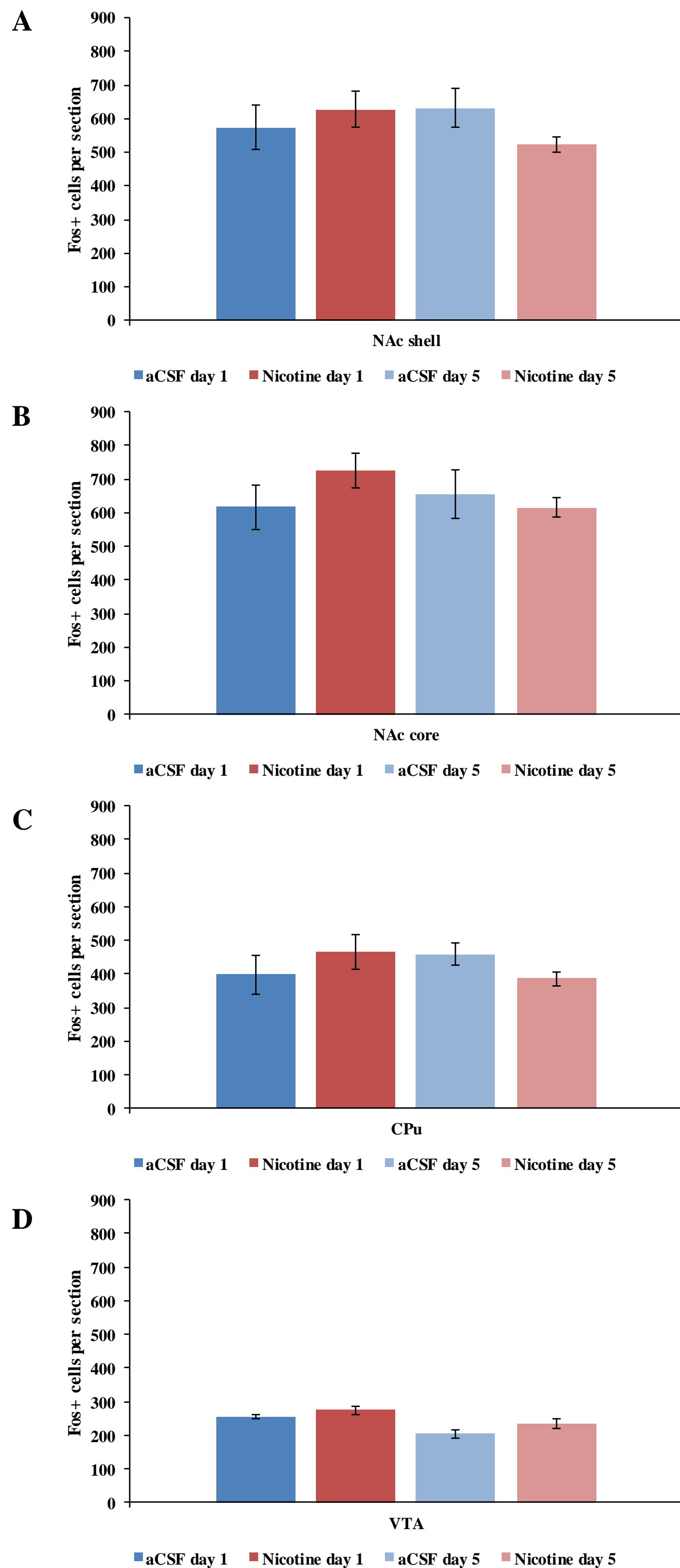

Figure 6.5 – Fos+ cells in the NAc shell (A), NAc core (B), CPu (C) and VTA (D) after acute and chronic nicotine ICSA into CA1 of the dHPC. Animals were either administered with acute nicotine (n = 7; dark red bars) and saline (n = 4; dark blue bars) or chronic nicotine (n = 7; light red bars) and saline (n = 4; light blue bars). Error bars = ± SEM.

