

A functional characterisation of retrosubstantia nigra dopaminergic neurons

Luke Alexander Bryden

Trinity College

University of Oxford



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Abstract

Characterising the function of the brain in health is critical if the changes that occur during disease states are to be fully understood. This is particularly true of the midbrain dopaminergic system, which is compromised in disorders such as Parkinson's disease and drug addiction. Much research concerning the midbrain dopaminergic system focuses on the two largest cell groups, namely those situated in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). Caudal of the SNc and VTA is the third, relatively understudied group of dopaminergic neurons in the retrorubral field (RRF). The overarching goal of my thesis work was to characterise the function of RRF dopaminergic neurons in the mouse, in terms of their molecular makeup, connectivity and electrophysiological activity.

Molecular markers expressed by RRF dopaminergic neurons shared features of both the SNc and VTA. Like the SNc, some neurons in the RRF express Sox6 but lack Otx2; like the VTA, some neurons in the RRF express calbindin-1 and calretinin. Moreover, I determined that while many RRF dopaminergic neurons express calbindin-1 and few express aldehyde dehydrogenase 1a1, the opposite is true for the caudal SNc. Thus, this combination of molecular markers is useful for delineating the border between the rostral RRF and caudal SNc. Intermingled amongst dopaminergic neurons in the RRF were many putative GABAergic and other non-dopaminergic neurons, indicating a diversity of cell types within this region.

Cell type-selective anterograde and retrograde tracing of RRF dopaminergic neurons revealed their brain-wide connectivity. Examination of axonal projections uncovered a dense innervation of the ventrolateral striatum (VLS) and interstitial nucleus of the posterior limb of the anterior commissure (IPAC), in addition to a sparse innervation of the dorsolateral striatum (DLS) and olfactory tubercle. Although the projections of RRF dopaminergic neurons share features of both the nigrostriatal and mesolimbic pathways, the prominence of projections in ventral forebrain structures suggests that RRF neurons contribute more to the mesolimbic pathway. I observed that neurons providing monosynaptic inputs to RRF dopaminergic neurons are located in many brain regions. The densest clusters of input neurons were located in the bed nucleus of the stria terminalis (BNST), IPAC, and central amygdala. Overall, the results of these tracing experiments indicate that while the axonal projections of RRF dopaminergic neurons terminate in the striatum, their most prominent inputs arise from the extended amygdala. In the case of the IPAC, there appears to be prominent reciprocal connectivity with RRF dopaminergic neurons.

Electrophysiological *in vivo* recording experiments demonstrated that the spontaneous firing properties of identified RRF dopaminergic neurons are broadly similar to their counterparts in SNc and VTA, specifically a slow firing rate (< 10 spikes/s) and relatively long action potential duration (> 1 ms). Foot shocks applied to the hindpaw induced a brief pause (~220 ms) in the firing of both identified RRF and SNc dopaminergic neurons, followed by rebound activation. Together, the results from these electrophysiological recordings demonstrate that the firing properties of dopaminergic neurons in the RRF are typical of midbrain dopaminergic neurons and that they are equipped to represent aversive stimuli with a pause in firing activity.

Next, I used an optogenetic approach to mimic the brief pause in firing observed in response to foot shock. Anaesthetised *in vivo* electrophysiological validation experiments demonstrated that ArchT-transduced dopaminergic neurons reliably pause their firing in response to brief (250 ms), physiologically-relevant yellow laser pulses. In freely-moving mice, 'positive control' experiments in which VTA dopaminergic neurons were briefly (250 ms) paused did not induce place aversion in either a bias conditioned place preference test or real-time place preference test.

Collectively, the work in this thesis provides important new information on the substrates for RRF dopaminergic neuron function. I have shown that the functional properties of these neurons are partly overlapping with respect to neighbouring SNc and VTA dopaminergic neurons, in terms of molecular makeup, connectivity and electrophysiological activity. I conclude that RRF dopaminergic neurons are equipped to fulfil distinct and specialised roles in the brain as compared to their midbrain counterparts.

Dedication

I dedicate this thesis to my late grandmother, Margaret Annabel Emblen. You always fostered my curiosity and had the patience of many saints, for which I am forever grateful.

Contents

1	General Introduction	1
1.1	Anatomical boundaries of midbrain dopaminergic neurons	3
1.2	Molecular diversity of midbrain dopaminergic neurons	5
1.3	Axonal projections of midbrain dopaminergic neurons	9
1.4	Inputs to midbrain dopaminergic neurons	13
1.5	Firing properties of midbrain dopaminergic neurons	15
1.6	Behaviours associated with midbrain dopaminergic neuron function	20
1.7	Aims of research	28
2	Materials and Methods	29
2.1	Animals	29
2.2	Molecular marker mapping	30
2.2.1	Perfuse-fixation and tissue processing	30
2.3	Virus injection	33
2.4	Anterograde and retrograde tracing of RRF dopaminergic neurons	34
2.4.1	Anterograde tracing: surgical procedure	34
2.4.2	Retrograde tracing: surgical procedure	35
2.4.3	Perfuse-fixation and tissue processing	37
2.4.4	Quantification and localisation of transduced neurons	39
2.4.5	Anterograde tracing: mapping axonal projections	43

2.4.6	Retrograde tracing: mapping monosynaptic input neurons	44
2.5	Electrophysiology in anaesthetised mice	45
2.5.1	Preparation of anaesthetised mice	45
2.5.2	Single-unit electrophysiology: set-up	46
2.5.3	Single-unit electrophysiology: spontaneous activity	47
2.5.4	Single-unit electrophysiology: noxious electrical stimulation of hindpaw . . .	48
2.5.5	Single-unit electrophysiology: optogenetics	48
2.5.6	Single-unit electrophysiology: juxtacellular labelling of single neurons	49
2.5.7	Perfuse-fixation and tissue processing	49
2.5.8	Analysis of recordings: spontaneous activity	52
2.5.9	Analysis of recordings: noxious electrical stimulation of hindpaw	53
2.5.10	Analysis of recordings: optogenetics	54
2.5.11	Analysis of recordings: statistical approach	54
2.6	Place conditioning	55
2.6.1	Optic fibre implant surgery	55
2.6.2	Conditioned place preference	56
2.6.3	Real-time place preference	59
2.6.4	Timeline of place preference behaviour	60
2.6.5	Perfuse-fixation and tissue processing	62
3	Molecular markers of RRF dopaminergic neurons	63
3.1	Introduction	63
3.2	Aims	67
3.3	Methods	67
3.4	Results	69
3.4.1	Aldh1a1 and calbindin-1 expression patterns distinguish the border between the rostral RRF and caudal SNc	69

3.4.2	The expression profile of selected molecular markers in the RRF	75
3.4.3	Putative GABAergic and other non-dopaminergic neurons are interspersed amongst dopaminergic neurons in the RRF	84
3.4.4	Results summary	85
3.5	Discussion	86
3.5.1	Aldh1a1 and calbindin-1 expression patterns distinguish the border between the rostral RRF and caudal SNc	87
3.5.2	The expression profile of other midbrain dopaminergic neurons molecular markers in the RRF	89
3.5.3	Putative GABAergic and other non-dopaminergic neurons are interspersed amongst dopaminergic neurons in the RRF	92
4	Anterograde tracing of RRF dopaminergic neurons	94
4.1	Introduction	94
4.2	Aims	97
4.3	Methods	97
4.4	Results	99
4.4.1	GFP-transduced dopaminergic neurons are mostly restricted to the RRF . .	99
4.4.2	RRF dopaminergic neurons project to the striatum and olfactory tubercle . .	104
4.4.3	RRF dopaminergic neurons project to the interstitial nucleus of the posterior limb of the anterior commissure	109
4.5	Discussion	111
4.5.1	GFP-transduced dopaminergic neurons are mostly restricted to the RRF . .	112
4.5.2	RRF dopaminergic neurons project to the striatum and olfactory tubercle . .	113
4.5.3	RRF dopaminergic neurons project to the interstitial nucleus of the posterior limb of the anterior commissure	116
4.5.4	Conclusions and functional significance	118

5	Monosynaptic inputs to RRF dopaminergic neurons	121
5.1	Introduction	121
5.2	Aims	124
5.3	Methods	124
5.4	Results	124
5.4.1	Starter neurons were mostly restricted to the RRF	124
5.4.2	Brain-wide monosynaptic inputs to RRF dopaminergic neurons	128
5.4.3	Objectively defining the regions innervating RRF dopaminergic neurons . . .	132
5.5	Discussion	141
5.5.1	Starter neurons were mostly restricted to the RRF	142
5.5.2	Brain-wide monosynaptic inputs to RRF dopaminergic neurons	142
5.5.3	Objectively defining the regions innervating RRF dopaminergic neurons . . .	143
5.5.4	Conclusions and future direction	150
6	Electrophysiological properties of RRF neurons	151
6.1	Introduction	151
6.2	Aims	153
6.3	Methods	153
6.4	Results	154
6.4.1	Spontaneous firing properties of identified dopaminergic neurons are similar in the RRF, SNc and VTA	154
6.4.2	Most RRF and SNc dopaminergic neurons display suppression of firing followed by rebound activation in response to electrical stimulation of the hindpaw	159
6.4.3	Non-dopaminergic neurons in the RRF and caudal SNc have diverse responses to electrical stimulation of the hindpaw	166

6.4.4	A cholinergic neuron in the pedunculopontine nucleus was phasically activated in response to electrical stimulation of the hindpaw	168
6.5	Discussion	169
6.5.1	Spontaneous firing properties of identified dopaminergic neurons are similar in RRF, SNc and VTA	170
6.5.2	Most RRF and SNc dopaminergic neurons display suppression followed by rebound activation in response to electrical stimulation of the hindpaw . . .	171
6.5.3	Non-dopaminergic neurons in the RRF and caudal SNc have diverse responses to electrical stimulation of the hindpaw	176
6.5.4	A cholinergic neuron in the pedunculopontine nucleus is phasically activated in response to electrical stimulation of the hindpaw	177
7	An optogenetic strategy to explore the effect of physiologically-relevant pauses in midbrain dopaminergic firing on place preference behaviours	179
7.1	Introduction	179
7.2	Aims	182
7.3	Methods	183
7.3.1	Electrophysiological recordings during optogenetic manipulation	183
7.3.2	Behavioural experiments	184
7.4	Results	185
7.4.1	Electrophysiological experiments: brief (250 ms), physiologically-relevant laser pulses reliably suppresses the firing of midbrain dopaminergic neurons	185
7.4.2	Electrophysiological experiments: longer laser pulses less reliably suppress the firing of midbrain dopaminergic neurons	188
7.4.3	Place preference behaviour	196
7.4.4	Optic fibre placement	207
7.5	Discussion	209

7.5.1	Electrophysiological experiments: brief (250 ms), physiologically-relevant laser pulses reliably suppresses the firing of midbrain dopaminergic neurons	210
7.5.2	Electrophysiological experiments: longer laser pulses less reliably suppress the firing of midbrain dopaminergic neurons	212
7.5.3	Place preference behaviour: mice have a baseline bias in a conditioned place preference paradigm	215
7.5.4	Place preference behaviour: optogenetically suppressing the firing of VTA dopaminergic neurons does not induce conditioned place aversion or real-time place aversion	216
8	General Discussion	221
8.1	Summary	221
8.1.1	Chapter 3—Molecular markers of RRF dopaminergic neurons	222
8.1.2	Chapters 4 and 5—Anterograde projections and monosynaptic inputs of RRF dopaminergic neurons	223
8.1.3	Chapter 6—Electrophysiological properties of RRF neurons	225
8.1.4	Chapter 7—Briefly pausing the firing of VTA dopaminergic neurons does not induce place aversion	226
8.2	Theoretical and technical considerations	227
8.2.1	What distinguishes RRF dopaminergic neurons from those of the SNc and VTA?	227
8.2.2	The genetic tools used to define the axonal projections and monosynaptic inputs of RRF dopaminergic neurons	228
8.2.3	Species differences	233
8.3	Future direction	234
8.3.1	Can RRF dopaminergic neurons be more objectively defined?	234
8.3.2	Do the axonal projection targets of midbrain dopaminergic subgroups overlap?	235

8.3.3	How does the firing of RRF dopaminergic neurons relate to behaviour? . . .	236
8.4	Concluding statement	237
References		237

List of Figures

1.1	Schematic coronal sections of the midbrain dopaminergic system in the mouse. . . .	5
1.2	Molecularly-defined dopaminergic neuron subgroups have distinct axonal projections. . . .	12
1.3	<i>In vivo</i> firing patterns of midbrain dopaminergic neurons.	16
1.4	Methods for identifying midbrain dopaminergic neurons recorded <i>in vivo</i>	16
2.1	Experimental design for monosynaptic input tracing.	36
2.2	CPP setup	56
2.3	Experimental design for biased CPP.	58
2.4	Timeline of place preference behaviour.	61
3.1	Defining the borders of the RRF, SNc and VTA in the mouse according to the distribution of Aldh1a1 and calbindin-1 in the mouse.	73
3.2	Expression of Aldh1a1 and calbindin-1 in dopaminergic neurons.	73
3.3	Comparison of molecular marker-defined boundaries of dopaminergic neurons with the borders in the Paxinos and Franklin atlas.	75
3.4	RRF dopaminergic neurons express SatB1.	76
3.5	SatB1 is mostly expressed in dopaminergic VTA, SNc and RRF neurons.	77
3.6	Few RRF dopaminergic neurons express Otx2.	78
3.7	Otx2 expression in midbrain dopaminergic neurons is mostly limited to the VTA. . .	78
3.8	RRF dopaminergic neurons express Sox6.	80

3.9	Sox6 is expressed in lateral VTA, SNc and RRF dopaminergic neurons.	80
3.10	RRF dopaminergic neurons express calretinin.	81
3.11	Calretinin expression in midbrain dopaminergic neurons is mostly limited to the RRF and VTA.	82
3.12	RRF dopaminergic neurons express G-protein-regulated inward-rectifier potassium channel 2 (GIRK2).	83
3.13	GIRK2 is exclusively expressed by dopaminergic neurons in the midbrain.	83
3.14	Dopaminergic, putative GABAergic and other non-dopaminergic neurons are interspersed in the RRF.	85
4.1	Most GFP-transduced dopaminergic neurons were within the RRF.	100
4.2	The distribution of GFP-transduced dopaminergic neurons in the midbrain.	102
4.3	A summary of the transduction selectivity and targeting efficiency.	103
4.4	RRF dopaminergic projections were broadly similar across animals.	106
4.5	RRF dopaminergic projections to the striatum and olfactory tubercle.	107
4.6	RRF dopaminergic axons in the striatum were highly branched and contained varicosity-like structures.	108
4.7	RRF dopaminergic projections to rostral striatum are mainly located in the matrix and striosomal compartments.	109
4.8	RRF dopaminergic neurons project to the IPACL.	111
4.9	RRF dopaminergic axons in the IPAC were highly branched and contained varicosity- like structures.	111
5.1	Starter neurons were dopaminergic and mostly restricted to the RRF.	127
5.2	The distribution of starter neurons in the midbrain.	127
5.3	A summary of the starter neuron transduction.	128
5.4	Ectopic expression of RV-eGFP in glial cells.	129

5.5	The pattern of inputs to RRF dopaminergic neurons.	131
5.6	Sparse striatal and accumbal inputs to RRF dopaminergic neurons.	133
5.7	RRF dopaminergic neurons receive inputs from the lateral BNST and rostral IPACL.	135
5.8	The IPAC sends direct inputs to RRF dopaminergic neurons.	137
5.9	RRF dopaminergic neurons receive inputs from the CeA.	139
5.10	The STN and pSTN directly innervate RRF dopaminergic neurons.	140
5.11	RRF dopaminergic neurons receive direct inputs from the PBN.	141
6.1	Spontaneous <i>in vivo</i> spiking activity of identified midbrain dopaminergic neurons.	155
6.2	The spontaneous firing properties of identified RRF, SNc and VTA dopaminergic neurons are similar.	157
6.3	The relationship between the spontaneous firing properties of midbrain dopaminergic neurons and their anatomical location.	158
6.4	Individual responses of identified RRF and SNc dopaminergic neurons to noxious electrical stimulation of the hindpaw.	160
6.5	Population responses of identified RRF and SNc dopaminergic neurons to noxious electrical stimulation of the hindpaw.	161
6.6	Heterogeneity in response types and profiles of identified RRF dopaminergic neurons following noxious electrical stimulation of the hindpaw.	163
6.7	Decreased burst firing and increased interspike interval (ISI) during rebound for identified RRF and SNc dopaminergic neurons.	164
6.8	Firing dynamics of identified RRF and SNc dopaminergic neurons in response to noxious electrical stimulation of the hindpaw are not inter-related.	165
6.9	Firing dynamics of identified RRF and SNc dopaminergic neurons in response to noxious electrical stimulation of the hindpaw are not related to anatomical location.	165
6.10	Diverse responses of identified non-dopaminergic midbrain neurons to noxious electrical stimulation of the hindpaw.	167

6.11	A cholinergic neuron in the pedunculopontine nucleus (PPN) is activated by noxious electrical stimulation of the hindpaw.	169
7.1	Experimental setup for optogenetic recordings.	184
7.2	250 ms laser pulses silenced most ArchT-transduced midbrain dopaminergic neurons.	188
7.3	2 s laser pulses silenced or suppressed ArchT-transduced midbrain dopaminergic neurons.	190
7.4	Comparison of midbrain dopaminergic firing during 5 and 10 mW laser pulses.	191
7.5	10 s laser pulses silenced or suppressed ArchT-transduced midbrain dopaminergic neurons.	193
7.6	The firing rate of most ArchT-transduced midbrain dopaminergic neurons was silenced or suppressed by 30 s laser pulses.	194
7.7	Some neurons displayed atypical responses to 30 s laser pulses.	195
7.8	The firing activity of a non-responsive neuron during 30 s laser pulses.	196
7.9	Mice develop a baseline preference for one (white) chamber in the CPP paradigm.	199
7.10	GFP and ArchT transduction in midbrain dopaminergic neurons.	200
7.11	Briefly suppressing the firing of VTA dopaminergic neurons does not affect CPP behaviour.	201
7.12	Suppressing the firing of VTA dopaminergic neurons for 2 s does not affect CPP behaviour.	203
7.13	Briefly suppressing the firing of VTA dopaminergic neurons does not affect RTPP behaviour.	205
7.14	Briefly suppressing the firing of VTA dopaminergic neurons does not induce transient changes in ongoing movement.	206
7.15	Suppressing the firing of VTA dopaminergic neurons for 2 s does not affect RTPP behaviour.	207
7.16	Placement of optic fibre above the VTA: brief (250 ms) pulse experiments.	209

7.17	Placement of optic fibre above the VTA: longer (2 s) pulse experiments.	209
8.1	A summary of RRF dopaminergic neuron connectivity.	225

List of Tables

2.2 Primary antibodies used to delineate brain regions projecting to RRF dopaminergic neurons. 45

3.2 Primary antibodies used for molecular marker mapping. 68

3.4 Molecular marker expression summary table 86

4.2 A summary of the brain regions innervated by retrorubral field (RRF) dopaminergic neurons. 105

5.2 A comparison of areas projecting to RRF dopaminergic neurons. 130

6.1 Summary Table of the spontaneous firing properties of identified non-dopaminergic neurons. 168

Abbreviations

6-OHDA 6-hydroxydopamine

A-P anterior-posterior distance from Bregma

AAV adeno-associated virus

ACC anterior cingulate cortex

Aldh1a1 aldehyde dehydrogenase 1a1

AMCA aminomethylcoumarin acetate

ANOVA analysis of variance

AP action potential

Arch archaerhodopsin

ArchT archaerhodopsin T

AVP arginine vasopressin

BDA biotinylated dextran amine

BLA basolateral amygdala

BNST bed nucleus of the stria terminalis

CeA central amygdala

ChAT choline acetyltransferase

ChR2 channelrhodopsin-2

CI confidence interval

CLi caudal linear nucleus of the raphe

CPA conditioned place aversion

CPP conditioned place preference

CTB cholera toxin subunit B

D-V dorsal-ventral distance from the brain surface

D₂-SPN D₂ receptor-expressing spiny projection neuron

DAT dopamine transporter

DLS dorsolateral striatum

DMS dorsomedial striatum

DOPAL 3,4-dihydroxyphenylacetaldehyde

EA extended amygdala

ECoG electrocorticogram

FoxP2 Forkhead box protein P2

GABA γ -aminobutyric acid

GFAP glial fibrillary acidic protein

GFP green fluorescent protein

GIRK2 G-protein-regulated inward-rectifier potassium channel 2

GPe external globus pallidus

GPi internal globus pallidus

GRP gastrin-releasing peptide

HCN hyperpolarisation-activated cyclic nucleotide-gated channel

IPAC interstitial nucleus of the posterior limb of the anterior commissure

IQR interquartile range

ISI interspike interval

LC locus coeruleus

LDTg laterodorsal tegmentum

LFP local field potential

LHb lateral habenula

Lhx6 LIM homeobox protein 6

M-L medial-lateral distance from the midline

MAO monoamine oxidase

MOR μ opioid receptor

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MT medial terminal nucleus

NAc nucleus accumbens

NAcLatS nucleus accumbens lateral shell

NAcMedS nucleus accumbens medial shell

NAcVentS nucleus accumbens ventral shell

NDNF Neuron Derived Neurotrophic Factor

NDS normal donkey serum

Nkx2-1 NK2 Homeobox 1

NMDA N-methyl-D-aspartate

NOS nitric oxide synthase

NpHR *Natronomonas pharaonis* halorhodopsin

OT olfactory tubercle

Otx2 orthodenticle homolog 2

PAG periaqueductal gray

PBN parabrachial nucleus

PBP parabrachial pigmented nucleus of the VTA

PBS phosphate buffered saline

PBS-azide phosphate-buffered saline containing 0.05% sodium azide

PBS-T phosphate-buffered saline with 0.3% (v/v) Triton X-100 and 0.03% sodium azide

PD Parkinson's disease

PFA paraformaldehyde in 0.1 M phosphate buffer, pH 7.4

PFC prefrontal cortex

PHA-L *Phaseolus vulgaris* leucoagglutinin

PKA protein kinase A

PPN pedunclopontine nucleus

PSTH peri-stimulus histogram

pSTN paraventricular nucleus

PV parvalbumin

PVH paraventricular hypothalamus

RGS robust gaussian surprise

RLi rostral linear nucleus

RMTg rostromedial tegmental nucleus

RPE reward prediction error

RRF retrorubral field

RTPA real-time place aversion

RTPP real-time place preference

RV-eGFP pseudotyped RG-deleted rabies virus

SatB1 special AT-rich sequence binding protein 1

SD standard deviation

SEM standard error of the mean

SNc substantia nigra pars compacta

SNL substantia nigra pars lateralis

SNr substantia nigra pars reticulata

Sox6 SRY-Box Transcription Factor 6

SPN spiny projection neuron

STN subthalamic nucleus

SWA slow-wave activity

TH tyrosine hydroxylase

TTX tetrodotoxin

VLS ventrolateral striatum

VMS ventromedial striatum

VP ventral pallidum

VTa ventral tegmental area

WGA-HRP wheat germ agglutinin conjugated to horseradish peroxidase

Zdhhc2 zinc finger DHHC domain containing 2

ZI zona incerta

Chapter 1

General Introduction

Research efforts continue to uncover nuances in the function of the midbrain dopaminergic system, reframing the perception of its role in the brain. Much of this effort derives from the association between dopaminergic dysfunction and neurological disease. Degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) is the defining feature of Parkinson's disease (PD) (Hassler, [1938](#); Hirsch et al., [1988](#)), underlining the importance of dopamine in movement control. On the other hand, elevated dopaminergic activity is associated with schizophrenia (Bogerts et al., [1983](#); Howes et al., [2013](#)) and drug addiction (Di Chiara and Imperato, [1988](#); Volkow et al., [2017](#); Wise and Robble, [2020](#)). The involvement of dopamine dysfunction in a variety of seemingly disparate disorders demonstrates the multifaceted role of the midbrain dopaminergic system. The work in this thesis will be carried out in mice, and thus any literature referred to will be in the mouse unless otherwise stated.

First described in the rat, midbrain dopaminergic neurons are parcellated into three main cell groups: A8, A9 and A10 (Dahlström and Fuxe, [1964](#)). These three groups are most commonly referred to as the retrorubral field (RRF), SNc and ventral tegmental area (VTA), respectively. It is important to note that the RRF, SNc and VTA are not solely composed of dopaminergic neurons as they contain non-dopaminergic and dual transmitter neuron populations (mouse: Kim et al., [2015](#); rat: Nair-Roberts et al., [2008](#); Morales and Root, [2014](#)). The VTA contains the largest proportion of

midbrain dopaminergic neurons (>50%), followed by the SNc (30–40%) and the RRF (10%) in the mouse (Nelson et al., 1996) and rat (Nair-Roberts et al., 2008). In the non-human primate and humans, however, the SNc population is expanded, containing ~70% of all midbrain dopaminergic neurons (François et al., 1999; Bentivoglio et al., 2005). The functional properties of dopaminergic neurons in the SNc and VTA have been extensively characterised in terms of connectivity, physiology, molecular makeup and roles in behaviour (reviewed in: Björklund and Dunnett, 2007; Bromberg-Martin et al., 2010; Paladini and Roeper, 2014; Coddington and Dudman, 2019; Schultz, 2019; Poulin et al., 2020). In comparison, knowledge of RRF dopaminergic neuron function is much less mature, leaving much to be discovered.

The RRF is the most cell group of the dopaminergic midbrain which have been described in the rat (Nair-Roberts et al., 2008), mouse (Fu et al., 2012; see Fig 1.1), non-human primate (Felten et al., 1974; François et al., 1999) and human brain (Hirsch et al., 1988; McRitchie et al., 1996). At its most rostral aspect, the RRF emerges as a cluster of dopaminergic neurons that form a mediolateral bridge between the parabrachial pigmented nucleus of the VTA (PBP) and the caudal SNc in the rat (Deutch et al., 1988) and mouse (Fu et al., 2012). At its intermediate and caudal aspects, the RRF forms an entirely distinct band of dopaminergic neurons above the rostral pedunculopontine nucleus (PPN).

There is an increasingly well-developed and nuanced understanding of the SNc and VTA dopaminergic systems as new techniques have emerged to permit finer dissection of the properties of these cell groups. However, such dissection has not been applied to the RRF. A PubMed search highlights the knowledge disparity between SNc/VTA and RRF dopaminergic systems: “substantia nigra pars compacta dopamine neurons” returns 3214 results; “ventral tegmental area dopamine neurons” returns 4216 results; “retrotrubral field dopamine neurons” returns 81 results (21 April 2021).

It is increasingly apparent that the midbrain dopaminergic system is functionally heterogeneous (Lammel et al., 2014; Engelhard et al., 2019). Therefore, uncovering the functional properties of RRF dopaminergic neurons is important, particularly in light of the scarcity of studies focusing on this

cell group. A more advanced understanding of RRF dopaminergic neurons may also provide pointers towards the role of this cell group in disease states. For example, it is known that aldehyde dehydrogenase 1a1 (Aldh1a1)-expressing dopaminergic neurons are particularly vulnerable to cell death in PD (McCaffery and Drager, 1994; Liu et al., 2014; Poulin et al., 2014), a small proportion of which are within the RRF (Poulin et al., 2014). However, little is known about the functional properties of these neurons or whether they are as equally vulnerable to cell death as Aldh1a1-expressing SNc dopaminergic neurons.

1.1 Anatomical boundaries of midbrain dopaminergic neurons

The dopaminergic input to distinct forebrain regions can be traced back to topographically distinct clusters of midbrain neurons with varying degrees of overlap (Björklund and Dunnett, 2007), suggesting that anatomically dividing the dopaminergic midbrain into A8, A9 and A10 is functionally relevant (Dahlström and Fuxe, 1964). However, the dopaminergic neurons of the RRF, SNc (including its most lateral extension in the substantia nigra pars lateralis (SNL)) and VTA are continuous with one another, precluding the delineation of clear anatomical boundaries between them. There are, however, features of these cell groups that aid in approximating their boundaries. For example, the dopaminergic neurons of the ventral SNc are densely packed in a medioventral orientation, transitioning to a mediolateral orientation in the dorsal SNc and more loose arrangement in the VTA, in both the mouse (Fu et al., 2012) and rat (Björklund and Lindvall, 1975; Fallon and Moore, 1978).

Although the SNc and VTA dopaminergic cell groups are often treated as distinct, it is worth noting that early studies in the primate (Lynd-Balta and Haber, 1994) and rat (Fallon and Moore, 1978) determined that, in terms of axonal projections, it may be more useful to consider these midbrain dopaminergic neurons as ‘dorsal tier’ (projecting mainly to ventral forebrain structures) and ‘ventral tier’ (projecting mainly to dorsal striatum) subgroups (Fallon and Moore, 1978). The dorsal tier includes dorsal SNc and VTA dopaminergic neurons, and the ventral tier is the ventral

SNC, ventral VTA and displaced dopaminergic neurons in the substantia nigra pars reticulata (SNr) (Fallon and Moore, 1978; Lynd-Balta and Haber, 1994). Functional differences between these two divisions support the anatomical classification of the dorsal and ventral tiers, in terms of axonal projection patterns in the striatum (rat: Fallon and Moore, 1978; Veening et al., 1980; Gerfen et al., 1987a; non-human primate: Lynd-Balta and Haber, 1994) and differential vulnerability in PD. The degeneration of dopaminergic neurons in PD occurs earliest and most severely in the ventral tier of dopaminergic neurons (Gibb and Lees, 1991; Damier et al., 1999b). Within this framework, dopaminergic neurons of the RRF belong to the dorsal tier in the primate (François et al., 1999).

Mice were the species of choice for all experiments in this thesis. Mice were chosen for two main reasons: i) there is a well-established dopamine transporter (DAT)-Cre knock-in mouse that allows dopaminergic neurons to be selectively accessed; ii) much current research on midbrain dopaminergic neurons is carried out in mice, making for easier comparisons between the results of this thesis and the literature. The coronal section schematics in Figure 1.1 illustrate anatomical organisation of the main dopaminergic cell groups in the caudal midbrain of the mouse, according to Paxinos and Franklin (2013). The rostral RRF interfaces with the caudal SNC laterally and the PBP medially (Figure 1.1B, C). As is the case throughout the midbrain, the dopaminergic neurons at this level are spatially contiguous, meaning it is hard to objectively define the boundaries of the RRF, SNC and VTA. However, there are some subtle differences between these cell groups that aid in approximating their anatomical boundaries. The generally mediolateral orientation of neurons in the RRF is distinct from the dorsoventral orientation of those in the SNC. Furthermore, the PBP is very cell-sparse at this level, distinguishable from the cell-dense RRF (Fu et al., 2012). The caudal RRF occupies a dorsal position in the midbrain and is separate from the caudal linear nucleus of the raphe (CLi) medially, making it clearly distinguishable as a cell group (Figure 1.1D–F).

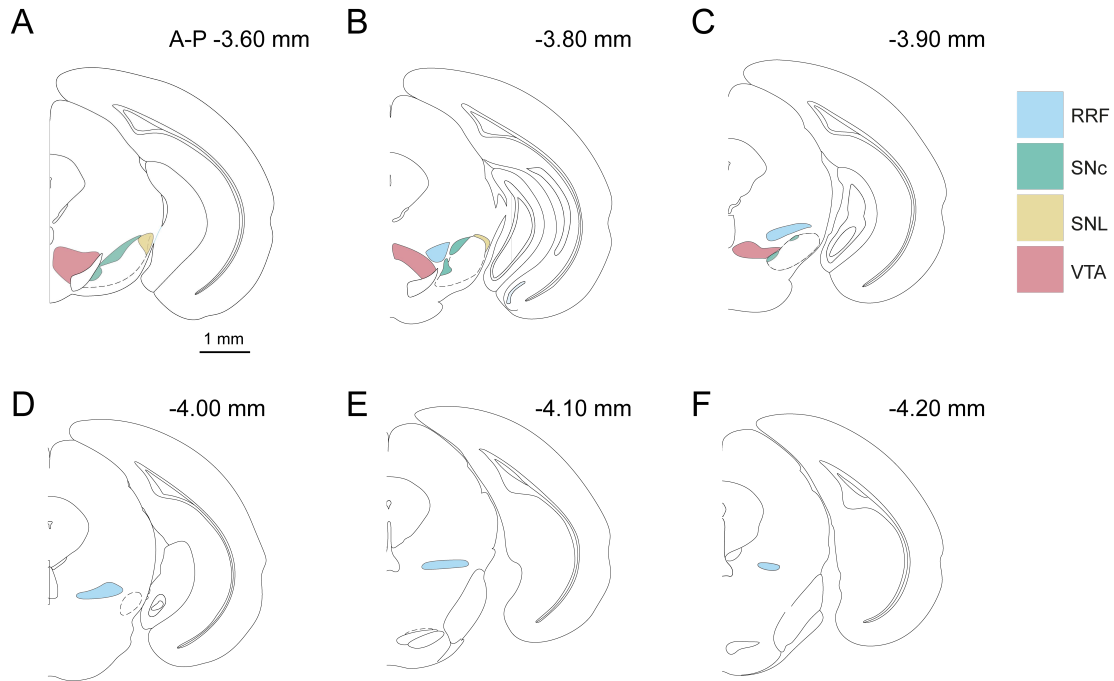


Figure 1.1: Schematic coronal sections of the midbrain dopaminergic system in the mouse. The midbrain dopaminergic cell groups are colour-coded, according to the key (top right). The SNr outline is indicated by the dashed line. The anterior-posterior (A-P) position is the distance from Bregma in mm. These schematics are adapted from Paxinos and Franklin (2013). SNL, substantia nigra pars lateralis.

1.2 Molecular diversity of midbrain dopaminergic neurons

Determining the molecular signature of a group of neurons is an essential aspect of their classification (Zeng and Sanes, 2017), without which many experimental approaches would be imprecise and untargeted. In the basal ganglia, for example, prototypic neurons of the external globus pallidus (GPe) that express LIM homeobox protein 6 (Lhx6), parvalbumin (PV) and NK2 Homeobox 1 (Nkx2-1) project mainly to the subthalamic nucleus (STN), whereas arkypallidal neurons of the GPe express Forkhead box protein P2 (FoxP2) and project mainly to the striatum (rat: Mallet et al., 2012; Abdi et al., 2015). These two populations of neurons are intermingled in the GPe but have distinct developmental, physiological and behavioural profiles (Dodson et al., 2015). Uncovering molecular markers in this way provides an avenue for selective genetic access to a cell type that is intermingled amongst others in a region, allowing for precise and targeted

manipulations. Moreover, defining a cell type based on a molecular marker provides a proxy for assessing other functional properties (e.g. firing properties and roles in behaviour).

In the rat, co-expression of DAT and tyrosine hydroxylase (TH) is one method to define midbrain neurons as dopaminergic (Shimada et al., 1992; Augood et al., 1993; Cerruti et al., 1993), distinct from TH-expressing noradrenergic/adrenergic neurons of the brainstem that lack DAT (Lorang et al., 1994). This is, however, not a complete definition because DAT expression varies throughout the dopaminergic midbrain. SNc dopaminergic neurons express higher levels of DAT than those of the VTA in the rat (Sanghera et al., 1994; Freed et al., 1995) and mouse (Lammel et al., 2008), and some VTA dopaminergic neurons lack DAT expression entirely (mouse: Lammel et al., 2008; human: Lammel et al., 2008; Reyes et al., 2013). TH expression is a robust marker for identifying midbrain dopaminergic neurons because TH-expressing neurons in the midbrain do not express the enzyme dopamine- β -hydroxylase (mouse: Fu et al., 2012; rat: Hartman et al., 1972), which is required to convert dopamine to noradrenaline. Moreover, midbrain dopaminergic neurons are anatomically separate from the TH-expressing noradrenergic/adrenergic cell groups of the brain stem, and can thus be easily distinguished. TH and DAT expression therefore provide an access point for profiling the molecular heterogeneity of midbrain dopaminergic neurons. The distinct axonal projections, roles in behaviour and differential vulnerability in PD raises the question: do the functional differences between dopaminergic neuron subpopulations map on to different molecular profiles?

The calcium-binding protein calbindin-D-28K (hereafter referred to as calbindin-1) is an example of a molecular marker that is differentially expressed in the midbrain, with significance in terms of dopaminergic neuron physiology and disease vulnerability. Calbindin-1 is expressed in most dopaminergic neurons of the VTA but is lacking in the ventral SNc in the mouse (Liang et al., 1996; Fu et al., 2012), rat (Gerfen et al., 1987a; Rogers, 1992) and non-human primate (Dopeso-Reyes et al., 2014), thus providing a useful marker for delineating these structures. Calbindin-1 is also expressed in the dorsal SNc in the rat (Gerfen et al., 1987a; Rogers, 1992) and the

non-human primate (Dopeso-Reyes et al., 2014). In mice, calbindin-1 expression in the dorsal SNc is modest in some studies (Liang et al., 1996) and minimal in others (Fu et al., 2012), which may be due to the difficulty in defining the border between dorsal SNc and lateral VTA in this species. In addition to having a distinct anatomical distribution, calbindin-1-expressing dopaminergic neurons have different electrophysiological characteristics to calbindin-1-negative neurons. Calbindin-1-positive dopaminergic neurons in the SNc fire slower than calbindin-1-negative neurons both *in vitro* (Neuhoff et al., 2002) in the mouse and *in vivo* (Brown et al., 2009) in the rat, whereas in the VTA the opposite is true, at least in mice (Neuhoff et al., 2002). These anatomical and electrophysiological differences may also be relevant to understanding the pathology of PD as calbindin-1-expressing dopaminergic neurons are less susceptible to degeneration in PD (Yamada et al., 1990), and in mouse (German et al., 1992; Liang et al., 1996) and non-human primate PD models (German et al., 1992).

Before the routine use of high-throughput technologies for determining the gene expression profiles of cell groups, molecular markers were often pre-selected, thus painting an incomplete picture of molecular heterogeneity. More recently, high-throughput gene expression profiling in the mouse (Chung et al., 2005; Brichta et al., 2015) and rat (Grimm et al., 2004; Greene et al., 2005) has allowed the gene expression profiles of SNc and VTA dopaminergic neurons to be directly compared. Specifically, transcripts enriched in the SNc include zinc finger DHHC domain containing 2 (*Zdhhc2*), special AT-rich sequence binding protein 1 (*SatB1*) and SRY-Box Transcription Factor 6 (*Sox6*), and in the VTA enriched transcripts include gastrin-releasing peptide (GRP) and orthodenticle homolog 2 (*Otx2*) (Chung et al., 2005; Brichta et al., 2015). Interestingly, *Sox6* and *Otx2* are vital determinants of SNc and VTA specification, respectively, during development (Panman et al., 2014). Although insightful, the binary distinction between the SNc and VTA in these studies presumes that they are homogeneous groups of neurons and therefore misses any within-region heterogeneity.

A more holistic approach is single-cell gene expression profiling of a cross-section of midbrain

dopaminergic neurons, followed by clustering analysis. This unbiased method results in five clusters of dopaminergic neurons (Poulin et al., 2014; La Manno et al., 2016), each with a unique combination of enriched transcripts and anatomical distribution. Importantly, although some clusters are confined to the SNc or VTA, other clusters are found within both groups. One cluster that is mostly confined to the ventral SNc expresses *Aldh1a1*, neuron-derived neurotrophic factor (*Ndnf*) and *Sox6* but does not express calbindin-1 (*Calb1*). A neighbouring cluster of neurons that is present in both the dorsal SNc and lateral VTA expresses *Ndnf* and *Sox6* but lacks *Aldh1a1* (Poulin et al., 2014). It could be posited that these molecularly-distinct ventral and dorsal cell groups represent the ventral and dorsal tiers outlined in early anatomical studies (rat: Fallon and Moore, 1978; non-human primate: Lynd-Balta and Haber, 1994). The dopaminergic neurons of the VTA fall into four clusters with differential gene expression. The first cluster of VTA dopaminergic neurons overlaps with neurons in the SNc (*a priori* dorsal tier neurons), whereas the other three clusters encompass more medial and ventral VTA dopaminergic neurons (Poulin et al., 2014). For example, dopaminergic neurons expressing *Aldh1a1*, calbindin-1 and *Otx2* are confined to the ventromedial VTA.

Within these single-cell expression profiling studies, some hints emerge as to where RRF dopaminergic neurons fit within these five clusters. Within the cluster of dopaminergic neurons that map mostly onto neurons of the ventral SNc (*Aldh1a1*⁺, *Ndnf*⁺, *Sox6*⁺), a small number are found scattered in the RRF (Poulin et al., 2014). Similarly, within the cluster of neurons that map mostly onto the dorsal SNc and rostral-dorsal VTA (*Aldh1a1*⁻, *Ndnf*⁺, *Sox6*⁺), some are represented in the RRF (Poulin et al., 2014). However, the molecular marker profile of RRF dopaminergic neurons from this study is incomplete (i.e. most RRF dopaminergic neurons are not represented in the five molecularly-distinct clusters), suggesting that only a small proportion of the single neurons that contributed to the clustering analysis were derived from the RRF.

1.3 Axonal projections of midbrain dopaminergic neurons

Midbrain dopaminergic neurons project to many forebrain regions, with the densest terminal fields in the striatum, amygdala and cortex (Fallon, 1988; Björklund and Dunnett, 2007). Mapping the relationship between the location of dopaminergic cell bodies in the midbrain and their axonal projection pattern offers an insight into the functional divisions of the midbrain dopaminergic system. In rodent studies, targeted deposits of retrograde tracers into subdivisions of the striatum and amygdala reveal that, on the macroscale, the medial-lateral and anterior-posterior position of midbrain dopaminergic neurons is organised topographically with respect to forebrain axonal targets in the mouse (Lerner et al., 2015) and rat (Fallon and Moore, 1978). On the other hand, a study in the non-human primate found little evidence for mediolateral or anterior-posterior topography (Lynd-Balta and Haber, 1994).