6.5 Chapter discussion

The main aim of this chapter was to quantify *c-fos* expression in selected brain regions following acute or chronic ICSA of nicotine into the dHPC. Fos quantification was carried out in the NAc shell and core, CPu and VTA to investigate nicotine-induced activity in key regions of the mesocorticolimbic system and HPC-VTA loop. Investigating the effect of acute and chronic nicotine explored regional neural activation after a single nicotine ICSA session compared to repeated ICSA sessions. This would determine if acute and chronic nicotine mediates its effects through the same neural mechanisms. In addition, Fos activity in the NAc and CPu was quantified to determine if there was a shift in neural activation from session 1 to habit-based learning by session 5.

In the current study, acute and chronic nicotine self-administration into the dHPC did not trigger Fos expression in the NAc shell and core, CPu or VTA (Figure 6.5). After the first session of intra-dHPC ICSA there was no change in *c-fos* activity, by the end of the fifth session nicotine appeared to decrease Fos expression in all regions of interest (Figure 6.5). This effect could be due to nAChR desensitisation, but a larger sample size is required to confirm this effect. As predicted, animals demonstrated high levels of lever-pressing behaviour during ICSA session 1 only in the nicotine-active condition. Animals were expected to increase nicotine-active lever-pressing behaviour and lever discrimination by session 5. In addition, repeated aCSF exposure was expected to have less effect over time. However, the behavioural data demonstrated increased variability and aCSF effect from session 3 onwards (Figure 6.4). The relationship between learning to lever press and Fos expression is not well correlated – high levels of lever pressing did not result in high levels of Fos expression (Figure 6.4 and 6.5).

Although CA1 of the dHPC was targeted, a number of cannula placements were located in CA2 (n = 6) (Figure 6.2). In the chronic study, the majority of animals in the aCSF group had

cannulae located in CA2 (n = 3), rather than CA1 (n = 1). As previously discussed, compared to other subregions CA2 is structurally and functionally distinct (Chevaleyre and Siegelbaum, 2010; Mercer *et al.*, 2012; Piskorowski and Chevaleyre, 2012). This could explain the unexpected increased in aCSF lever-pressing behaviour and variability from session 3 onwards. However, it would not explain the low levels of lever-pressing behaviour in sessions 1 and 2. During ICSA session 1, animals in the acute and chronic nicotine groups demonstrated high levels of lever-pressing behaviour. In the chronic nicotine study, animals demonstrated a strong preference towards the nicotine-active lever during session 1 – this preference was maintained across 5 ICSA sessions (Figure 6.4). In the acute nicotine group, cannula placements were located within CA1 (n = 3) and CA2 (n = 3), while the majority of placements for chronic nicotine animals were located in CA1 (n = 6). This would explain the difference in lever-pressing behaviour between groups on session 1. Acute animals with CA1 placements demonstrated a preference for the nicotine-active lever (presses = 13) compared to CA2 (presses = 7). However, larger sample size is required to confirm this.

It was hypothesised that intra-dHPC nicotine would induce neural activation in the VTA and NAc. However, nicotine did not induce Fos expression in either of these regions. It was further hypothesised that neural activation would shift from the NAc in session 1 to the CPu by session 5. However, Fos expression in the NAc and CPu was not affected by the degree of learning that had taken place. As previously discussed, the downward arc of the HPC-VTA loop is believed to carry novelty signals from the HPC to the VTA through the NAc (Legault, Rompré and Wise, 2000; Floresco, Todd and Grace, 2001). However, neural activity in the NAc and VTA did not change following intra-dHPC nicotine ICSA. As discussed above, acute nicotine enhances dHPC-dependent learning during context fear conditioning, yet this effect disappeared following 12 days of chronic nicotine exposure (Gould and Wehner, 1999; Gould and Higgins, 2003; Davis *et al.*, 2005). It could be suggested that intra-dHPC nicotine enhances learning behaviour during ICSA testing. Animals in the chronic nicotine group