In the dorsal-ventral plane, this topography is reversed, such that ventrally located SNc dopaminergic neurons project mostly to the dorsal striatum in the mouse (Farassat et al., 2019), rat (Fallon and Moore, 1978) and the non-human primate (Lynd-Balta and Haber, 1994; François et al., 1999), whereas dorsally located dopaminergic neurons (SNc and lateral VTA) project to the ventral striatum (rat: Fallon et al., 1978; non-human primate: Lynd-Balta and Haber, 1994; François et al., 1999) and amygdala (Fallon et al., 1978). The inverse dorsoventral topography of the SNc overlaps in the medial SNc, where dopaminergic neurons project to both the dorsal and ventral striatum. In the VTA, this dorsal-ventral topography is also evident as ventral VTA dopaminergic neurons project to dorsal target regions and vice versa (rat: Breton et al., 2019). This inverse dorsoventral topography of midbrain projections is also evident in human diffusion tensor imaging experiments (Chowdhury et al., 2013; Zhang et al., 2017), suggesting this anatomical relationship is evolutionarily conserved.

Similar methodologies have demonstrated that the prelimbic, anterior cingulate and entorhinal cortices, nucleus accumbens (NAc) and hippocampus have dopaminergic terminal fields that originate in the VTA (mouse: Lammel et al., 2008; rat: Scatton et al., 1980; Swanson, 1982; Verney

et al., 1985; Gasbarri et al., 1994). Although there is some topographical organisation of VTA dopaminergic neurons with respect to projection-target, they are mostly intermingled (mouse: Lammel et al., 2008; Rodríguez-López et al., 2017; Farassat et al., 2019; Tang et al., 2020; rat: Swanson, 1982; Breton et al., 2019). Systematic retrograde tracing from subdivisions of the striatum, amygdala and neocortex results in dopaminergic neuron labelling patterns in the VTA that are distinct but largely overlapping in the rat (Breton et al., 2019), meaning that cell body location in the VTA cannot be used as a proxy for projection target.

In the rat, dopaminergic terminal fields in the cortex are limited to the anterior cingulate, prelimbic and entorhinal cortices (Fallon, 1981; Swanson, 1982; Chandler et al., 2013), as well as a sparse projection to primary motor cortex (Hosp et al., 2015). The VTA and medial SNc are the primary sources of dopaminergic input to these cortical regions. In the non-human primate (Berger et al., 1986; Lewis et al., 1987; Williams and Goldman-Rakic, 1993; Williams and Goldman-Rakic, 1998; Zubair et al., 2021) and humans (Gaspar et al., 1989), however, dopaminergic terminal fields are widespread throughout most of the cortex and originate from all divisions of the dopaminergic midbrain (Williams and Goldman-Rakic, 1998), pointing towards an expanded role of cortical dopamine in these species. The expansion of cortical dopamine in the non-human primate and humans is paralleled by widespread dopaminergic innervation of the thalamus, whereas in the rat dopaminergic axons are limited to the ventral thalamus and epithalamus (García-Cabezas et al., 2009).

Although projection-defined dopaminergic neurons are intermingled, most studies using dual/triple-site retrograde tracing found little evidence for axon collateralisation across multiple brain regions and conclude that most midbrain dopaminergic neurons project to a single target region in the mouse (Lammel et al., 2008; Rodríguez-López et al., 2017; Farassat et al., 2019) and rat (Fallon, 1981). However, there are populations in the medial SNc (rat: Fallon, 1981) and VTA (Aransay et al., 2015; Beier et al., 2015) that have prominent axon collaterals. Transducing single dopaminergic neurons with a fluorescent protein (such as with the Sindbis-pal-eGFP viral vector) is

a powerful method for investigating axon collateralisation, which has demonstrated that while some VTA dopaminergic neurons have extensive collateralisation across multiple target regions in the rat (Aransay et al., 2015), neighbouring SNc neurons have very little extensive collateralisation (Matsuda et al., 2009). Specifically, some VTA dopaminergic neurons that project to the ventral striatum have axon collaterals that project caudally to brainstem nuclei (Aransay et al., 2015), whereas SNc neurons projecting to the striatum have only minor collaterals in the GPe and no extensive collateralisation in other sites (Matsuda et al., 2009). Cell type-selective anterograde tracing has demonstrated that VTA dopaminergic neurons projecting to the nucleus accumbens lateral shell (NAcLatS) have axon collaterals in the dorsal striatum (Beier et al., 2015), indicating that experiments using the dual/triple-site retrograde tracing method (Lammel et al., 2008; Farassat et al., 2019) may have missed evidence of collateralisation due to the limits of the methodology.

Harnessing an intersectional genetics approach, it has been shown that molecularly-defined clusters of midbrain dopaminergic neurons have distinct axonal projection patterns (Poulin et al., 2018), as illustrated in Figure 1.2. For example, ventral SNc dopaminergic neurons (*Aldh1a1*⁺, *Calb1*⁺) project to dorsolateral striatum (DLS) (Figure 1.2A), whereas dorsal tier neurons (*Aldh1a1*⁺, *Calb1*⁺) project to the ventromedial striatum (VMS) (Figure 1.2B). Moreover, these transcriptionally-distinct clusters of neurons have differential vulnerability in mouse models of PD, such that dopaminergic neurons of the ventral SNc (*Aldh1a1*⁺, *Calb1*⁺) are vulnerable to degeneration whereas the other clusters are relatively resistant (Poulin et al., 2014).

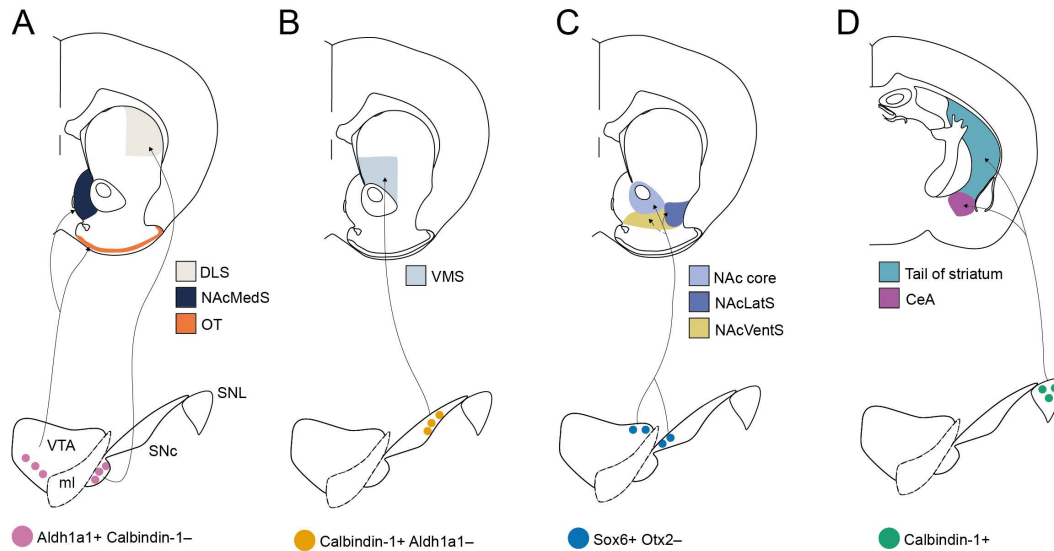


Figure 1.2: Molecularly-defined dopaminergic neuron subgroups have distinct axonal projections. **A**, Aldh1a1-expressing dopaminergic neurons of the VTA project to the nucleus accumbens medial shell (NAcMedS) and olfactory tubercle (OT), and those of the ventromedial SNc project to the DLS. **B**, Calbindin-1-expressing dopaminergic neurons of the dorsal SNc project to the VMS. **C**, Sox6-expressing dopaminergic neurons of the dorsolateral VTA and medial SNc project to the NAc core, NAcLatS and nucleus accumbens ventral shell (NAcVentS). The dashed arrow represents a minor projection. **D**, Calbindin-1-expressing dopaminergic neurons of the SNL project to the tail of the striatum and central amygdala (CeA). These schematics are based on the results of a projection-mapping study using an intersectional genetics approach (Poulin et al., 2018). Coronal schematics adapted from Paxinos and Franklin (2013). ml, medial lemmiscus.

Where do RRF dopaminergic neurons fit into this schema? The axonal projection targets of RRF neurons have been mapped with ‘classical’ anterograde and retrograde tracers. Injections of classical, non cell type-selective anterograde tracers into the RRF and surrounding neuropil results in the sparse labelling of axonal projections throughout the striatum in the rat (Gerfen et al., 1987a; Deutch et al., 1988) and the non-human primate (François et al., 1999). Retrograde tracing in the non-human primate (François et al., 1999) and rodents (mouse: Farassat et al., 2019; rat: Deutch et al., 1988) confirms that some of these axonal projections derive from dopaminergic (i.e. TH-positive) neurons in the RRF, and that the most prominent RRF dopaminergic projection is to the ventrolateral striatum (VLS).

Beyond the striatum, a combination of classical anterograde and retrograde tracing in the rat has shown that many other ventral forebrain regions receive dopaminergic innervation from the RRF, including the bed nucleus of the stria terminalis (BNST), amygdala and ventral pallidum (Deutch et al., 1988), and in the non-human primate innervation of the STN has been demonstrated (François et al.,

2000). In addition to these distal projections, there is evidence that RRF dopaminergic neurons project to the nearby VTA in the rat (Deutch et al., 1988) and cats (Arts et al., 1996). Studies tracing the axons of RRF dopaminergic neurons have so far evidenced a subset of their projections. However, although the range of projection sites targeted with retrograde tracers was broad, it was not exhaustive; therefore, a brain-wide map of RRF dopaminergic neuron axonal projections is lacking.

1.4 Inputs to midbrain dopaminergic neurons

Characterising the circuits into which midbrain dopaminergic neurons are embedded is crucial if their function is to be understood. As described in the previous section, determining the regions that receive dopaminergic projections is one aspect of this; as important is mapping the source of direct inputs to dopaminergic neurons. The inputs to midbrain dopaminergic neurons arise from many areas of the brain (mouse: Watabe-Uchida et al., 2012; Beier et al., 2015; rat: Zahm et al., 2011), a significant proportion of which are excitatory glutamatergic inputs from both cortical and subcortical regions (rat: Geisler et al., 2007). In addition to this excitatory input, γ -aminobutyric acid (GABA)ergic spiny projection neurons (SPNs) of the dorsal and ventral striatum, especially those in the striosomal compartment, densely innervate the SNc and VTA (mouse: Watabe-Uchida et al., 2012; Beier et al., 2015; rat: Nauta et al., 1978; Gerfen, 1985; Bolam and Smith, 1990; Heimer et al., 1991).

Cell type-selective rabies virus-based retrograde tracing methods have identified the brain-wide monosynaptic inputs to SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012; Beier et al., 2015). The inputs to SNc and VTA neurons are diverse and overlapping, but some brain regions are quantitatively biased in their targetting of either the SNc or VTA. SNc dopaminergic neurons receive relatively dense inputs from brain regions associated with motor function, such as the STN, GPe and dorsal striatum, whereas VTA neurons are more densely innervated by the dorsal raphe (Watabe-Uchida et al., 2012). Some studies have shown that projection-defined subpopulations of midbrain dopaminergic neurons have distinct inputs. VTA dopaminergic neurons projecting to the NAcLatS have markedly more inputs from the NAcLatS (i.e. reciprocally

connected), NAc core, anterior cortex and dorsal striatum than other projection-defined subpopulations (Beier et al., 2015); and dopaminergic neurons projecting to the tail of the striatum receive much fewer inputs from the ventral forebrain than other populations of dopaminergic neurons (Menegas et al., 2015).

Another feature of inputs to midbrain dopaminergic neurons is reciprocal connectivity, meaning some subgroups of neurons receive a large input from their respective projection sites. Reciprocal connectivity has been demonstrated for dopaminergic neurons projecting to the dorsomedial striatum (DMS) and DLS in the mouse (Lerner et al., 2015), and to prefrontal cortex (PFC) in the rat (Carr and Sesack, 2000). Moreover, Aldh1a1-expressing SNc dopaminergic neurons project to the dorsal aspects of the rostral and intermediate striatum and receive a reciprocal pattern of SPN inputs (Wu et al., 2019). Others have shown, however, that the concept of reciprocity is not common throughout the dopaminergic midbrain and most dopaminergic neurons receive a similar set of inputs (Menegas et al., 2015).

It is important to be mindful that although input mapping studies quantify the density of inputs to midbrain dopaminergic neurons, they do not provide insight into the strength or nature of the synaptic connection. The laterodorsal tegmentum (LDTg) (Omelchenko and Sesack, 2005) and periaqueductal gray (PAG) (Omelchenko and Sesack, 2010) form both putatively excitatory (asymmetric) and putatively inhibitory (symmetric) synapses with VTA dopaminergic neurons in the rat. Moreover, the excitatory input from the LDTg targets NAc-projecting dopaminergic neurons, whereas the inhibitory input targets PFC-projecting dopaminergic neurons (Omelchenko and Sesack, 2005). Thus, not only is the source of inputs to midbrain dopaminergic neuron subpopulations projection site-dependent (Beier et al., 2015; Menegas et al., 2015), but also for some input sources the nature of their synapses with dopaminergic neurons is projection site-dependent.

Direct inputs to SNc and VTA dopaminergic neurons have been studied with both electron microscopy and rabies virus-based tracing. The direct inputs to RRF dopaminergic neurons, on the other hand, are not precisely defined. Classical anterograde and retrograde tracing studies have

shown that the RRF is innervated by brain regions that are known to also provide direct inputs to SNc and VTA dopaminergic neurons, such as the rostromedial tegmental nucleus (RMTg) (Kaufling et al., 2009), NAc (Nauta et al., 1978; Heimer et al., 1991), GPe (von Krosigk et al., 1992) and extended amygdala (Wallace et al., 1989, 1992; Zahm et al., 2011). Compared with the SNc/VTA, the RRF receives more terminals from the BNST and central amygdala (Zahm et al., 2011) and more noradrenergic input in the rat (Mejías-Aponte et al., 2009). This suggests the inputs to the RRF are distinct; however, none of these studies unequivocally determined whether the inputs formed synapses with dopaminergic neurons (rather than non-dopaminergic neurons) within the RRF.

1.5 Firing properties of midbrain dopaminergic neurons

Extracellular electrophysiological recordings (i.e. single units) of midbrain dopaminergic neurons *in vivo* reveal a slow firing rate (2–10 spikes/s) and broad (> 1.1 ms threshold-to-trough) action potential waveform (Ungless and Grace, 2012) (Figure 1.3A and B, left panels). Under general anaesthesia, dopaminergic neurons spontaneously discharge at ~5 spikes/s in the rat (Guyenet and Aghajanian, 1978; Grace and Bunney, 1984a; Ungless et al., 2004; Brown et al., 2009) and mice (Schiemann et al., 2012; Janezic et al., 2013), which is remarkably similar to the firing rate seen at rest in awake animals (mouse: Dodson et al., 2016; rat: Hyland et al., 2002). This suggests there is good preservation of firing properties in the presence of some anaesthetic agents. Although the criteria used to putatively identify midbrain dopaminergic neurons are largely accurate, there are neurons in the VTA that have a broad (> 1.1 ms) action potential waveform and slow firing rate (< 10 spikes/s) that are non-dopaminergic in the rat (Ungless et al., 2004). Therefore, cell-filling followed by immunolabelling (Figure 1.4A), or recordings coupled with optogenetics (Figure 1.4B), are needed for unequivocal identification of cell type. In addition to these techniques, systemic administration of the D₁/D₂ agonist apomorphine reduces the firing rate of midbrain dopaminergic neurons and is an established method for cell type identification (mouse: Sanghera et al., 1984; rat: Bunney et al., 1973).

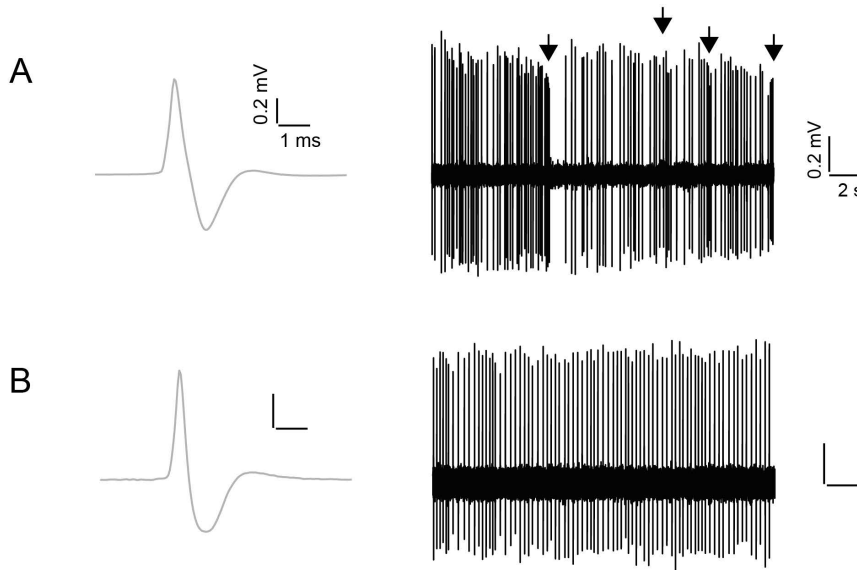


Figure 1.3: *In vivo* firing patterns of extracellularly-recorded identified midbrain dopaminergic neurons in urethane-anaesthetised mice. **A**, An irregularly firing identified midbrain dopaminergic neuron with occasional burst firing activity (arrows indicate bursts). **B**, A regularly firing identified midbrain dopaminergic neuron. Left panels are the action potential waveforms of the respective neurons. These plots are samples of my own recording data.

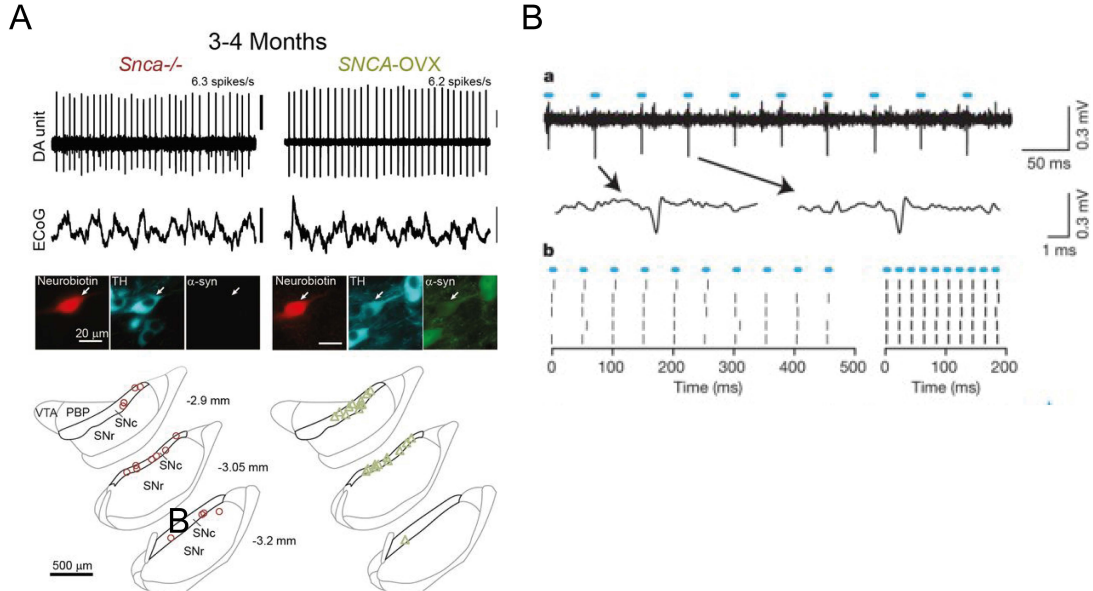


Figure 1.4: Methods for identifying midbrain dopaminergic neurons recorded *in vivo*. **A**, *In vivo*, juxtacellular labelling of extracellularly-recorded neurons with Neurobiotin permits *post hoc* confirmation of dopaminergic identity (Neurobiotin⁺ tyrosine hydroxylase (TH)⁺) and anatomical location. Adapted from Janezic et al. (2013), Figure 6. **B**, Extracellular recording of a midbrain dopaminergic neuron transduced with channelrhodopsin-2 (ChR2). Application of blue (473 nm) laser pulses evokes time-locked firing of transduced neurons, termed 'opto-tagging'. This permits recorded neurons to be identified as dopaminergic because ChR2 expression is restricted to midbrain dopaminergic neurons (injection of a Cre-dependent AAV encoding ChR2 into the midbrain of DATr-Cre mice in this case). Adapted from Cohen et al. (2012), Figure 3.

The majority of midbrain dopaminergic neurons fire in an irregular pattern (Figure 1.3A, right panel); other subpopulations fire regularly, or in episodic bursts superimposed over an irregular background rate (mouse: Figure 1.3B, right panel; rat: Tepper et al., 1995). An *in vivo* burst starts with an initial interspike interval (ISI) of < 80 ms for the first two spikes, increasing to an ISI of > 160 ms for the last two spikes of the burst in the rat (Grace and Bunney, 1983, 1984b). The intra-burst firing frequency is often >20 spikes/s in the rat (Hyland et al., 2002), which is markedly higher than the tonic rate between bursts. There is also a notion that some dopaminergic neurons are in fact ‘silent’ under resting conditions from experiments in the rat, meaning they do not discharge spikes. In the rat, manipulating inputs to midbrain dopaminergic neurons is postulated to bring silent neurons ‘on-line’, leading to more recorded neurons per electrode track (Floresco et al., 2001, 2003), and early *in vivo* intracellular recordings also showed that application of glutamate causes silent neurons to fire (Grace and Bunney, 1984a). However, the idea of ‘silent’ midbrain dopaminergic neurons has been refuted by another study in which very few (~2%) neurons were silent (rat: Dai and Tepper, 1998). Dopaminergic neurons that switch from silent to active *in vivo* in awake animals have not been observed, so it is unclear whether switching is a phenomenon that occurs during behaviour.

Midbrain dopaminergic neurons are autonomously active (i.e. they do not require inputs to fire), which is underlain by multiple membrane conductances. Their intracellular electrophysiological activity oscillates between a slow depolarising current before the action potential and a long afterhyperpolarisation (rat: Grace and Bunney, 1984a), both of which contribute to the slow and irregular firing pattern. The dynamics of firing are orchestrated by many membrane conductances, including subthreshold sodium currents (Khaliq and Bean, 2010), various voltage-gated calcium channels (Nedergaard et al., 1993; Mercuri et al., 1994; Chan et al., 2007; Puopolo et al., 2007), calcium-activated potassium channels (Grace and Bunney, 1984a; Shepard and Bunney, 1988; Harris et al., 1989), non-selective cation channels (Kim et al., 2007), A-type potassium channels (Liss et al., 2001) and hyperpolarisation-activated cation channels (Neuhoff et al., 2002). The membrane

conductances that govern the firing of midbrain dopaminergic neurons vary between subpopulations. In SNc dopaminergic neurons, calcium currents underpin the slow depolarisation necessary for intrinsic pacemaker activity, which is insensitive to the blockade of voltage-gated sodium channels with tetrodotoxin (TTX) (rat: Kang and Kitai, 1993), whereas the pacemaker activity of VTA dopaminergic neurons is underpinned by sodium currents (Khaliq and Bean, 2010). Within the SNc subgroup of dopaminergic neurons in the rat, there is marked heterogeneity between neurons in the A-type potassium and H-type cationic currents, allowing fine tuning of pacemaker activity (Amendola et al., 2012). Such variation in membrane conductances both between and within subgroups of midbrain dopaminergic neurons may contribute to the variation in baseline firing rate and pattern observed *ex vivo* and *in vivo*.

In the mouse and rat, it has been demonstrated that burst firing in midbrain dopaminergic neurons is essential for eliciting the phasic increase in striatal dopamine release (rat: Gonon, 1988; Suaud-Chagny et al., 1992) that underpins synaptic plasticity in SPNs (mouse: Shen et al., 2008; Yagishita et al., 2014; rat: Reynolds et al., 2001; Fisher et al., 2017), which is in turn required for behavioural reinforcement (mouse: Tsai et al., 2009; Zweifel et al., 2009; rat: Reynolds et al., 2001; Steinberg et al., 2013). Burst firing does not occur spontaneously *in vitro* in the rat (Grace and Onn, 1989) and cannot be induced with current injections (Richards et al., 1997), indicating a synaptic mechanism for this firing mode. Indeed, burst firing requires N-methyl-D-aspartate (NMDA) receptor activation that is provided by glutamatergic input in the rat (Overton and Clark, 1992; Chergui et al., 1993). This synaptic mechanism confers extrinsic control over burst firing, which is essential for the temporal encoding of behaviour, such as in response to unpredicted reward in mice (Cohen et al., 2012) and the non-human primate (Mirenowicz and Schultz, 1994). Indeed, selective deletion of NMDA receptors from dopaminergic neurons impairs learning in cue-reward association tasks (Zweifel et al., 2009). However, it is opportune to mention that deletion of NMDA receptors from dopaminergic neurons reduces, but does not abolish, burst firing in freely moving mice (Zweifel et al., 2009), suggesting additional mechanisms for burst firing.

The STN is a source of glutamatergic input that promotes burst firing in SNc dopaminergic neurons in the rat (Smith and Grace, 1992; Chergui et al., 1994), whereas for VTA dopaminergic neurons this input is provided by the PPN and LDTg (Floresco et al., 2003; Lodge and Grace, 2006; Dautan et al., 2016). In addition to these excitatory mechanisms, reducing GABAergic conductances disinhibits dopaminergic neurons and induces burst firing in the rat (Lobb et al., 2010) and computational models (Lobb et al., 2011). This means that sources of inhibitory input could induce burst firing in dopaminergic neurons by reducing GABAergic transmission. The dependence of burst firing on inputs (or lack thereof) means there is variation in this mode of activity across the midbrain, such that dopaminergic neurons of the VTA fire more of their action potentials in bursts than their counterparts in the SNc in the rat (Grenhoff et al., 1988; Marinelli and White, 2000). Within the VTA, dopaminergic neurons located ventromedially fire more bursts than those located laterally (Brischoux et al., 2009), which may correspond to high-bursting dopaminergic neurons that project to the PFC in the rat (Chiodo et al., 1984). Indeed, projection target is a crucial determinant of firing properties such as burst firing (Lammel et al., 2008; Farassat et al., 2019), suggesting that the circuits into which different subpopulations of dopaminergic neurons embed are functionally tuned.

On the other end of the activity spectrum, some dopaminergic neurons cease firing in response to aversive stimuli and/or reward omission in mice (Cohen et al., 2012) and the non-human primate (Schultz et al., 1993). Optogenetic activation of VTA GABAergic neurons pauses the firing of some dopaminergic neurons (Cohen et al., 2012; Tan et al., 2012), suggesting local circuitry may be involved in generating pauses. Moreover, brain regions that are proposed to relay aversion signals to midbrain dopaminergic neurons in the rat are glutamatergic (Coizet et al., 2006, 2010) and form excitatory synapses with mostly non-dopaminergic elements in the midbrain (Comoli et al., 2003). Therefore, it is likely that a di-synaptic mechanism is necessary to induce a pause in dopaminergic neuron firing via GABAergic neurons in the midbrain, as well as via GABAergic neurons in the RMTg of the mouse (Stamatakis and Stuber, 2012) and rat (Jhou et al., 2009).

Much of the work to describe the firing properties of midbrain dopaminergic neurons comes from recordings of SNc and VTA neurons. Extracellular recordings of putative RRF dopaminergic neurons in awake the non-human primate show that they are slow firing and have a broad action potential waveform (Schultz, 1986; Romo and Schultz, 1990; Ljungberg et al., 1992; Mirenowicz and Schultz, 1994; Fiorillo et al., 2013b), in line with their counterparts in SNc and VTA. However, to date, the firing properties of *identified* RRF dopaminergic neurons have not been described.

1.6 Behaviours associated with midbrain dopaminergic neuron function

Degeneration of nigrostriatal dopaminergic neurons (mainly located in the SNc) is the cardinal pathology of PD (Ehringer and Hornykiewicz, 1960), leading to poor control over movement. The symptoms of PD, such as bradykinesia and tremor, can be recapitulated when the ventromedial midbrain is lesioned in the non-human primate (Poirier, 1960; Péchadre et al., 1976) and alleviated when dopamine is repleted with L-DOPA (Goldstein et al., 1973). Moreover, selective degeneration of dopaminergic terminals in the dorsal striatum impairs action initiation in the rat (Carli et al., 1985). These observations naturally point towards a role for midbrain dopaminergic neurons in controlling movement. Early work correlating the activity of midbrain dopaminergic neurons to behaviour initially focused on variables related to movement. In reaching tasks, some putative dopaminergic neurons in the non-human primate midbrain transiently increased their firing rate during self-initiated movements, while a smaller population decreased their firing rate (Schultz et al., 1983; Schultz, 1986).

Movement-related changes in firing rate of putative midbrain dopaminergic neurons in the non-human primate are modest (Romo and Schultz, 1990). When food-deprived monkeys learn to associate a stimulus with the availability of food (reward), more pronounced and time-locked phasic increases in the firing rate of dopaminergic neurons occur in response to the reward than in

response to any associated movements. Moreover, the phasic increase in firing rate shifts from the reward itself early in learning to the cue predicting reward later in learning (Romo and Schultz, 1990; Ljungberg et al., 1992; Mirenowicz and Schultz, 1994). It is inherently challenging to parse the influence of stimuli and movement on firing dynamics in these studies. Reward availability and cue presentation both elicit movement in the non-human primate (Ljungberg et al., 1992) and thus stimuli and action are not orthogonal. Therefore, it is difficult to disentangle what is encoded by phasic changes in the firing rate of dopaminergic neurons.

It is proposed that the firing rate dynamics of midbrain dopaminergic neurons across learning represent a reward prediction error (RPE) signal in mice (Cohen et al., 2012; Eshel et al., 2016) and the non-human primate (Schultz et al., 1997), providing a neurophysiological basis for many behavioural theories of reinforcement learning (Pavlov, 1927; Rescorla and Wagner, 1972) and temporal-difference learning (Sutton, 1988; Sutton and Barto, 1998). Selectively activating dopaminergic neurons is sufficient to promote behavioural conditioning (Tsai et al., 2009), implying a causative role for this signal in reinforcement learning. Consistent with RPE signalling, some dopaminergic neurons pause their firing in response to aversive stimuli (mouse: Cohen et al., 2012; rat: Ungless et al., 2004; Brischoux et al., 2009; Brown et al., 2009), and in response to reward omission (mouse: Cohen et al., 2012; non-human primate: Schultz et al., 1993). This negative RPE signal fits neatly into computational models of reinforcement learning (Schultz et al., 1997) and is sufficient to drive learning in the rat (Chang et al., 2016). It should be noted that RPE-related phasic firing rate changes in midbrain dopaminergic neurons are not binary, but rather linearly related to the magnitude of the prediction error, in terms of response onset latency, duration and amplitude, as has been shown in the non-human primate (Bayer et al., 2007).

Although RPE signalling by dopaminergic neurons is a prevailing theory of their function, it is increasingly appreciated that the relationship between midbrain dopaminergic neuron activity and behaviour is heterogeneous (Lammel et al., 2014), extending beyond reward-guided learning. Indeed, not all dopaminergic neurons encode an RPE signal (Kaufmann, 2017), though possibly

those of the lateral VTA are more uniform in that respect (Eshel et al., 2016). In the non-human primate, prominent populations (25–45%) of midbrain dopaminergic neurons do not change their firing rate in response to variables in reward-guided learning tasks (Schultz, 1986; Romo and Schultz, 1990; Ljungberg et al., 1992). Furthermore, in the non-human primate, more dopaminergic neurons in the VTA (~50%) respond to reward than those in the SNc (~20%) during learning (Schultz et al., 1993); in mice, only 35% of SNc dopaminergic neurons respond to reward during learning (Da Silva et al., 2018). This distinction between VTA and SNc neurons is also present in the activity of dopaminergic axons in the NAc and dorsal striatum, respectively, wherein axons from the VTA are more reward-responsive than those from the SNc (Howe and Dombeck, 2016). The prominent populations of VTA and SNc neurons not responding to reward suggests a function beyond RPE encoding for some midbrain dopaminergic neurons.

Outside of a reward context, some midbrain dopaminergic neurons respond to non-appetitive, novel stimuli that are by nature salient (Bromberg-Martin et al., 2010). Subgroups of dopaminergic neurons in the VTA and SNc respond to alerting neutral stimuli with a phasic burst of action potentials in the non-human primate (Ljungberg et al., 1992) and cats (Strecker and Jacobs, 1985; Horvitz et al., 1997) in a manner that is not related to general arousal (Strecker and Jacobs, 1985) but rather to the attentional properties of stimuli. Indeed, the phasic burst of dopaminergic neuron activity in response to salient stimuli is attenuated after a small number of stimulus presentations (Ljungberg et al., 1992), indicating that stimulus novelty is crucial in determining this type of activity.

If the firing rate of a neuron phasically increases in response to both appetitive and aversive stimuli, this can be interpreted as representing motivational salience (Bromberg-Martin et al., 2010). In contrast, an increase and decrease, respectively, is in line with representing motivational value. In the anaesthetised preparation, some midbrain dopaminergic neurons increase their firing rate in response to noxious (putatively aversive) stimuli, whereas others decrease their firing rate in the mouse (Tan et al., 2012), rat (Chiodo et al., 1980; Ungless et al., 2004; Coizet et al., 2006; Brischoux

et al., 2009; Brown et al., 2009; Coizet et al., 2010) and non-human primate (Schultz and Romo, 1987). This suggests that there are different subpopulations of midbrain dopaminergic neurons that encode motivational salience and motivational value. Studies in which recorded neurons were juxtacellularly labelled (followed by immunolabelling for TH to unequivocally identify them as dopaminergic) have determined that most dopaminergic neurons in the SNc of the rat do not respond to a foot pinch stimulus and only a small subpopulation decrease their firing rate (Brown et al., 2009). However, in the SNc of the behaving non-human primate, the firing rate of dopaminergic neurons in the medial SNc represents motivational value, whereas the firing rate of those in the lateral SNc represents motivational salience (Matsumoto and Hikosaka, 2009). This medial vs lateral distinction may map onto neurons that project to the DMS and DLS, respectively, as has been shown in mice (Lerner et al., 2015). Lateral SNc neurons encoding motivational salience (Matsumoto and Hikosaka, 2009; Lerner et al., 2015) may also encompass the SNL. Indeed, the activity of SNL dopaminergic neurons (Steinberg et al., 2020) and their axons in the tail of the striatum (Menegas et al., 2015, 2017) increases in response to both aversive and appetitive stimuli.

In the VTA of the rat, some neurons that are putatively dopaminergic and activated by a foot pinch stimulus are actually non-dopaminergic (immunonegative for TH) when identified *post-hoc* (Ungless et al., 2004). In the rat, those identified dopaminergic neurons that increase their firing rate in response to a noxious stimulus are located in the ventromedial VTA (Brischoux et al., 2009), and those that either do not respond or decrease their firing rate are in the lateral VTA (Ungless et al., 2004; Brischoux et al., 2009). These studies taken together suggest that the firing rate of dopaminergic neurons in the ventromedial portion of the VTA represents motivational salience, whereas those in the lateral portion of the VTA represent motivational value.

The distinction between ventromedial and lateral VTA dopaminergic neurons is also evident in awake mice. Ventromedial and lateral VTA dopaminergic neurons have axonal projections to the NAcMedS and NAcLatS, respectively (de Jong et al., 2019). The activity of dopaminergic axons in the NAcMedS (measured using calcium imaging) increases in response to both aversive foot shock

and appetitive reward, whereas the activity of those in the NAcLatS decreases and increases in response to aversive foot shock and appetitive reward, respectively (de Jong et al., 2019). This anatomical segregation of responses aligns with the previous anaesthetised recording work in the rat (Brischoux et al., 2009). During fear conditioning, some VTA dopaminergic neurons (precise anatomical location not determined) phasically increase their firing rate in response to foot shock (Tang et al., 2020). This heterogeneous activity profile is also present when milder aversive stimuli are used. In head-fixed mice, VTA dopaminergic neurons decreased their firing rate in response to an air puff delivered to the face (Matsumoto et al., 2016), whereas in another study the opposite was observed (Hughes et al., 2020). In the latter study, the phasic increase in firing rate in response to air puff was related to the kinematics of a startle response (Hughes et al., 2020), suggesting the neuronal response was more tightly linked to resultant behaviour than motivational salience per se.

In behavioural tasks, the aversive value of stimuli is often not quantified and thus not parsed from the other qualities of the stimulus, such as novelty or salience. This makes it difficult to interpret the effect of aversive stimuli on dopaminergic neuron firing dynamics. For example, in many head-fixed behavioural paradigms, the aversiveness of an air puff may be less intense than the value of the reward in food/water-deprived animals. Fiorillo et al. (2013a) showed that when the aversiveness of an air puff is intense enough for a monkey to forgo reward in order to avoid it, the increase in firing rate of dopaminergic neurons (which could be regarded as representing motivational salience) is followed immediately by a decrease in firing rate. This pattern is interpreted as reflecting an initial sensory arousal response, followed by value coding immediately after. Therefore, in a reward context, the value of an aversive stimulus is encoded in the secondary phase of firing dynamics.

In addition to data from behavioural tasks, the role of midbrain dopaminergic neurons in aversive conditioning is evident from place preference experiments. Optogenetic inhibition of VTA dopaminergic neurons induces both real-time place aversion and conditioned place aversion (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014), indicating that artificially suppressing the firing of these neurons is sufficient to drive aversive conditioning. Similarly, optogenetic inhibition of SNc

dopaminergic neurons induces real-time place aversion (Ilango et al., 2014). However, it is important to note that in these optogenetic experiments dopaminergic neurons were suppressed for seconds (up to 30 s), whereas in behavioural tasks they tend to be suppressed for < 500 ms following an aversive stimulus (Matsumoto et al., 2016). Such artificial reductions in dopaminergic activity make it difficult to interpret the significance of these findings. This leads one to ask whether optogenetic suppression with shorter (i.e. < 500 ms), more physiologically-relevant stimulation parameters would also induce place aversion.

In the past decade, numerous reports have highlighted that phasic changes in the activity of midbrain dopaminergic neurons may be related to movement, both in untrained and instrumentally-conditioned animals. In mice that are not food/water-deprived, some dopaminergic SNc and VTA neurons pause during spontaneous movement (Dodson et al., 2016; Coddington and Dudman, 2018; Da Silva et al., 2018), whereas other populations in the SNc (Da Silva et al., 2018), VTA and SNL (Dodson et al., 2016) exhibit a pre-movement increase in firing rate. In food/water-deprived animals, an increase in the firing rate of some SNc dopaminergic neurons precedes action initiation (Jin and Costa, 2010; Da Silva et al., 2018), and SNc dopaminergic axons in the dorsal striatum are phasically activated before and during spontaneous movement (Howe and Dombeck, 2016). This diversity in firing rate dynamics around movement and action may be reflective of discrete functional subpopulations (Engelhard et al., 2019), or perhaps also reflective of the different types of movement/action studied. A recent study indicated that diversity with which VTA dopaminergic neurons represent movement may reflect both these factors, in that discrete subpopulations of neurons encode the direction of force generation with distinct firing patterns (Hughes et al., 2020). In the NAc, phasic dopamine release is contingent on goal-directed action initiation in the rat (Hamid et al., 2016; Syed et al., 2016), pointing towards a role of mesolimbic dopamine signalling in invigorating action. Taken together, these studies indicate that dopaminergic signalling is a gatekeeper of movement, which is further supported by optogenetic studies demonstrating that activation and suppression of SNc dopaminergic neurons promotes and

impairs, respectively, action initiation (Da Silva et al., 2018).

In a heterogeneous midbrain dopaminergic system, a question that emerges is whether individual dopaminergic neurons encode a single behavioural variable, or whether they multiplex information which is contextualised downstream. Most studies have focused on one aspect of dopaminergic function during behaviour; however, a recent study incorporated multiple behavioural variables in one task (Engelhard et al., 2019). Interestingly, before the reward outcome, the activity of spatially separate populations of VTA dopaminergic neurons reflects distinct subsets of sensory, motor and cognitive variables. In the outcome period, the phasic response to reward predominates across these populations in a more homogeneous manner. Others have suggested that the reward and movement-related activity of dopaminergic neurons (Da Silva et al., 2018), and their axons in the striatum (Howe and Dombeck, 2016), are represented by largely separate populations.

How the activity of RRF dopaminergic neurons varies during behaviour is mostly unknown. An important question would be whether RRF dopaminergic neurons are similar to SNc and VTA neurons in their encoding of RPE. Studies in the non-human primate have shown that putative RRF dopaminergic neurons are activated by rewards in a similar manner to neurons in the SNc or VTA (Schultz, 1986; Ljungberg et al., 1992; Mirenowicz and Schultz, 1994; Fiorillo et al., 2013b), indicating that they share the reward-encoding property. There have, however, been no *in vivo* electrophysiological recordings of *identified* RRF dopaminergic neurons during behaviour or in response to external stimuli.

Degeneration of midbrain dopaminergic neurons in PD causes profound deficits in motor function. Although neurons within the ventral SNc are most susceptible to degeneration, there is also a marked loss of RRF dopaminergic neurons (Hirsch et al., 1988; Damier et al., 1999b). In animal models, the neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) cause the death of some midbrain dopaminergic neurons, leading to impaired motor behaviours (Bové and Perier, 2012). In the rat 6-OHDA model, the largest loss of dopaminergic neurons occurs in the SNc, followed by the RRF

and VTA following intraventricular (Rodríguez et al., 2001) or intrastriatal injections (Healy-Stoffel et al., 2014) of the neurotoxin. Similarly, in the MPTP model, systemic injections of MPTP in mice (German et al., 1996) and non-human primates (German et al., 1988) induces the largest loss of dopaminergic neurons in the SNc, followed by the RRF and VTA. Taken together, these clinical and animal studies suggest some RRF dopaminergic neurons are susceptible to degeneration in PD. Selective lesioning of RRF dopaminergic neurons with 6-OHDA in cats leads to deficits in motor behaviour (Arts and Cools, 1998), providing evidence that loss of RRF dopaminergic neurons leads to behavioural changes in addition to the archetypal loss of SNc dopaminergic neurons.

1.7 Aims of research

The vast amount of research effort dedicated to the study of the midbrain dopaminergic system has focused on SNc and VTA neurons. This research has uncovered the functional diversity and heterogeneity of these midbrain dopaminergic neuron subgroups. Much less is known, however, about a third group of dopaminergic neurons in the RRF. In order to gain an insight into the functional properties of these neurons, the work in this thesis was focused on characterising their anatomical features, electrophysiological activity and potential roles in behaviour in mice. The specific aims were to:

- Describe the molecular marker profile of RRF dopaminergic neurons (Chapter 3).
- Map the anterograde projections of RRF dopaminergic neurons in the forebrain (Chapter 4).
- Map the monosynaptic inputs to RRF dopaminergic neurons (Chapter 5).
- Characterise the electrophysiological properties of RRF dopaminergic neurons and the effect of a noxious stimulus on their firing activity (Chapter 6).
- Determine whether optogenetically pausing the firing of VTA dopaminergic neurons induces place aversion (Chapter 7).

Chapter 2

Materials and Methods

2.1 Animals

All experiments were carried out in mice in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom) and performed during the light phase of a 12/12-h light/dark cycle (lights on 07:00–19:00). Conducting experiments during the light phase is not optimal because, since mice are nocturnal, it is outside of their active period. This issue is particularly pertinent for behavioural experiments. However, it was not possible for a reverse light/dark cycle to be implemented in the host animal facility, and thus this confound was unavoidable. Mice received food and water *ad libitum*.

Adult C57Bl/6J mice and mice expressing Cre recombinase under the control of the promoter of the DAT gene (DAT^{IRESCre}, The Jackson Laboratory, 006660; Bäckman et al., 2006) were used for all experiments. The knock-in mice, henceforth referred to as DAT-Cre mice, were heterozygous for the *Slc6a3*-internal ribosome entry site (IRES)-Cre transgene and were maintained on a C57BL/6J background. DAT-Cre mice were used when the experimental aims necessitated the selective expression of an opsin for optogenetic manipulations (channelrhodopsin-2 (ChR2); archaerhodopsin T (ArchT)), a fluorescent reporter protein for anterograde tracing, or a helper virus for monosynaptic input tracing from dopaminergic neurons. Both male and female mice were used. C57Bl/6J and DAT-Cre mice were housed with their littermates of the same sex in groups of 2–5,

though in some instances, where a DAT-Cre litter produced a single male, these were housed singly. Details regarding the number of mice used, their age range and sex can be found in the relevant methods section.

2.2 Molecular marker mapping

2.2.1 *Perfuse-fixation and tissue processing*

Adult male C57Bl/6J mice were administered a lethal dose of sodium pentobarbitone (0.3 ml; 20% w/v), and then transcardially perfused with ~20 ml of 0.1 M phosphate buffered saline (PBS), pH 7.4, followed by ~50–60 ml of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PFA). Brains were then removed and stored in 4% (w/v) PFA overnight at 4°C and then stored in phosphate-buffered saline containing 0.05% sodium azide (PBS-azide) (Sigma) until they were sectioned 12 h to 1 week later.

Fixed brains were sectioned to a thickness of 50 μm in the coronal plane with a vibrating-blade microtome (VT1000S; Leica Microsystems). The midbrain was sectioned into four series, meaning that in a single series the distance between adjacent sections was 200 μm . All midbrain sections were incubated in a 10% normal donkey serum (NDS) blocking solution (Jackson ImmunoResearch) for 1 h before incubation in primary antibodies.

Midbrain sections were incubated overnight at room temperature in a solution of phosphate-buffered saline with 0.3% (v/v) Triton X-100 and 0.03% sodium azide (PBS-T) and 1% NDS (Jackson ImmunoResearch) containing guinea pig anti-TH (1:1000; 213-104, Synaptic Systems). Midbrain sections were then washed for 5 minutes in PBS, three times, followed by overnight incubation at room temperature in PBS-T containing Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch). Sections were then washed for 5 minutes in PBS, three times, after which they were mounted on glass slides with aqueous mounting medium (Vectashield, Vector Laboratories) and viewed under an epifluorescence

microscope (Axioscope.A1, Zeiss). Sections were manually inspected and the anterior-posterior distance from Bregma (A-P) was determined, guided by TH immunoreactivity and a standard brain atlas (Paxinos and Franklin, 2013). The aim was to have a series of sections containing the RRF at rostral (A-P -3.80 and -3.90 mm), intermediate (A-P -4.00 and -4.10 mm) and caudal (A-P -4.20/-4.25 mm) levels, in addition to a more rostral section between A-P -3.30 and -3.60 mm to represent the 'classical' midbrain dopaminergic neuron cell groups (i.e. SNc and VTA). Given the small size of the RRF, this often required sections from different series to be pooled in order to have a full representation of all desired A-P levels.

Once a pool of sections was gathered with all desired A-P levels represented, it was incubated overnight at room temperature in a solution of PBS-T and 1% NDS (Jackson ImmunoResearch) containing rabbit anti-aldehyde dehydrogenase 1a1 (Aldh1a1) (1:500; AB24343, Abcam) and mouse anti-calbindin-1 (1:500; CB300, Swant), and then washed in PBS for 5 minutes, three times. Following this, sections were incubated overnight at room temperature in PBS-T containing Cy3-conjugated donkey anti-rabbit IgG (1:1000; 711-165-152, Jackson ImmunoResearch) and AlexaFluor 488-conjugated donkey anti-mouse IgG (1:500; 715-545-151, Jackson ImmunoResearch), and then washed in PBS for 5 minutes, three times. Sections were then mounted on glass slides with aqueous mounting medium (SlowFade™ Diamond Antifade Mountant, Invitrogen). Once it was determined that Aldh1a1 and calbindin-1 were useful for defining the border between caudal SNc and rostral RRF (and thus defining the outer boundaries of RRF, SNc and VTA), the series were incubated in primary antibodies raised against other molecular markers known to be enriched in midbrain dopaminergic neurons (Poulin et al., 2014).

Alongside TH, Aldh1a1 and calbindin-1 primary antibodies (described above), a series was incubated in one of the following primary antibodies: goat anti-special AT-rich sequence binding protein 1 (SatB1) (1:1000; SC-5989, Santa Cruz), goat anti-orthodenticle homolog 2 (Otx2) (1:1000; AF1979, RD Systems), goat anti-calretinin (1:500; CG1, Swant) or goat anti-G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) (1:500; AB65096, Abcam). Sections were then washed

in PBS for 5 minutes, three times, followed by overnight incubation at room temperature in PBS-T containing Cy3-conjugated donkey anti-goat (1:1000; 705-165-147, Jackson ImmunoResearch). For the incubation with rabbit anti-SRY-Box Transcription Factor 6 (Sox6) (1:4000; AB30455, Abcam), anti-Aldh1a1 could not be used as this was also produced in the rabbit. Therefore, only anti-TH (guinea pig) and anti-calbindin-1 (mouse) were included in the incubation. Sox6 signal was optimal when a AlexaFluor 647-conjugated secondary antibody was used (donkey anti-rabbit IgG; 1:500; 711-605-152, Jackson ImmunoResearch); Cy3-conjugated donkey anti-guinea pig IgG (1:1000; 706-165-148, Jackson ImmunoResearch) and AlexaFluor 488-conjugated donkey anti-mouse IgG (1:500; 715-545-151, Jackson ImmunoResearch) were used to visualise TH and calbindin-1 immunoreactivity, respectively. These sections were then washed in PBS for 10 minutes, three times.

In a separate series, sections were incubated overnight at room temperature in goat anti- $\alpha 1$ GABA_A receptor subunit (1:250; SC-7348, Santa Cruz) to visualise putative midbrain GABAergic neurons, alongside guinea pig anti-TH (1:1000; 213-104, Synaptic Systems) to visualise dopaminergic neurons and rabbit anti-NeuN (1:500; R-3770-100, Biosensis) to visualise all neuronal cell bodies. After washing in PBS for 5 minutes, three times, these sections were then incubated overnight at room temperature in a solution containing the relevant fluorophore-conjugated secondary antibodies: AlexaFluor 488-conjugated donkey anti-goat IgG (1:500; 705-545-003, Jackson ImmunoResearch), Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch) and Cy3-conjugated donkey anti-rabbit IgG (1:1000; 711-165-152, Jackson ImmunoResearch). Sections were then washed in PBS for 5 minutes, three times. Sections were then mounted on glass slides with aqueous mounting medium (SlowFadeTM Diamond Antifade Mountant, Invitrogen).

Sections were imaged using a confocal microscope (LSM 880, Axio Imager 2, Zeiss) with a 20x objective lens (NA = 0.8). For each section, the dopaminergic neurons in one hemisphere were imaged. Due to the sparsity of dopaminergic neurons in the RRF, z-stack images were taken with the optical

section set to 1.6 μm across all channels imaged, and a z-stack interval of 1.2 or 1.3 μm (z-series thickness of 35–40 μm). Prior to acquisition, the excitation-emission wavelengths were adjusted to ensure minimal/no overlap between channels in order to avoid crosstalk between signals. Following acquisition, the z-stacked images were subject to maximum intensity projection in ZEN Software (2.3 blue edition, 2016) to convert the 3D data into a single 2D image using the orthogonal projection method, and the resulting tiled 2D images were then stitched together.

2.3 Virus injection

Mice were anaesthetised using 3% (v/v) isoflurane (Abbott; delivered in O_2 at 2 L/min) for induction and 1–2% isoflurane for maintenance. Depth of anaesthesia was regularly assessed by checking for the absence of a hindlimb withdrawal reflex in response to a firm pinch of the hindpaw. The head was then shaved and the mouse placed in a stereotaxic frame (David Kopf Instruments) on a homeothermic heating mat (Harvard Apparatus). Prior to surgery, perioperative analgesia was administered systemically (buprenorphine, 0.08 mg/kg, s.c.; Ceva). Also, local anaesthetic (0.5% bupivacaine hydrochloride, 0.02 mL, s.c.; Aspen) was delivered around the incision site, and corneal dehydration was prevented using a carbomer eye gel (Optixcare). All surgeries were performed aseptically.

All viruses were stored at -80°C and thawed just before the injection surgery. To target the injection of viruses to midbrain dopaminergic neurons, the skull was exposed and a small craniotomy made above the target region(s), avoiding damage to the dura mater where possible. Saline solution (0.9% w/v NaCl) was applied to the craniotomy to prevent the exposed cortex dehydrating. Before surgery, a calibrated glass micropipette (7087-07, Blaubrand® intraMark, Brand) was made using a vertical puller (PE-2, Narishige), creating a long, narrow tip (broken back to a flat tip; inner diameter of tip 17–20 μm). To fill the micropipette with virus solution, tubing (inner diameter 0.08 cm; Tygon Lab Tubing 06407-70, Cole-Parmer) was attached to the open end of the micropipette and the pulled end was lowered into the virus solution. Then, a 1 or 5 mL

syringe was attached to the tubing via a Luer Lock adapter and the syringe plunger was manually withdrawn, which applied suction and drew up the virus solution into the micropipette. The filled micropipette was lowered into position using a stereotaxic arm, with the depth taken from the dura mater. The virus solution was pressure injected at a rate of $\leq 66 \text{ nLmin}^{-1}$, using air applied with a 1 or 5 mL syringe. To minimise reflux, the micropipette was left in position for 5 minutes before being slowly retracted. The virus was always injected in the right hemisphere, unless experimental aims necessitated bilateral injection (i.e. optogenetic behavioural experiments, Chapter 7).

Information about the viruses used, volumes for injection and stereotaxic coordinates is outlined in the relevant Methods Sections (below) and results Chapters.

2.4 Anterograde and retrograde tracing of RRF dopaminergic neurons

2.4.1 Anterograde tracing: surgical procedure

To trace the anterograde projections of RRF dopaminergic neurons, DAT-Cre mice were unilaterally injected with an adeno-associated virus (AAV) containing a Cre-inducible sequence encoding a cell-filling green fluorescent protein (GFP) reporter (AAV2-CAG-FLEX-GFP, 2.0×10^{12} gc/mL; UNC Vector Core) under stereotaxic guidance, as described in Section 2.3. This reporter fills the soma and distal axonal projections with GFP, permitting anterograde projections to be mapped. Thirty-three nL of virus was injected in the hindbrain at coordinates A-P -4.50, medial-lateral distance from the midline (M-L) +1.10, dorsal-ventral distance from the brain surface (D-V) -3.20 mm. By combining a small volume of virus solution and caudal injection coordinates, it was possible to limit most of the virus transduction to RRF dopaminergic neurons, with minimal spread to the neighbouring SNc and VTA. This strategy enabled the axonal projections of RRF dopaminergic neurons to be traced.

2.4.2 Retrograde tracing: surgical procedure

To trace the monosynaptic inputs to RRF dopaminergic neurons, I took advantage of a genetically-targetable rabies virus-based transynaptic tracing system. The approach involved sequential injection of a Cre-dependent ‘helper virus’ (AAV5-DIO-TVA^{V5}-RG; bicistronically expressing TVA receptor fused to a V5 tag, and the rabies glycoprotein (RG); Ährlund-Richter et al., 2019) and a ‘modified rabies virus’ (EnvA-SAD Δ G-EGFP; pseudotyped with EnvA, RG deleted, and expressing enhanced GFP (eGFP); Fürth et al., 2018; Ährlund-Richter et al., 2019) in DAT-Cre mice (Figure 2.1a). Firstly, the helper virus (33–66 nL) was unilaterally injected (as described in Section 2.3) into the hindbrain at the following coordinates: A-P -4.45, M-L +1.10, D-V -3.20 mm. As with anterograde tracing (see Section 2.4), injecting the virus into the hindbrain led to the virus spreading enough to transduce RRF dopaminergic neurons while minimising the spread to more rostral midbrain dopaminergic neurons (i.e. SNc/VTa). Mice were maintained for 21 d after this first surgery to allow time for neural transduction, Cre-mediated recombination and generation of ‘starter’ neurons expressing all the necessary components for transsynaptic labelling of presynaptic neurons (Figure 2.1b). For the second step in the sequence, 33–66 nL of modified rabies virus was injected at the slightly more rostral coordinates of A-P -4.30, M-L +1.10 and D-V -3.20 mm. Following this step, mice were maintained for 7 d, allowing time for the modified rabies virus to infect the starter neurons and transsynaptically label input neurons (Figure 2.1c).

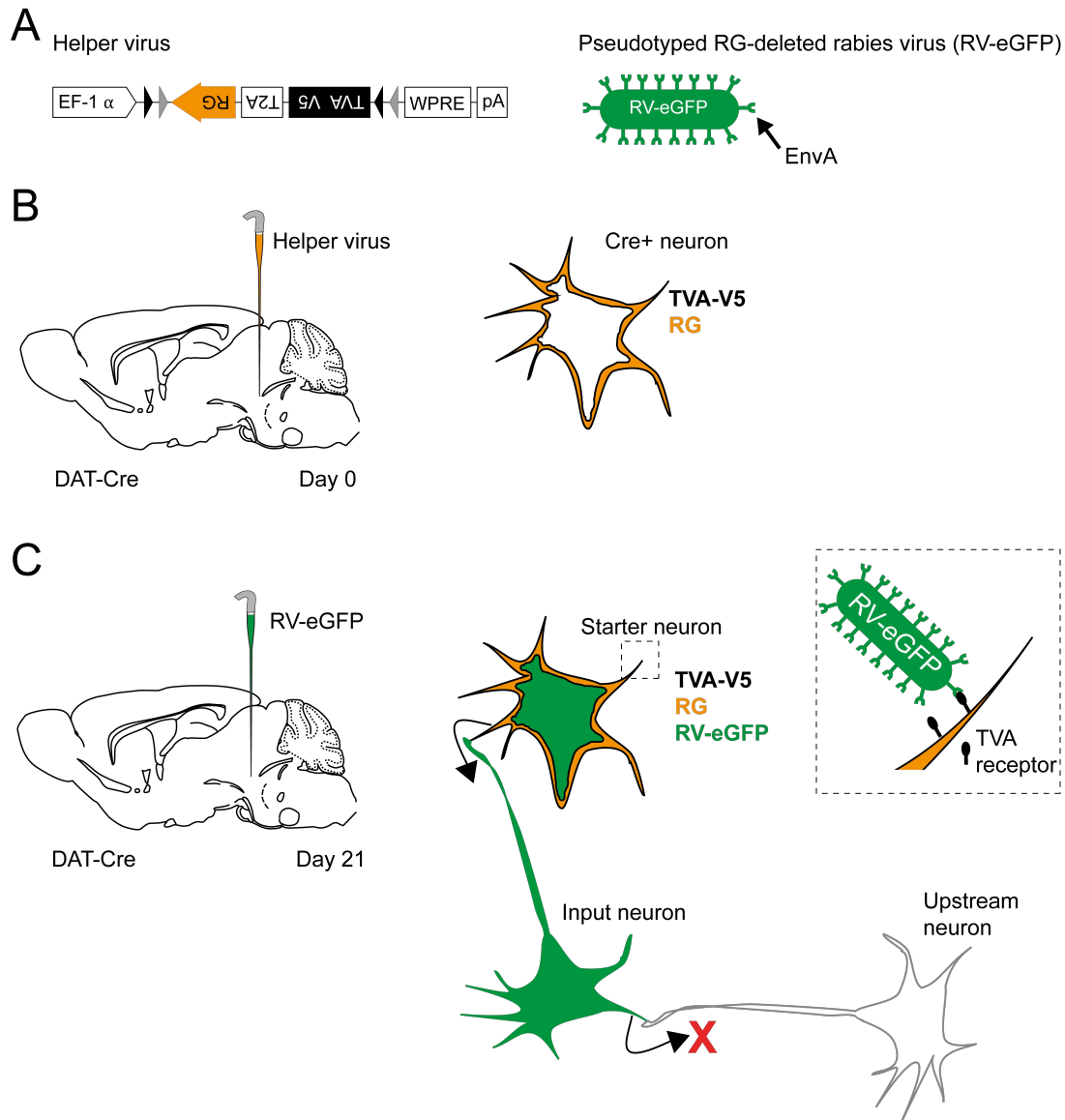


Figure 2.1: Experimental design for monosynaptic input tracing using a helper and modified rabies virus system. **A, Left,** The Cre-dependent helper virus construct, encoding the avian TVA receptor (necessary for modified rabies virus (RV-eGFP) infection) fused to a V5 tag (for immunohistochemical labelling of transduced neurons) and RG (complements with the modified rabies virus, permitting presynaptic spread). **Right,** The modified rabies virus construct (RV-eGFP), coated with an envelope protein (EnvA) to target RV-eGFP infection to cells that express the cognate TVA receptor on their surface. **B,** Helper virus was injected into DAT-Cre mice. Infection of Cre⁺ (i.e. dopaminergic) neurons results in TVA^{V5} and RG expression. **C,** RV-eGFP was injected 21 d later. RV-eGFP infection is limited to Cre⁺ cells that express the TVA receptor on their surface (dashed inset), generating starter neurons. Complementation with RG enables the RV-eGFP to spread (arrow) to presynaptic neurons (input neurons), labelling them with GFP. Transsynaptic spread to neurons upstream of the monosynaptic input neurons does not occur because these upstream neurons do not contain RG, which is essential for presynaptic spread. Therefore, RV-eGFP expression is limited to the monosynaptic inputs to dopaminergic neurons. Modified from Ährlund-Richter et al., 2019.

2.4.3 *Perfuse-fixation and tissue processing*

Mice were administered a lethal dose of sodium pentobarbitone (0.3 mL; 20% w/v), and then transcardially perfused with ~20 mL of 0.1 M PBS, pH 7.4, followed by ~50–60 mL of 4% (w/v) PFA in 0.1 M phosphate buffer, pH 7.4. The brains were removed and stored in 4% (w/v) PFA overnight at 4°C and then stored in PBS-azide until they were sectioned 12 h to 1 week later.

Fixed brains were embedded in agar (3% w/v dissolved in dH₂O) before being sectioned to a thickness of 50 µm in the coronal plane with a vibrating-blade microtome (VT1000S; Leica Microsystems). Free-floating brain sections were collected into four series, meaning that in a single series the A-P distance between adjacent sections was 200 µm. The midbrain from each series was isolated and processed separately from the rest of the brain (forebrain and hindbrain sections). All brain sections were incubated in a 10% NDS (Jackson ImmunoResearch) blocking solution for 1 h before primary antibodies were applied.

2.4.3.1 *Tissue processing: anterograde tracing experiment*

For the anterograde tracing experiment, midbrain sections were incubated overnight at room temperature in a solution of PBS-T and 1% NDS (Jackson ImmunoResearch) containing guinea pig anti-TH (1:1000; 213-104, Synaptic Systems) and rat anti-GFP (1:500; 04404-84, clone GF090R, Nacalai Tesque) to immunolabel dopaminergic neurons and to enhance the native GFP fluorescence, respectively. The primary antibody solution also contained rabbit anti-Aldh1a1 (1:500; AB24343, Abcam) for distinguishing caudal SNc from rostral RRF (see Chapter 3 for rationale).

Forebrain and hindbrain sections were incubated overnight at room temperature in a solution of PBS-T and 1% NDS (Jackson ImmunoResearch) containing: rat anti-GFP (1:500; 04404-84, clone GF090R, Nacalai Tesque) for visualising the location of anterograde projections expressing the GFP reporter; guinea pig anti-TH (1:1000; 213-104, Synaptic Systems) for delineating the boundaries of the striatum, the olfactory tubercle (OT) and the ventral border of the interstitial nucleus of the posterior limb of the anterior commissure (IPAC); goat anti-Forkhead box protein P2 (FoxP2) (1:2000; EB05226,

Everest Biotech) for labelling some striatal neurons; rabbit anti- μ opioid receptor (MOR) (1:10 000; 24216, Immunostar) for labelling the striosome compartment of the rostral striatum and the dorsal IPAC. Note, although the anti-MOR antibody was used at a 1:10 000 dilution, the optimal dilution is in fact 1:5000.

Sections were then washed in PBS for 5 minutes, three times, before incubation overnight at room temperature in solutions containing appropriate fluorophore-conjugated secondary antibodies. Midbrain sections: Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch); AlexaFluor 488-conjugated donkey anti-rat IgG (1:500; 712-545-153, Jackson ImmunoResearch); Brilliant Violet 421TM-conjugated donkey anti-rabbit (1:500; 406410, BioLegend); forebrain sections: Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch); AlexaFluor 488-conjugated donkey anti-rat IgG (1:500; 712-545-153, Jackson ImmunoResearch); AMCA-conjugated donkey anti-goat (1:500; Jackson ImmunoResearch); Cy3-conjugated donkey anti-rabbit (1:1000; 711-165-152, Jackson ImmunoResearch). Following this final incubation, sections were washed in PBS for 5 minutes, three times, and mounted on glass slides with aqueous mounting medium (SlowFadeTM Diamond Antifade Mountant, Invitrogen).

2.4.3.2 Tissue processing: retrograde tracing experiment

For the retrograde tracing experiment, midbrain sections were incubated overnight at room temperature in a solution of PBS-T and 1% NDS (Jackson ImmunoResearch) containing the following primary antibodies: guinea pig anti-TH (1:1000; 213-104, Synaptic Systems) to immunolabel dopaminergic neurons; chicken anti-V5 tag (V5; 1:2000; ab9113, Abcam) to immunolabel starter neurons; rat anti-GFP (1:500; 04404-84, clone GF090R, Nacalai Tesque) to enhance the native fluorescence in starter neurons and input neurons expressing pseudotyped RG-deleted rabies virus (RV-eGFP). The primary antibody solution also contained rabbit anti-Aldh1a1 (1:500; AB24343, Abcam) for distinguishing caudal SNc from rostral RRF.

Forebrain and hindbrain sections were incubated overnight at room temperature in a solution

of PBS-T and 1% NDS (Jackson ImmunoResearch) containing rat anti-GFP (1:500; 04404-84, clone GF090R, Nacalai Tesque) for visualising the location of RV-eGFP-expressing input neurons.

Sections were then washed in PBS for 5 minutes, three times, before incubation overnight at room temperature in solutions containing appropriate fluorophore-conjugated secondary antibodies: Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch); Cy3-conjugated donkey anti-chicken IgG (1:1000; 703-165-155, Jackson ImmunoResearch); AlexaFluor 488-conjugated donkey anti-rat IgG (1:500; 712-545-153, Jackson ImmunoResearch); Brilliant Violet 421TM-conjugated donkey anti-rabbit (1:500; 406410, BioLegend). Following this final incubation, sections were washed in PBS for 5 minutes, three times, and mounted on glass slides with aqueous mounting medium (SlowFadeTM Diamond Antifade Mountant, Invitrogen).

2.4.4 Quantification and localisation of transduced neurons

Following immunohistochemistry and prior to quantification, the entire midbrain of each mouse (i.e. all series) was mounted on glass slides and viewed under an epifluorescence microscope (Axioscope.A1, Zeiss). The distribution of GFP-expressing neurons (anterograde tracing experiment) or starter neurons (V5⁺ GFP⁺; retrograde tracing experiment) in the midbrain was visually inspected. The sections were taken forward for quantification if it was determined qualitatively that the GFP-expressing/starter neurons were mostly limited to the RRF. Specifically, it was first determined that transgene expression was present within the caudal (A-P -4.15 to -4.25 mm) and intermediate RRF (A-P -4.00 to -4.15 mm). In sections containing the rostral RRF (A-P -3.80 to -4.00 mm), the expression of Aldh1a1 was used to delineate the RRF from the adjacent caudal SNc. If transgene expression was limited to the RRF, with minimal expression in the caudal SNc, then the subject was taken forward for stereological quantification. If there were large numbers of transgene-expressing neurons within the caudal SNc and in more rostral sections (i.e. rostral of the RRF in the 'classical' midbrain) then the subject was determined to be unsuitable for

analysing the connectivity of RRF dopaminergic neurons and not analysed further.

Of the four midbrain series from each mouse, series 1 and 3, or 2 and 4, were imaged for quantification, meaning the section interval for quantification was two (i.e. 100 μm between adjacent sections). Unbiased cell counts of transduced neurons were determined using a version of design-based stereology, the 'modified optical fractionator' (Abdi et al., 2015; Dodson et al., 2015). Imaging for stereology was performed using Stereo Investigator software (version 2019.1.4, MBF Bioscience). Tissue images were acquired using an epifluorescence microscope (Axio Imager.M2, Zeiss) equipped with an ORCA Flash-4.0 LT digital CMOS camera (Hamamatsu), Apotome.2 (Zeiss), and a Colibri 7 LED light source (Type R,[G/Y]B-UV, Zeiss). Fluorescence channels were imaged using the following filter cubes: AMCA (excitation 299–392 nm, beamsplitter 395 nm, emission 420–470 nm); AlexaFluor-488 (excitation: 450–490 nm, beamsplitter 495 nm, emission 500–550 nm); Cy3 (excitation 532–558 nm, beamsplitter 570 nm, emission 570–640 nm); and Cy5 (excitation 625–655 nm, beamsplitter 660 nm, emission 665–715 nm). Images for each channel were acquired separately and sequentially in order to prevent crosstalk of signals between channels.

To quantify GFP-expressing neurons for the anterograde tracing experiments, all sections within a series containing GFP-expressing neurons were imaged in the AlexaFluor 488 and Cy5 channels to test for the expression of GFP and TH, respectively. To quantify starter neurons in the retrograde tracing experiments, all sections within a series containing V5-expressing neurons were imaged in the AlexaFluor-488, Cy3 and Cy5 channels to test for the expression of GFP, V5 and TH, respectively. Additionally, the entire RRF was imaged in order to determine the total number of dopaminergic (i.e. TH-expressing) neurons within it. Before quantification, the outer borders of the midbrain dopaminergic cell groups were defined (using a 5/10x objective). Aldh1a1 immunoreactivity was used in order to distinguish the borders between some midbrain dopaminergic neuron cell groups, but was not imaged. In 'classical' midbrain sections (A-P -3.40 to -3.70 mm), the SNc (including the substantia nigra pars lateralis (SNL)) was defined as being lateral to the medial lemniscus and guided by the Aldh1a1-expressing dense cluster of dopaminergic

neurons in its ventromedial aspects; and the VTA was defined as being medial to the medial lemniscus. In sections containing rostral RRF (A-P -3.80 to -3.95 mm), the outer borders of the caudal SNc were defined based on two main criteria: i) a dense cluster of Aldh1a1-expressing dopaminergic neurons in the ventral aspects of the SNc; and ii) the dorsoventral orientation of many SNc dopaminergic neurons, compared with the generally mediolateral orientation of RRF neurons (Fu et al., 2012). The rostral RRF was defined as the dense cluster of Aldh1a1-sparse dopaminergic neurons dorsal and medial with respect to the SNc; and the parabrachial pigmented nucleus of the VTA (PBP) was defined as the sparse cluster of dopaminergic neurons medial to the RRF (see Chapter 3 for rationale). All dopaminergic neurons in sections containing the intermediate (A-P -4.00 to -4.15 mm) and caudal (A-P -4.20 to -4.25 mm) RRF were defined as being RRF dopaminergic neurons, except for those in the rostral linear nucleus (RLi)/caudal linear nucleus of the raphe (CLi) close to the midline.

After the outer borders of each midbrain cell group were traced, each cell group was imaged using the optical fractionator workflow in Stereo Investigator using 2D counting frame (600 x 600 μm). Each 2D counting frame had two perpendicular inclusion lines and two perpendicular exclusion lines for counting purposes (see below). Within each counting frame, z-stacked images were acquired using a 20x objective lens (NA = 0.8; Plan-Apochromat) in 1 μm steps at depths of 2–12 μm from the top of the section, in order to capture all signal in the Z-axis; the 10 μm of imaged tissue is termed the ‘optical disector’. The top 2 μm of the section was not imaged to prevent any surface irregularities from distorting image analysis.

Neurons were counted offline. A neuron was only counted if its nucleus came into focus within the optical disector, or if the top of its nucleus was within the optical disector; if the bottom of a neuron’s nucleus (i.e. at the top of the image stack) was present within the optical disector then it was not counted. For a given molecular marker, X, a neuron was designated as immunopositive, X^+ , or immunonegative, X^- . A neuron was only defined as immunonegative for a given molecular marker (X^-) if other nearby neurons in the same optical section were immunopositive. This was to

avoid false negatives in the unlikely case the antibody to the given molecular marker had insufficiently penetrated the tissue. Within the RRF, all neurons expressing TH were counted (TH⁺) for both the anterograde and retrograde tracing experiments, in order to determine the proportion of RRF dopaminergic neurons immunopositive for the given molecular markers (i.e. GFP for anterograde tracing and V5/RV-eGFP for retrograde tracing).

For anterograde tracing experiments, stereological quantification involved counting all GFP-expressing neurons, both TH-expressing (TH⁺GFP⁺) and TH-negative (TH⁻GFP⁺). Stereological quantification of starter neurons for the retrograde tracing experiments involved counting all neurons expressing V5 and rabies-encoded GFP (V5⁺GFP⁺), both TH⁺ and TH⁻. A high level of precision in the cell counts was achieved by ensuring the Coefficient of Error (CE; using the Gundersen method) was ≤ 0.1 with a smoothness factor of $m = 1$ for each of the important marker combinations (outlined above). The CE is an estimate of sampling precision that is independent of biological variation (West et al., 1991; Gundersen et al., 1999; West, 2012). Practically, this level of precision was achieved by counting two midbrain series from each mouse (series 1 and 3, or 2 and 4; section interval of 2).

From the counting data, the total number of dopaminergic neurons in the RRF was determined. The penetration of the viral transduction strategy was calculated as the percentage of TH-expressing neurons in the RRF that also expressed GFP in the anterograde tracing experiment (TH⁺GFP⁺), or V5 and rabies-encoded GFP (TH⁺V5⁺GFP⁺; i.e. dopaminergic starter neurons) in the retrograde tracing experiment. For the anterograde and retrograde tracing experiments, the specificity of viral transduction was determined as the proportion of GFP-expressing or starter (V5⁺GFP⁺) neurons, respectively, that also expressed TH. To objectively determine how well circumscribed to the RRF the viral transduction was, the number of GFP-expressing dopaminergic neurons (TH⁺GFP⁺) or dopaminergic starter neurons (TH⁺V5⁺GFP⁺) in the RRF was expressed as a percentage of all TH⁺GFP⁺/TH⁺V5⁺GFP⁺ neurons for anterograde and retrograde tracing experiments, respectively. This was termed the transduction efficiency.

Images for figures were acquired with a confocal microscope (LSM 880, Axio Imager 2, Zeiss) with z-stacking (optical section set to 1.6 μm across all channels imaged, and a z-stack interval of 1.2 or 1.3 μm ; z-series thickness of 35–40 μm). Following acquisition, the z-stacked images were subject to maximum intensity projection in ZEN Software (2.3 blue edition, 2016) to convert the 3D data into a single 2D image using the orthogonal projection method, and the resulting tiled 2D images were then stitched together.

2.4.5 Anterograde tracing: mapping axonal projections

For each mouse, serial coronal brain sections were examined for the presence of axonal projections. Sections containing axonal arborisations, indicative of terminal sites, were then imaged using a confocal microscope (LSM 880, Axio Imager 2, Zeiss) with a 20x objective lens (NA = 0.8). A relatively thick optical section of 8 μm (matched across channels) was used in order to image the projection pattern within a given section; a thinner optical section would have sampled a relatively small proportion of fibres. Prior to acquisition, the excitation-emission wavelengths were adjusted to ensure minimal/no overlap between channels in order to avoid crosstalk between signals. The acquired images were then compared in order to qualitatively describe the projection pattern in each animal.

For more detailed analyses of projections in a subset of regions, z-stack images were taken with the optical section set to 2.7 μm across all channels imaged, and a z-stack interval of 2.1–2.3 μm (z-series thickness of 35–40 μm). Following acquisition, the z-stacked images were subject to maximum intensity projection in ZEN Software (2.3 blue edition, 2016) to convert the 3D data into a single 2D image using the orthogonal projection method, and the resulting tiled 2D images were then stitched together.

To examine the axonal arbors within forebrain regions at high magnification, z-stack images were acquired with a 63x objective lens (NA = 1.46). The optical section was set to 1.2 μm across all channels and images were acquired with a z-stack interval of 0.4 μm (z-series thickness 35–48 μm). Following

acquisition, the z-stacked images were subject to maximum intensity projection in ZEN Software (2.3 blue edition, 2016) to convert the 3D data into a single 2D image using the orthogonal projection method.

2.4.6 Retrograde tracing: mapping monosynaptic input neurons

For mapping the distribution of RV-eGFP⁺ input neurons in each mouse, serial coronal sections were imaged with a confocal microscope (LSM 880, Axio Imager 2, Zeiss) fitted with a 5x objective lens (NA = 0.16) at 200 μ m intervals. These images were then examined and used to generate the qualitative table of input regions (i.e. regions in which RV-eGFP⁺ were observed; Table 5.2). These images were not displayed in the results.

For each mouse, serial coronal sections were then imaged with a confocal microscope with a 10x objective lens (NA = 0.45; optical section thickness 39 μ m) to create a comparative map of the brain regions that send projections to RRF dopaminergic neurons. Brain regions displayed in this comparative figure are a representative sample of the brain regions in which monosynaptic input neurons were observed. Across mice, sections were matched in the rostrocaudal plane where possible.

To examine the distribution of input neurons more precisely, molecular markers were selected that aided in delineating the borders of brain regions of interest. For the qualitative purposes of this work, one mouse (LAB338) was chosen for this aspect of the analysis because it had the highest proportion of starter neurons in the RRF. After examining the distribution of RV-eGFP expression in this mouse, a list was made of putative brain regions containing labelled cells. Based on this, a selection of markers to objectively define these brain regions was chosen. Table 2.2 below outlines the markers used to define each brain region and the respective antibodies used:

Antigen (host species; dilution)	Source and catalog number	Brain region(s)	Secondary antibody
Calbindin-1 (mouse; 1 in 500)	Swant CB300	Striosomes and NAc core	Cy5-conjugated donkey anti-mouse (Jackson ImmunoResearch, 715-175-150)
FoxP2 (goat; 1 in 2000)	Everest Biotech EB05230	CeA and STN	Cy5-conjugated donkey anti-goat (Jackson ImmunoResearch, 705-175-147)
MOR (rabbit; 1 in 10 000)	Immunostar 24216	IPAC	Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch, 711-165-152)
Somatostatin (mouse; 1 in 250)	GeneTex GTx71935	BNST and CeA	Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch, 715-165-150)
TH (guinea pig; 1 in 1000)	Synaptic Systems 213-104	LC	Cy5-conjugated donkey anti-guinea pig (Jackson ImmunoResearch, 706-175-148)

Table 2.2: Primary antibodies used to delineate brain regions projecting to RRF dopaminergic neurons. BNST, bed nucleus of the stria terminalis; CeA, central amygdala; FoxP2, forkhead box protein P2; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LC, locus coeruleus; MOR, μ opioid receptor; NAc, nucleus accumbens; STN, subthalamic nucleus; TH, tyrosine hydroxylase.

Following incubation in these antibodies, z-stack images of the selected brain regions were then taken with a confocal microscope (LSM 880, Axio Imager 2, Zeiss) fitted with a x20 objective lens (NA = 0.8).

2.5 Electrophysiology in anaesthetised mice

2.5.1 Preparation of anaesthetised mice

General anaesthesia was induced with 3–4% isoflurane (Abbott; delivered in O₂ at 2 L/min) and maintained with urethane (ethyl carbamate dissolved in dH₂O, 10% (w/v); initial dose 1.55 mg/kg, i.p.; Sigma Aldrich). Isoflurane was used for induction prior to injection of urethane because in my

experience this leads to the most rapid onset of sustained anaesthesia, whereas urethane alone often leads to a tardy onset. Isoflurane was not applied after the animal was placed in the ear bars of the stereotaxic frame (David Kopf Instruments), thus the recordings of single units (see below) were deemed to be mostly within purely urethane anaesthesia, based on a isoflurane half-life of 30–150 minutes in mice (Wyrwicz et al., 1987; Hohlbaum et al., 2017). However, it is possible that some single units recorded early in the day may have been under the influence of both urethane and isoflurane. When mice no longer had a hindlimb withdrawal reflex in response to a firm pinch of the hindpaw, their head was shaved and they were placed in a stereotaxic frame (David Kopf Instruments). Air enriched with O₂ (2 L/min) was supplied throughout the recording session to counter any respiratory depression due to urethane anaesthesia. Depth of anaesthesia was regularly assessed by checking for the absence of a hindlimb withdrawal reflex in response to firm pinching of the hindpaw; if a reflex occurred, the mouse was administered a supplementary dose of urethane (0.15 mg/kg, i.p.). Core body temperature was maintained at $36.5 \pm 0.5^{\circ}\text{C}$ using a homeothermic heating mat (Harvard Apparatus), and corneal dehydration was prevented by using a carbomer eye gel (Optixcare).

An electrocorticogram (ECoG) was recorded over left secondary motor cortex (A-P +2.5mm, M-L +2.5 mm) with a 0.9 mm diameter stainless steel screw inserted through a small craniotomy, juxtaposed to the dura mater and referenced against a screw positioned above the contralateral cerebellum. Raw ECoG signals were band-pass filtered (0.3–1500 Hz, -3 dB limits) and amplified (2000X; DPA-2FS filter/amplifier; NPI Electronic Instruments) before acquisition.

2.5.2 Single-unit electrophysiology: set-up

A $\sim 1.5 \text{ mm}^2$ craniotomy was made above the RRF (centred at A-P -3.50 mm, M-L +1.0 mm). Saline (0.9% w/v NaCl) was frequently applied to prevent the exposed cortex from dehydrating. Extracellular action potentials fired by single neurons (i.e. single-unit activity) were recorded with borosilicate glass electrodes (broken back to give a tip diameter of 1.3–1.7 μm ; 10–25 M Ω in situ

impedance; GC120F-10, Harvard Apparatus; made using a vertical puller, PE-2, Narishige) filled with 0.5 M NaCl containing Neurobiotin (1.5% w/v, Vector Laboratories). Using stereotaxic guidance, electrodes were lowered into the midbrain using a micromanipulator (IVM-1000, Scientifica). Electrode signals were 10X amplified via the active bridge circuitry of an ELX-01MX amplifier (NPI Electronic Instruments). Signals were filtered at 0.3–5 kHz and subject to a further 100x amplification (DPA-2FS filter/amplifier; NPI Electronic Instruments). ECoG and single-unit activity were sampled at a rate of 20 kHz using a Power1401 analog-digital convertor (Cambridge Electronic Design) and Spike2 acquisition and analysis software (version 8.13, Cambridge Electronic Design). A Humbug (Quest Scientific) was used to eliminate mains noise at 50 Hz. To ensure minimal bias when searching for neurons, a relatively generous criteria of ≥ 0.9 ms biphasic action potential duration (interval between the point the upward slope is $> 15\%$ and the minimum of the trough) and ≤ 11 spikes/s firing rate was used to identify neurons as putatively dopaminergic (Ungless and Grace, 2012).