demonstrated high rates of lever-pressing behaviour in session 1, which was maintained across 5 testing sessions. Previously, nicotine-induced learning was reported to disappear following chronic nicotine exposure, but in the current chronic nicotine study lever-pressing rates did not diminish. However, animals were exposed to 5 sessions of nicotine ICSA over 10 days compared to 12 continuous days of nicotine testing – 5 sessions may not be enough to produce this effect. On the other hand, perhaps ICSA intra-dHPC nicotine does not have the same effect as intra-dHPC during context fear conditioning and chronic ICSA nicotine exposure may maintain high levels of lever-pressing behaviour across a longer period of time.

Due to the unexpected aCSF effect, increased variability and small group size, it is unclear whether animals readily administer nicotine into CA1 of the dHPC. However, it is clear that Fos expression in regions of the mesocorticolimbic system and HPC-VTA loop are not correlated with lever-pressing behaviour (Figure 6.4 and 6.5). This suggests that other brain regions and systems are involved, or the effect of intra-dHPC nicotine is mediated by the HPC itself. Nicotine is known to increase the frequency of hippocampal theta oscillations (4 – 12 Hz), particularly in CA3 (Yamamoto, 1998; Lu and Henderson, 2010). Theta rhythms are believed to be involved in increased attention, which could drive animals to increase lever-pressing behaviour (Ekstrom *et al.*, 2005). After intra-CA1 nicotine, Fos expression could be quantified in hippocampal subregions (excluding CA1) to determine if nicotine triggers local neural activation.

Dorsal CA1 is reciprocally connected to the retrosplenial area (Table 6.1) (Cenquizca and Swanson, 2007). The retrosplenial area has been linked to reward processing, reward memory, allocentric spatial memory and spatial working memory (Vann and Aggleton, 2002; Ikemoto, Witkin and Morales, 2003; Keene and Bucci, 2009; Smith, Barredo and Mizumori, 2012). Ikemoto *et al.* (2003) reported that Fos expression was increased in the retrosplenial area following intra-VTA infusion of carbachol. The retrosplenial area receives projections from dorsal CA1, but also receives DAergic projections from VTA neurons (Loughlin and Fallon,

1984; Berger *et al.*, 1985; Ikemoto, Witkin and Morales, 2003). In addition, non-dopaminergic input is provided to the retrosplenial area by the nucleus of the diagonal band and raphe nuclei – regions which also project to the HPC (van Groen and Wyss, 1990). Dorsal CA1 projects to the tenia tecta, a region which has also been implicated in reward-related learning (Cenquizca and Swanson, 2007; Maddux and Holland, 2012). Although the direct infusion of nicotine into the tenia tecta did not affect trace fear conditioning, this region may mediate effects of nicotine as part of a wider circuitry (Raybuck and Gould, 2010). These structures may be involved in the reward-related learning that occurs following self-administration of intra-dHPC nicotine. Therefore, it would be of interest to quantify Fos expression in the retrosplenial area and tenia tecta following intra-dHPC nicotine infusion. This would determine if intra-dHPC nicotine mediates its effects through either of these regions, and whether levels of neural activation change between acute and chronic nicotine exposure.

Chapter 7: General Discussion

7.1 Summary of aims and results

This thesis set out to investigate the role of mesopontine cholinergic input to midbrain DA neurons utilising Dtx-UII. As discussed in Chapter 4, if selective cholinergic lesions in the pVTA had been achieved using Dtx-UII, behavioural testing could have been performed, allowing for the development of a better understanding of the functions of cholinergic projections from the pPPTg and LDTg to the pVTA. For example, it would have been possible to investigate the effect of cholinergic drugs in rats with Dtx-UII pVTA lesions. Intra-pVTA ICSA of nicotine in rats with cholinergic denervation of the pVTA could have aided the understanding of the role of the pVTA in nicotine addiction and in reward-related behaviours more generally. Given that the aim was to develop a better understanding of cholinergic activity in the VTA an alternative approach was adopted, using nicotine as a means of cholinergic activation.

7.1.1 Summary of main findings

Chapter 3

- Dtx-UII did not destroy cholinergic input to the pVTA from the pPPTg or LDTg at any of the time points analysed
- In the pVTA, at each of the time points investigated, there was no evidence of neurodegeneration or non-selective damage following Dtx-UII infusion
- At each time point, PPTg and LDTg cholinergic cell density remained unchanged after pVTA Dtx-UII

Chapter 4

- Acute and chronic systemic nicotine had no visible effect on Fos expression in the VTA, NAc shell and core, CPu or dHPC
- Systemic nicotine administration was confirmed by assessing locomotor behaviour

Chapter 5

- ICSA of nicotine into the VTA triggered neural activation the NAc shell and core, CPu and dHPC, with no difference observed between acute and chronic administration
- Nicotine-induced Fos expression in the NAc and CPu was not affected by the degree of learning that had taken place
- Intra-VTA nicotine resulted in low levels of lever-pressing behaviour during ICSA session 1. By session 5, after repeated nicotine exposure, rats demonstrated increased lever pressing and lever discrimination
- Lever-pressing behaviour was independent from nicotine-induced neural activation – the number of nicotine-active lever presses did not correlate with *c-fos* expression

Chapter 6

- Acute and chronic nicotine ICSA into the dHPC did not induce Fos expression in the NAc shell and core, CPu or VTA
- In the nicotine group, Fos expression decreased from session 1 to session 5. However, larger sample sizes are required to confirm this effect
- Neural activation in the NAc and CPu was not affected by the degree of learning that had taken place
- Animals willingly self-administer acute and chronic nicotine directly into the dHPC
- Acute and chronic nicotine groups displayed high levels of lever-pressing behaviour during ICSA session 1
- In the chronic nicotine study, animals demonstrated a strong preference towards the nicotine active lever during session 1, which was maintained across 5 ICSA sessions
- There was unexpected increase in lever-pressing behaviour in the aCSF group after session 3. This could be due to CA1/CA2 placements, and a larger sample number is required to investigate this effect

7.1.2 Methodological considerations

There are a number of important methodological considerations that should be taken into account. In Chapter 5, the location of pVTA cannulae placements were distributed across the aVTA, pVTA and a/pVTA border. All animals, with the exception of one, demonstrated lever-pressing behaviour as previously reported in pVTA ICSA (Ikemoto, Qin and Liu, 2006; Farquhar, Latimer and Winn, 2012). Animals with aVTA cannula placements displayed lever-pressing behaviour – it is likely that nicotine diffused to the pVTA which mediated this behavioural effect. To reduce diffusion to distal sites, small volumes of nicotine were infused (75 nl) (McBride, Murphy and Ikemoto, 1999), but due to the small size of the rat VTA, and interconnectivity between VTA subregions, diffusion of nicotine most likely occurred. The infused drug will mix with local fluids, rather than displacing them, which will spread 1 – 2 mm from the injection site (Wise and Hoffman, 1992).

In a minority of brains, *c-fos* immunohistology stained blood vessels due to incomplete perfusion. To reliably quantify Fos expression it was essential that brains were successfully perfused to prevent *c-fos* binding to endogenous IgG. Any brains that were not perfused successfully were omitted from the study. Previous studies used a matched pair design, where Fos activity was expressed and analysed as percentage difference rather than absolute difference (Porter, 2008). This accounted for changes in baseline Fos expression between groups and staining intensity variations. In each Chapter, all sections were processed for Fos at the same time to reduce intensity of staining variances (Chapter 4, 5 and 6). Baseline levels of Fos were stable to analyse data as absolute difference.