2.5.3 Single-unit electrophysiology: spontaneous activity

The spontaneous firing properties of dopamine neurons were determined from single-unit activity recorded during robust cortical slow-wave activity (SWA) because the firing pattern of midbrain dopamine neurons has been shown to be dependent on brain state (Brown et al., 2009; Walczak and Błasiak, 2017). ECoG data were Fourier-transformed with a frequency resolution of 0.2 Hz, and the ratio in the SWA band (0.5–2 Hz) to the power in the gamma band (30–80 Hz) was calculated as a power ratio. Contiguous epochs of ECoG data (minimum duration 10 s) in which the power ratio was > 13 were then concatenated (Janezic et al., 2013). The unit activity within the concatenated data was then used for analysis of spontaneous firing properties. Note, the peri-stimulus histograms (PSTHs) from the paw and optogenetic stimulation experiments (see Methods sections 2.5.4 and 2.5.5, below) were constructed without consideration of brain state. Brain state affects firing pattern but not firing rate (Brown et al., 2009), thus is unlikely to affect unit activity dynamics in response to

paw/optogenetic stimulation. The mean average recording duration for spontaneous activity was 165.4 ± 104 s (mean (standard deviation)).

2.5.4 Single-unit electrophysiology: noxious electrical stimulation of hindpaw

Following recording of spontaneous activity, a noxious electrical stimulus (5 mA, 2 ms pulse duration, delivered at 0.5 Hz; Coizet et al. (2006)) was applied to the hindpaw contralateral to the recording site using a stimulus isolator (A365, World Precision Instruments). The stimulation was applied via two 0.2 mm silver wires (45071, Alfa Aesar) inserted underneath the skin of the hindpaw, one in the mid-plantar surface of the foot and the other two-thirds of the distance between the mid-plantar surface and the ankle joint. The delivery of the precisely timed electrical stimuli was controlled via Spike2 using a custom configuration file. For each neuron, a minimum of 57 stimulations were applied; the mean number of stimulations was 104 (19).

2.5.5 Single-unit electrophysiology: optogenetics

DAT-Cre mice were injected (see Section 2.3) unilaterally (coordinates: A-P -4.00, M-L +1.20, D-V, -3.20 mm) with AAV2-CAG-FLEX-ArchT-GFP (330 nL, 2.0×10^{12} gc/mL ; UNC Vector Core) and maintained for 10–24 weeks before electrophysiological recordings. To assess the effect of laser stimulation on ArchT-transduced dopaminergic neurons, a 200 μ m optic fibre (0.37 NA; MFP_200/230/2000-0.37_2m_FC-X, Doric Lenses) was inserted above the RRF (A-P -4.00 mm, M-L +1.2 mm, D-V -3.2 mm) and coupled to a 561 nm diode-pumped solid-state laser (CL561-100-0, CrystaLaser). The laser was connected to a Power1401 analog-digital convertor (Cambridge Electronic Design) and the power was controlled using custom-written configuration files in Spike2. The power of the laser was calibrated before each recording session and set to 5 mW output at the tip, measured using an optical sensor coupled to an optical power meter (S121C (sensor), PM100D (power meter), Thor Labs). The single-unit recordings were carried out as described in the above Section 2.5.2, except that the electrode was angled at 10° in the anterior

direction.

The stimulation parameters used were as follows: i) stim A: 250 ms ON, 2 s OFF; 50 trials ii) stim B: 2 s ON, 5 s OFF; 20 trials iii) stim C: 10 s ON, 20 s OFF; 5 trials iv) stim D: 30 s ON, 30 s OFF; 2–10 trials.

2.5.6 *Single-unit electrophysiology: juxtacellular labelling of single neurons*

Following acquisition of recording data, the single recorded neuron was filled with Neurobiotin using the juxtacellular method (Pinault, 1996; Brown et al., 2009). This was achieved by slowly moving the electrode close to the neuron until the amplitude of the action potential was at least 1 mV and applying an iontophoretic current (1–10 nA anodal current, 200 ms duration, 50% duty cycle). The amplitude of the applied current was tuned to a level that entrained the firing of the neuron to the current pulse. ‘Driving’ the neuron in this manner indicates a high probability that the neuron is being successfully labelled, in that Neurobiotin will be entering the cell during the current pulses. A neuron was entrained for 2–5 minutes, which is sufficient to label the somata and proximal dendrites; the Neurobiotin was then left to transport along neuronal processes for 1–12 h. This method allows for the *post hoc* localisation and neurochemical identification of the recorded neuron in perfusion-fixed tissue.

2.5.7 *Perfuse-fixation and tissue processing*

At the end of an experiment, mice were administered a lethal dose of sodium pentobarbitone (0.3 mL; 20% w/v), and then transcardially perfused with ~20 mL of 0.1 M PBS, pH 7.4, followed by ~35 mL of 4% (w/v) PFA in 0.1 M phosphate buffer, pH 7.4. Brains were then removed and stored in 4% (w/v) PFA overnight at 4°C and stored in PBS-azide until they were sectioned 12 h later.

Fixed brains were sectioned to a thickness of 50 µm in the coronal plane with a vibrating-blade microtome (VT1000S; Leica Microsystems) and the midbrain was isolated. The brain was sectioned into four or six series, meaning that in a single series the A-P distance between adjacent sections was

200 or 300 μm , respectively. To identify the location of the Neurobiotin-labelled neurons, free-floating sections were incubated for 4 h/overnight at room temperature in Cy3-conjugated streptavidin (1:1000 dilution; PA43001, GE Healthcare). The sections were then washed in PBS for 5 minutes, three times. Each series was then mounted on glass slides with aqueous mounting medium (Vectashield, Vector Laboratories) and viewed under an epifluorescence microscope (Axioscope.A1, Zeiss) to locate the brain section(s) containing the Neurobiotin-labelled neuron(s) (i.e. the neurons that were positive for the Cy3-conjugated streptavidin).

To determine whether the Neurobiotin-labelled neurons were dopaminergic, their immunoreactivity for TH was assessed using indirect immunofluorescence. The sections containing labelled neurons were incubated overnight in PBS-T containing a mouse anti-TH antibody (1:1000; T2928, Sigma Aldrich), or in some cases a rabbit anti-TH antibody (1:1000; AB152, Chemicon). In optogenetic experiments (see Section 2.5.5), sections containing labelled neurons were also incubated in a chicken anti-GFP antibody (1:500; GFP1020, Aves Labs) alongside the anti-TH antibody. These sections were then washed in PBS for 5 minutes, 3 times, followed by overnight incubation in PBS-T containing an aminomethylcoumarin acetate (AMCA)-conjugated donkey anti-mouse/anti-rabbit IgG (1:500; 715-155-151/711-155-152, Jackson ImmunoResearch) at room temperature, or in some rare cases a Cy5-conjugated donkey anti-rabbit IgG (1:500; 711-605-152, Jackson ImmunoResearch). Where appropriate, an AlexaFluor 488-conjugated donkey anti-chicken IgG (1:500; Code: 703-545-155, Jackson ImmunoResearch) was also included in order to enhance the native GFP signal in transduced neurons. Sections were then washed in PBS for 5 minutes, three times, and mounted on glass slides with aqueous mounting medium (Vectashield, Vector Laboratories).

Each Neurobiotin-labelled neuron was imaged using a confocal microscope (LSM 880, Axio Imager 2, Zeiss), with a 40x objective (NA = 1.4; oil immersion) with z-stacking for 3D reconstruction of the labelled somata (optical section thickness, 1.6 μm ; z-stack interval, 1.2–1.3 μm ; z-series thickness, 35–45 μm). A labelled neuron was defined as an identified dopaminergic

neuron if it was immunoreactive for TH. A labelled neuron that was amongst dopaminergic neurons but immunonegative for TH was defined as TH-negative as long as it was surrounded by other neurons that were immunopositive for TH in the same optical section. This was to ensure that it was truly TH-negative, not just immunonegative as a result of poor tissue penetration by the anti-TH antibody. In addition, the entire hemisphere containing the labelled neuron was imaged using a 10x objective (NA = 0.45), in order to determine its precise anatomical coordinates. All confocal images were processed in ZEN Software (2.3 blue edition, 2016) to adjust brightness and contrast. The whole hemisphere image was compared with the mouse brain atlas (Paxinos and Franklin, 2013) to determine the A-P, M-L and D-V location of the labelled neuron. TH immunoreactivity and anatomical landmarks were used for additional guidance. These coordinates were used for generating the schematic images showing the locations of labelled neurons in the midbrain (Chapters 6 and 7).

Neurobiotin-labelled dopaminergic neurons located A-P -4.00 mm to -4.30 mm were defined as being within the RRF. For labelled neurons in sections containing the rostral RRF (A-P -3.85 to -3.90 mm), sections were incubated overnight at room temperature in PBS-T containing rabbit anti-Aldh1a1 (1:500; AB24343, Abcam), and then washed in PBS for 5 minutes, three times. This was followed by overnight incubation at room temperature in PBS-T containing, depending on the channel available, AlexaFluor 488/Cy5-conjugated donkey anti-rabbit IgG (1:500; A21206, Invitrogen /711-605-152, Jackson ImmunoResearch), and then sections were washed in PBS for 5 minutes, three times before mounting on to glass slides with aqueous mounting medium (Vectashield, Vector Laboratories; or SlowFadeTM Diamond Antifade Mountant, Invitrogen, in cases where a Cy5-conjugated secondary antibody was used). Aldh1a1 immunoreactivity was used to distinguish caudal SNc from rostral RRF (see Chapter 3 for rationale). A Neurobiotin-labelled neuron was defined as being in the RRF if it was within the dorsal, more medial, Aldh1a1-sparse group of TH-positive neurons, or in the SNc if it was within the ventral, Aldh1a1-dense group of TH-positive neurons. Also, the orientation of dopaminergic neurons was used to guide cell group

definitions, as caudal SNc neurons tend to be orientated in the dorsal-ventral axis, whereas RRF neurons tend to be orientated in the medial-lateral axis (Fu et al., 2012).

For the single case of a neuron located in the pedunculopontine nucleus (PPN), the section was incubated overnight at room temperature in PBS-T containing goat anti-choline acetyltransferase (ChAT) (1:500; AB144P, Millipore), and then washed in PBS for 5 minutes, three times. This was followed by overnight incubation at room temperature in PBS-T containing AlexaFluor647-conjugated donkey anti-goat IgG (1:500; 705-605-147, Jackson ImmunoResearch), and then sections were washed in PBS for 5 minutes, three times, before mounting on to glass slides with aqueous mounting medium (SlowFadeTM Diamond Antifade Mountant, Invitrogen).

2.5.8 Analysis of recordings: spontaneous activity

Mean firing rates for midbrain dopaminergic neurons were calculated in spikes/s from recordings made during the concatenated SWA data (see Section 2.5.3 above). The variability of firing was calculated by comparing the standard deviation (SD) of adjacent interspike intervals (ISIs) within a spike train and then obtaining a mean value of this variability for each neuron, denoted CV2 (Holt et al., 1996). Lower CV2 values indicate a more regular firing pattern and vice versa. Burst firing properties of midbrain dopamine neurons were calculated using a robust gaussian surprise (RGS) method (Ko et al., 2012). This statistical method normalises the spiking activity of a neuron, generating a central distribution, and detects sets of spikes within that data with consecutive ISIs that lie outside of the negative tail of the central distribution (i.e. a cluster of spikes with low ISIs). A burst was defined as at least three spikes/burst. Data in all graphs was expressed as mean $\pm$ standard error of the mean (SEM) for normally distributed data and median (interquartile range (IQR) = $IQ_1 - IQ_3$) for non-normally distributed data, where IQ_1 is the first quartile and IQ_3 is the third quartile. The firing rate within a burst (intra-burst firing rate) was calculated by dividing the number of spikes within a burst by the burst duration.

2.5.9 Analysis of recordings: noxious electrical stimulation of hindpaw

The effect of electrical hindpaw stimulation on the firing of neurons was assessed by generating PSTHs, centred around the onset of hindpaw stimulation with a bin size of 20 ms. Data in all PSTH graphs is expressed as mean $\pm$ SEM. A neuron was defined as being significantly modulated by the stimulus if two consecutive 20 ms bins deviated from the mean baseline firing rate (500 ms prior to stimulation onset) by ≥ 1.96 SD. The time of this relative to the onset of hindpaw stimulation was defined as the response onset. Response profile criteria were defined as follows: **non-responsive** if the firing rate of the neuron was not significantly changed following stimulation onset; **short-latency inhibition/excitation** was defined as a significant reduction/increase in firing rate within 200 ms of stimulation onset followed by return to baseline firing rate; **short latency inhibition with rebound excitation** was defined as a significant reduction in firing rate within 200 ms of stimulation onset, followed by significant excitation within 500 ms of stimulation onset; **long-latency excitation** was defined as a significant increase in firing rate between 200 and 500 ms post-stimulation. These criteria were decided based on manual observation of response dynamics prior to any statistical analysis. When calculating parameters such as the period between laser offset and rebound firing a bin size of 5 ms was used.

When plotting pre-stimulation vs post-stimulation graphs, the pre-stimulation period was -500 to 0 ms and the post-stimulation was 0 to 150 ms. This post-stimulation period was selected as it captured the peak of the short-latency response. To quantify the effect of the stimulation on burst firing and ISI measurements during rebound, the pre-stimulation (baseline) period (500 ms prior to stimulation onset) was compared with the rebound period (300 ms after onset of rebound firing) using the RGS method (Ko et al., 2012). These data were graphed as Gardner-Altman estimation plots (Ho et al., 2019) in Python (anaconda python 3.6; 'dabest' package: <https://github.com/ACCLAB/DABEST-python>).

2.5.10 Analysis of recordings: optogenetics

The effect of optogenetic stimulation on the firing of neurons was assessed by generating PSTHs, centred around the onset of the laser pulse. The bin sizes for data analysis and graphing were as follows: i) 20 ms for stim A (250 ms ON, 2 s OFF) ii) 200 ms for stim B (2 s ON, 5 s OFF) iii) 200 ms for stim C (10 s ON, 20 s OFF) iv) 500 ms for stim D (30 s ON, 30 s OFF). The activity of the neuron during the optogenetic stimulation was considered significantly changed if the trial-averaged firing rate during the stimulation crossed the threshold of baseline mean $\pm$ 1.96 SD.

To quantify the effect of optogenetic stimulation on firing rate, the rate in the pre-stimulation (baseline) period (500 ms prior to stimulation onset) was compared with the mean firing rate during the stimulation period. These data were graphed as Gardner-Altman estimation plots (Ho et al., 2019) in Python (anaconda python 3.6; 'dabest' package: <https://github.com/ACCLAB/DABEST-python>).

2.5.11 Analysis of recordings: statistical approach

Before the spontaneous firing data was statistically analysed, the normality of the data distribution and equality of variances for each group were assessed using the Shapiro-Wilk test (assumption of normality rejected if $p \leq 0.05$) and Brown-Forsythe test (assumption of equal variance rejected if $p \leq 0.05$), respectively. If data were normally distributed and group variances were equal, a Student's *t*-test for comparing two groups or a One-way analysis of variance (ANOVA) for comparing multiple groups was used. If the data were not normally distributed or groups had unequal variance, then non-parametric equivalents were used, namely the Mann Whitney *U* test or Kruskal-Wallis One-way ANOVA on Ranks, respectively. SigmaStat (Systat Software Inc., Version 14) was used for testing normality, equality of variance, and carrying out statistical comparisons. For graphical representation of the spontaneous firing properties data, if the property in question was normally distributed it was graphed as a swarm plot with the mean indicated by a line with SEM bars overlaid. If the data was not normally distributed, swarm plots with the first quartile, median and third quartile (indicated by a boxplot) were used. For correlation analyses, a Pearson's correlation coefficient (*R*) and its associated

statistical significance (p value) were calculated in MATLAB (MathsWorks) using in0built functions.

All graphs were generated in MATLAB using custom-written scripts unless otherwise stated. The activity of individual example neurons was plotted as firing rate in spikes/s. For activity averaged across multiple neurons, firing rate in each bin was z-score transformed using the following formula:

$$\frac{x - \mu}{\sigma}$$

x = firing rate in bin; μ = trial-averaged firing rate in the baseline period (500 ms before light onset); σ = SD of baseline firing rate.

Before plotting, a Gaussian smoothing function was applied to the PSTH data (5-point filter; 70 ms half-width).

2.6 Place conditioning

2.6.1 Optic fibre implant surgery

For optogenetic behavioural experiments, DAT-Cre mice were injected with an AAV encoding a Cre-inducible inhibitory opsin, ArchT. As a control for heat, viral transduction, surgical effects etc, a group of mice were injected with an AAV encoding a Cre-inducible GFP reporter protein. Mice were prepared for surgery as described in Section 2.3. Firstly, AAV2-CAG-FLEX-GFP (330 nL, 2.0×10^{12} gc/mL ; UNC Vector Core) control virus or AAV2-CAG-FLEX-ArchT-GFP (330 nL, 2.0×10^{12} gc/mL ; UNC Vector Core) were stereotactically injected bilaterally into the VTA (A-P -3.00 M-L +/-0.5 D-V -4.00 mm).

Following virus injection, optic fibres (200 μ m, 0.37 NA; MFC_200/230-0.37_10mm_ZF1.25(G)_FLT, Doric Lenses) were implanted bilaterally over the VTA. Each fibre was angled at 10° in the mediolateral plane and implanted through small craniotomies (A-P -3.10 to -3.20 M-L +/-1.25 D-V -3.86 mm), which targeted the tip of the optic fibre above the VTA at approximately M-L +/-0.6 mm. Optic fibres were implanted sequentially and

secured in place with dental adhesive resin cement (Super-Bond CB, Sun Medical Co, Ltd.). Following this, the cement was built up around the fibres to ensure they were fixed and supported in place. Behavioural experiments were conducted 4–5 weeks after virus injection and optic fibre implantation.

2.6.2 Conditioned place preference

The conditioned place preference (CPP) apparatus consisted of three compartments separated by manual guillotine-style doors (Figure 2.2; MED-CPP-3013, Med Associates). The two conditioning compartments were separated by a central, ‘neutral’ compartment. One of the conditioning compartments had white walls and a mesh flooring (6 mm² holes) and the second had black walls with grid flooring (3 mm bars, spaced 5 mm apart), providing both visual and tactile stimuli to distinguish the compartments. The light levels in each chamber were measured each day prior to behavioural testing and adjusted to 30–40 lux.

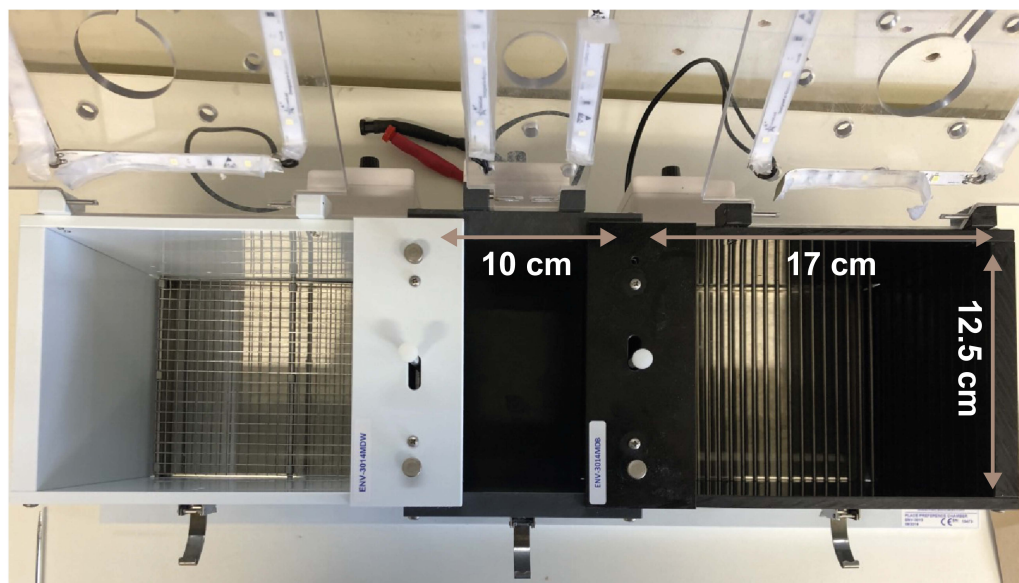


Figure 2.2: CPP setup. A photograph of the setup used for all relevant behavioural testing.

All behavioural experiments were conducted between 12:00 and 19:00 and the equipment was cleaned with 10% ethanol between each mouse. Mice were not food/water-deprived at any point during behavioural testing. CPP was comprised of six sessions on consecutive days: three pre-test days (Figure 2.3A), conditioning day 1, conditioning day 2 (Figure 2.3B), and a post-test (Figure 2.3C).

For the pre-test sessions, the mouse was placed in the central compartment and allowed to explore the entire apparatus unrestricted for 15 minutes. Because most mice displayed a baseline (pre-test) preference for the white chamber (see Results Section 7.4.3.1), and the hypothesis was that inhibition of VTA dopaminergic neurons would induce place aversion, the white chamber was paired with laser ON during conditioning (Figure 2.3B) in a biased CPP protocol. A bias for a chamber was defined as spending 25% more time in one chamber than the other.

On each of the conditioning days, the mouse was connected to two fibre-optic patch cords (MFP_200/230/900-0.37_2m_FC-ZF1.25(F), Doric Lenses) via ferrule adapters (ADAL1, Thor Labs). Each ferrule adapter was surrounded by a layer of black heat-shrink to limit light scatter from the coupling joint. The patch cord cables were threaded through an electrical rotary joint that counterbalanced the tension and rotation generated by the mice (SVTS C series, Servotecnica). The patch cords were coupled to a 561 nm diode-pumped solid-state laser (CL561-100-0, CrystaLaser) via another rotary joint (FRJ_1x2i_FC-2M3, Doric Lenses) to permit yellow light delivery on one of the conditioning days (white chamber). On the other conditioning day, the mouse was tethered but no light delivered (black chamber). The order of conditioning sessions was counterbalanced such that for some mice light delivery in the white chamber was on day 1 and for others it was on day 2 (Figure 2.3B). The laser power was calibrated before each behavioural session and set to 5 mW output at the tip, measured using an optical sensor coupled to an optical power meter (S121C (sensor) PM100D (power meter), Thor Labs). During the laser ON conditioning session, 250 ms or 2 s pulses of light were delivered with probabilistic inter-pulses intervals of 3–7s. In order that the number of pulses delivered in the 250 ms or 2 s laser ON experiments were closely matched, the duration of the conditioning session was 30 and 40 minutes, respectively. In both types of experiment, ~343 pulses were delivered per conditioning session. Light delivery was controlled using custom-written software to deliver TTLs (Arduino). The post-test day was identical to the pre-test day: the mice were placed in the central compartment and allowed to explore the entire apparatus unrestricted for 15 minutes (Figure 2.3C; note, the mouse was not tethered to the patch

cords).

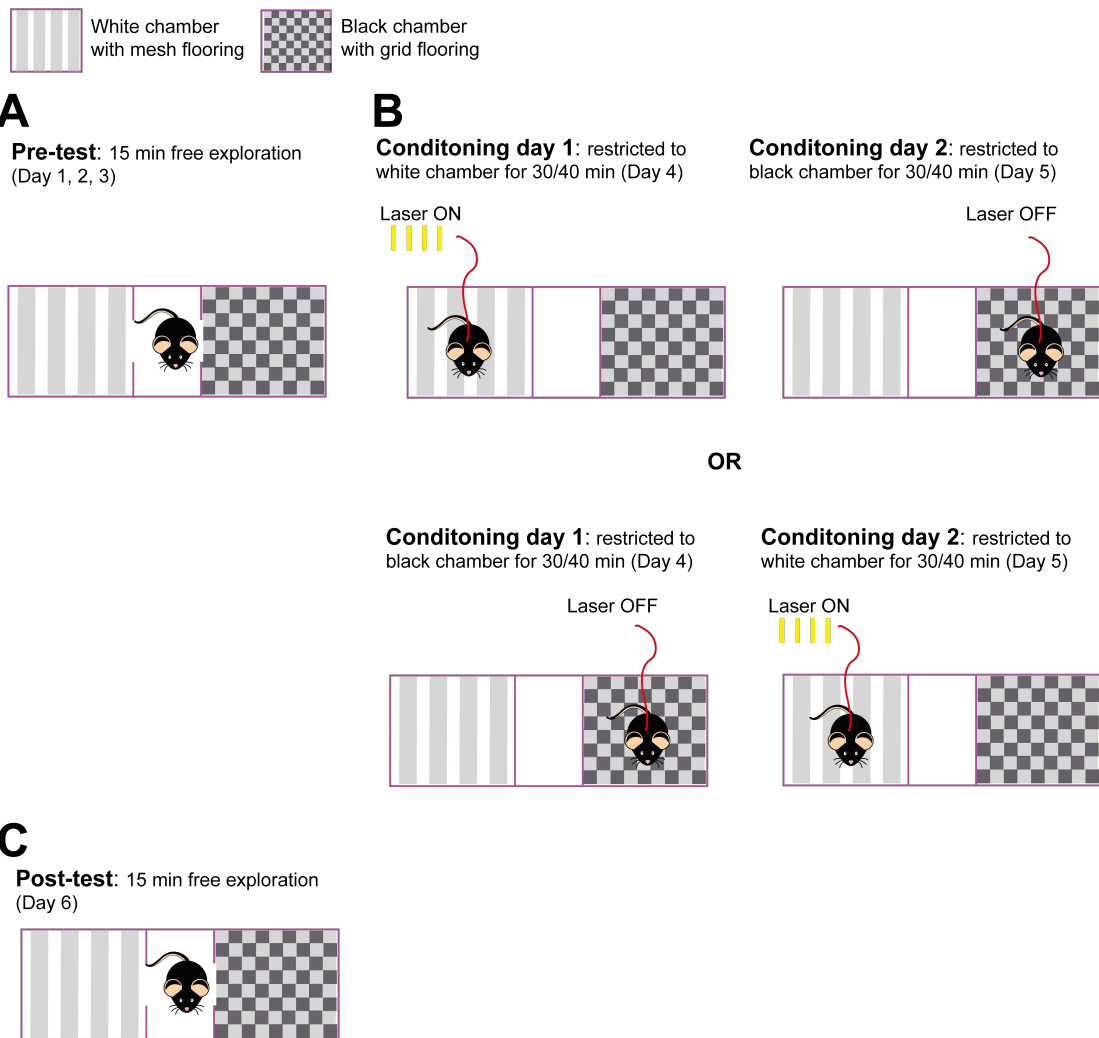


Figure 2.3: Experimental design for biased CPP. **A**, On the three pre-test days the mouse was allowed to freely explore all three compartments. **B**, Laser pulses were delivered in the white chamber on conditioning day 1 (top) or conditioning day 2 (bottom). **C**, For the post-test day, the mouse was allowed to freely explore all three compartments.

During the pre-test and post-test sessions, the location of the mouse in the CPP apparatus was monitored with an overhead camera (GS3-U3-23S6M-C, FLIR Systems; videos acquired using StreamPix, version 7). The time spent in each compartment during the pre-test and post-test sessions was determined offline using the ezTracker package (<https://github.com/DeniseCaiLab/ezTrack>; Pennington et al., 2019) in Python (Anaconda for Python 3.6). Briefly, this software tracks the centre of mass of the mouse and allows the time spent in each compartment to be calculated.

To calculate whether optogenetic inhibition of VTA dopaminergic neurons induced a change in preference for the conditioned (laser ON) chamber (white), the amount of time spent in the conditioned chamber in the post-test was divided by the amount of time spent in the conditioned chamber in the last pre-test day (post/pre). If there was no change in preference for the conditioned chamber then the preference score would be 1; if, for example, the mouse spent 50% less time in the conditioned chamber following conditioning then the preference score would be 0.5. These data were graphed as Gardner-Altman estimation plots for data with two groups, or Cumming estimation plots for three or more groups (Ho et al., 2019). All graphing was in Python (Anaconda for Python 3.6; 'dabest' package: <https://github.com/ACCLAB/DABEST-python>).

2.6.3 Real-time place preference

The same mice were used for CPP and real-time place preference (RTPP). One week was left between the last session of CPP and the RTPP day (Figure 2.4). The CPP and RTPP contexts were entirely different, meaning it is unlikely any conditioning that occurred during CPP was generalised to the RTPP environment.

RTPP was assessed in an open field (60 x 60 cm) divided into two identical sides: one side was paired with bilateral yellow (561 nm) light delivery to the VTA (laser ON) and in the other side no light delivery occurred (laser OFF), counterbalanced. The mouse was connected to two fibre-optic patch cords (as described in the above Section 2.6.2) and placed in the side of the open field that was not paired with light delivery, and allowed to freely explore the whole open field for 15 minutes. Every time the mouse moved from the laser OFF side to the laser ON side, 250 ms or 2 s laser pulses were delivered with probabilistic inter-pulses intervals of 3–7 s, and when the mouse moved from the laser ON to laser OFF side the light delivery was terminated. The trigger for the stimulation to be turned on or off was via a manual switch built-in to the Arduino circuit board (see Section 2.6.2). As for the CPP experiment, the mice were monitored using an overhead camera and the time spent in each side was determined offline using the ezTracker package (<https://github.com/DeniseCaiLab/ezTrack>;

Pennington et al., 2019) in Python (Anaconda for Python version 3.6). In addition to time spent, the software measures distance travelled. These data were graphed as Cumming estimation plots (Ho et al., 2019) in Python.

2.6.3.1 Real-time place preference: measuring acceleration

In addition to the optic fibre patch cords, the mouse was fitted with a head-mounted accelerometer (SEN-13762, Sparkfun) during RTPP experiments. Serial data from the accelerometer was sampled at 100 Hz and relayed to a Teensy 3.6 Arduino board (Sparkfun) via a differential I2C breakout board (PCA9615, Sparkfun). The code for this acquisition was adapted from a pre-existing library (<https://github.com/bolderflight/MPU9250>). Note, this ‘accelerometer’ also capture other motion parameters, but these were not analysed for the purposes of this research.

Acceleration data was analysed in MATLAB (MathWorks) using custom analysis scripts. For each group, PSTH graphs of z-score transformed acceleration data were generated (bin size, 500 ms) centred around laser onset. The plots show the group mean with the SEM represented by the shaded area above and below.

2.6.4 Timeline of place preference behaviour

For the conditioning experiments, the same mice were used for both CPP and RTPP experiments. Furthermore, these mice were used for the experiments in which 250 ms and 2 s laser pulses were delivered during CPP/RTPP. An example behavioural timeline is outlined in Figure 2.4 below.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1	Pre-test (1)	Pre-test (2)	Pre-test (3)	Conditioning (laser ON; 250 ms pulses)	Conditioning (laser OFF)	Post-test	
2							
3	RTPP (250 ms pulses)						
4							
5	Pre-test (1)	Pre-test (2)	Pre-test (3)	Conditioning (laser OFF)	Conditioning (laser ON; 2s pulses)	Post-test	
6							
7	RTPP (2 s pulses)						

Figure 2.4: Timeline of place preference behaviour. Each behavioural protocol (CPP/RTPP) was interleaved with a week in which no behavioural testing occurred. This was to extinguish any possible carry-over associations between protocols. **Note**, for CPP, some mice received laser stimulation on the first conditioning day, whereas other mice received stimulation on the second conditioning day (i.e. counterbalanced).

A full dataset (i.e. $n = 6$ and 7 for GFP control and ArchT groups, respectively) was gathered for the experiments in which 250 ms laser pulses were delivered during conditioning/RTPP. However, the group sizes were smaller ($n = 3$ and 4 for GFP control and ArchT groups, respectively) for the experiments in which 2 s laser pulses were delivered because the cannulae of the optic fibres snapped during attachment to the optic fibre cable in some cases. In these cases, the 2 s laser protocol could not be carried out and the animals were killed and perfused (see below). It was assumed that this happened because the cement was not built-up sufficiently around the joint between the optic fibre and the ferrule, leading to breakages after repeat attachment and detachment to the patch cord.

2.6.5 Perfuse-fixation and tissue processing

At the end of behavioural experiments, mice were administered a lethal dose of sodium pentobarbitone (0.3 mL; 20% w/v), and then transcardially perfused with ~20 mL of 0.1 M PBS, pH 7.4, followed by ~35 mL of 4% (w/v)PFA in 0.1 M phosphate buffer, pH 7.4. The implant was carefully removed to avoid tissue damage, and then the brain was removed and stored in 4% (w/v) PFA overnight at 4°C, followed by storage in PBS-azide until sectioning 12 h to 1 week later.

Fixed brains were sectioned to a thickness of 50 μm in the coronal plane with a vibrating-blade microtome (VT1000S; Leica Microsystems) and the midbrain was isolated. The brain was sectioned into 4 series, meaning that in a single series the A-P distance between adjacent sections was 200 μm . Free-floating brain sections were then incubated in 10% (v/v) normal-donkey serum in PBS-T for 1 h followed by overnight incubation at room temperature in the following primary antibodies, also in PBS-T: guinea pig anti-TH (1:2000; 213-104, Synaptic Systems) to immunolabel dopaminergic neurons; rat anti-GFP (1:500; GF090R, Nacalai Tesque) to enhance the GFP signal in GFP/ArchT-transduced neurons; mouse anti-glial acidic fibrillary protein (GFAP; 1:1000; G3893, Sigma Aldrich) to immunolabel the glial scar left by the optic fibre tracks. These sections were then washed in PBS for 5 minutes, three times, followed by overnight incubation in PBS-T at room temperature containing the following fluorophore-conjugated secondary antibodies: Cy5-conjugated donkey anti-guinea pig IgG (1:500; 706-605-148, Jackson ImmunoResearch); AlexaFluor 488-conjugated donkey anti-rat IgG (1:500; 712-545-153, Jackson ImmunoResearch); Cy3-conjugated donkey anti-mouse IgG (1:500; 715-165-151, Jackson ImmunoResearch). Next, these sections were washed in PBS for 5 minutes, three times, and then mounted on glass slides with aqueous mounting medium (SlowFade™ Diamond Antifade Mountant, Invitrogen).

For each mouse, the section(s) containing the deepest optic fibre track (i.e. where the optic fibre tip had been) was imaged using a confocal microscope with a 10x objective (NA = 0.45). The anatomical location of the tip was then determined, guided by (Paxinos and Franklin, 2013).

Chapter 3

Molecular markers of RRF dopaminergic neurons

3.1 Introduction

The first description of catecholaminergic neurons in the mesencephalon subdivided them into A8, A9 and A10 regions in the rat (Dahlström and Fuxe, 1964). These are now commonly known as the midbrain dopaminergic neurons of the RRF, SNc and VTA, respectively. It is not trivial to objectively define the boundaries of these nuclei based on anatomical landmarks in rats (Dahlström and Fuxe, 1964) or mice (Fu et al., 2012), and even with selective molecular markers for dopaminergic neurons, it is still challenging to define these cell groups (Fu et al., 2012). This raises the question: what are the most useful and comprehensive criteria (neuronal properties) for defining a circumscribed group of neurons? In addition to location within the brain, the process of classifying neurons can be approached at four main levels: neuronal connectivity, morphological properties, physiological properties, and molecular makeup (Zeng and Sanes, 2017). These levels have been explored for some midbrain dopaminergic neurons, comparing both between and within the VTA and SNc (Fu et al., 2012; Ungless and Grace, 2012; Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015; Poulin et al., 2020). The RRF, on the other hand, has received less attention

in such classification schemes.

Knowing the molecular makeup of midbrain dopaminergic neurons is central to understanding their function. This makeup can be conceptualised as a ‘molecular ground state’ (Fishell and Heintz, 2013), and by defining this state, the changes that occur in disease can be mapped to generate a ‘molecular barcode’ for a disease state. Degeneration of midbrain dopaminergic neurons in Parkinson’s disease (PD) occurs most markedly in the ventral tier of the SNc (Hassler, 1938; Fearnley and Lees, 1991; Damier et al., 1999b), suggesting that the pathological burden is not borne equally across all dopaminergic neurons. The molecular underpinnings of this selective vulnerability are an ongoing focus of research (Brichta and Greengard, 2014; Poulin et al., 2014), a key aspect of which is understanding the functional distinctions between the midbrain dopaminergic neuron subgroups.

Pooling together SNc dopaminergic neurons and comparing their gene expression profile with that of VTA dopaminergic neurons reveals differentially expressed molecular markers. For example, expression of insulin-like growth factor (*Igf1*) and the transcriptional regulators SRY-Box Transcription Factor 6 (*Sox6*) and special AT-rich sequence binding protein 1 (*Satb1*) are all enriched in SNc dopaminergic neurons, whereas gastrin-releasing peptide (*Grp*) and the transcriptional regulator orthodenticle homolog 2 (*Otx2*) are enriched in VTA dopaminergic neurons (Grimm et al., 2004; Chung et al., 2005; Brichta et al., 2015). Furthermore, single-cell gene expression profiling has helped to identify midbrain dopaminergic neuron subtypes based on the presence or absence of a selection of these and other molecular markers (Poulin et al., 2014; La Manno et al., 2016), with relevance for understanding selective vulnerability in disease (Poulin et al., 2014) and projection targets (Poulin et al., 2018). However, this depth of understanding is lacking for RRF dopaminergic neurons.

The calcium-binding protein calbindin-D-28k (calbindin-1) is another example of a protein differentially expressed in midbrain dopaminergic neurons; it is present in many neurons of the RRF, VTA and dorsal SNc but largely absent from the ventral SNc (Liang et al., 1996; Fu et al., 2012). There is evidence that dopaminergic neurons expressing calbindin-1 are more resistant to death in

PD (Yamada et al., 1990; Lavoie and Parent, 1991; German et al., 1992). This observation is in line with the most severe loss of dopaminergic neurons observed in the ventral SNc (Gibb and Lees, 1991; Damier et al., 1999b). The ventral striatum is innervated by calbindin-1-expressing dopaminergic neurons—a *priori* resistant to cell death—of the dorsal SNc and parabrachial pigmented nucleus of the VTA (PBP) (Poulin et al., 2018), whereas the dorsal striatum is devoid of such innervation (Dopeso-Reyes et al., 2014). Again, this fits with the understanding of PD pathology, in that loss of dopaminergic innervation to the dorsal striatum is comparatively more severe (Kish et al., 1988). The evidence that calbindin-1 itself confers neuroprotection is, however, mixed (Airaksinen et al., 1997; Tan et al., 2000; Yuan et al., 2013), and therefore it may serve merely as a marker of resistant dopaminergic neurons. In addition to somatic expression of calbindin-1 in midbrain dopaminergic neurons, it is also present in the neuropil of the substantia nigra pars reticulata (SNr) (McRitchie et al., 1996; Hontanilla et al., 1997; Morel et al., 2002) and is therefore a useful marker for defining its borders.

In an expression pattern that is somewhat inverse to that of calbindin-1, some dopaminergic neurons in ventral SNc and VTA express the ‘detoxifying enzyme’ aldehyde dehydrogenase 1a1 (Aldh1a1) (McCaffery and Drager, 1994; Poulin et al., 2014). Again in an inverse manner to calbindin-1, these dopaminergic neurons are particularly vulnerable to cell death in PD (McCaffery and Drager, 1994; Liu et al., 2014; Poulin et al., 2014). Aldh1a1 detoxifies the reactive aldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is produced following dopamine metabolism by the actions of monoamine oxidase (MAO) (Marchitti et al., 2007). Deletion of Aldh1a1 worsens the degeneration of dopaminergic neurons in PD models (Liu et al., 2014), which parallels the downregulation of Aldh1a1 observed in the SNc of PD brains (Galter et al., 2003; Werner et al., 2008). Moreover, overexpression of Aldh1a1 is somewhat neuroprotective (Liu et al., 2014). More recently, Aldh1a1 has been shown to mediate the non-canonical synthesis of GABA in some dopaminergic neurons of the SNc and VTA (Kim et al., 2015). Anatomically, Aldh1a1-expressing dopaminergic neurons project throughout the most dorsal aspects of the dorsal striatum, confined

mainly to the rostral and intermediate striatum (McCaffery and Drager, 1994; Sgobio et al., 2017; Poulin et al., 2018). Aldh1a1 is also present in a small population of VTA dopaminergic neurons that project to the nucleus accumbens medial shell (NAcMedS) (McCaffery and Drager, 1994; Poulin et al., 2018; Wu et al., 2019). Taken together, studies of Aldh1a1 and calbindin-1 in midbrain dopaminergic neurons suggest that these two markers have distinct yet overlapping expression patterns with relevance to their axonal projections and vulnerability to death in PD. However, their expression pattern has not been studied concurrently in the midbrain.