It is possible that a different IEG would be better suited to indicate nicotine-induced activation in the VTA and terminal regions. Okuno *et al.* (2011) reported that the threshold for Fos induction is considered high compared to other IEGs (Okuno, 2011). Zif268 (or Erg1) is a IEG regulatory transcription factor responsible for the transcription of late response genes and is

more responsive to synaptic activities at physiological levels (Lonergan *et al.*, 2010). Or activity-regulated cytoskeleton-associated protein (*Arc/Arc3.1*) is directly involved in cellular changes at the cytoskeleton and is transported to the dendrites where Arc protein synthesis takes place (Link *et al.*, 1995; Wallace *et al.*, 1998). This IEG is particularly useful for investigating synaptic plasticity and postsynaptic changes supporting memory formation (Guzowski *et al.*, 2000). However, the activation of *Arc* is more time sensitive than *c-fos*. RNA levels of *Arc* peak between 0 – 8 min after insult, which rapidly returns to baseline levels after 16 min (Vazdarjanova *et al.*, 2002).

7.2 Further discussion

7.2.1 Can nicotine act as a reward outside of the 'reward system'?

Acute and chronic ICSA of nicotine into the VTA significantly activated *c-fos* through regions of the mesocorticolimbic system and HPC-VTA loop. Conversely, nicotine self-administration directly into the dHPC did not mediate its effects through the same systems. This suggests that other brain regions and systems are involved, or intra-dHPC nicotine mediates its effects within the HPC itself. Intra-dHPC nicotine resulted in high levels of nicotine-active lever-pressing behaviour across five sessions of ICSA, but where does intra-dHPC nicotine mediate its effect? What regions are recruited to support this behaviour?

As mentioned in the previous Chapter, nicotine is known to increase the frequency of hippocampal theta oscillations (4 – 12 Hz), particularly in CA3 (Yamamoto, 1998; Lu and Henderson, 2010). Theta and gamma rhythms are essential for co-ordinating information required for cognitive function and working memory (Wulff *et al.*, 2009). In the HPC, theta rhythms are believed to be involved in increased attention (Ekstrom *et al.*, 2005). Previous studies have identified that hippocampal theta oscillations are modulated by projections from the medial septum diagonal band area (MS/DB) (Stewart and Fox, 1990; Dutar *et al.*, 1995; Vinogradova, 1995). GABAergic projections from the MS/DB preferentially project to CA3,

while cholinergic MS/DB projections are distributed throughout the HPC (Freund and Antal, 1988; Yamano and Luiten, 1989; Gulyás, Görcs and Freund, 1990; Miettinen and Freund, 1992; Freund and Buzsáki, 1996). The disruption of the cholinergic projections from the MS/DB to the hippocampus severely attenuates theta power, spatial learning and reduces the specificity of hippocampal place cells (Shapiro *et al.*, 1989; Lee *et al.*, 1994; Brazhnik, Muller and Fox, 2003; Rogers and Kesner, 2003; Sandin *et al.*, 2004). Conversely, administration of cholinesterase inhibitor physostigmine, leads to increased acetylcholine release and promotes theta oscillations (Yoder and Pang, 2005)). Numerous studies have reported that the release of acetylcholine enhances the dynamics of memory formation. It has been shown that the impairment of the parahippocampal cholinergic system produces deficits in recognition, spatial and working memory encoding – but not memory retrieval (Aigner and Mishkin, 1986; Tang, Mishkin and Aigner, 1997). In the hippocampal formation, acetylcholine release has been reported to enhance encoding through increased theta oscillations (Bland and Oddie, 2001; Siok *et al.*, 2006; Tsanov, 2015). Perhaps intra-dHPC nicotine increases theta rhythms in the HPC, which leads to increased nicotine self-administration. The nature of "reward" tends to be associated with physical things – natural rewards (such as food) or "unnatural" rewards (such as drugs). Conditioned rewards are taken to be substitutes for these – previously neutral stimuli that have acquired the properties of reward by association. But other states can be rewarding – being alert, attentive and switched on are preferable to being dull, listless and lethargic. As such, heightened attention through hippocampal theta could be pleasurable or rewarding – without a physical reward being present. This goes against the idea of a "reward system" or "pleasure centres" which mediates all reward or pleasure. It would be interesting to quantify Fos expression in subregions of the HPC, especially CA3, after intra-CA1 nicotine ICSA to determine if nicotine triggers local neural activation. Furthermore, it could be beneficial to quantify Fos expression in the MS/DB, to determine whether intra-dHPC nicotine increases neural activation in this region which leads to increased theta oscillations.

As discussed in Chapter 6, CA1 of the dHPC is reciprocally connected to the tenia tecta and retrosplenial cortex (RSC – also known as retrosplenial area). The tenia tecta has been implicated in reward-related learning (Cenquizca and Swanson, 2007; Maddux and Holland, 2012). Although the direct infusion of nicotine into the tenia tecta did not affect trace fear conditioning, this region may mediate effects of nicotine as part of a wider circuitry (Raybuck and Gould, 2010). Therefore, it would be interesting to quantify Fos expression in the tenia tecta following intra-dHPC nicotine infusion. The RSC has also been linked to reward processing, reward memory, allocentric spatial memory and spatial working memory (Vann and Aggleton, 2002; Ikemoto, Witkin and Morales, 2003; Keene and Bucci, 2009; Smith, Barredo and Mizumori, 2012). This large cortical region can be separated into granular (RSG) and agranular (RSA) cortices (Vogt and Peters, 1981; Vogt and Miller, 1983; van Groen and Wyss, 1990, 1992; Vann, Aggleton and Maguire, 2009). In particular, the dHPC is reciprocally connected to the RSG (Aggleton and Brown, 1999; Maddock, 1999; Aggleton and Pearce, 2001; Aggleton *et al.*, 2010). The VTA supplies DAergic projections to both granular and agranular cortices of the RSC (Loughlin and Fallon, 1984; Berger *et al.*, 1985; Ikemoto, Witkin and Morales, 2003; Aransay *et al.*, 2015). Based on these results, it would be interesting to determine the effect of nicotine ICSA into the VTA or dHPC on the neural activity of the RSC, or to determine the effect of nicotine self-administration directly into the RSC measuring Fos expression in the VTA and dHPC. The RSC is one of the largest cortical regions in the rat, extending ~ 8 mm along the rostral-caudal axis of the rat brain (Robinson *et al.*, 2014). Regions of this size would be difficult to reliably quantify *c-fos* activity or surgically cannulate – this would require the implantation of several cannulae along the length of the structure. Therefore, it would be more beneficial to specifically target regions of the RSC, such as RSG area 29, which are reciprocally connected to the dHPC. This would determine if intra-dHPC nicotine mediates its effects through either of the RSG, and whether levels of neural activation change between acute and chronic nicotine exposure.

7.2.2 What is the relationship between c-fos expression, behaviour and learning?

In Chapters 5 and 6, *c-fos* activation and lever-pressing behaviour were not well correlated. Intra-VTA nicotine resulted in low levels of lever-pressing behaviour during session 1, which increased across 5 sessions (with increased lever discrimination). Nicotine significantly increased Fos expression by the same degree during ICSA session 1 and 5. In Chapter 6, intra-dHPC nicotine did not trigger *c-fos* activation in session 1 or 5, but animals demonstrated high levels of lever-pressing behaviour. It was hypothesised that levels of Fos expression would increase as animals learned to lever press. However, it would appear that *c-fos* expression and behaviour are unrelated. In order to confirm that nicotine-induced neural activation is independent from lever-pressing behaviour it would be useful to increase group sizes – especially in the intra-dHPC group. In addition, it would be beneficial to identify the specific neurochemical identity of activated neurons to confirm that Fos expression and behaviour are not correlated. Double immunohistology could be carried out for *c-fos* with either: - DA (in the VTA), GABA, or glutamate markers. This would also determine whether nicotine-induced neural activation was excitatory or inhibitory.