At its more caudal aspects, the RRF is a dorsal band of midbrain dopaminergic neurons with little spread in the dorsal-ventral axis and is relatively easy to distinguish as a circumscribed cell group based on the expression of TH (Deutch et al., 1988; François et al., 1999; Fu et al., 2012). However, the rostral aspect of RRF is adjacent to the caudal SNc laterally and PBP medially, meaning it is challenging to delineate. Molecular markers differentially expressed between the rostral RRF, caudal SNc and PBP would be useful for objectively defining these abutting borders. The most widely cited anatomical atlas of the mouse brain is that of Paxinos and Franklin (2013). However, it is unclear how the subregions of the dopaminergic midbrain are objectively demarcated in this atlas, indicating clearer criteria are required. Some progress was made relatively recently (Fu et al., 2012), but further criteria would aid in the delineation of anatomical borders, particularly between the caudal SNc and rostral RRF. To this end, I sought to determine a molecular marker(s) that is differentially expressed between the caudal SNc and rostral RRF. Although the molecular makeup of RRF dopaminergic neurons has not been extensively studied, it is known that some RRF neurons express calbindin-1 (Rogers, 1992; Liang et al., 1996; Fu et al., 2012) and a minority express Aldh1a1 (McCaffery and Drager, 1994; Poulin et al., 2014; Wu et al., 2019). Investigating the expression patterns of these two markers was of particular interest given their contrasting distribution profile in the SNc and VTA, as outlined above. Moreover, comprehensive comparisons of molecular marker expression between the RRF and other midbrain dopaminergic neuron subgroups are lacking. To address this gap, I chose a panel of pertinent molecular markers and

determined their expression in the caudal dopaminergic midbrain of mice using immunohistochemistry.

3.2 Aims

- Determine whether Aldh1a1 and calbindin-1 expression in dopaminergic neurons could serve as immunohistochemical markers for determining the borders between the rostral RRF, caudal SNc and PBP.
- Examine the expression pattern of other molecular markers in RRF dopaminergic neurons, focusing on a selection of proteins known to be enriched in SNc and/or VTA dopaminergic neurons.

3.3 Methods

Adult male (n = 4) and female (n= 2) C57Bl/6J mice aged 3–5 months were transcardially perfused and their brains were subsequently sectioned in the coronal plane at 50 μ m. Indirect immunofluorescence was used to visualise dopaminergic neurons using tyrosine hydroxylase as a primary marker. Additionally, the expression of other molecular markers in dopaminergic neurons was assessed: Aldh1a1, calbindin-1, SatB1, Otx2, Sox6, calretinin and G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) (Table 3.2). Furthermore, putative GABAergic neurons were visualised by immunoreactivity for the α 1 subunit of the GABA_A receptor. See Materials and Methods Section 2.2 for more details.

Antigen (host species; dilution)	Source and catalog number	Specificity reference
Aldh1a1 (rabbit; 1 in 500)	Abcam AB24343	Berglöf et al. (2007); Poulin et al. (2018): high degree of overlap between Aldh1a1 immunoreactivity and tdTomato expression in Aldh1a1-CreER ^{T2} ;Ai9 mice.
Calbindin-1 (mouse; 1 in 500)	Swant CB300	Airaksinen et al. (1997): no immunoreactivity in midbrain sections from calbindin-1 KO mice.
Calretinin (goat; 1 in 500)	Swant CG1	Schurmans et al. (1997) and Product Description: no immunoreactivity in brain sections from calretinin KO mice.
GABA _A receptor α 1-subunit (goat; 1 in 250)	Santa Cruz SC-7348	Ferrer and Puig (2003); Product Description: the antibody recognised a single protein band in a Western blot analysis using mouse brain lysate.
GIRK2 (goat; 1 in 500)	Abcam AB65096	Chabrat et al. (2019); Product Description: the antibody recognised a single protein band in a Western blot analysis using mouse brain lysate.
NeuN (rabbit; 1 in 500)	Biosensis R-3770-100	Roberts et al. (2020): specifically expressed in neurons and not nearby astrocytes.
Otx2 (goat; 1 in 1000)	R&D Systems AF1979	El Nagar et al. (2018): no protein recognised in Western blot analysis or immunohistochemistry with brain tissue from Otx2 conditional KO mice.
SatB1 (goat; 1 in 1000)	Santa Cruz SC-5989	Peng et al. (2017): SatB1 KO from retinal ganglion results in loss of SatB1 immunoreactivity.
Sox6 (rabbit; 1 in 4000)	Abcam AB30455	Product Description: no immunoreactivity in Sox6 KO brain tissue.
TH (guinea pig; 1 in 1000)	Synaptic Systems 213-104	González-Cabrera et al. (2017) and Farassat et al. (2019); Present study: clear somatic expression in midbrain neurons and axons in striatum, identical to other high quality anti-TH antibodies.

Table 3.2: A summary of the primary antibodies used in this Chapter and information about their specificity. KO, knockout.

3.4 Results

3.4.1 *Aldh1a1 and calbindin-1 expression patterns distinguish the border between the rostral RRF and caudal SNc*

Calbindin-1 (Liang et al., 1996; Fu et al., 2012) and Aldh1a1 (McCaffery and Drager, 1994; Liu et al., 2014; Poulin et al., 2014; Wu et al., 2019) are expressed by some dopaminergic neurons in the SNc, VTA and RRF and are present in relatively distinct subpopulations. In the midbrain, somatic Aldh1a1 expression is confined mostly ($\sim 75\%$) to dopaminergic neurons (Kim et al., 2015). Similarly, somatic calbindin-1 expression is nearly exclusively confined to dopaminergic neurons in the rat midbrain (Rogers, 1992). I chose to test for the expression of these markers in RRF dopaminergic neurons using immunohistochemistry.

I selected sections cut at 100 μm intervals from -3.80 mm to -4.20 mm with respect to Bregma (Figure 3.1B–F; guided by Paxinos and Franklin (2013)) to give a representative sample of the caudal dopaminergic midbrain. I also included a section at -3.60 mm from Bregma that contained the ‘classical’ and widely studied SNc and VTA for comparison (Figure 3.1A). At -3.60 mm from Bregma, the SNc and VTA were separated by the medial lemniscus and therefore their boundaries could be clearly defined (Figure 3.1A). The GABAergic SNr was defined by its size, shape and location, and by the presence of dense calbindin-1 immunoreactivity in the neuropil, as well as its paucity of TH-expressing and Aldh1a1-expressing neurons (Figure 3.1). In the VTA, Aldh1a1-positive dopaminergic neurons were sparse and mainly confined to the medial and ventral aspects (Figure 3.1A, middle panel). In contrast, Aldh1a1-positive dopaminergic neurons were dense within the ventromedial SNc, becoming sparser in the dorsal and lateral SNc. Dopaminergic neurons in the substantia nigra pars lateralis (SNL) did not express Aldh1a1. In a somewhat inverse pattern, calbindin-1 was present in many VTA dopaminergic neurons but almost entirely absent in neurons of the SNc (Figure 3.1A, right panel). Notably, calbindin-1 was expressed in many SNL dopaminergic neurons, making it a useful marker for delineating this region and defining its

borders with respect to the SNc.

The rostral RRF becomes visible at approximately -3.80 mm from Bregma, shown in Figure 3.1B. However, it is difficult to demarcate the boundaries of the RRF as it is continuous with the caudal SNc laterally and the PBP medially. Since *Aldh1a1* was enriched in the SNc more rostrally (Figure 3.1A, middle panel), I sought to determine whether it could serve as a useful marker for defining the border between the RRF and SNc in more caudal sections. A dense cluster of *Aldh1a1*-positive dopaminergic neurons marked the ventral portion of the SNc at -3.80 mm from Bregma (Figure 3.1B, middle panel), whereas *Aldh1a1*-positive neurons in the overlying putative RRF were more sparsely distributed. Though this expression pattern was useful for defining the border between these two regions, the demarcation is not entirely clear because there were also a sparse group of *Aldh1a1*-positive neurons in the dorsolateral aspects of the putative SNc near those of the overlying putative RRF. In a somewhat inverse pattern, calbindin-1 expressing dopaminergic neurons were abundant in the rostral RRF but almost entirely absent from the caudal SNc. Thus, a combination of *Aldh1a1* and calbindin-1 expression allow the border between the rostral RRF and caudal SNc to be objectively defined with acceptable clarity.

The boundary of the SNL at -3.80 mm (Figure 3.1B) was easily defined as it was anatomically separate from the RRF/SNc and, as was the case in a more rostral section (Figure 3.1A), many of its dopaminergic neurons expressed calbindin-1 (Figure 3.1B, right panel). It was more challenging to define the border between the medial RRF and the PBP at -3.80 mm from Bregma (Figure 3.1B) as both of these regions contained dopaminergic neurons that express calbindin-1 and *Aldh1a1*. However, the dopaminergic neurons of the PBP were sparsely distributed at this level, unlike the denser clusters of neurons in the RRF and SNc (Figure 3.1B, left panel). This difference in cell density permitted a border to be drawn between the RRF and PBP; however, this could perhaps be considered more arbitrary as it was not based on a differential expression of molecular markers.

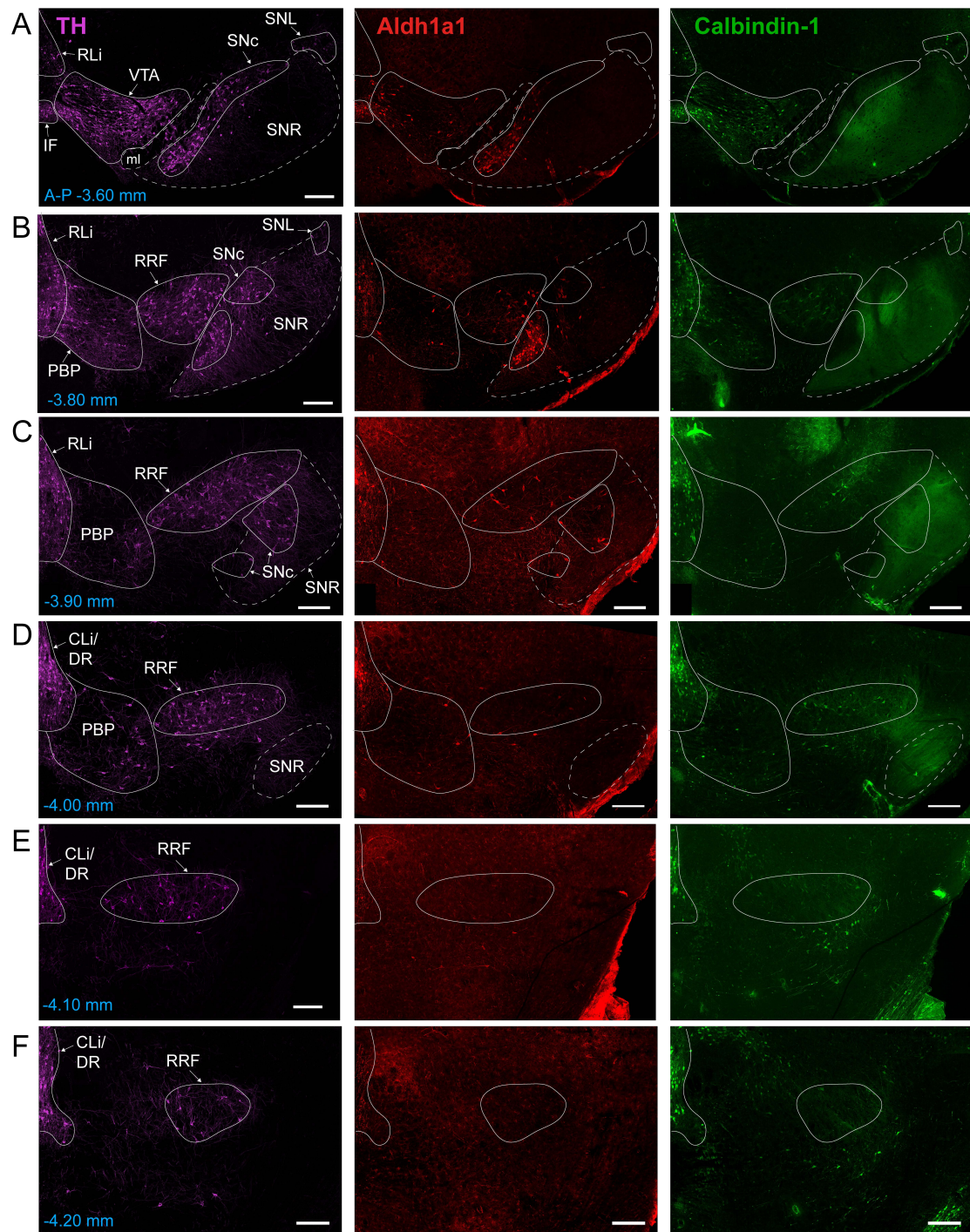
At -3.90 mm from Bregma (Figure 3.1C) the rostral RRF becomes larger, forming a 'dorsal bridge' between the PBP medially and the SNc laterally. As was the case at -3.80 mm from Bregma,

SNc dopaminergic neurons often expressed Aldh1a1 but lacked calbindin-1, allowing a border to be drawn with the overlying RRF. Again, the border between the RRF and PBP was more difficult to objectively define on the basis of Aldh1a1/calbindin-1 expression and so the sparser distribution of dopaminergic neurons in the PBP was used to loosely define it.

Moving more caudally, at -4.00 mm from Bregma the SNc is no longer present (Figure 3.1D), as demonstrated by the near total absence of Aldh1a1-positive dopaminergic neurons (Figure 3.1D, middle panel). The dopaminergic neurons of the RRF were predominant at this coronal level, alongside a few sparsely distributed dopaminergic neurons in the PBP more medially (Fig 3.1D, left panel). Both the RRF and PBP contained some calbindin-1-expressing dopaminergic neurons (Fig 3.1D, right panel). The SNr was much smaller at this level, as shown by the smaller area of calbindin-1 immunopositive neuropil (Figure 3.1D, right panel).

The PBP is no longer present at -4.10 mm from Bregma and most dopaminergic neurons are clustered together in the RRF (Figure 3.1E, left panel). As at -4.00 mm from Bregma (Figure 3.1D), some RRF dopaminergic neurons expressed calbindin-1 (Figure 3.1E, right panel), and there were very few RRF dopaminergic neurons expressing Aldh1a1 (Figure 3.1E, middle panel). Interestingly, there were some dopaminergic neurons ventral to the RRF cluster that could not be readily assigned to a known cell group (Figure 3.1E, left panel). At this level the SNr is absent, as shown by the lack of calbindin-1-dense neuropil (Figure 3.1E, right panel).

The most caudal aspect of the RRF is at around -4.20 mm from Bregma, where a small and sparsely distributed group of dopaminergic neurons were observed (Figure 3.1F, left panel). This level is distinguished from more rostral sections as the SNr is absent and the cluster of dopaminergic neurons is small, cell sparse, and laterally located. No dopaminergic neurons expressed Aldh1a1 in the caudal RRF (Figure 3.1F, middle panel), and only a few expressed calbindin-1 (Figure 3.1F, right panel). As at -4.10 mm from Bregma, a small number of ventrally-located dopaminergic neurons were present which could not be assigned to a known cell group (Figure 3.1F, left panel).



(Figure legend on next page)

Figure 3.1: Defining the borders of the RRF, SNc and VTA according to the distribution of Aldh1a1 and calbindin-1 expression in the mouse. **A–F** (left panels) TH expression throughout the anterior-posterior (A-P) extent of the caudal midbrain defined the dopaminergic neurons of the RRF, SNc and VTA in representative coronal sections from one mouse. **A**, Aldh1a1 (middle panel) was expressed in SNc neurons, most densely in the ventromedial portion, and sparsely expressed in the VTA. Somatic calbindin-1 (right panel) expression was observed throughout the VTA and SNL but was largely absent from the SNc. Dense calbindin-1 expression in the neuropil was observed in the SNr. **B, C**, Aldh1a1 (middle panel) was often expressed in caudal SNc neurons, most densely in the ventromedial portion, and sparsely expressed in the RRF and PBP. Calbindin-1 (right panel) was largely absent from SNc neurons but expressed by neurons throughout the RRF, PBP (**B, C**) and SNL (**B**). **D, E** Aldh1a1 (middle panel) was expressed sparsely in the RRF (**D, E**) and PBP (**D**). Calbindin-1 (right panel) was often expressed in neurons throughout the RRF (**D, E**) and PBP (**D**). **F**, In the caudal RRF, Aldh1a1 (middle panel) was absent and a minority of neurons expressed calbindin-1 (right panel). Scale bars, 200 μ m. Distances shown in bottom left of each left panel are anterior-posterior (A-P) distances with respect to Bregma for each section. Dopaminergic cell groups are defined by solid white lines; the SNr and medial lemniscus (ml) are defined by dashed white lines. The medial lemniscus (ml) is a white matter tract containing a few scattered dopaminergic (TH-positive) neurons. CLi, caudal linear nucleus of the raphe; DR, dorsal raphe; IF, interfascicular nucleus of the VTA; PBP, parabrachial pigmented nucleus of the VTA; RLi, rostral linear nucleus of the VTA.

Next, I tested whether the somatic expression of these markers was mostly limited to dopaminergic neurons within the midbrain nuclei (Figure 3.2A, B). Close examination suggested that the majority of neurons expressing Aldh1a1 (Figure 3.2A) and calbindin-1 (Figure 3.2B) were indeed dopaminergic. Taken together, these results so far demonstrate that the expression patterns of Aldh1a1 and calbindin-1 are useful for distinguishing the borders of the rostral RRF (Aldh1a1^{LOW}, calbindin-1^{HIGH}) and caudal SNc (Aldh1a1^{HIGH}, calbindin-1^{LOW}). In future Chapters, this delineation will permit the functional characteristics of dopaminergic neurons to be confidently ascribed to the RRF vs the SNc/VTA. And, pertinent to this Chapter, the expression pattern of other molecular markers in the RRF can be mapped (see below).

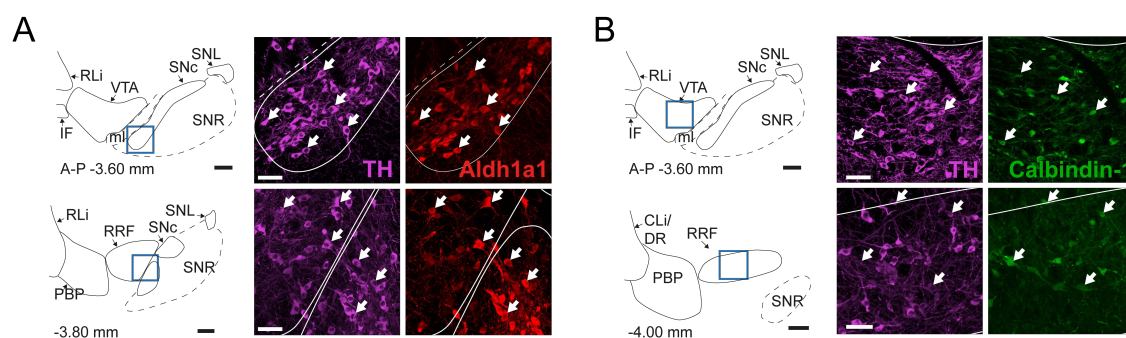


Figure 3.2: Expression of Aldh1a1 and calbindin-1 in the midbrain is mostly restricted to dopaminergic neurons. Images in this Figure are a higher magnification of those shown in Figure 3.1. **A, B Left**, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. **Middle, Right**, Confocal micrographs showing the location of Aldh1a1-expressing (**A**) and calbindin-1-expressing (**B**) dopaminergic (white arrows) neurons in the midbrain. Scale bars, 50 μ m.

Next, I sought to compare my delineation of the RRF, SNc and VTA dopaminergic cell groups with the widely cited atlas of Paxinos and Franklin (2013). In this atlas, the criteria used to define midbrain dopaminergic cell groups are unclear. It should be noted that the anterior-posterior distance (from Bregma) for each section in Figure 3.1 was determined using the Paxinos atlas (guided by anatomical landmarks), but the borders of the RRF, SNc and VTA were drawn independently based on the rationale outlined above. The use of calbindin-1 and Aldh1a1 to delineate the borders of the SNc and VTA approximated the Paxinos atlas for -3.60 mm from Bregma (Figure 3.3A). Similarly, using the expression of Aldh1a1 (Figure 3.1B and C, middle panel) and calbindin-1 (Figure 3.1B and C, right panel) to guide the definition of RRF, SNc and VTA at -3.80 mm (Figure 3.3B) and -3.90 mm (Figure 3.3C) from Bregma approximated the borders displayed in the Paxinos atlas. The PBP is poorly defined at -3.80 and -3.90 mm from Bregma in Paxinos and Franklin (2013), meaning it was difficult to compare the definition in the present study with the atlas. The presence of the PBP on the section I defined as -4.00 mm from Bregma (Figure 3.3D left panel) differs from the same level in the Paxinos atlas (Figure 3.3D right panel) where the PBP is absent. I was relatively confident the PBP cell group was present as the sparsity of the cells within this region was similar to -3.90 mm from Bregma; and I was confident this section (Figure 3.3D) was not -3.90 mm from Bregma as the SNr was smaller in this section than at -3.90 mm from Bregma, which is the same as the atlas (Fig 3.3D, right panel). However, it is possible that the dopaminergic neurons I have defined as being within the PBP are in fact a medial aspect of the RRF. At -4.10 mm (Figure 3.3E, left panel) and -4.20 mm from Bregma (Figure 3.3F, left panel), my outline for the RRF approximated the Paxinos atlas (Figure 3.3E and F, right panels).

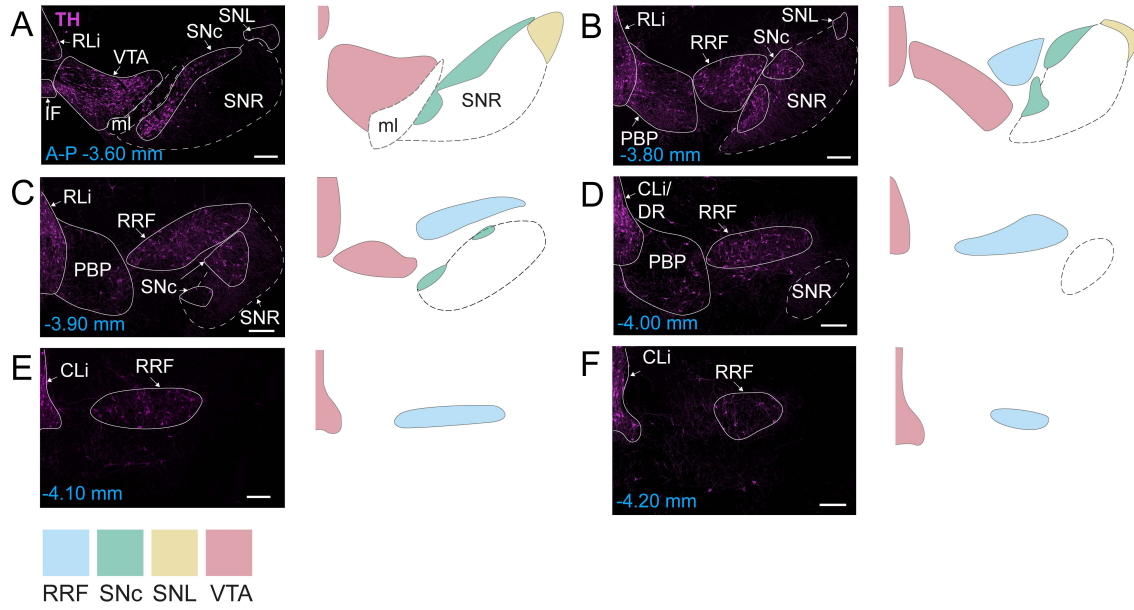


Figure 3.3: Comparison of molecular marker-defined boundaries of dopaminergic neurons with the borders in the Paxinos and Franklin atlas. **A–F**, Left panels show the borders of RRF, SNc, VTA and SNL as defined by the expression of Aldh1a1 and calbindin-1 (Figure 3.1). Right panels show the borders defined in Paxinos and Franklin (2013). The distance from Bregma for sections in the left panels (bottom left of each panel) was matched to the same plane in Paxinos and Franklin (2013). Scale bars, 200 μ m. Scale of confocal micrographs on the left and corresponding Paxinos atlas Figures on the right were matched. For the colour key, the definition of VTA includes: the CLi, the RLi, the PBP and the paranigral nucleus of the VTA (PN); note, no objective method was devised to delineate the PN from the overlying PBP in immunohistochemistry experiments, hence these two regions were grouped together under the umbrella of ‘VTA’ (A, left).

3.4.2 The expression profile of selected molecular markers in the RRF

Now that I had determined a combination of calbindin-1 and Aldh1a1 immunoreactivity was helpful in delineating the border between rostral RRF (Aldh1a1^{LOW}, calbindin-1^{HIGH}) and caudal SNc (Aldh1a1^{HIGH}, calbindin-1^{LOW}), it was possible to explore the distribution of other molecular markers in RRF dopaminergic neurons. Each of these will be considered below in a qualitative manner. To this end, I selected caudal midbrain sections containing the rostral (A-P -3.80 to -3.90 mm from Bregma), intermediate (A-P -4.00 to -4.10 mm from Bregma) and caudal RRF (A-P -4.20 to -4.25 mm from Bregma), as well as a section containing the rostral midbrain (A-P -3.30 to -3.60 mm from Bregma). This gave a representative sample of the different levels of the RRF, as well as a section containing the classical SNc and VTA.

3.4.2.1 RRF dopaminergic neurons express SatB1

SatB1 is a DNA-binding protein involved in regulating cellular senescence in dopaminergic neurons (Riessland et al., 2019). As has been previously demonstrated (Grimm et al., 2004; Chung et al., 2005; Huang et al., 2011; Brichta et al., 2015), the transcription factor SatB1 was expressed in many dopaminergic neurons of the VTA (Figure 3.4A and 3.5A) and SNc (Figure 3.4A, B and 3.5B, C). Here, I show that many RRF dopaminergic neurons also express SatB1 (Figure 3.4B–D and 3.5C, D) and that, qualitatively, SatB1 immunoreactivity is not a specific marker of any midbrain dopaminergic cell group. SatB1 was mostly expressed in dopaminergic neurons in the VTA, SNc and RRF, though there were examples of SatB1 expression in non-dopaminergic neurons (Figure 3.5, green arrows).

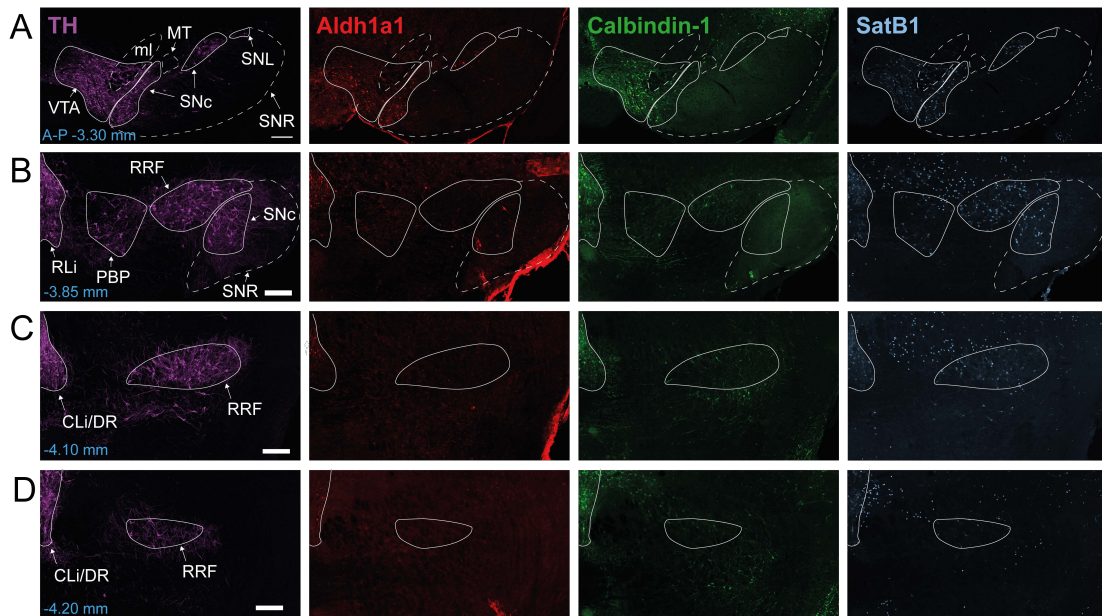


Figure 3.4: RRF dopaminergic neurons express SatB1. The expression patterns of Aldh1a1 and calbindin-1 were used to define the borders of the RRF, SNc and VTA. The expression of SatB1 was examined in the ‘classical’ midbrain (A), in addition to sections containing the rostral (B), intermediate (C) and caudal (D) RRF. Scale bars, 200 μ m. Distances shown in bottom left of each left panel are A-P distances with respect to Bregma for each section. Dopaminergic cell groups are defined by solid white lines; the SNr, ml and medial terminal nucleus (MT) are defined by dashed white lines.

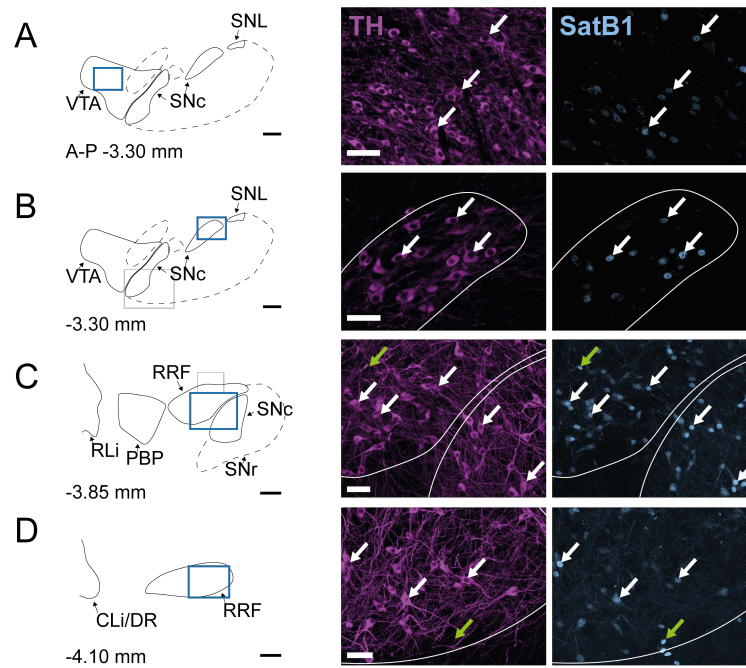


Figure 3.5: SatB1 is mostly expressed in dopaminergic VTA, SNc and RRF neurons. Images and outlines in this Figure are a higher magnification of those shown in Figure 3.4. **A–D Left**, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. **Middle, Right**, Confocal micrographs showing the location of SatB1-expressing dopaminergic neurons (white arrows) and SatB1-expressing non-dopaminergic neurons (green arrows). Scale bars, 50 μ m.

3.4.2.2 Few RRF dopaminergic neurons express *Otx2*

During development, the transcription factor *Otx2* is involved in specifying a subset of VTA dopaminergic neurons, and continues to be expressed in post-mitotic neurons (Panman et al., 2014). In the present study, *Otx2* was expressed in rostral VTA dopaminergic neurons (Figure 3.6A and 3.7A) but was mostly absent from SNc dopaminergic neurons (Figure 3.6A and 3.7B). In a more caudal section, the expression of *Otx2* was mostly limited to non-dopaminergic neurons within the PBP (Figure 3.6B and 3.7C). Within the RRF, *Otx2* was expressed predominately in non-dopaminergic neurons (Figure 3.7D). Overall, this qualitative examination suggests that *Otx2* expression in midbrain dopaminergic neurons is mostly limited to the rostral VTA. In the caudal midbrain, *Otx2* expression in the PBP and RRF is mostly limited to non-dopaminergic neurons and is thus not a marker of dopaminergic neurons in these regions.

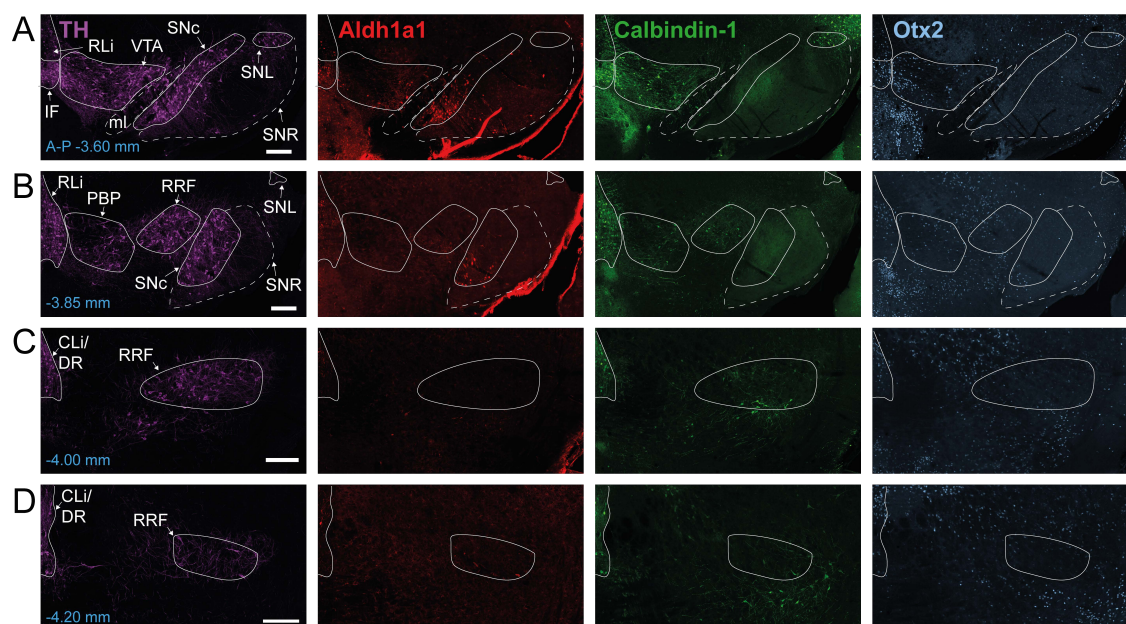


Figure 3.6: Few RRF dopaminergic neurons express Otx2. The expression patterns of Aldh1a1 and calbindin-1 were used to define the borders of the RRF, SNc and VTA. The expression of Otx2 was examined in the ‘classical’ midbrain (A), in addition to sections containing the rostral (B), intermediate (C) and caudal (D) RRF. Scale bars, 200 μ m. Dopaminergic cell groups are defined by solid white lines; the SNr and ml are defined by dashed white lines.

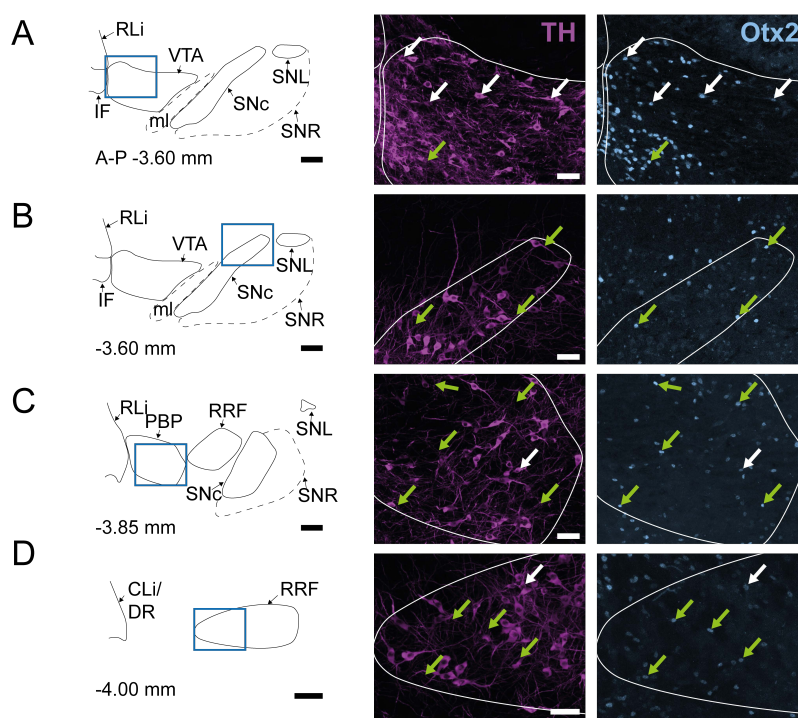


Figure 3.7: Otx2 expression in midbrain dopaminergic neurons is mostly limited to the VTA. Images and outlines in this Figure are a higher magnification of those shown in Figure 3.6. A–D *Left*, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. *Middle, Right*, Confocal micrographs showing the location of Otx2-expressing dopaminergic neurons (white arrows) and Otx2-expressing non-dopaminergic neurons (green arrows) in the midbrain. Scale bars, 50 μ m.

3.4.2.3 RRF dopaminergic neurons express Sox6

In a manner that is spatially distinct from Otx2, the transcription factor Sox6 is important for specifying SNc dopaminergic neurons during development (Panman et al., 2014). In the present study, the expression of this marker was examined in dopaminergic neurons of the SNc, VTA and RRF (Figure 3.8). Sox6 was expressed in dorsolateral VTA (Figure 3.9A) and SNc dopaminergic neurons (Figure 3.9B, C). In the medial VTA, Sox6 expression in dopaminergic neurons was rare (Figure 3.8). In addition to this established expression pattern (Panman et al., 2014), Sox6 was also observed in rostral (Figure 3.9C) and intermediate RRF dopaminergic neurons (Figure 3.9D). This suggests there is a medial-lateral gradient of Sox6 expression in midbrain dopaminergic neurons, and that Sox6 is a molecular marker common to both SNc and RRF neurons. Note, Aldh1a1 immunoreactivity was not examined in these sections (Figure 3.8) because both the anti-Aldh1a1 and anti-Sox6 primary antibodies were raised in rabbit; alternative antibodies of suitably high quality raised in other species were not available. However, calbindin-1 immunoreactivity alone allowed for the border between the rostral RRF and caudal SNc to be confidently defined (Figure 3.8B).

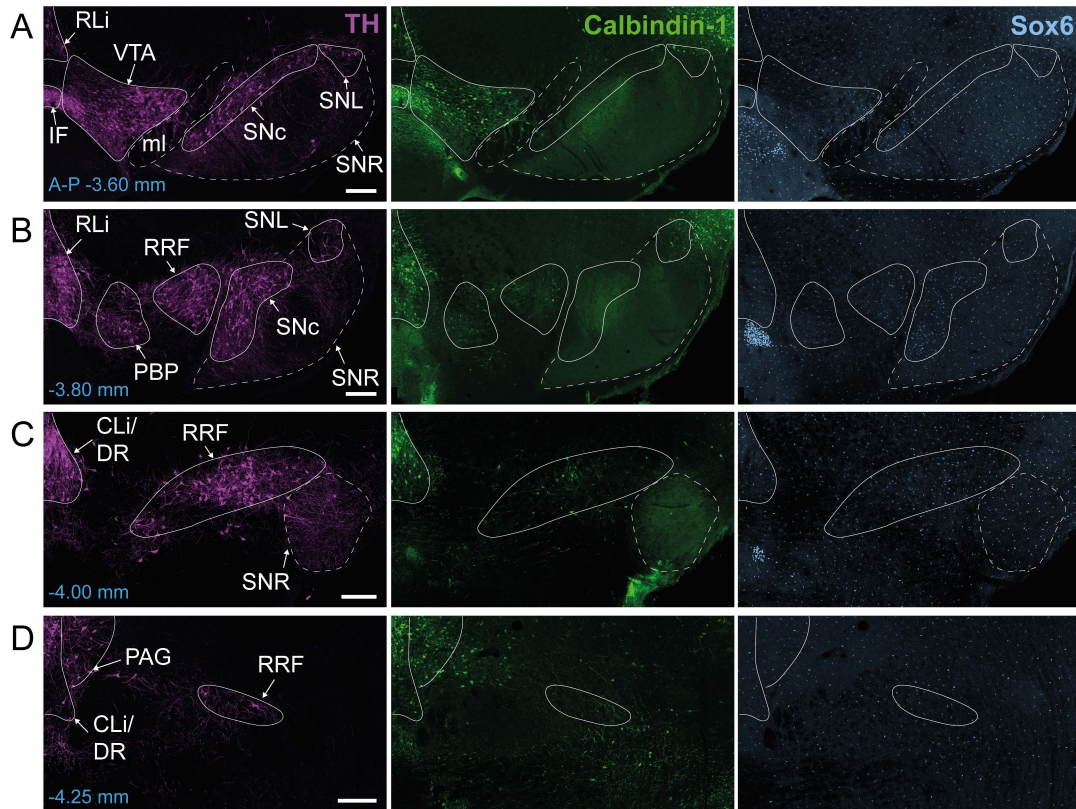


Figure 3.8: RRF dopaminergic neurons express Sox6. The expression pattern of calbindin-1 was used to define the borders of the RRF, SNc and PBP. The expression of Sox6 was examined in the ‘classical’ midbrain (A), in addition to sections containing the rostral (B), intermediate (C) and caudal (D) RRF. Scale bars, 200 μ m. Dopaminergic cell groups are defined by solid white lines; the SNr and ml are defined by dashed white lines.

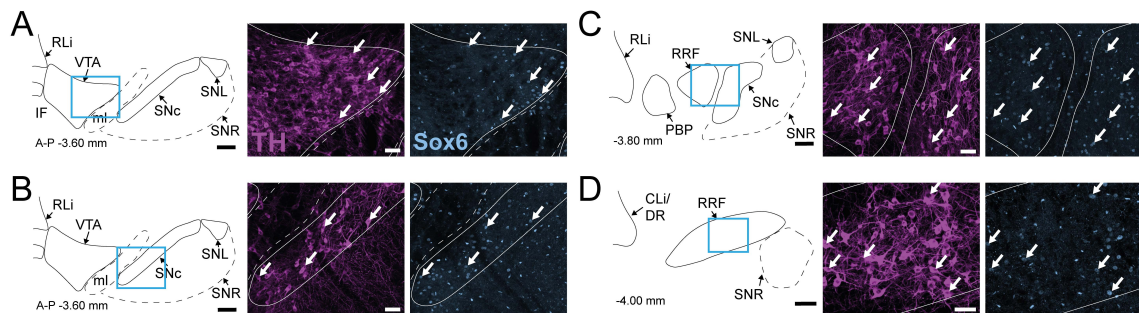


Figure 3.9: Sox6 is expressed in lateral VTA, SNc and RRF dopaminergic neurons. Images and outlines in this Figure are a higher magnification of those shown in Figure 3.8. **A–D Left**, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. **Middle, Right**, Confocal micrographs showing the location of Sox6-expressing dopaminergic neurons (white arrows). Scale bars, 50 μ m.