Following systemic or intracranial nicotine administration, it was hypothesised that neural activation would shift from the NAc in session 1 to the CPu by session 5 – from the novelty of session 1 to habit-based learning by the final session (Chapter 4, 5 and 6). In Chapter 4, acute and chronic systemic nicotine did not activate the shell and core of the NAc or CPu. In Chapter 5, acute and chronic intra-VTA nicotine triggered neural activation in the shell and core of the NAc, and CPu. Conversely, in Chapter 6, neither acute nor chronic intra-dHPC nicotine activated the NAc or CPu. These results suggest that nicotine-induced Fos expression in the NAc and CPu was not affected by the degree of learning that had taken place. As well as habit-based learning, the CPu has been implicated in motor-memory and instrumental conditioning (Yin, Knowlton and Balleine, 2004; Everitt and Robbins, 2005; Faure, Haberland and El Massioui, 2005; Yin *et al.*, 2005; Belin *et al.*, 2009). Increased neural activation in the

CPu could be due to motor activation itself. During ICSA the animals work to lever press, which could lead to increased Fos expression in the CPu. However, if CPu activation was triggered by ICSA habit-based learning, motor memory or motor activation, increased Fos expression would have been observed following ICSA into the VTA and dHPC. As discussed in Chapter 4, CPu Fos activation triggered by intra-VTA nicotine could be due to nicotine diffusion to the neighbouring substantia nigra (SN), which provides substantial DA input to the dorsal striatum (in which the CPu is located) (Panagis *et al.*, 1996). There was no visible difference in CPu *c-fos* activity between more medial or laterally positioned VTA cannulae (Chapter 5). Therefore, it is unlikely that increased Fos expression in the CPu is due to nicotine diffusion to the SN.

Within the CPu, the medial portion primarily receives projections from the prefrontal cortex (PFC), and the lateral portion is innervated by the sensorimotor cortex (Maroteaux *et al.*, 2014). The lateral CPu is primarily involved in habit-based learning, but the medial CPu is associated with action-outcome associations implicated in goal-directed behaviours (Yin, Knowlton and Balleine, 2006; Yin, Ostlund and Balleine, 2008; Balleine, Liljeholm and Ostlund, 2009). Previous studies have reported that learning-associated neural activation shifts from the medial portion of the CPu, to the lateral portion with progressive training (Thorn *et al.*, 2010). When replicating this work to increase sample size, it would be interesting to quantify Fos expression in specific regions of the CPu, to determine if nicotine-induced activation is limited to areas of the CPu innervated by the SN. This could determine whether CPu activation is triggered by nicotine diffusion from the VTA to the SN.

7.2.3 Does acute and chronic nicotine induce differential neural activation?

In the current study, there were no obvious differences between acute and chronic nicotine exposure (Chapter 4, 5 and 6). In Chapter 4, acute and chronic systemic nicotine did not activate the VTA, NAc shell and core, CPu or dHPC. In Chapter 5, acute and chronic nicotine

self-administration into the VTA increased Fos expression in the NAc shell and core, CPu and dHPC. Conversely, in Chapter 6 both acute and chronic intra-dHPC nicotine self-administration failed to activate the regions of interest. As discussed in Chapter 4, repeated nicotine exposure is known to trigger complex receptor alterations and neuroadaptations that result in the onset of nicotine dependence and addiction. The neurochemical mechanisms that lead to nicotine dependence are somehow initiated by the activation of VTA DA neurons that ultimately results in increased DA release in the NAc (Benwell and Balfour, 1992; Pontieri *et al.*, 1996; Balfour *et al.*, 2000; Balfour, 2004). These studies demonstrate that nicotine has a differential impact on specific brain regions depending on the level of nicotine exposure.

Huang *et al.* (2015) investigated the timing between doses of nicotine on brain function, specifically analysing the resting state functional connectivity (rsFC) between brain regions (Fedota and Stein, 2015; Huang *et al.*, 2015). On the final day of chronic nicotine exposure, animals were challenged with a second dose of nicotine 3, 6, 12 or 24 h after the initial dose, and the rsFC response was measured using functional magnetic resonance imaging (fMRI) (Huang *et al.*, 2015). This technique measured increase or decrease in circuit strength in response to nicotine, implicating different regional connectivity at different time points. When animals received a second dose of nicotine 3 or 6 h after the initial dose of nicotine, the connectivity between the RSC and HPC dramatically increased (Huang *et al.*, 2015). This increased rsFC returned to baseline levels by 12 and 24 h. The rsFC between the HPC-VTA remained near baseline levels at 3, 12 and 24 h, but significantly decreased at 6 h (Huang *et al.*, 2015). These results suggest that the connectivity between the RSC and dHPC are involved in mediating the effects of nicotine. Furthermore, these results demonstrate a time sensitive effect between circuit strength, brain region connectivity and nicotine administration. This technique could be applied to identify the immediate effects of initial nicotine exposure, to determine the effects of a single administration on circuit strength between brain regions. Then, to understand the neuroadaptations and connectivity changes which occur after repeated

exposure in target brain regions. It is important to understand the neural circuitry involved and changes that occur between acute and chronic nicotine, to determine how these alterations ultimately lead to changes in behaviours.

By furthering our knowledge of the structural organisation and functions of the VTA, NAc and HPC, our understanding of the implications of drug addiction will expand. This will lead to a better comprehension of the interactions between nicotine and the interrelated neural systems. Furthermore, this could aid the understanding of the molecular mechanisms and neuroadaptations that occur from a single exposure of nicotine leading to repeated nicotine use, nicotine dependence and addiction. It is important to study the specific connections between these nuclei, which will in turn advance our understanding of pathologies of various mental disorders and neurological diseases, as well as their role in addiction – possibly uncovering new potential targets for therapeutic intervention.

7.3 Future directions

The main direction for future work is to replicate the intra-dHPC ICSA study, to increase the power and reduce variability of behavioural results. This could highlight a clear nicotine-active effect in CA1 of the dHPC across 5 ICSA sessions, and reduce the unexpected effect of aCSF that was observed. Following on from this, it would be interesting to analyse the length of time between the first and second drug infusion – also known as the inter-press interval (IPI) which is an indirect measure of drug reinforcement (Farquhar, Latimer and Winn, 2012). If a drug is positively reinforcing, animals will lever press to receive the drug a second time (Farquhar, Latimer and Winn, 2012). It is assumed that the latency between the two infusions reflects the degree to which the drug is reinforcing.

Although Fos activity was quantified in key regions of the mesocorticolimbic system and HPC-VTA loop, neural activation could also be measured in the olfactory tubercle (OT), prefrontal cortex (PFC) (mesocorticolimbic system) or ventral pallidum (VP) (HPC-VTA

loop). This could confirm that intra-dHPC nicotine does not mediate its effects through either of these systems, or confirm that intra-VTA nicotine does. As discussed above, nicotine-induced neural activity could be quantified in regions associated with the dHPC, such as the RSC or tenia tecta. As previously mentioned, the VTA and dHPC is divided into discrete subregions. Zhao-Shea *et al.* (2011) reported that the level of *c-fos* expression was different across VTA subregions. It is particularly difficult to differentiate between VTA subregions or subnuclei (Pang, Kiba and Jayaraman, 1993), which is why VTA Fos expression was taken as a whole for the current study. But, it may be interesting to determine the level of nicotine-induced neural activity in specific subregions of the pVTA (PIF, PBP and PN) or dHPC (CA1, CA2, CA3 and DG). This could also confirm if intra-dHPC nicotine mediates its effects through the HPC itself.

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