3.4.2.4 RRF dopaminergic neurons express calretinin

Calretinin, also known as calbindin-D-29K or calbindin-2, is a calcium-binding protein expressed in some midbrain dopaminergic neurons (Liang et al., 1996). In the present study, calretinin was

observed to be widely expressed in VTA (Figure 3.10A and 3.11A) and medial SNc but largely absent from the dorsal/lateral SNc (Figure 3.10A and 3.11B). Most calretinin-expressing neurons in the VTA were dopaminergic whereas most in the SNc were non-dopaminergic (Figure 3.11A, B). In the caudal SNc, no calretinin-expressing dopaminergic neurons were observed (Figure 3.10B and 3.11C). This qualitative examination suggests calretinin expression in rostral midbrain dopaminergic neurons is mostly confined to the VTA. Within the RRF, expression of calretinin in dopaminergic neurons was relatively common, alongside expression in non-dopaminergic neurons (Figure 3.10C, D and 3.11D).

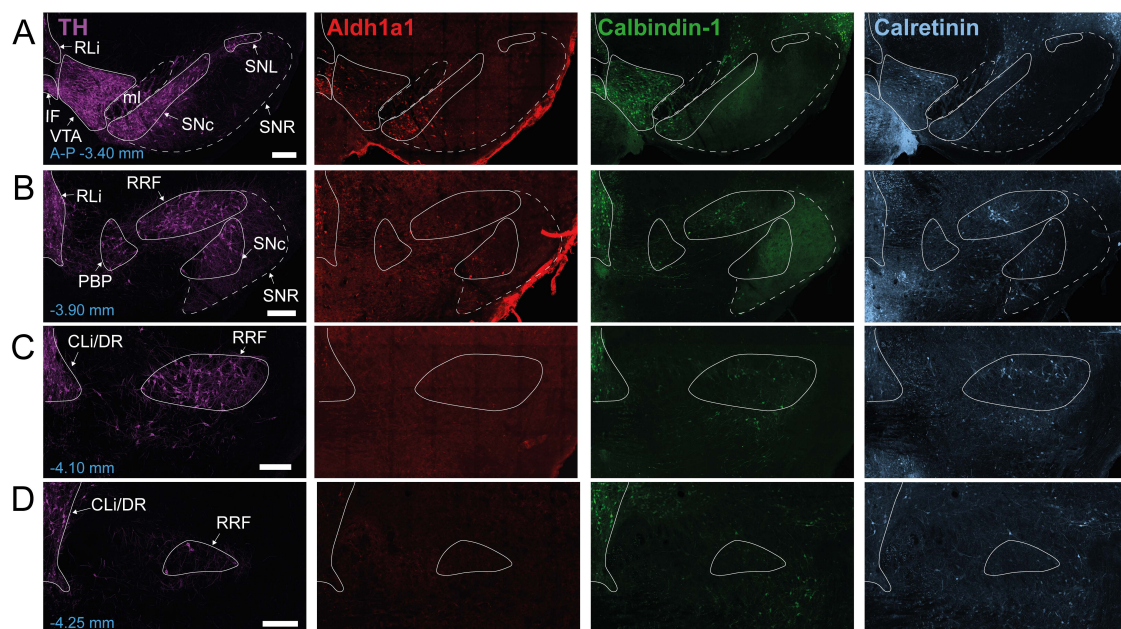


Figure 3.10: RRF dopaminergic neurons express calretinin. The expression patterns of Aldh1a1 and calbindin-1 were used to define the borders of the RRF, SNc and PBP. The expression of calretinin was examined in the 'classical' midbrain (A), in addition to sections containing the rostral (B), intermediate (C) and caudal (D) RRF. Scale bars, 200 μm. Dopaminergic cell groups are defined by solid white lines; the SNr and ml are defined by dashed white lines.

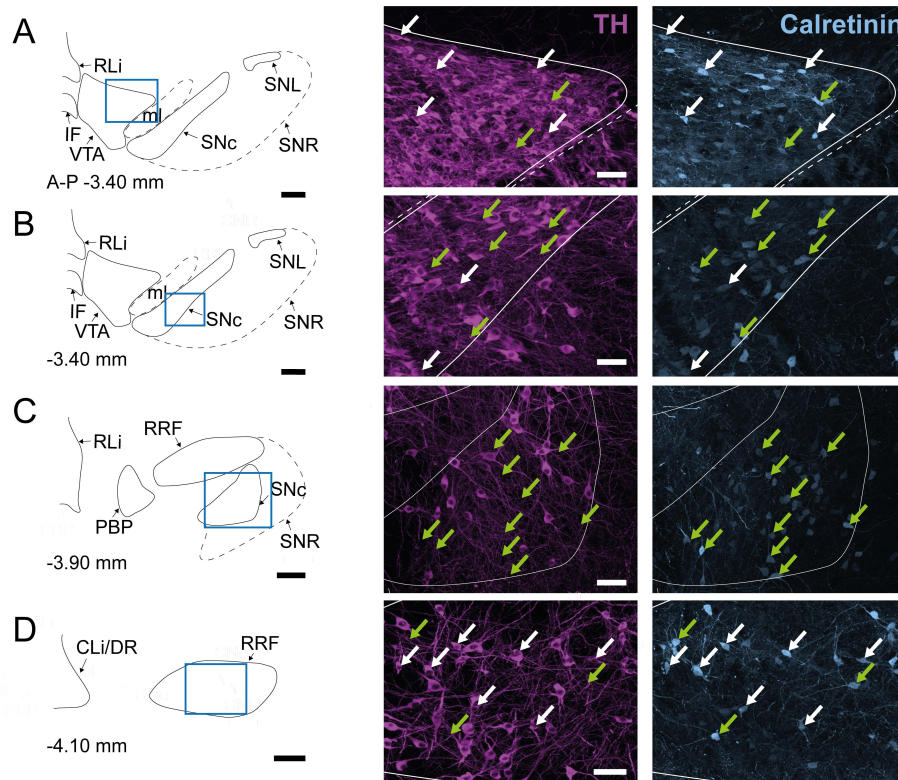


Figure 3.11: Calretinin expression in midbrain dopaminergic neurons is mostly limited to the RRF and VTA. Images and outlines in this Figure are a higher magnification of those shown in Figure 3.10. **A–D Left,** Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. **Middle, Right,** Confocal micrographs showing the location of calretinin-expressing dopaminergic neurons (white arrows) and calretinin-expressing non-dopaminergic neurons (green arrows). Scale bars, 50 μ m.

3.4.2.5 RRF dopaminergic neurons express GIRK2

GIRK2 is a potassium channel expressed by some midbrain dopaminergic neurons (Chung et al., 2005; Fu et al., 2012; Reyes et al., 2012) that is involved in controlling membrane excitability (Lacey et al., 1987). Expression of GIRK2 was observed throughout the dopaminergic midbrain (Figure 3.12), in the somata and neurites of neurons (Figure 3.13). Although it was difficult to define the cytoplasmic expression of GIRK2 in neuronal somata due to the dense labelling of the neuropil, the nucleus of GIRK2-expressing neurons had diminished immunoreactivity and thus aided in identifying GIRK2-positive neurons. GIRK2 was expressed in the SNc (Figure 3.12A, B), VTA (Figure 3.12A, B) and RRF (Figure 3.12C), and therefore it did not serve as a marker for demarcating the different midbrain subregions. SNL neurons rarely expressed GIRK2, setting it apart from the other dopaminergic midbrain subregions (Figure 3.12A, B). Qualitative examination suggested that

GIRK2 was expressed exclusively in dopaminergic neurons of the VTA (Figure 3.13A), SNc (Figure 3.13B) and RRF (Figure 3.13C).

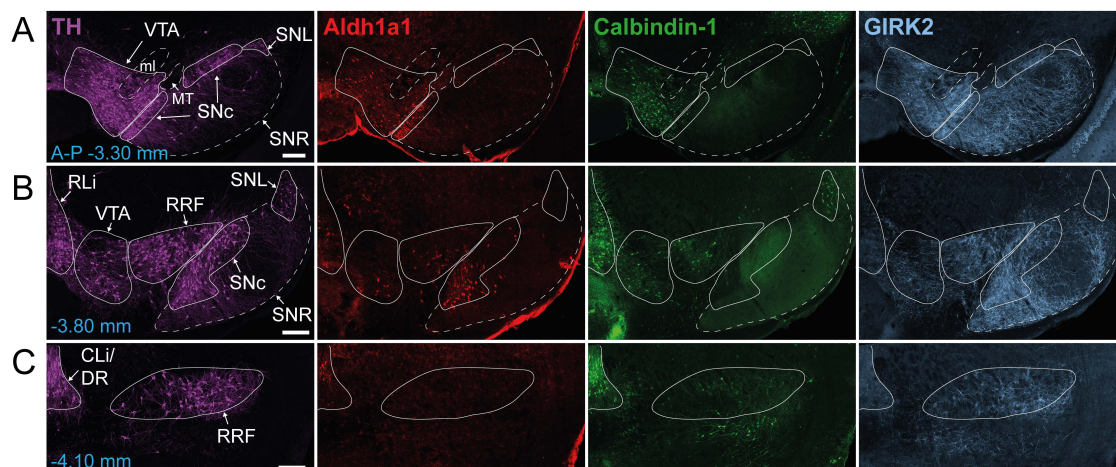


Figure 3.12: RRF dopaminergic neurons express GIRK2. The expression patterns of Aldh1a1 and calbindin-1 were used to define the borders of the RRF, SNc and PBP. The expression of GIRK2 was examined in the ‘classical’ midbrain (A), in addition to sections containing the rostral (B) and intermediate (C) RRF. Scale bars, 200 μ m. Dopaminergic cell groups are defined by solid white lines; the SNr, ml and MT are defined by dashed white lines.

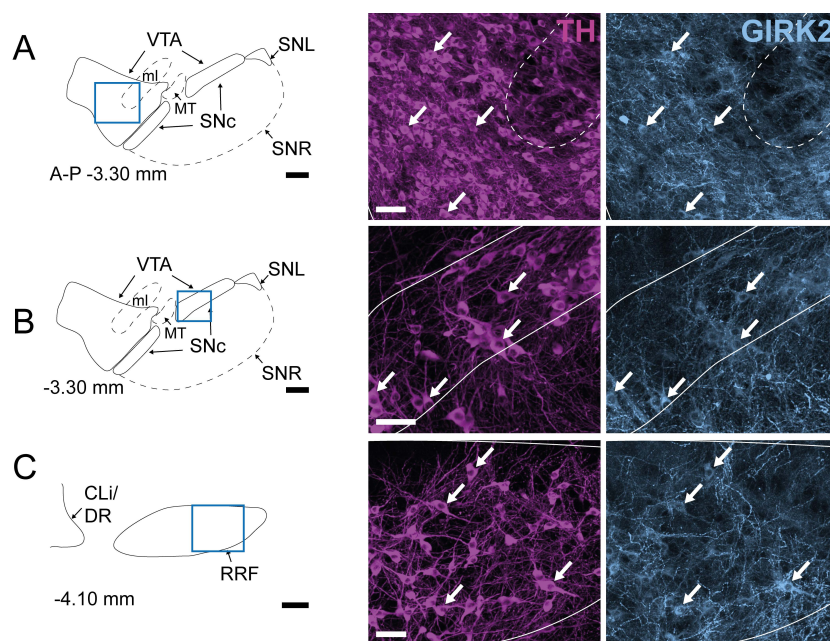


Figure 3.13: GIRK2 is exclusively expressed by dopaminergic neurons in the midbrain. Images and outlines in this Figure are a higher magnification of those shown in Figure 3.12. A–C Left, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. Middle, Right, Confocal micrographs showing the location of GIRK2-expressing dopaminergic (white arrows) in the VTA (A), SNc (B) and RRF (C). Scale bars, 50 μ m.

3.4.3 Putative GABAergic and other non-dopaminergic neurons are interspersed amongst dopaminergic neurons in the RRF

The preceding results sections in this Chapter demonstrated that there were non-dopaminergic neurons interspersed amongst their dopaminergic counterparts in the midbrain (i.e. expression of molecular markers within non-dopaminergic elements presumed to be neurons). Moreover, previous work has shown that the VTA, SNc and RRF contain significant populations of non-dopaminergic neurons (i.e. glutamatergic and GABAergic) in the rat (Nair-Roberts et al., 2008). There are a lack of antibodies available for immunohistochemistry experiments that selectively label the soma of GABAergic or glutamatergic neurons in the midbrain, hence most previous work involved *in situ* hybridisation using RNA probes. However, in mice expressing green fluorescent protein (GFP) in GABAergic neurons (GAD67-GFP mice), an antibody that recognises the α subunit of the GABA_A receptor labels 81–92% of GFP-positive neurons. Moreover, only 3–7% of dopaminergic neurons express the $\alpha 1$ subunit, meaning it serves as a putative marker of a major subset of midbrain GABAergic neurons (Fritschy and Mohler, 1995; Tan et al., 2010). To determine whether these neurons were present in the RRF, sections were incubated in an antibody raised against the $\alpha 1$ subunit of the GABA_A receptor alongside the pan-neuronal marker NeuN and dopaminergic neuron marker TH. In the VTA (Figure 3.14A) and SNc (Figure 3.14B), putative GABAergic neurons and other non-dopaminergic neurons (i.e. TH⁻, $\alpha 1$ ⁻, NeuN⁺) were interspersed between TH-expressing dopaminergic neurons. Similarly, the RRF contained $\alpha 1$ -expressing neurons that were a separate population to the TH-expressing dopaminergic neurons. Alongside $\alpha 1$ expression in non-dopaminergic neurons, there were rare examples of $\alpha 1$ expression in dopaminergic neurons (Figure 3.14A, C). Expression of the $\alpha 1$ subunit was limited to neuronal cell bodies, as shown by overlap with NeuN immunoreactivity (Figure 3.14A–C).

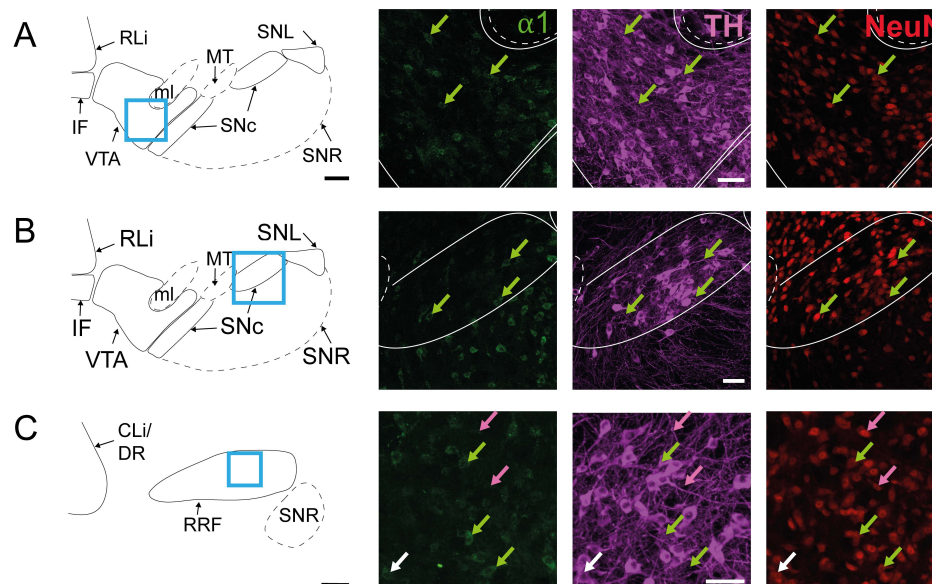


Figure 3.14: Dopaminergic and putative GABAergic neurons are interspersed in the RRF. **A–C Left**, Schematic outlines of midbrain dopaminergic cell groups. The blue boxed area indicates the location of the corresponding confocal micrographs within the midbrain. Scale bars, 200 μ m. Confocal micrographs showing the locations of putative GABAergic neurons ($\alpha 1^+$; green arrows) and other non-dopaminergic neurons (TH^- , $\alpha 1^-$, $NeuN^+$; pink arrows) interspersed between dopaminergic (TH^+) neurons in the VTA (**A**), SNc (**B**) and RRF (**C**). A minority of dopaminergic neurons also expressed the $\alpha 1$ receptor (**C**, white arrow). The pan-neuronal marker NeuN shows that $\alpha 1$ expression is limited to neurons. Scale bars, 50 μ m.

3.4.4 Results summary

Below is a table summarising the expression of molecular markers in dopaminergic neurons within the RRF, SNc and VTA:

Molecular marker	Region	Relative expression
Aldh1a1	RRF	**
	SNc	****
	VTA	***
Calbindin-1	RRF	***
	SNc	*
	VTA	****
Calretinin	RRF	***
	SNc	*
	VTA	***
GIRK-2	RRF	****
	SNc	****
	VTA	****
Otx2	RRF	*
	SNc	*
	VTA	**
SatB1	RRF	**
	SNc	***
	VTA	**
Sox6	RRF	**
	SNc	***
	VTA	*

Table 3.4: The relative expression of molecular markers in midbrain dopaminergic neurons. Relative expression is in terms of proportion of neurons positive for marker. *, very few; **, few; ***, many; ****, the majority.

3.5 Discussion

Dopaminergic neurons in the rostral aspect of the RRF and the caudal aspect of the SNc are continuous with one another and it is therefore non-trivial to delineate the border between these two cell groups. This led to the main aim of this Chapter, which was to determine a molecular marker/combination of markers that allowed the borders between the rostral RRF and caudal SNc to be objectively defined. This aim was achieved by observing a dichotomous pattern of Aldh1a1 and calbinin-1 expression in the rostral RRF and caudal SNc using immunohistochemistry. This also permitted a qualitative examination of other molecular markers in RRF dopaminergic neurons. Objectively defining the borders of the RRF was a pertinent issue for the work in this thesis because the overarching aim was to characterise the functional properties of RRF dopaminergic neurons. In the following results chapters, Aldh1a1 immunoreactivity is used to delineate the border between the rostral RRF and caudal SNc for anterograde tracing (Chapter 4), retrograde tracing (Chapter 5)

and electrophysiology experiments (Chapter 6). Without the aid of this molecular marker, I would have had less confidence in assigning functional properties specifically to RRF dopaminergic neurons because defining the borders of the rostral RRF would have been subjective.

It should be reiterated that the work in this Chapter is qualitative rather than quantitative, thus any conclusions drawn are not based on cell counts but rather based on detailed visual inspection. For the qualitative examination of expression, sections representing the ‘classical’ midbrain (i.e. containing SNc and VTA only; between -3.00 and -3.65 mm from Bregma) and all levels of the RRF were selected. Although this fulfilled the aim of finding molecular markers for distinguishing the RRF from the SNc, the distribution of other molecular markers were described in a purely qualitative manner. In future, unbiased stereological quantification of marker expression in the RRF, SNc and VTA would allow for a more robust comparison of the relative expression of markers.

3.5.1 Aldh1a1 and calbindin-1 expression patterns distinguish the border between the rostral RRF and caudal SNc

Consistent with previous immunohistochemical and gene expression studies, Aldh1a1 and calbindin-1 were found to have non-stochastic distribution patterns in midbrain dopaminergic neurons. Specifically, Aldh1a1 was abundant in the ventromedial portions of the SNc and the ventral VTA but sparsely expressed in the dorsal VTA (McCaffery and Drager, 1994; Liu et al., 2014; Poulin et al., 2014; La Manno et al., 2016; Wu et al., 2019) and the RRF (Wu et al., 2019). However, Aldh1a1 was by no means absent from the RRF and therefore did not clearly define the border with the SNc. In a somewhat inverse pattern, calbindin-1 was abundant in the VTA and SNL and present at intermediate levels in the RRF, whereas it was almost entirely absent from the SNc. This is largely in keeping with previous studies (Liang et al., 1996; Airaksinen et al., 1997; Fu et al., 2012). Although it has been previously demonstrated that many RRF dopaminergic neurons express calbindin-1 (Liang et al., 1996; Airaksinen et al., 1997; Fu et al., 2012) and that a small subset express Aldh1a1 (Poulin et al., 2014; Wu et al., 2019), this is the first time these markers have been compared together.

In combination, their relatively inverse expression patterns can be used to objectively define the border between the rostral RRF and caudal SNc.

In PD, ventral tier SNc dopaminergic neurons are most susceptible to cell death (German et al., 1992; Damier et al., 1999b), corresponding to neurons that are typically in calbindin-poor zones (Damier et al., 1999a,b). Ventral SNc dopaminergic neurons express Aldh1a1 and are particularly susceptible to cell death in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Poulin et al., 2014). Conversely, calbindin-1-expressing dopaminergic neurons are spared in PD (Yamada et al., 1990) and non-human primate models (Dopeso-Reyes et al., 2014). In combination with the observed pattern of cell loss in human post-mortem studies (Yamada et al., 1990; German et al., 1992; Damier et al., 1999b), it appears Aldh1a1 and calbindin-1 expression are at least proxies for selective vulnerability of SNc/VTA dopaminergic neurons in PD. It is interesting to reflect on whether this is also holds for RRF dopaminergic neurons. In the MPTP model, loss of dopaminergic neurons occurs mainly in the ventral SNc in mice (Poulin et al., 2014) and non-human primates (German et al., 1992), whereas cell loss in the RRF and VTA is limited. One could postulate that the higher levels of calbindin-1 and lower levels of Aldh1a1 expression in VTA and RRF dopaminergic neurons confer some level of neuroprotection. Indeed, there is some evidence that calbindin-1 is neuroprotective in the MPTP model of PD (Yuan et al., 2013) by buffering intracellular calcium and preventing cell death (Jung et al., 2019). Moreover, calbindin-1 is upregulated in surviving VTA dopaminergic neurons (Ng et al., 1996).

Defining the borders of the rostral RRF and caudal SNc (A-P -3.80 to -3.95 mm from Bregma) using a combination of Aldh1a1 and calbindin-1 immunoreactivity closely matched the borders described in the Paxinos and Franklin (2013) atlas. Unlike in Paxinos and Franklin (2013), however, there was often a sparse group of ventromedially-located dopaminergic neurons on coronal sections containing the intermediate RRF (at around -4.00 mm from Bregma). These were designated as being within the PBP because their sparsity and ventral location is not consistent with the structure of the RRF at this level. Anatomical landmarks and the Paxinos and Franklin (2013)

atlas itself were used as guides when determining the A-P coordinate, but it is possible that I incorrectly matched these sections to ~ -4.00 mm from Bregma when in fact they were slightly more rostral.

At -3.90 mm from Bregma, it is possible that some dopaminergic neurons designated as being within the rostral RRF were in fact part of the caudal SNc. Although I determined that calbindin-1 immunoreactivity was useful for defining neurons as belonging to the RRF, it should be noted that previous work has reported that dorsal SNc neurons often express calbindin-1 in rats (Gerfen et al., 1987b) and non-human primates (Lavoie and Parent, 1991; German and Manaye, 1993). Furthermore, a cluster of dorsally-located dopaminergic neurons at around -3.90 mm from Bregma were defined as being within the caudal SNc in previous work (Fu et al., 2012), whereas in the present study they were defined as being within the RRF. However, in mice much fewer ($\sim 20\%$) SNc dopaminergic neurons express calbindin-1 (Liang et al., 1996). It is also important to note that differentiating the dorsal SNc and lateral PBP is non-trivial, and thus some dorsal SNc neurons described in previous studies (Gerfen et al., 1987b; Lavoie and Parent, 1991; German and Manaye, 1993) may have in fact been within the PBP. This highlights the difficulty in defining the borders of the different midbrain dopaminergic nuclei. Notwithstanding, the combination of markers proposed in the present study provide an objective means for differentiating the rostral RRF and caudal SNc.

3.5.2 The expression profile of other midbrain dopaminergic neurons molecular markers in the RRF

Previous work has shown that the transcription factors SatB1 and Otx2 are differentially expressed in SNc and VTA dopaminergic neurons. Broadly, *Satb1* and *Otx2* gene expression is higher in SNc (Chung et al., 2005; Poulin et al., 2014; Brichta et al., 2015) and VTA dopaminergic neurons (Chung et al., 2005; Di Salvio et al., 2010a,b; Panman et al., 2014; Poulin et al., 2014; La Manno et al., 2016), respectively. In particular, SatB1 is enriched in ventral SNc dopaminergic neurons, whereas Otx2 is most common in those of the ventromedial VTA (Poulin et al., 2014). However,

immunohistochemistry experiments have demonstrated that 95% of *both* SNc and VTA dopaminergic neurons express SatB1 (Huang et al., 2011). Although the results of the present study are not able to provide quantitative information about SatB1 or Otx2 expression to compare to previous studies, SatB1 expression in dopaminergic neurons was observed in all midbrain regions, whereas Otx2 was found to be limited to dopaminergic neurons in the rostral VTA; any midbrain Otx2 expression outside of the VTA was found to be mostly in non-dopaminergic neurons. The present study demonstrates that some RRF dopaminergic neurons express SatB1, whereas very few express Otx2. This would suggest that the expression profile for these transcription factors in RRF dopaminergic neurons is more similar to SNc than VTA neurons.

The results of this Chapter show that the transcription factor Sox6 was expressed in lateral VTA and SNc neurons, which is largely in keeping with previous work (Panman et al., 2014). Also, I show for the first time that Sox6 is also expressed in RRF dopaminergic neurons. During development, Sox6 is required for specifying SNc dopaminergic neurons (Panman et al., 2014), whereas Otx2 is required for specifying a subset of VTA neurons (Di Giovannantonio et al., 2013). Although very little is known about the development and specification of RRF dopaminergic neurons, the presence of Sox6 and scarcity of Otx2 expression suggests that the developmental profile of these neurons has more in common with their counterparts in the SNc than VTA.

Similar to calbindin-1 (Yamada et al., 1990; Lavoie and Parent, 1991; German et al., 1992; Yuan et al., 2013), there is some evidence that calretinin is a marker of dopaminergic neurons resistant to cell death in PD (Mouatt-Prigent et al., 1994). In the present study, calretinin was observed to have a broadly similar pattern of expression in midbrain dopaminergic neurons as calbindin-1, which is in line with a previous report (Liang et al., 1996). Specifically, calretinin was expressed in many VTA and RRF dopaminergic neurons but was less commonly expressed in SNc dopaminergic neurons. Previous studies of gene expression in the VTA and SNc have determined that *Calb2* (calretinin) is higher in VTA dopaminergic neurons (Chung et al., 2005; Poulin et al., 2014), and immunohistochemical studies have demonstrated that proportionally similar (~40%) numbers of

VTA and RRF dopaminergic neurons express calretinin (Liang et al., 1996). Thus, it would seem that the expression of the calcium binding proteins calbindin-1 and calretinin in RRF dopaminergic neuron is more similar to the VTA than SNc, which is in contrast to the distribution of the transcription factors Otx2 and Sox6.

GIRK2 is a potassium channel highly expressed in midbrain dopaminergic neurons (Schein et al., 1998; Liss et al., 1999) and is involved in controlling their excitability (Lacey et al., 1987). A point mutation in the mouse GIRK2 channel leads to progressive loss of SNc dopaminergic neurons (Liao et al., 1996) and aberrant electrophysiological properties of surviving neurons (Liss et al., 1999). Early studies suggested that GIRK2 expression was more prevalent in the SNc compared with the VTA of mice (Chung et al., 2005) and rats (Eulitz et al., 2007). However, further gene expression (Greene et al., 2005; Poulin et al., 2014; Brichta et al., 2015) and immunohistochemistry studies in mice (Schein et al., 1998; Fu et al., 2012; Reyes et al., 2012) and humans (Reyes et al., 2012) did not support this observation and found high levels of GIRK2 expression in dopaminergic neurons in the SNc and VTA. In line with the present study, GIRK2 expression was observed in RRF dopaminergic neurons in mice (Schein et al., 1998; Fu et al., 2012). This pan-midbrain expression of GIRK2 in dopaminergic neurons is in line with the qualitative observations made in the present study. This would suggest that GIRK2 is not a marker of any subregion of the midbrain dopaminergic system. However, the early immunohistochemistry study in rats demonstrated strong GIRK2 labelling in the SNc and lateral VTA but weaker labelling in the medial VTA (Eulitz et al., 2007), which is not observed in mice (Reyes et al., 2012). This suggests some species differences in the expression of GIRK2. As observed in the present study, previous reports have found GIRK2 to be almost exclusively expressed in dopaminergic neurons within the midbrain (Schein et al., 1998; Reyes et al., 2012).

3.5.3 Putative GABAergic and other non-dopaminergic neurons are interspersed amongst dopaminergic neurons in the RRF

In line with previous reports in rats (Nair-Roberts et al., 2008; Yamaguchi et al., 2013; Morales and Root, 2014), I demonstrated that non-dopaminergic (i.e. TH⁻) neurons are present in the SNc, VTA and RRF, interspersed between dopaminergic neurons. As others have noted (Nair-Roberts et al., 2008), there is a lack of suitably selective antibodies to reveal glutamatergic and GABAergic neuronal somata for immunohistochemistry experiments. However, there is evidence that a subset of VTA GABAergic neurons express the GABA_A receptor $\alpha 1$ subunit (Tan et al., 2010, 2012). In the present study, neurons immunopositive for the $\alpha 1$ subunit, and thereby designated as putatively GABAergic, were also observed in the RRF. This is comparable to previous *in situ* hybridisation work in the rat (Nair-Roberts et al., 2008), wherein GAD-positive GABAergic neurons were intermingled amongst TH-positive dopaminergic neurons in the RRF. Dopaminergic neurons expressing the $\alpha 1$ subunit of the GABA_A receptor were rarely observed, in line with previous work (Tan et al., 2010, 2012). Unlike for the VTA and SNc, very little is known about the function of GABAergic neurons in the RRF. However, in light of previous work in the rat showing that the majority of projections to the hippocampus from the RRF are non-dopaminergic (Gasbarri et al., 1996), it would be interesting to further investigate the role of these putative RRF GABAergic neurons. Amongst dopaminergic and putative GABAergic neurons there were also non-dopaminergic neurons which could not be assigned to a cell type. Based on previous *in situ* hybridisation work in rats, some of these non-dopaminergic neurons are likely to be glutamatergic (Nair-Roberts et al., 2008; Yamaguchi et al., 2013; Morales and Root, 2014).

The diversity of cell types in the RRF highlights the difficulty in interpreting data from studies using non-selective neuronal tracers to map the outputs (rats: Gerfen et al., 1987a; Deutch et al., 1988; non-human primates: François et al., 1999) and inputs (rat: Zahm et al., 2011) of the RRF. In these studies, it is likely that many of the neurons traced were non-dopaminergic, and thus it is clear that

anterograde and retrograde tracing with selective tracers is needed to map the outputs and inputs of RRF dopaminergic neurons with precision. This will be outlined in Chapters 4 (anterograde) and 5 (retrograde) of this thesis.

In summary, the results of this Chapter give a broad overview of molecular marker expression in RRF dopaminergic neurons. As has been demonstrated previously, Aldh1a1 (Poulin et al., 2014; Wu et al., 2019) is sparsely expressed, whereas calbindin-1 (Liang et al., 1996; Fu et al., 2012), calretinin (Liang et al., 1996) and GIRK2 (Schein et al., 1998; Fu et al., 2012) are abundantly expressed in dopaminergic neurons of the RRF. The present study has demonstrated that in addition to these previously described markers, SatB1 and Sox6 are expressed in RRF dopaminergic neurons, whereas Otx2 is scarcely present. In addition, this Chapter highlights that a combination of calbindin-1 and Aldh1a1 immunoreactivity allow the border between the rostral RRF and caudal SNc to be objectively delineated.

Chapter 4

Anterograde tracing of RRF dopaminergic neurons

4.1 Introduction

Mapping the axonal projections of neurons is a cornerstone of neuroscience. Without knowing where a group of neurons project to, any understanding of their function would be incomplete. In the RRF, SNc and VTA, dopaminergic neurons are intermingled with non-dopaminergic neurons (rat: Nair-Roberts et al., [2008](#); mouse: Chapter [3](#)). Unlike the SNc and VTA, there are proportionally more GABAergic neurons relative to dopaminergic neurons in the RRF, at least in the rat (Nair-Roberts et al., [2008](#)). With this in mind, understanding the functional characteristics of RRF dopaminergic neurons requires methods with cell type-selective precision.

The axons of midbrain dopaminergic neurons are found throughout the brain and are most dense in the striatum (Björklund and Dunnett, [2007](#)). In early studies, mapping these projections was achieved using classical anterograde and retrograde tracing techniques. More recently, ‘driver’ mice expressing Cre recombinase in dopaminergic neurons (e.g. DAT-Cre mice; Bäckman et al., [2006](#)) have permitted selective tracing of dopaminergic axons. These mice can be crossed with ‘reporter’ mice or intracerebrally-injected with a recombinase-dependent adeno-associated virus

(AAV) to permit the selective expression of fluorescent proteins in dopaminergic neurons and subsequent axonal tracing (Beier et al., 2015; Lerner et al., 2015; Menegas et al., 2017; Poulin et al., 2018; Beier et al., 2019; Wu et al., 2019).

SNC dopaminergic neurons send dense axonal projections to the striatum, thus forming the nigrostriatal pathway. Most neurons in the ventral SNC, including those that extend into the substantia nigra pars reticulata (SNr), preferentially target the most dorsal aspects of the striatum, whereas those in the dorsal SNC tend to project to the ventral striatum and amygdala (mouse: Poulin et al., 2018; rat: Fallon and Moore, 1978; Fallon et al., 1978; non-human primate: Lynd-Balta and Haber, 1994; François et al., 1999). Dopaminergic neurons in the medial SNC share features of both the ventral and dorsal subgroups, projecting to both dorsal and ventral striatum in the mouse (Poulin et al., 2018) and non-human primate (Lynd-Balta and Haber, 1994). Moving to the VTA, dopaminergic neurons project to the nucleus accumbens (NAc), olfactory tubercle (OT), anterior cingulate cortex (ACC) and prefrontal cortex (PFC), as well as the hippocampus and amygdala (mouse: Lammel et al., 2008; Poulin et al., 2018; rat: Scatton et al., 1980; Swanson, 1982; Verney et al., 1985; Gasbarri et al., 1994). These projections form the mesolimbic (VTA → NAc, OT and amygdala) and mesocortical pathways (VTA → ACC and PFC). The somata of VTA dopaminergic neurons projecting to different targets in the mouse (Lammel et al., 2008; Rodríguez-López et al., 2017; Farassat et al., 2019) and rat (Swanson, 1982; Breton et al., 2019) are largely intermingled, and therefore the location of dopaminergic neuron cell bodies is not a clear cut proxy for projection target.

In the rat, the axonal projections of RRF neurons (of mixed cell types) overlap with those of SNC and VTA neurons and can thus be conceptualised as contributing to both the nigrostriatal and mesolimbic pathways (Deutch et al., 1988). Indeed, injection of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) into the RRF leads to axonal labelling throughout the striatum, NAc, bed nucleus of the stria terminalis (BNST), amygdala, OT, pyriform and entorhinal cortices, with the densest innervation in the ventrolateral striatum (rat: Deutch et al., 1988; non-human

primate: François et al., 1999). A caveat of using anterograde tracers such as PHA-L is that injection into the RRF will undoubtedly lead to uptake by non-dopaminergic neurons, particularly given the prominent populations of non-dopaminergic neurons in this region (Nair-Roberts et al., 2008). This means that any conclusion about the dopaminergic origin of axons labelled with PHA-L cannot easily be made unless combined with TH immunolabelling of traced axons. There is also the concern that under certain experimental conditions PHA-L can be taken up by axons passing through the injection site (Cliffer and Giesler, 1988; Schofield, 1990), thereby labelling the axons of neurons that lie outside of the region of interest. Furthermore, although tracers such as PHA-L and biotinylated dextran amine (BDA) are transported principally in the anterograde direction, there are numerous reports of retrograde staining of somata distal to the injection site (e.g. Shu and Peterson, 1988; Rajakumar et al., 1993; Bevan et al., 1994; Dolleman-Van der Weel et al., 1994), indicating retrograde transport. Overall, these caveats make it difficult to interpret data from studies using classical neural tracers.

Retrograde tracing can confirm the dopaminergic origin of some of the projections observed in anterograde tracer studies by determining the dopaminergic identity of retrogradely-labelled neurons. Indeed, injections of the retrograde tracer wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into various RRF projection sites showed that some retrogradely-labelled neurons in the RRF were dopaminergic (Deutch et al., 1988; François et al., 1999). Although these studies were thorough, involving the injection of retrograde tracer into many forebrain regions, they do not provide a comprehensive brain-wide map of RRF dopaminergic neuron projections. Moreover, the injection of retrograde tracers involves relatively bulky deposits and thus the precise pattern of projections within a region cannot be determined. This is illustrated by studies showing that the axonal projections of RRF neurons in the striatum are largely confined to the matrix compartment in the rat (Gerfen et al., 1987a), cat (Jimenez-Castellanos and Graybiel, 1987) and non-human primate (Langer and Graybiel, 1989), but to what extent these projections are dopaminergic has not been determined. One reason this has not been determined is that targeting

retrograde tracer deposits to the striosome or matrix compartment is not practically possible. Thus, the use of cell type-selective anterograde tracing techniques would allow the dopaminergic component of this projection to be unequivocally determined.

Selective expression of fluorescent proteins in midbrain dopaminergic neurons using genetic approaches permits the anterograde tracing of their brain-wide projections. This approach has been used to map the projections of SNc and VTA dopaminergic neurons with great detail (Lerner et al., 2015; Menegas et al., 2018; Poulin et al., 2018; Wu et al., 2019). However, the projections of RRF dopaminergic neurons have not been mapped with cell type-selective approaches and thus the output sites of these neurons are not precisely defined. In this Chapter, I circumvented the issues arising from the use of classical anterograde and retrograde tracers by selectively transducing RRF dopaminergic neurons to express a green fluorescent protein (GFP) reporter protein. I then qualitatively mapped the GFP-expressing axonal projections. Overall, the projection pattern of RRF dopaminergic neurons overlapped with the classical nigrostriatal (SNc to dorsal striatum) and mesolimbic (VTA to ventral striatum) pathways. However, the dense dopaminergic projections observed in the ventrolateral striatum (VLS) suggests an emphasis should be placed on RRF output being more closely aligned to the mesolimbic pathway.

4.2 Aims

- To selectively transduce RRF dopaminergic neurons with a fluorescent reporter protein while minimising transduction of SNc and VTA neurons.
- To map the anterograde axonal projection pattern of RRF dopaminergic neurons.

4.3 Methods

DAT-Cre mice (n = 3 females) were intracerebrally-injected with a Cre-inducible AAV containing a sequence encoding a cell-filling GFP reporter (AAV2-CAG-FLEX-GFP; UNC Vector Core) under

stereotaxic guidance. Thirty-three nL of virus was injected just caudal to the RRF at coordinates anterior-posterior (A-P) -4.50, medial-lateral (M-L) +1.10 and dorsal-ventral (D-V) -3.20 mm relative to Bregma (see Materials and Methods Section 2.3 and 2.4.1). Mice were killed and perfused 4 weeks after virus injection. The whole brains were sectioned and processed for immunohistochemistry (see Materials and Methods Section 2.4.3), followed by quantification of transduced (GFP-expressing) neurons using rigorous stereology (West, 1999, 2012) and qualitative mapping of anterograde projections (see Materials and Methods Section 2.4.4 and 2.4.5). It is important to note that the success rate of this targeting strategy was variable; the three mice in this Chapter came from a surgical cohort that had a success rate of 40%, whereas other cohorts had much lower success rates. Targeting was deemed unsuccessful when too many SNc/VTA dopaminergic neurons were transduced, or if no/too few RRF dopaminergic neurons were transduced (i.e. transduction strategy failed altogether). Overall, the success rate for anterograde tracing experiments was 23% (3 out of 13 mice). The reason why it was difficult to consistently target GFP expression to the RRF (i.e. avoid transduction of neurons in the SNc/VTA) was difficult to ascertain. However, there are two possible reasons I postulated. Firstly, difficulty defining the location of Bregma may have led to inconsistent stereotaxy. Secondly, the measurement divisions on the micropipettes used to inject the virus solution were marked manually, and therefore there may have been variability between subjects in terms of the amount of virus solution injected. That is, based on the information provided by the manufacturer, a length of 1 mm along the micropipette holds 66 nL of liquid, thus marks were made at 1 mm increments along the length of the micropipette in order to monitor how much virus was injected into the brain during surgery (see Materials and Methods Section 2.3 for details). However, this may have been suboptimal because marking the divisions by hand will have undoubtedly led to inconsistent volumes being injected. Data are presented as mean (SD) unless otherwise stated.

4.4 Results

The overarching aim of this Chapter was to leverage powerful modern technologies to advance understanding of the axonal projections of RRF dopaminergic neurons. To this end, I successfully developed an anterograde labelling strategy in DAT-Cre mice to restrict the expression of GFP to mostly RRF dopaminergic neurons in order to trace their brain-wide axonal projections (Figure 4.1B). Injection of a Cre-inducible virus encoding GFP into the hindbrain (Figure 4.1A) ensured the caudal dopaminergic neurons of the RRF were captured while keeping the number of transduced SNc and VTA dopaminergic neurons to a minimum.

4.4.1 *GFP-transduced dopaminergic neurons are mostly restricted to the RRF*

As is shown in Figure 4.1 (D–F), most of the transduced (GFP-expressing) dopaminergic neurons were within the RRF. Aldehyde dehydrogenase 1a1 (Aldh1a1) expression was used to define the border between the RRF and caudal SNc (Figure 4.1), based on the observation that Aldh1a1 is often expressed in SNc neurons but sparsely expressed by adjacent rostral RRF neurons (see Chapter 3). There were, however, a small group of transduced neurons in the caudal SNc, identified as being within the cluster of Aldh1a1-expressing dopaminergic neurons (Figure 4.1C). The distribution of transduced dopaminergic neurons within the RRF was relatively sparse (Figure 4.1C–F). As noted in Chapter 3 Section 3.4, there was a small group of ventrally located dopaminergic neurons below the RRF in the intermediate (Figure 4.1E) and caudal RRF (Figure 4.1F) that were difficult to assign to a cell group. However, these cells were defined as being within the RRF for unbiased stereology (see below) as I was confident the sections contained no other midbrain dopaminergic nuclei (i.e. SNc or parabrachial pigmented nucleus of the VTA (PBP)). It may be useful to conceptualise these cells as displaced RRF dopaminergic neurons, in a similar manner to the SNc dopaminergic neurons often observed within the SNr of rats (Deutch et al., 1986; González-Hernández and Rodríguez, 2000) and mice (Chapter 3). Defining these cells as ventrally-displaced RRF dopaminergic neurons is a

tentative classification at this stage and requires further experimentation to confidently define them as such. For example, electrophysiological recording and anatomical tracing would allow for their similarity to RRF dopaminergic neurons to be assessed.

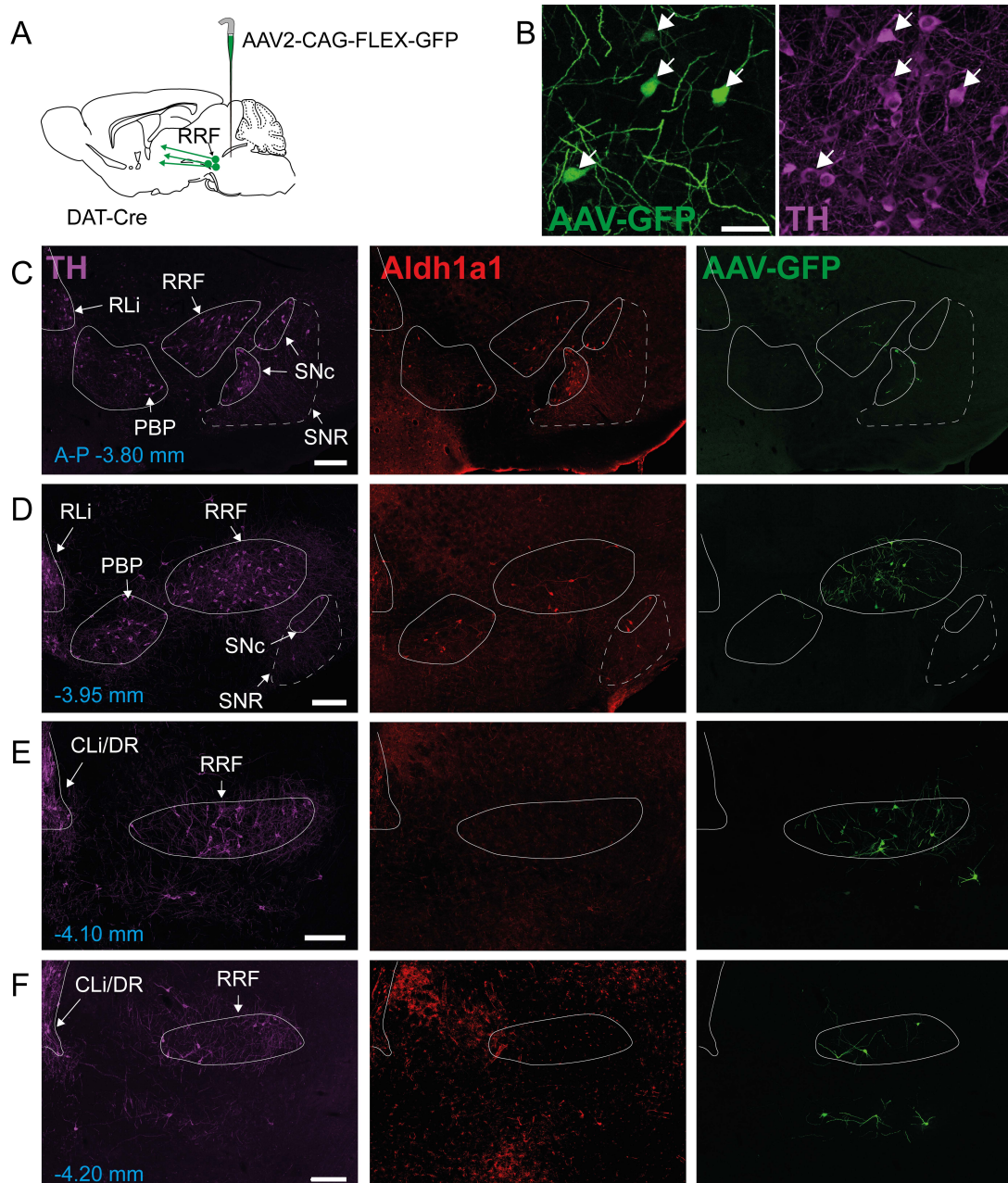


Figure 4.1: Most GFP-transduced dopaminergic neurons were within the RRF. **A**, Schematic representation of virus injection strategy. **B**, Confocal micrographs from the RRF showing the selective expression of GFP in dopaminergic (TH-expressing) neurons. Scale bar, 50 μ m. **C–F**, Confocal micrographs showing the distribution of transduced neurons in the rostral (**C**), intermediate (**D**, **E**) and caudal (**F**) RRF, in addition to a small number in the caudal SNc (**C**). Aldh1a1 immunoreactivity (*middle*) was used to delineate the border between the RRF and caudal SNc (**C**, **D**). Scale bars, 200 μ m. A-P, anterior-posterior distance with respect to Bregma; CLi, caudal linear nucleus of the raphe; DR, dorsal raphe; PBP, parabrachial pigmented nucleus; RLi, rostral linear nucleus of the VTA.

To rigorously quantify the number and distribution of transduced dopaminergic neurons in the

three DAT-Cre mice, an unbiased stereological approach was chosen. As was the case for Figure 4.1, the border between the rostral RRF and caudal SNc and VTA was defined based on Aldh1a1 immunoreactivity and the relative locations of the counted neurons within the optical disector were mapped (Figure 4.2). This gave an insight into the mediolateral and dorsoventral spread of transduced neurons within a given section. Very few transduced neurons were found within midbrain sections rostral to the RRF (Figure 4.2A–C), demonstrating that the transduction of midbrain dopaminergic neurons was mainly limited to the caudal midbrain. Also, few caudal SNc neurons were transduced in sections containing the rostral RRF (Figure 4.2A–C), demonstrating that the transduction of midbrain dopaminergic neurons was mainly limited to the RRF. Transduction was spread throughout the RRF, although there was clustering in the intermediate RRF (A-P -4.00 to -4.15 mm from Bregma), and fewer cells were labelled in the most medial and most lateral aspects of the RRF (Figure 4.2A–C). When the proportion of RRF dopaminergic neurons transduced with GFP was examined along the rostrocaudal axis, it was clear that proportionally more neurons were transduced in more caudal sections (Figure 4.2D). Across animals, the distribution of GFP-transduced neurons was broadly similar, although there were clear differences. Specifically, there were more transduced neurons in the rostral RRF of mouse LAB271 (Figure 4.2A) compared with the other two mice (Figure 4.2B, C), and transduced neurons were absent from the most lateral and medial aspects of the intermediate RRF (A-P -4.00 to -4.15 mm) in LAB277 and 278, respectively (Figure 4.2B, C). As a whole, ~22% of all RRF dopaminergic neurons were transduced with GFP (penetrance; Figure 4.2E).

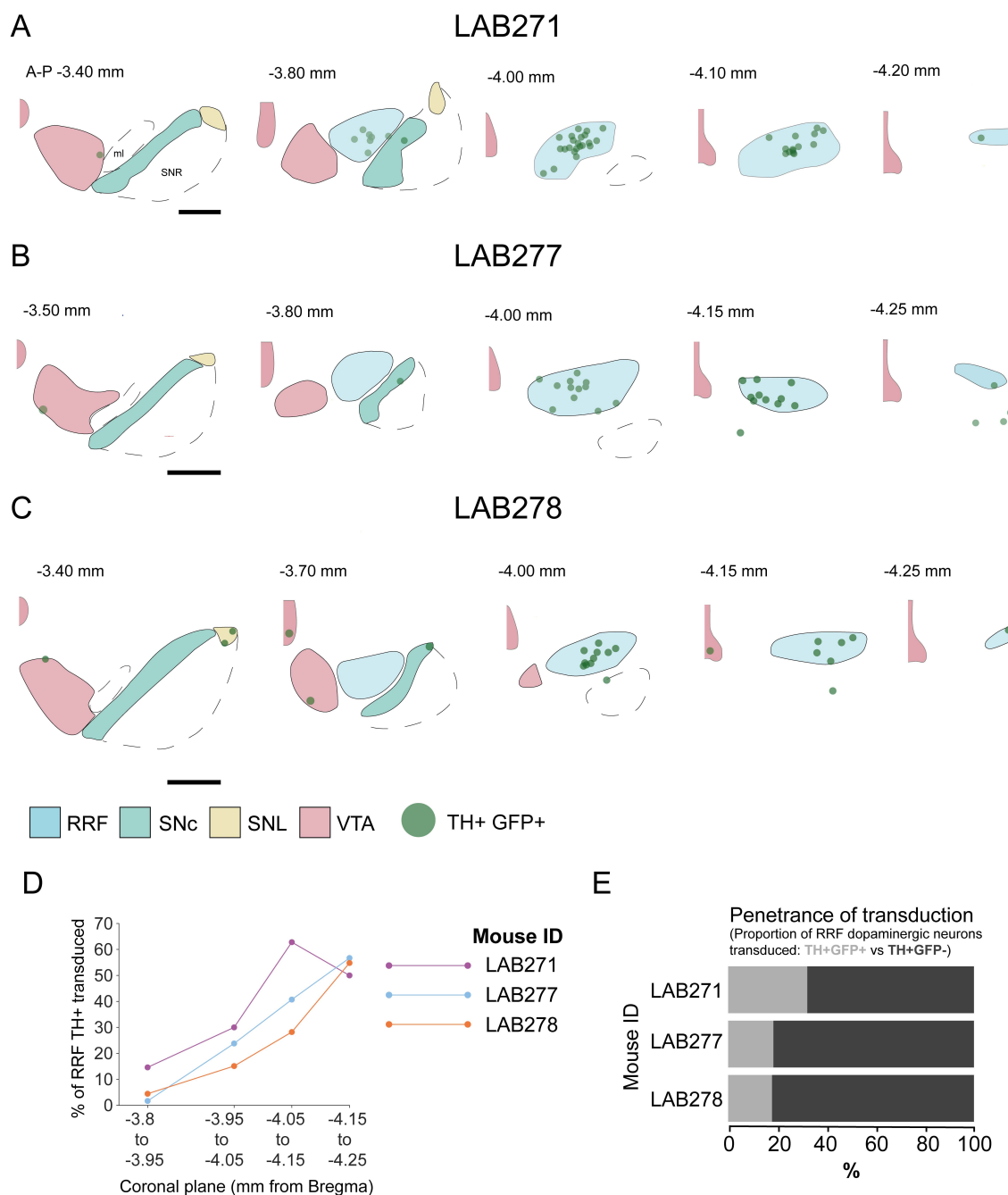


Figure 4.2: The distribution of GFP-transduced dopaminergic neurons in the midbrain. **A–C**, Coronal midbrain schematics showing the distribution of GFP-transduced dopaminergic neurons (green dots) at all rostrocaudal planes in which transduced neurons were located for each mouse. Each dot represents one TH⁺GFP⁺ neuron; the dots displayed represent neurons counted in the optical disector (10 μ m thick) in each tissue section from a series. Note, the dots below the RRF were defined as GFP-transduced RRF dopaminergic neurons for the purposes of stereology. Scale bars, 500 μ m. For the colour key, the definition of VTA includes: the CLi, the RLi, the PBP and the paranigral nucleus of the VTA (PN). SNL, substantia nigra pars lateralis. **D**, The percentage of RRF dopaminergic neurons transduced with GFP along the rostrocaudal axis. Note that the proportion increased the more caudal the sampled level. **E**, The penetrance of transduction for each mouse. Group average: 22.8% (6.6).

In addition to quantifying the penetrance of transduction (the proportion of RRF dopaminergic neurons transduced), I also stereologically determined the selectivity (the proportion of transduced

neurons that were dopaminergic; Figure 4.3A) and targeting efficiency (the proportion of transduced neurons in the RRF vs SNc/VTA) of viral transduction (Figure 4.3B). Although the targeting efficiency was high (> 80% of transduced neurons in the RRF), 20% of transduced dopaminergic neurons lay outside of the RRF (Figure 4.3). This means a small (but not negligible) number of neurons contributing to the brain-wide axonal projections were SNc or VTA in origin. As noted above, the distribution of GFP-expressing neurons in the RRF was sparse (Figure 4.1). This was quantified by the penetrance of GFP expression being fairly low ($\sim 22\%$; Figure 4.2E), meaning only a minority of RRF dopaminergic neurons were captured. In the present study, it was not practically possible to increase the targeting efficiency and penetrance of GFP expression as there seemed to be a trade-off between the two measures: the more caudal the injection of the virus, the better the targeting efficiency but the lower the penetrance. As expected, the virus was selectively expressed in dopaminergic neurons (Figure 4.1B and 4.3A); less than 2% of transduced neurons were TH negative on average.

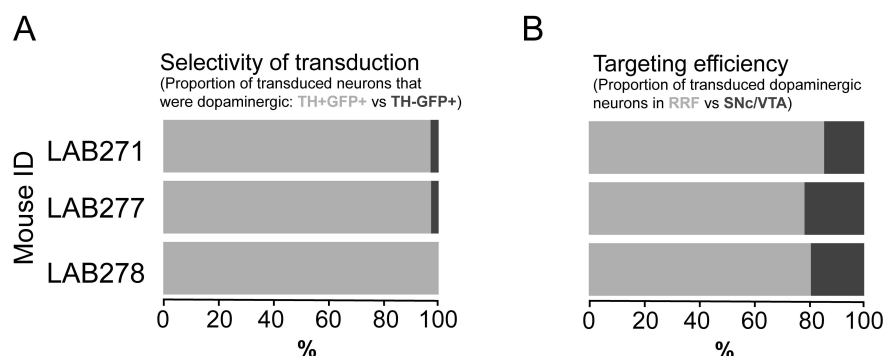


Figure 4.3: A summary of the transduction selectivity and targeting efficiency for each mouse. **A**, Selectivity of transduction, 98.1% (1.3). **B**, Targeting efficiency, 81.5% (3).

These data so far outline a careful examination of the viral strategy used to define the anterograde projections of RRF dopaminergic neurons. Although transduction was sparse, most GFP-expressing dopaminergic neurons ($\sim 80\%$) were located in the RRF in three mice. From this, the projections of these RRF dopaminergic neurons could be mapped.

4.4.2 RRF dopaminergic neurons project to the striatum and olfactory tubercle

After it was established that the transduction of dopaminergic neurons was relatively specific to the RRF (i.e. >80% of transduced neurons located within the RRF), serial sections from the whole brain of each mouse were examined for axonal projections. GFP⁺ fibres with axonal arborisation and varicosities were defined as putative termination sites (see below Figure 4.6). Although the projection pattern across the three mice studied was broadly similar, there were some notable differences between subjects. The regions in which RRF dopaminergic projections were observed are summarised in Table 4.2 below. For mouse LAB278 there were no GFP⁺ fibres in the OT (Figure 4.4B, C) or interstitial nucleus of the posterior limb of the anterior commissure (IPAC) (Figure 4.4D) but there was a projection to the rostral aspect of the striatum (Figure 4.4A), setting it apart from the other two subjects. Moreover, projections to the intermediate striatum were notably sparser in LAB278 compared with the other two subjects (Figure 4.4C). Note, putative termination sites were not observed in hindbrain structures (i.e. caudal of the RRF) of any animal, thus the list in Table 4.2 is exhaustive.

RRF dopaminergic projections were observed throughout the rostrocaudal extent of the striatum (Figure 4.4 and Figure 4.5), mainly restricted to its lateral aspect. In the rostral striatum of a representative mouse, GFP⁺ fibres were sparse and mostly limited to the dorsolateral striatum (DLS) (Figure 4.5Ai and Aii). There was also a sparse projection in the adjacent insular cortex of this mouse (Figure 4.5Aiii), however this was not common to the other two mice (Figure 4.4B).

Projection site	Mouse ID		
	LAB271	LAB277	LAB278
Striatum, rostral aspect	-	-	+
Insular cortex	+	-	-
DLS (rostral)	+	+	+
VLS (rostral)	+	+	+
OT (rostral)	+	+	-
DLS (intermediate)	+	+	+
VLS (intermediate)	+	+	+
VMS (intermediate)	+	+	-
OT (intermediate)	+	+	-
Tail of striatum (caudal)	+	+	+
OT (caudal)	+	+	-
IPACL (intermediate)	+	+	-
IPACL (caudal)	+	+	-

Table 4.2: A summary of the brain regions innervated by RRF dopaminergic neurons. The presence (+) or absence (-) of GFP⁺ fibres was recorded but no attempt was made to further qualitatively score these from low to high innervation. This table can be compared to Figure 4.4. The most rostral aspect of striatum was defined as coronal sections in which the striatum first appears (A-P +1.70 to +1.80 mm from Bregma). The following are the A-P range of sections defined as rostral, intermediate and caudal striatum with respect to Bregma: +1.54 to +0.38 mm; +0.38 mm to -0.46 mm; -0.46 to -1.94 mm. DLS, dorsolateral striatum; IPACL, lateral aspect of the interstitial nucleus of the posterior limb of the anterior commissure; OT, olfactory tubercle; VLS, ventrolateral striatum; VMS, ventromedial striatum.

At the level of the intermediate striatum (Figure 4.5Bi), GFP⁺ fibres were dense in the VLS (Figure 4.5Bii) and overlying centrolateral striatum. Additionally, axonal arborisations were observed in the OT, which was defined based on dense TH immunoreactivity (Figure 4.5Biii). Similar to the rostral striatum (Figure 4.5Ai), the medial aspects of the intermediate striatum were almost entirely devoid of projections (Figure 4.5Bi). It was thus concluded that the striatal projection pattern of RRF dopaminergic neurons was restricted to its lateral aspects, with the densest projections within the VLS.

In addition to RRF dopaminergic projections in the rostral (Figure 4.5Ai) and intermediate striatum (Figure 4.5Bi), GFP⁺ fibres were also observed in caudal forebrain sections (Figure 4.5Ci) within the tail of the striatum (Figure 4.5Cii). Although these projections were in the medial aspects of the tail of the striatum close to the border with the caudal external globus pallidus (GPe), they were still at a relatively lateral location in the brain. GFP⁺ fibres were detected in the nigrostriatal tract in the same section (Figure 4.5Ciii).

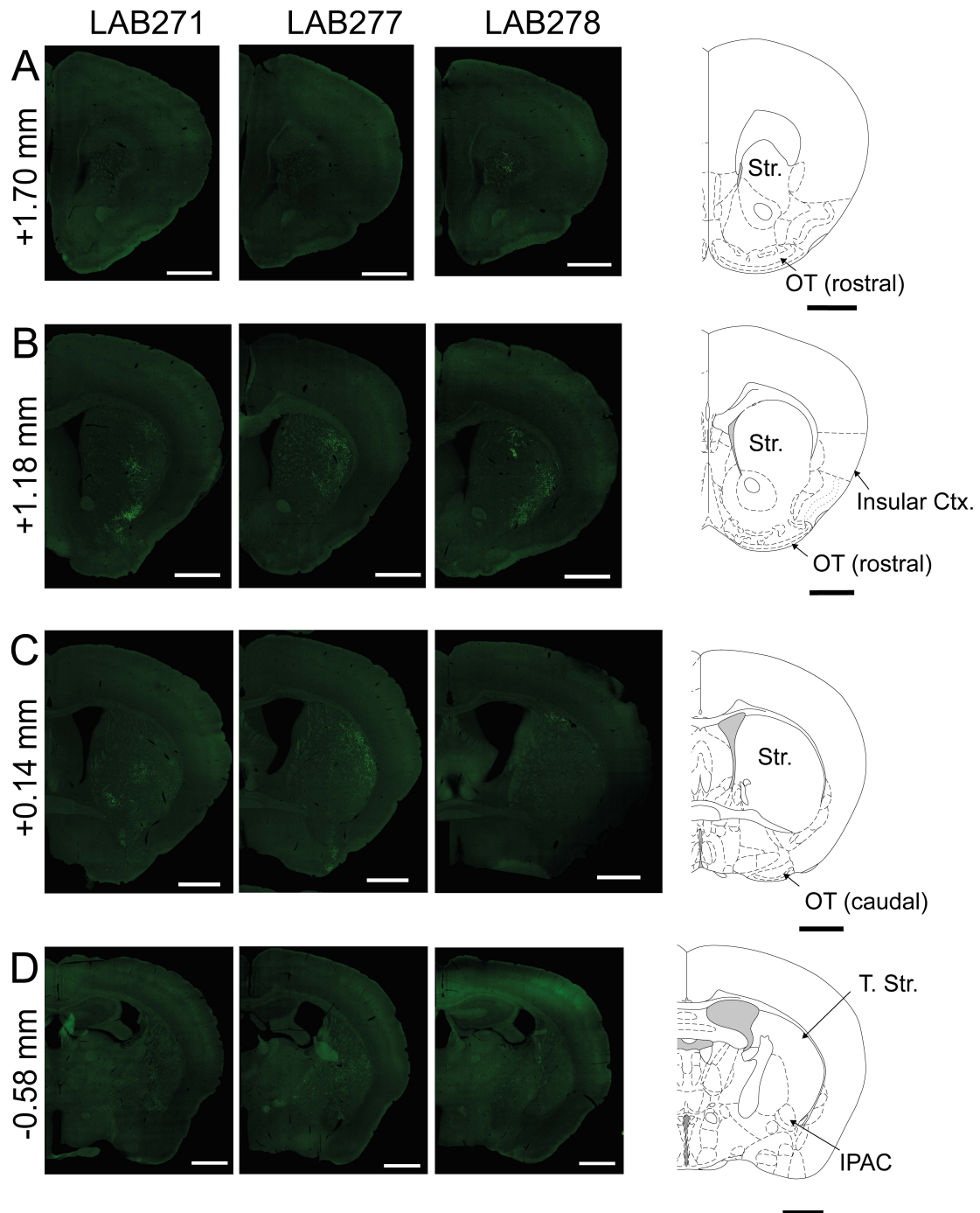


Figure 4.4: RRF dopaminergic projections were broadly similar across animals but with notable differences. **A–D**, Confocal micrographs at approximately the same coronal level for each mouse is shown alongside the relevant Paxinos and Franklin (2013) atlas reference figure (*right*). The A-P distance relative to Bregma is shown on the left of each panel of micrographs. Scale bars, 1 mm. **A**, GFP-labelled axons were observed in the rostral aspect of the striatum only in mouse LAB278. **B**, GFP-labelled axons were observed in the DLS and VLS of all mice, whereas axons were observed in the OT of LAB271 and 277 but not LAB278. Only LAB271 had GFP-labelled axons in the insular cortex (see Figure 4.5Aiii for higher magnification image). **C**, GFP-labelled axons were observed in the intermediate striatum (mostly lateral) of all mice but were notably sparser in LAB278. **D**, GFP-labelled axons were observed in the tail of striatum (T. Str.) of all mice, whereas axons in the IPAC were only observed in LAB271 and 277.

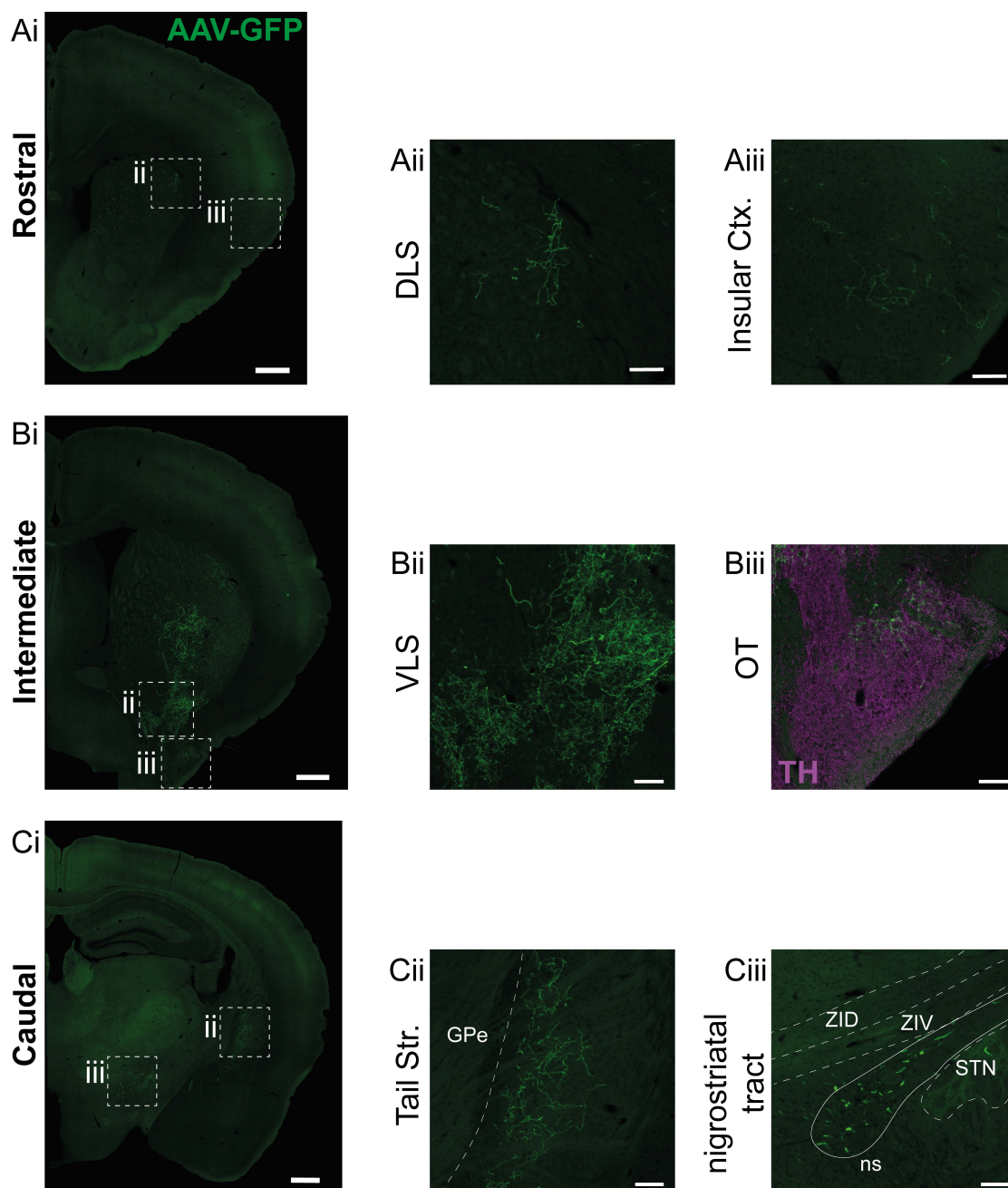


Figure 4.5: RRF dopaminergic projections to the striatum and olfactory tubercle. Sections from one mouse (LAB271) containing the rostral (**A**), intermediate (**B**) and caudal striatum (**C**) were examined for highly branched, putative axon terminations. **A**, Dorsolateral striatum (DLS) was sparsely innervated (**i**). **B**, Ventrolateral striatum (VLS) was densely innervated (**i**) and the underlying olfactory tubercle (OT) was sparsely innervated (**ii**). Note, TH immunoreactivity (magenta) delineated the borders between the OT and adjacent regions. **C**, The tail of the striatum was innervated (**i**). Axon labelling was also observed in the nigrostriatal tract (ns) between the areas containing the zona incerta (ZI) and subthalamic nucleus (STN) (**ii**). The dashed boxes indicate the areas shown in the *middle* and *right* panels for **A–C**. Scale bars: *left*, 500 μ m; *middle, right*, 100 μ m. Ctx., cortex; GPe, external globus pallidus; ZID, zona incerta, dorsal part; ZIV, zona incerta, ventral part.

Closer inspection of the axonal arborisations in the DLS (Figure 4.6A) and VLS (Figure 4.6B) revealed varicosities, suggesting that the dopaminergic innervation to these regions from the RRF was equipped for dopamine release. TH immunoreactivity in these regions was dense and therefore

precluded the analysis of colocalisation with GFP. However, in a ventral region of the lateral OT, GFP⁺ fibres were located in an area of sparser TH immunoreactivity (Figure 4.6C). In this area, GFP⁺ fibres were also immunoreactive for TH, indicating they were dopaminergic. Note, GFP⁺ dopaminergic fibres also contained varicose-like structures (Figure 4.6C), indicating swellings observed in GFP⁺ fibres were likely varicosities and not an artefact of GFP expression in transduced neurons.

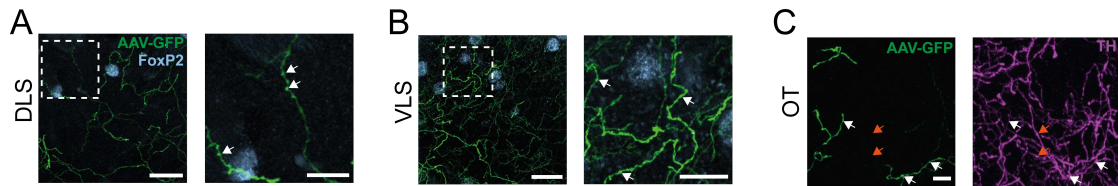


Figure 4.6: RRF dopaminergic axons in the striatum were highly branched and contained varicosities. **A**, **B**, Axon fibres of RRF dopaminergic neurons (GFP-labelled) in the DLS (**A**) and VLS (**B**) contained multiple varicosities (white arrows). Forkhead box protein P2 (FoxP2) immunoreactivity labels the nuclei of some striatal neurons and was included for perspective. The dashed box in the *left* panel of **A** and **B** indicates the area shown in the *right* panel higher magnification images. Scale bars: *left*, 20 μ m; *right*, 10 μ m. **C**, GFP⁺ axon fibres in the lateral OT had varicosities (white arrows) and expressed TH. GFP⁺ TH⁺ axon fibres also had varicosities (orange arrows). Scale bar, 5 μ m.

4.4.2.1 RRF dopaminergic neurons innervate both the matrix and striosomal compartments

The striatum is a highly complex structure, wherein inputs and outputs associated with motor, sensory and associative processes are integrated within specialised subdomains (Bolam et al., 2000; Hintiryan et al., 2016). One anatomical reflection of input/output segregation is the striosome-matrix (also known as patch-matrix) organisation of the striatum (Smith et al., 2016). In the present study, one aim was to determine whether dopaminergic inputs from the RRF display preferential targeting of the striosome or matrix compartments. To this end, immunoreactivity for μ opioid receptor (MOR) in forebrain serial sections was examined. This receptor is specifically expressed in the striosomal compartment of the rostral striatum (Tajima and Fukuda, 2013); markers of striosomes in caudal striatum were not examined and thus the rostral striatum was the focus of the present study. Examination of sections containing the rostral striatum revealed that RRF dopaminergic projections innervate both the matrix (Figure 4.7A) and striosomal

compartments (Figure 4.7B).

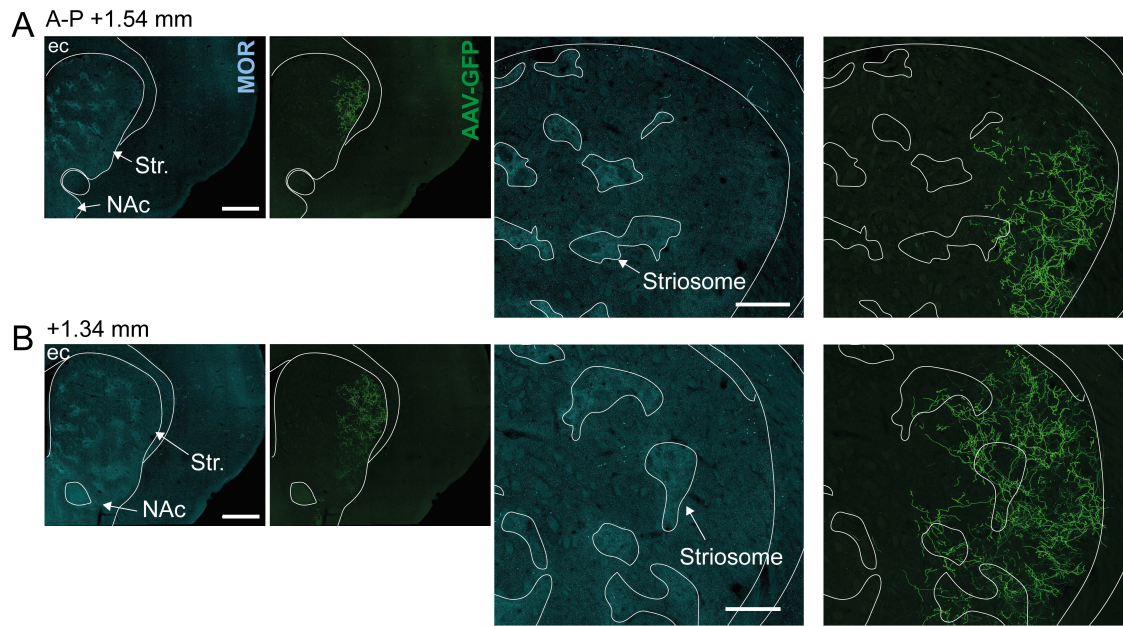
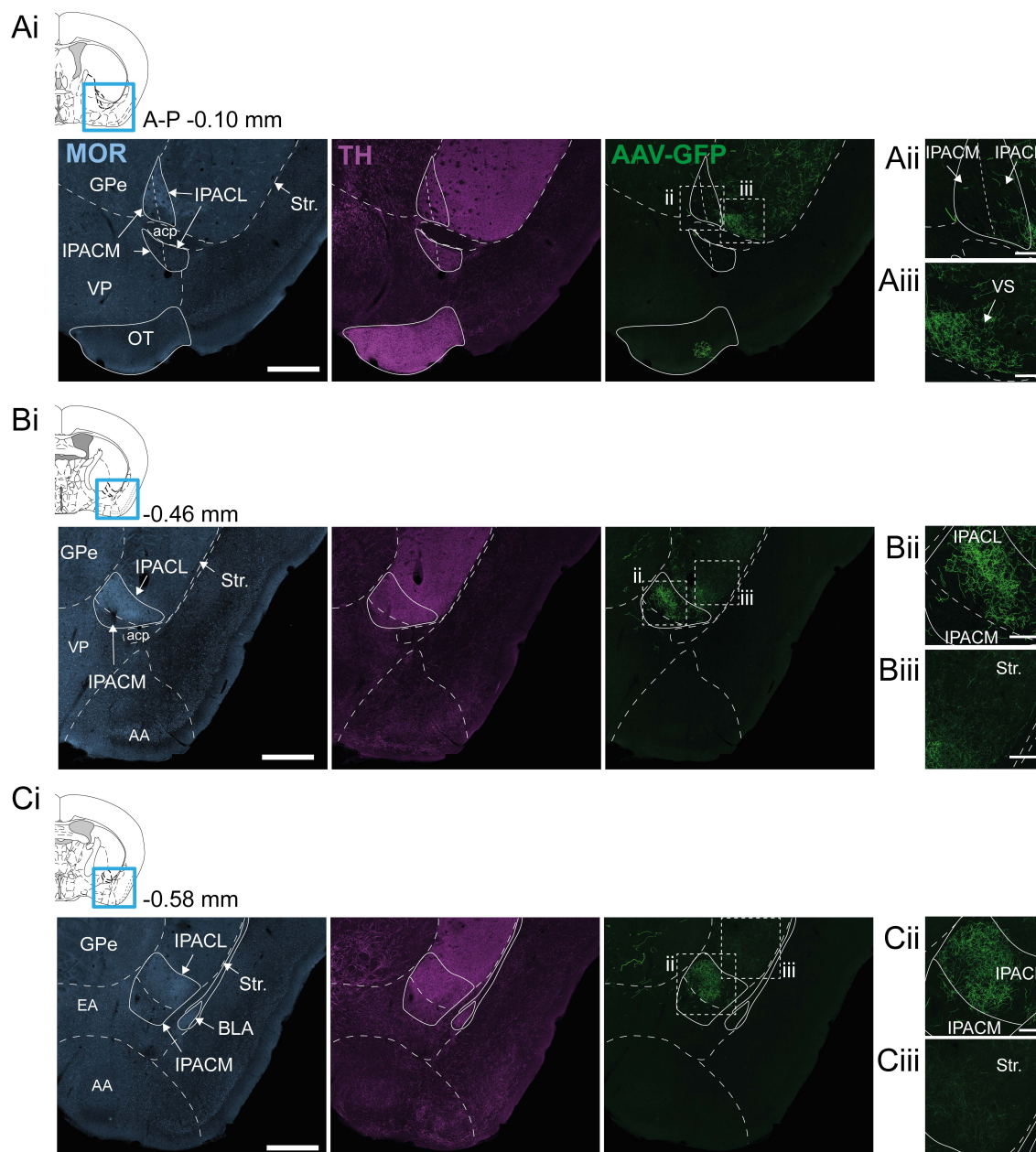


Figure 4.7: RRF dopaminergic projections to rostral striatum are located in the matrix (A) and striosomal compartments (B). Scale bars: *left*, 500 μm ; *right*, 200 μm . ec, external capsule; NAc, nucleus accumbens; Str., striatum. Mouse ID, LAB277.

4.4.3 RRF dopaminergic neurons project to the interstitial nucleus of the posterior limb of the anterior commissure

The IPAC is a ventral forebrain structure that straddles the ventral striatum and extended amygdala (Alheid et al., 1999; Shammah-Lagnado et al., 1999). Like the striatum, the IPAC has dense TH expression, a reflection of dopaminergic input from the midbrain (Yamaguchi et al., 2018). In the present study, serial sections containing the caudal aspect of the intermediate forebrain had dense GFP⁺ fibres close to the anterior commissure and GPe (Figure 4.8B, C). This dense innervation seemed to be within the IPAC, defined according to Paxinos and Franklin (2013). However, sometimes matching of sections to those in the Paxinos and Franklin (2013) atlas was non-trivial and thus a more objective delineation of the IPAC was sought. A recent study demonstrated that MOR is enriched in the dorsal IPAC and delineates its border with the GPe medially and the striatum laterally (Tanaka et al., 2019). Here, I replicate this observation and show that RRF dopaminergic neurons indeed project to the MOR-enriched IPAC (Figure 4.8), specifically in its

intermediate aspects (A-P -0.10 to -0.58 mm from Bregma). In sections containing the rostral aspect of the intermediate IPAC (Figure 4.8Ai), GFP⁺ fibres were sparse (Figure 4.8Aii), whereas in the adjacent ventral striatum fibres were dense (Figure 4.8Aiii), in line with observations described above (Figure 4.5Bii).



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Figure 4.8: RRF dopaminergic neurons project to the IPACL at intermediate levels. MOR immunoreactivity delineates the dorsal, medial and lateral border of the IPAC, and TH immunoreactivity delineates the ventral border. **A**, The rostral aspect of the intermediate IPACL (**Ai**) was sparsely innervated (**Aii**), abutted by dense innervation of the ventral striatum (VS; **Aiii**). **B–C**, The central aspect of the intermediate IPACL (**Bi**) and the caudal IPACL (**Ci**) received dense innervation from RRF dopaminergic neurons (**Bii**, **Cii**), whereas the underlying medial IPAC (IPACM) was scarcely innervated. The intermediate striatum received sparse innervation at these rostrocaudal levels (**Biii**, **Ciii**). The dashed, boxed-off areas in **i** indicate the areas from which the higher magnification images in **ii** and **iii** were taken. Scale bars, 500 μm (panel **i**) and 100 μm (panels **ii** and **iii**). The inset schematics are from Paxinos and Franklin (2013); the blue box indicates the area of the section shown in panel **i**. AA, anterior amygdaloid area (including cortical-amygdala transition zone); acp, anterior commissure, posterior part; BLA, basolateral amygdala; EA, extended amygdala; GPe, external globus pallidus; OT, olfactory tubercle; VP, ventral pallidum. Mouse ID, LAB277.

In sections containing the caudal aspects of the intermediate IPAC, the border between the lateral IPAC (IPACL) and medial IPAC (IPACM) was defined by a gradient of MOR immunoreactivity, and the ventral border with the ventral pallidum (VP) (Figure 4.8Bi) or extended amygdala (Figure 4.8Ci) defined by a gradient of TH immunoreactivity. GFP⁺ fibres were dense in the IPACL, whereas the medial IPACM contained very little innervation (Figure 4.8Bi, Ci). Dorsal to the IPAC in these sections, the RRF projections to the striatum were relatively sparse (Figure 4.8Biii, Ciii).

The GFP⁺ fibres within the IPAC were highly branched and contained varicose structures (Figure 4.9), similar to the pattern observed in the striatum (Figure 4.6A, B) and OT (Figure 4.6C). This suggests a bona fide innervation as opposed to fibres passing through the area.

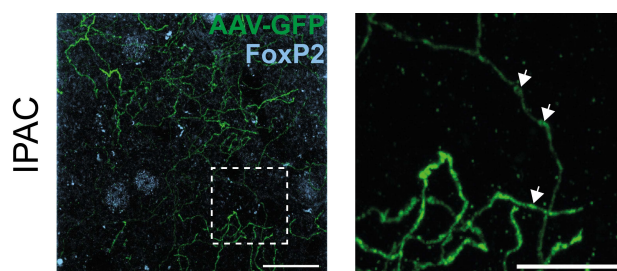


Figure 4.9: RRF dopaminergic axons in the IPAC were highly branched and contained varicosities. Axon fibres of RRF dopaminergic neurons in the IPAC contained multiple varicosities (white arrows). FoxP2 labels the nuclei of some neurons and was included for perspective. The dashed box in the *left* panel indicates the area shown in the *right* panel higher magnification image. Scale bars: *left*, 20 μm ; *right*, 10 μm . Mouse ID, LAB271.

4.5 Discussion

To my knowledge, this is the first selective anterograde tracing of RRF dopaminergic neurons. Broadly, axonal projections were observed in the striatum, olfactory tubercle and IPAC. In these

structures, the projections were mainly confined to the lateral aspects, with particularly dense innervation of the VLS and IPACL. Compared with those of the SNc and VTA, the somata of dopaminergic neurons in the RRF occupy a relatively dorsal position, and have a similar mediolateral spread to SNc neurons. In the present study, the observation that RRF projections were densest in ventral and lateral regions of the forebrain suggests a mediolateral and inverse dorsoventral topography of ascending axons, which is in keeping with the pattern observed for SNc and VTA dopaminergic neurons in the rat (Fallon and Moore, 1978).

4.5.1 *GFP-transduced dopaminergic neurons are mostly restricted to the RRF*

Limiting the transduction of dopaminergic neurons to the RRF while avoiding ‘spillover’ into the neighbouring SNc/VTA proved challenging. However, a method was established that resulted in ~80% of the transduced neurons being located within the RRF. The trade-off of this method was that only ~20% of RRF dopaminergic neurons were transduced. Although this level of transduction did result in clear, dense labelling of axonal arbors, it is possible that some projection sites were missed. This is an especially important consideration given that in two mice (LAB277 and 278), the most medial and lateral aspects of the RRF were sparsely transduced compared with the central RRF.

Studies with classical anterograde and retrograde tracers have demonstrated that dopaminergic neurons in the SNc, VTA (mouse: Lammel et al., 2008; Farassat et al., 2019; rat: Fallon and Moore, 1978; Björklund and Dunnett, 2007) and RRF (rat: Deutch et al., 1988) are organised topographically with respect to projection target. In the present study, proportionally more RRF dopaminergic neurons were transduced in more caudal sections, steadily increasing along a rostrocaudal gradient. Moreover, transduced neurons tended to be clustered in the centre of the RRF. It is therefore possible that the projection sites of dopaminergic neurons in the rostral, medial and lateral aspects of the RRF were underrepresented in the present study. However, it was determined that although the transduction of dopaminergic neurons was sparse in these three mice, they would be useful for

mapping the projection targets of RRF dopaminergic neurons.

4.5.2 RRF dopaminergic neurons project to the striatum and olfactory tubercle

Previous work using the classical anterograde tracer PHA-L revealed that the RRF projects to many forebrain regions, including the dorsal and ventral striatum, the amygdala, BNST and OT, and that the densest projection is to the VLS in the rat (Deutch et al., 1988) and cat (Jimenez-Castellanos and Graybiel, 1987). A non-exhaustive selection of these projection sites have been confirmed to receive dopaminergic input from the RRF using the retrograde tracer WGA-HRP in the rat (Deutch et al., 1988) and non-human primate (François et al., 1999). The present study shows that the dense projection to the VLS is indeed dopaminergic, and that the density of innervation observed in previous work was not exaggerated by a parallel non-dopaminergic projection from the RRF. The present study shows for the first time that the innervation of the lateral OT from the RRF (Jimenez-Castellanos and Graybiel, 1987; Deutch et al., 1988) is in fact dopaminergic. Previous investigators have suggested that there may be a rostrocaudal topography of the midbrain dopamine system, in that RRF neurons are located in the caudal midbrain and project to caudal forebrain structures compared with the SNc and VTA (rat: Deutch et al., 1988; non-human primate: François et al., 1999). In this Chapter, RRF dopaminergic projections were found at rostral, intermediate and caudal levels of the striatum, suggesting this topography does not necessarily hold in the mouse.

Injection of non-selective anterograde tracers into a midbrain nucleus provides insight into the projection sites of *all* cell types therein, the dopaminergic contribution of which can be examined by follow-up experiments with retrograde tracers (Deutch et al., 1988; François et al., 1999). However, this does not resolve the pattern of dopaminergic projections in a brain region, and thus subtle features of dopaminergic innervation cannot be determined. On the other hand, the use of a dopaminergic-neuron-selective anterograde tracing method in the present study permitted such examination. As a result, it was possible to determine that the bulk of RRF dopaminergic projections the rostral DLS are in the extrastriosomal matrix compartment, although there was also

clear labelling of axons within MOR-rich striosomes, but to a lesser extent. This bias to the matrix compartment is largely in keeping with classical anterograde tracing studies in the rat (Gerfen et al., 1987a), cat (Jimenez-Castellanos and Graybiel, 1987) and non-human primate (Langer and Graybiel, 1989). However, this is the first unequivocal identification of RRF dopaminergic projections to the striatal matrix. It should be noted that in previous reports there was emphasis placed on the near-exclusive innervation of the matrix by neurons of the RRF, whereas I observed merely a bias. It is important to say, however, that I only distinguished the striosome and matrix compartments in the rostral striatum because MOR immunoreactivity does not distinguish them in the intermediate or caudal striatum (Tajima and Fukuda, 2013). Thus, it may be the case that RRF dopaminergic projections in the intermediate and caudal striatum target striosomes more specifically; selecting markers that distinguish the striosomal and matrix compartments in these parts of striatum, such as substance P, will be an important future experiment.

Moving from the striatum to amygdala and BNST, I found no evidence for RRF dopaminergic projections to any part of the amygdala (central amygdala (CeA), basolateral amygdala (BLA) or anterior amygdaloid structures), or any part of the BNST. This is in sharp contrast to previous work in the rat which demonstrated labelling of dopaminergic neurons in the RRF following deposits of retrograde tracers in the extended amygdala (EA), CeA, BLA and BNST (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002). However, these previous studies describe a minor dopaminergic component of the RRF input to the CeA (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002) and EA (Hasue and Shammah-Lagnado, 2002). It is thus possible that these amygdala-projecting RRF dopaminergic neurons were missed in the present study given the sparse level of GFP transduction. Interestingly, Hasue and Shammah-Lagnado (2002) found that the majority of BNST-projecting dopaminergic neurons were in the rostral aspect of the RRF, which had limited levels of transduction in the present study. In future, more complete transduction of the RRF (including its rostral aspect) may clarify whether there is a dopaminergic projection to the BNST from the RRF. It is also possible that the projection targets of RRF dopaminergic neurons in rats and

mice are different, which may explain why there is divergence between the previous work and the present study.

Interestingly, a recent study mapping the monosynaptic inputs to multiple cell types in the CeA and BLA of mice found no evidence for RRF inputs (dopaminergic or otherwise; Fu et al., 2020). However, it is important to note that in rabies-mediated transsynaptic tracing studies like this, relatively few neurons in the SNc, VTA or RRF are traced from starters in the NAc (Li et al., 2018), dorsal striatum (Wall et al., 2013) or amygdala (Fu et al., 2020), despite the established dopaminergic innervation of these structure (Björklund and Dunnett, 2007). This is potentially because dopamine release mostly occurs outside of classical, tightly-coupled active zones (Liu et al., 2018), and thus starter neurons are prohibitively far from dopaminergic axons for transsynaptic spread of the rabies virus (Wall et al., 2013). However, others do see numerous retrogradely traced SNc dopaminergic neurons from starters in the dorsal striatum (Guo et al., 2015). Thus, differences in the viral constructs used and other experimental conditions may alter the degree to which dopaminergic inputs are labelled. From the data in the present study, one could conclude that the dopaminergic input to the amygdala is not derived from the RRF in mice, though further studies are warranted, particularly given the sparse transduction of RRF dopaminergic neurons.

Anterograde tracing from the RRF with classical tracers in the rat uncovered projections to the ventral and mediodorsal nuclei of the thalamus, and the lateral hypothalamus (Deutch et al., 1988). It would appear these are likely non-dopaminergic as I did not observe any RRF dopaminergic projections in these regions, although a species difference between the rat and mouse could also be an explanation. However, previous work with classical retrograde tracers in the rat targeted to mediodorsal thalamus (Groenewegen, 1988) and different aspects of the lateral hypothalamus (Barone et al., 1981; Kita and Oomura, 1982; Hahn and Swanson, 2010) did not uncover projections from the RRF, but rather retrogradely-labelled cells were located in the SNc and VTA. Similarly, previous reports have identified projections from the RRF to the STN (rat: Deutch et al., 1988; non-human primate: François et al., 2000), which in the non-human primate were confirmed to be

dopaminergic (François et al., 2000). However, I observed no RRF projections to the STN. There were, however, projections in the nearby nigrostriatal tract, which are likely the axons coursing on their trajectory to the forebrain.

As described above, the axonal projections of RRF dopaminergic neurons were found exclusively in forebrain regions, and there was no evidence of terminals in hindbrain areas. However, previous work in the rat with classical tracers described a projection from the RRF to the reticular formation (von Krosigk and Smith, 1991; von Krosigk et al., 1992). Taken together with the results of the present study, it would appear that this projection from the RRF to the reticular formation is non-dopaminergic.

In the striatum, dopaminergic innervation is dense, meaning that, for example, all corticostriatal and thalamostriatal synapses are within $\sim 1 \mu\text{m}$ of a dopaminergic synapse (Moss and Bolam, 2008) and therefore subject to modulation by dopamine release (Rice and Cragg, 2008). In the present work, closer examination of RRF dopaminergic axons in the striatum revealed a highly branched plexus of axonal arborisations with evidence of varicosities. Although this does suggest that the axon labelling in the projection regions is equipped to release dopamine and modulate striatal output, it should be noted that dopamine release in striatum is highly complex and that it is estimated only $\sim 30\%$ of varicosities actively release dopamine (Liu et al., 2018). Thus, future work to more thoroughly examine the molecular composition of these varicosities is warranted. For example, colocalisation of active-zone components such as bassoon would point towards active release sites.

4.5.3 *RRF dopaminergic neurons project to the interstitial nucleus of the posterior limb of the anterior commissure*

The IPAC is a ventral forebrain structure that is considered part of the extended amygdala but shares neurochemical features of the striatum, such as a high density of TH and acetylcholinesterase (Alheid et al., 1999). Previous studies in the rat with classical retrograde tracers injected into the

IPAC and adjacent regions observed labelling of RRF neurons (Shammah-Lagnado et al., 1999; Hasue and Shammah-Lagnado, 2002; Yamaguchi et al., 2018), some of which confirmed to be dopaminergic (Hasue and Shammah-Lagnado, 2002; Yamaguchi et al., 2018). One issue with retrograde tracing is the spread of the tracer outside of the region of interest; given its small size and proximity to other dopamine-receiving areas, injecting retrograde tracers into the IPAC often leads to spillover into adjacent regions, such as the ventral pallidum (Hasue and Shammah-Lagnado, 2002) and striatum (Shammah-Lagnado et al., 1999). Thus, concluding which cell type(s) within the RRF project to the IPAC specifically is non-trivial. This is not to discredit previous conclusions, but rather to encourage cognisance of the drawbacks of certain methodologies.

Furthermore, depositing retrograde tracers into the IPAC does not reveal the fine anatomical features of the dopaminergic input from the RRF. In the present study, however, selective anterograde tracing of RRF dopaminergic neurons determined that the IPACL receives a dense dopaminergic projection from the RRF, whereas the IPACM receives very little innervation. Interestingly, the connectivity of the IPACL has features of both the central extended amygdala and the ventral striatum, suggesting it is a transitional area, whereas the connectivity of the IPACM has much more in common with the central extended amygdala (Shammah-Lagnado et al., 1999; Shammah-Lagnado et al., 2001). Taken together with the lack of GFP-labelled fibres in any aspects of the amygdala (see above discussion), the evidence provided in the present study tentatively suggests that RRF dopaminergic neurons are not involved in providing dopaminergic input to the amygdala. This is in departure from previous studies that observed retrogradely-labelled dopaminergic neurons in the RRF following injection of WGA-HRP into the central amygdala (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002) or BNST of the rat (Hasue and Shammah-Lagnado, 2002). However, it is possible that the RRF dopaminergic neurons projecting to the amygdala were ‘missed’ in the present study, given the sparse transduction of neurons within the RRF (i.e. ~20% of RRF dopaminergic neurons transduced). In future, transducing a larger proportion of RRF dopaminergic neurons would allow stronger conclusions to be drawn regarding

the presence or absence of projections to the amygdala.

In the present study, the IPAC was identified based on dense immunoreactivity for MOR, which distinguished it from the overlying intermediate/caudal striatum (Tanaka et al., 2019). Dense MOR expression in striosomes is limited to the rostral striatum (Tajima and Fukuda, 2013) and thus it appears unlikely that the area with enriched MOR immunoreactivity in the IPAC is a striosome. The ventral border of the IPAC with the VP rostrally and EA caudally was distinguished by dense TH immunoreactivity in the IPAC versus VP/EA. Although this border was less definitive than the MOR-defined dorsal border, I am confident the RRF dopaminergic projections observed in the IPAC did not also include the EA. Moreover, previous work has determined that nitric oxide synthase (NOS) is highly expressed in the ventral IPAC but weak in underlying the VP and EA (Zahm et al., 2014). In some pilot work determining molecular markers of the IPAC (not shown), I found that both TH and NOS immunoreactivity were equally useful for distinguishing the ventral border of the IPAC, hence TH and MOR were used in combination in the present study to delineate the IPAC. Also, the GFP⁺ fibres were parallel with the rostral BLA and thus in the IPAC, whereas the EA is located more ventrally and medially (Paxinos and Franklin, 2013).

4.5.4 Conclusions and functional significance

The use of a dopaminergic-neuron-selective anterograde tracing method in this study confirms some of the projection regions of RRF dopaminergic neurons previously determined using non-selective anterograde and retrograde tracers. Specifically, the results of this Chapter replicate the finding that diffuse projections occur throughout lateral striatum, with the densest projections in the ventrolateral aspect (rat: Deutch et al., 1988; cat: Jimenez-Castellanos and Graybiel, 1987; non-human primate: François et al., 1999). In contrast to previous work in the rat (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002), the present study found no evidence for dopaminergic projections from the RRF to any part of the amygdala. The precision of the selective anterograde tracing method used in this Chapter demonstrated for the first time that the RRF dopaminergic projections to the IPAC target

the IPACL, whereas the IPACM is scarcely innervated. Moreover, I determined that the bulk of RRF projections to the DLS in the rostral striatum are located within the matrix compartment.

As has been previously suggested in the rat (Deutch et al., 1988), the projection pattern of RRF dopaminergic neurons shares features of both the nigrostriatal and mesolimbic pathways, suggesting they are well placed to influence sensorimotor and limbic processing. Moreover, the present study demonstrates that RRF dopaminergic projections to the dorsal striatum are biased towards the matrix compartment. Given that the corticostriatal projections from the prelimbic and sensory/motor cortex preferentially innervate the striosome and matrix compartments, respectively (Gerfen, 1984; Donoghue and Herkenham, 1986), dopaminergic neurons of the RRF are particularly well positioned to influence sensorimotor processing in the dorsal striatum. The dynamics of dopamine activity in the striosome and matrix compartments of the striatum are complex. In the dorsal striatum, electrically-evoked dopamine release is higher in the matrix compared with striosomes, whereas the reverse is true in the ventral striatum (Salinas et al., 2016). Moreover, neuromodulators can further alter these dynamics. For example, substance P enhances electrically-evoked dopamine release in striosomes, reduces release in the border between the striosome and matrix compartments, and has little effect on release in the matrix, thereby weighting dopamine signals in a compartment-specific manner (Brimblecombe and Cragg, 2015). In future, understanding whether these features of dopamine signalling are common to dopamine released from RRF neurons will be interesting to explore.

RRF dopaminergic neurons projecting to the DLS may be involved in habit formation, which is known to require the DLS (Yin et al., 2004; Amaya and Smith, 2018) and involve dopamine activity (Faure et al., 2005). Additionally, there was a distinct lack of RRF axonal projections in the dorsomedial striatum (DMS) in the present study, suggesting that RRF dopaminergic neurons are not as closely involved in associative processing (Yin et al., 2005).

The role of the RRF dopaminergic innervation of the IPACL is more difficult to conceptualise because it is a poorly understood brain region. However, some studies have demonstrated that the

activity of neurons in the IPAC is related to learned and innate disgust in response to aversive tastants (Yasoshima et al., 2006; Tanaka et al., 2019). It is therefore possible that dopamine released in the IPAC from the terminals of RRF neurons is involved in modulating these disgust responses.

Moving to the ventral striatum, the dense projection observed in the VLS in the present study suggests that release of dopamine from RRF neurons is positioned to influence goal-directed behaviour (Natsubori et al., 2017; Tsutsui-Kimura et al., 2017). Midbrain dopaminergic neurons tend to project exclusively to one brain region without sending distal collaterals (mouse: Lammel et al., 2008; Rodríguez-López et al., 2017; Farassat et al., 2019; rats Fallon, 1981), which would indicate that RRF dopaminergic neurons projecting to the dorsal and ventral striatum are distinct subpopulations. In future, a closer examination of the input-output relationship of these two subpopulations would provide important insight about the brain circuitry into which they are embedded.

Chapter 5

Monosynaptic inputs to RRF dopaminergic neurons

5.1 Introduction

The functions of neurons are partly defined by their inputs. It follows that defining the inputs to a given cell type then informs on its function in the brain. The RRF, SNc and VTA receive diverse inputs from cortical and subcortical areas in the rat (Zahm et al., [2011](#)). Although the presence of dopaminergic neurons typically defines these midbrain nuclei, they are intermingled with glutamatergic neurons (rat: Yamaguchi et al., [2007](#); Nair-Roberts et al., [2008](#); Yamaguchi et al., [2013](#)) and GABAergic neurons (mouse: Tan et al., [2010](#); rat: Nair-Roberts et al., [2008](#)). Additionally, a small number of some midbrain dopaminergic neurons are equipped to corelease glutamate (Kawano et al., [2006](#); Yamaguchi et al., [2007](#); Hnasko et al., [2010](#)) or GABA (Tritsch et al., [2012](#); Kim et al., [2015](#)). The plurality of cell types in the midbrain necessitates that the tracing of their inputs uses tools and methods that permit selective access to the population of interest. The use of classical retrograde tracers, such as wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) or rabies virus, does not offer the desired selectivity because they are retrogradely transported to afferent neurons irrespective of the cell type(s) that were innervated. Moreover,

polysynaptic spread of WGA-HRP (Goshgarian and Buttry, 2014; Sillitoe, 2016) and rabies (Ugolini, 1995) from direct input neurons to other neurons makes defining the monosynaptic inputs to an area impossible with these tools. Thus, previous work in the rat mapping midbrain afferents using classical retrograde tracers (Swanson, 1982; Zahm et al., 2011) provides information about the inputs to all cell types in the midbrain, but any further level of detail regarding the cell type-selectivity of these inputs is not provided. Furthermore, precise targeting of the RRF and SNc with classical retrograde tracers is particularly difficult, owing to their narrow width.

The modified rabies virus-based transsynaptic tracing method (Wickersham et al., 2007) enables the monosynaptic inputs to a genetically-defined cell type to be mapped. (Note, the term ‘monosynaptic’ is contentious, as discussed in Chapter 8 Section 8.2.2.) This method is most useful when cell types in a region are heterogeneous, as is the case in the midbrain. Firstly, the ‘helper’ AAV virus encoding the avian TVA receptor and the rabies glycoprotein (G) is injected into the brain region of interest. The TVA cell-surface receptor and rabies G are expressed in a Cre-dependent manner, together defining a ‘starter’ neuron. Secondly, the glycoprotein gene-deleted rabies virus tagged with GFP (SAD Δ G-eGFP; henceforth referred to as RV-eGFP) is injected. Importantly, this modified rabies virus is coated with the envelope protein EnvA, rendering it unable to enter mammalian cells unless they express the cognate receptor TVA. Thus, it can only enter Cre⁺ neurons expressing TVA. Because the transduced Cre⁺ also contain G, the rabies virus can form a viral capsid and leave the starter neuron (presumably through the dendrites) and enter presynaptic input neurons. These input neurons themselves do not contain G, meaning that even though they produce RV-eGFP, they do not produce the G required to form the viral capsid and as a result RV-eGFP cannot leave the cell. Therefore, only the monosynaptic inputs to a defined cell type are labelled. This method has been used for identifying the monosynaptic inputs to dopaminergic neurons (Watabe-Uchida et al., 2012; Beier et al., 2015), glutamatergic neurons (Faget et al., 2016) and GABAergic neurons of the VTA (Beier et al., 2015; Faget et al., 2016), and dopaminergic neurons of the SNc (Watabe-Uchida et al., 2012; Lerner et al., 2015; Menegas et al., 2015). The inputs to

dopaminergic, glutamatergic and GABAergic neurons of the VTA are from a diverse array of brain regions and are qualitatively similar. However, within some input regions there are differences. For example, inputs to GABAergic VTA neurons from the paraventricular hypothalamus (PVH) are more often from oxytocin- and arginine vasopressin (AVP)-expressing neurons than is the case for dopaminergic VTA neurons (Beier et al., 2015). Quantitatively, dopaminergic neurons in the VTA receive more inputs from the striatum and external globus pallidus (GPe), glutamatergic VTA neurons receive more inputs from the cortex, and GABAergic VTA neurons receive more inputs from the lateral habenula (LHb) and laterodorsal tegmentum (LDTg) (Faget et al., 2016). These qualitative and quantitative differences illustrate the level of information revealed by a genetically-targetable input tracing method.

In addition to the whole brain inputs to SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012; Beier et al., 2015), virus-based approaches have allowed the input mapping from projection-defined subpopulations of dopaminergic neurons. For SNc dopaminergic neurons, this has revealed that there is reciprocal connectivity between subregions of dorsal striatum and the subgroups of SNc dopaminergic neurons from which they receive input (Lerner et al., 2015). Similarly, VTA dopaminergic neurons receive biased inputs depending on their projection target (Beier et al., 2015).

There are so far no data describing the pattern of inputs to RRF dopaminergic neurons, in contrast to the high level of detail uncovered for their counterparts in the SNc and VTA. Non-selective retrograde tracing has revealed that, as compared with the SNc and VTA, the RRF receives more input from the bed nucleus of the stria terminalis (BNST), central amygdala (Zahm et al., 2011) and noradrenergic cell groups in the rat (Mejías-Aponte et al., 2009). It has not been confirmed whether this distinct input pattern is selective for a particular cell type in the RRF. In this Chapter, I used a modified rabies virus-based transsynaptic tracing system (Fürth et al., 2018; Ährlund-Richter et al., 2019) to map the monosynaptic inputs to RRF dopaminergic neurons.

5.2 Aims

- To develop and validate a strategy that restricts most starter neurons to the dopaminergic neurons of the RRF.
- To map the whole-brain monosynaptic inputs to RRF dopaminergic neurons.

5.3 Methods

DAT-Cre mice (n = 1 male and 3 females) were intracerebrally-injected with a Cre-dependent ‘helper virus’ (AAV5-DIO-TVA^{V5}-RG) followed 3 weeks later by injection of a ‘modified rabies virus’ (RV-eGFP). This permits the tracing of monosynaptic inputs to Cre-expressing dopaminergic neurons (see Materials and Methods Section 2.4.2 for more details). At each stage, 33–66 nL of virus was injected, targeted just behind the RRF at coordinates anterior-posterior (A-P) -4.50/-4.30 (helper/modified rabies), medial-lateral (M-L) +1.10 and dorsal-ventral (D-V) -3.20 mm relative to Bregma (see Materials and Methods Section 2.3 and 2.4.2 for more details). Mice were killed and perfused 1 week after the RV-eGFP was injected. The whole brains were then sectioned and processed for immunohistochemistry (see Materials and Methods Section 2.4.3).

Starter neurons (V5⁺GFP⁺) in the midbrain were quantified using rigorous stereology (West, 1999, 2012) (see Materials and Methods Section 2.4.4) and the brain-wide distribution of input neurons was qualitatively mapped (see Materials and Methods Section 2.4.6). Data are presented as mean (SD) unless otherwise stated.

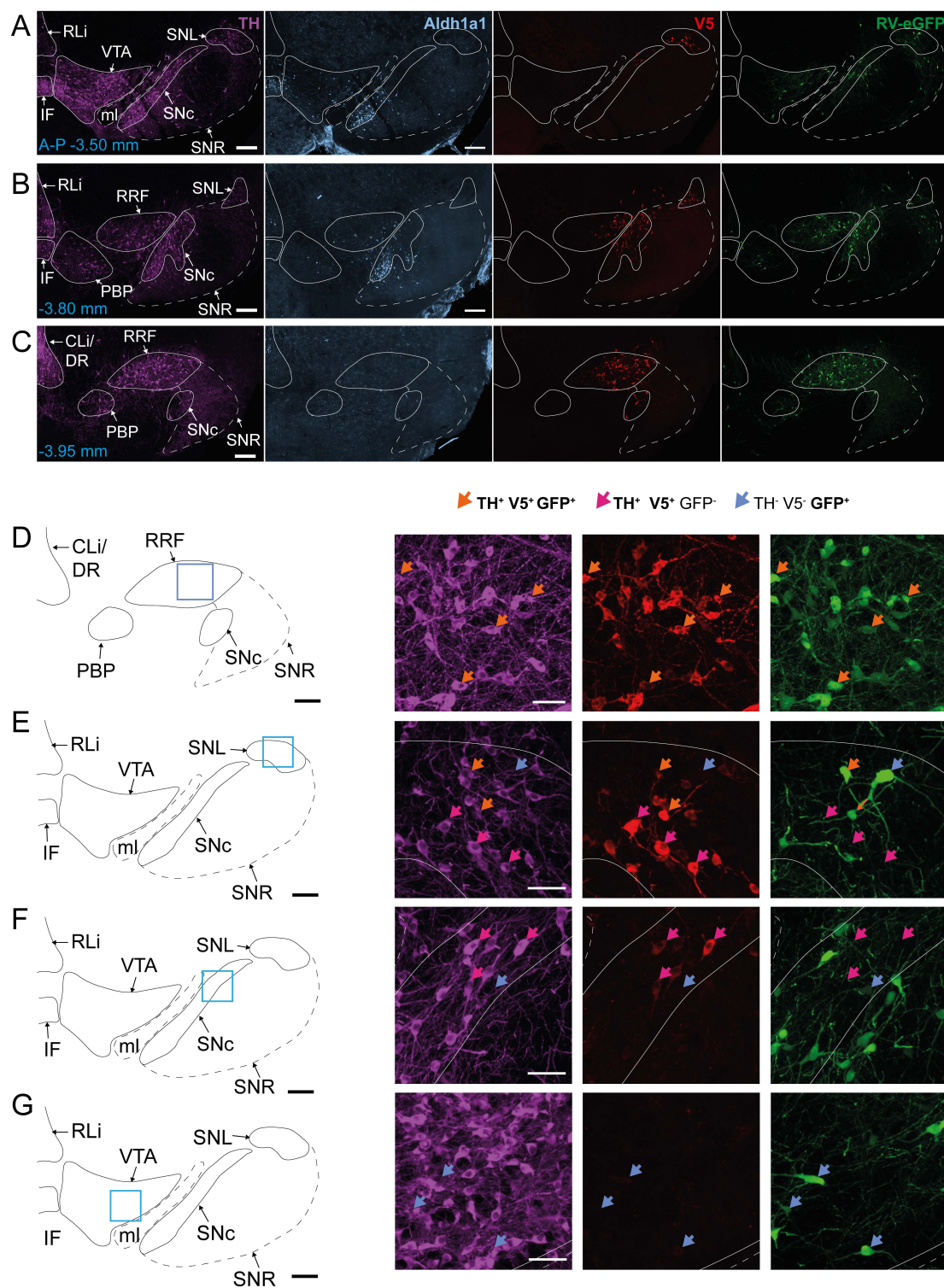
5.4 Results

5.4.1 Starter neurons were mostly restricted to the RRF

Starter neurons in the midbrain were stereologically quantified for three main reasons. Firstly, it was important that a high proportion of starter neurons (from which input neurons are

transsynaptically traced) were in the RRF with minimal transduction of neighbouring SNc and/or VTA neurons (transduction efficiency). Secondly, it was important to quantify the number of starter neurons in order to normalise the number of input neurons. (Note, this normalisation is not carried out in this Chapter but rather will be the focus of future work.) Thirdly, it was important to determine the selectivity of viral transduction by quantifying the proportion of starter neurons that were dopaminergic.

Starter neurons ($V5^+GFP^+$) also expressed TH and were thus midbrain dopaminergic neurons (Figure 5.1A–F). Some other neurons were $TH^+V5^+GFP^-$, which indicated Cre-mediated recombination of the helper virus but no subsequent transduction by the RV-eGFP (Figure 5.1E, F). These cells were not true starters (Ährlund-Richter et al., 2019), in the sense that they did not contribute to the transduction of neurons providing monosynaptic inputs because they contained no RV-eGFP. Hence, although in some confocal micrographs the area of V5 transduction seems wide (i.e. many V5-expressing neurons in the SNc and substantia nigra pars lateralis (SNL); Figure 5.1A, B), most of these neurons were not transduced with GFP and therefore were not true starter neurons. This is why the distribution of starter neurons appears better restricted to the RRF in the schematic maps (Figure 5.2) than the confocal micrographs (Figure 5.1). A third set of neurons expressing only RV-eGFP ($TH^-V5^-GFP^+$) were observed in the SNL (Figure 5.1E), SNc (Figure 5.1F) and VTA (Figure 5.1G), which represented neurons providing local direct inputs to the starter neuron population.



(Figure legend on next page)

Figure 5.1: Starter neurons were dopaminergic and mostly restricted to the RRF. **A–C**, Confocal micrographs showing the distribution of transduced neurons in the SNc/VTA (**A**), rostral RRF (**B**) and intermediate RRF (**C**). Aldehyde dehydrogenase 1a1 (Aldh1a1) immunoreactivity was used to delineate the border between the RRF and caudal SNc (**B** and **C**). Scale bars, 200 μ m (**A–C**). A-P, anterior-posterior distance with respect to Bregma; CLi, caudal linear nucleus of the raphe; DR, dorsal raphe; PBP, parabrachial pigmented nucleus of the VTA; RLi, rostral linear nucleus of the VTA; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticulata. **D–G**, Higher magnification images of **A** and **C** highlighting the transduction of midbrain neurons. Many dopaminergic starter neurons were observed in the RRF (**D**, orange arrows), and some were observed in the SNL (**E**, orange arrows) and SNc (not shown). There were also some dopaminergic neurons transduced with the helper virus (V5) but not the pseudotyped RG-deleted rabies virus (RV-eGFP; i.e. not true starters) in the SNL (**E**, pink arrows), SNc (**F**, pink arrows) and RRF (not shown). GFP-only neurons (i.e. local inputs to starter neurons) were located in the SNL (**E**, blue arrows), SNc (**F**, blue arrows) and VTA (**G**, blue arrows). The blue boxed off area on the schematics (*left*) represents the area shown in the accompanying confocal micrographs. Scale bars, 50 μ m (**D–G**).

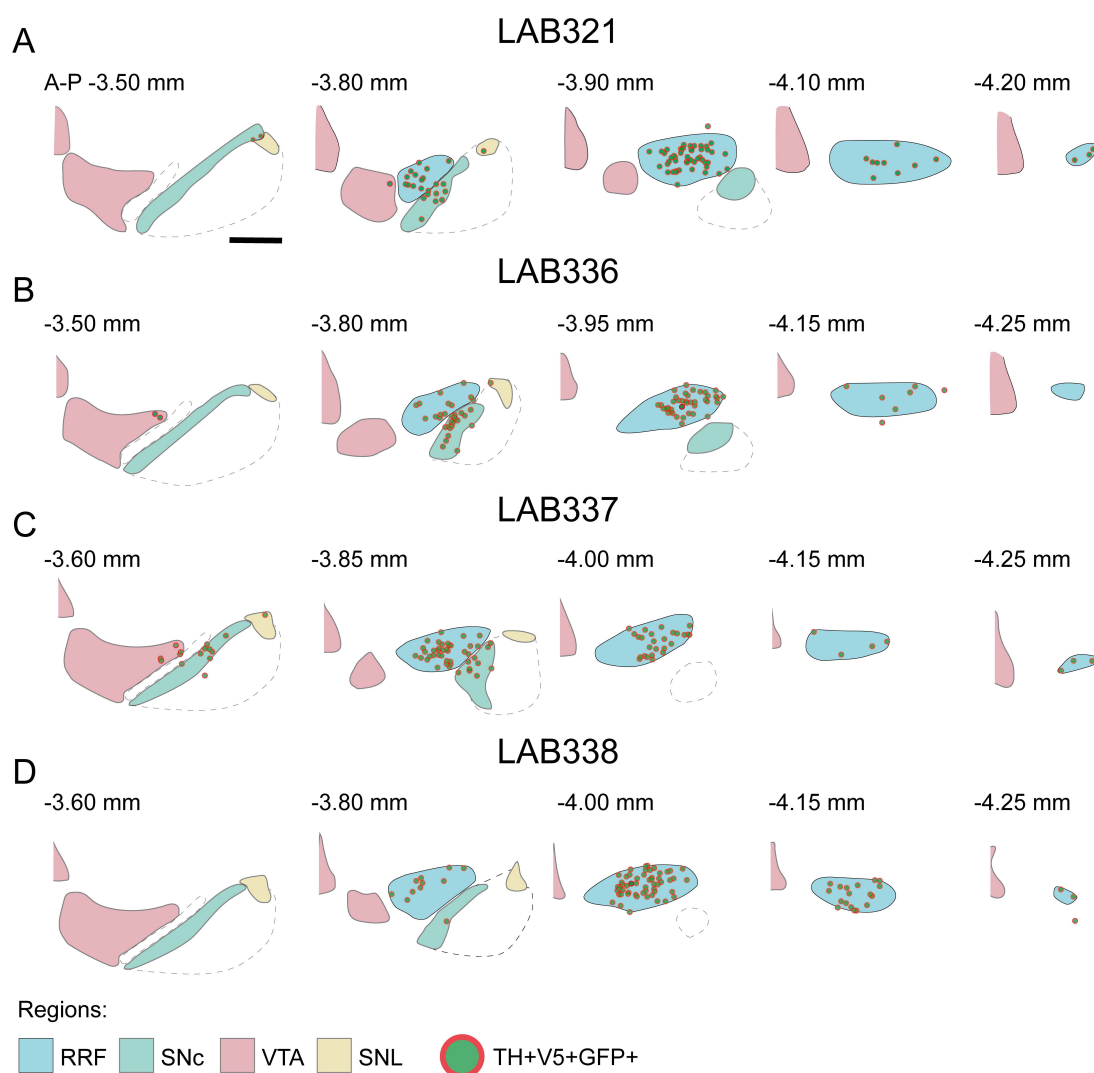


Figure 5.2: The distribution of starter neurons in the midbrain. **A–D**, Coronal midbrain schematics showing the distribution of dopaminergic starter neurons for each mouse (LAB321, 336–338). Each dot represents one neuron; the dots displayed represent neurons counted in one optical section (10 μ m) from serial midbrain sections. Note, the dots below the RRF were defined as RRF dopaminergic starter neurons for the purposes of stereology. Scale bar, 500 μ m. For the colour key, the definition of VTA includes: the CLi, the RLi, the PBP and the paranigral nucleus of the VTA (PN).

In addition to being highly selective ($\sim 99\%$ of transduced neurons were dopaminergic; Figure 5.3A), the penetrance of transduction was high, with $\sim 70\%$ of RRF dopaminergic neurons being transduced on average (Figure 5.3B). Furthermore, starter neurons were found to be sufficiently restricted to the RRF in four mice (targeting efficiency; Figure 5.3C), with $\sim 83\%$ of starter neurons being within the RRF. However, there was substantial variation in the success of targeting; in two mice the targeting of the RRF was deemed to be very specific ($> 88\%$), whereas in the other two mice the specificity was high but with a relatively large number of starter neurons ($> 20\%$) located outside the RRF in the SNc and VTA (Figure 5.3C). Thus, the brain-wide monosynaptic inputs are described and compared for all four mice, but more detailed examination of input regions was carried out in the mouse with the highest proportion of starter neurons in the RRF (Figure 5.3C, mouse LAB338).

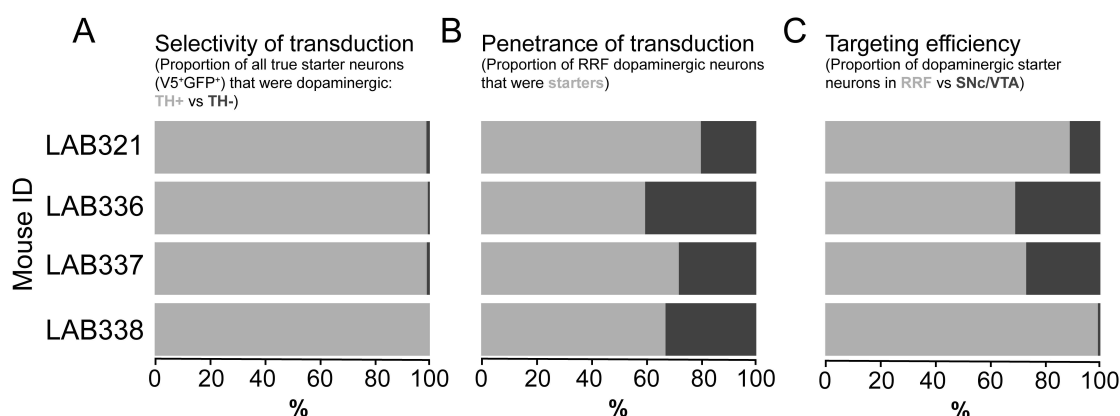


Figure 5.3: A summary of the midbrain transduction for each mouse. These results were obtained from the stereological counting data. Group average results: selectivity (A), 99.2% (0.47); penetrance (B), 69.6% (7.4); targeting efficiency (C), 82.5% (12.1).

So far, these results have met the first aim of this Chapter by demonstrating that starter cells were overwhelmingly dopaminergic and mostly within the RRF. From this, it was possible to proceed forward to fulfill the second aim and characterise the brain-wide distribution of the transsynaptically-traced monosynaptic input neurons.

5.4.2 Brain-wide monosynaptic inputs to RRF dopaminergic neurons

Serial sections from each mouse were examined for the presence of transsynaptically-labelled input neurons. It was noted that, in addition to input neurons, glial-like cells were also transduced with RV-

eGFP, mainly in the striatum (Figure 5.4). There is no evidence to suggest glial cells send long-range projections to neurons, so it seems unlikely the expression of RV-eGFP was due to transsynaptic spread from dopaminergic starters in the midbrain. Glia could instead be taking up material from neighbouring RV-eGFP⁺ neurons (e.g. via exosomes released from neurons, or glia engulfing damaged neurons). Thus, the data from these experiments are still valid because glial cells ectopically expressing RV-eGFP are easily distinguishable from transsynaptically-labelled neurons due to their small size and highly branched, diffuse processes (Figure 5.4). In future, testing for markers of astrocytes (e.g. S100 β) and microglia (e.g. Iba1) could help determine which class(es) of glia these cells belong to.

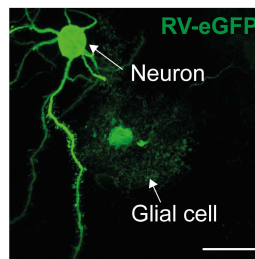


Figure 5.4: Ectopic expression of RV-eGFP in glial cells. Transsynaptically-labelled, RV-eGFP⁺ neurons (top) had clearly defined neurites emanating from a large cell body. RV-eGFP⁺ glial cells (bottom) had a diffuse ‘web’ of poorly-defined processes. This neuron and glial cell were located within the striatum. Scale bar, 20 μ m.

Qualitatively, the pattern of RV-eGFP⁺ input neuron labelling was similar in the four mice examined for this Chapter (Figure 5.5 and Table 5.2). The BNST, central amygdala (CeA) and interstitial nucleus of the posterior limb of the anterior commissure (IPAC) were noted to be densely packed with transsynaptically-labelled input neurons (Figure 5.5). The two mice in which starter neurons were particularly well restricted to the RRF (LAB321 and 338) had notably fewer striatal input neurons than the other two mice (Figure 5.5). In future, it will be crucial to quantify the number and density of input neurons (Watabe-Uchida et al., 2012) in order to make more robust and objective conclusions regarding these observations.

Input region	Mouse ID			
	LAB321	LAB336	LAB337	LAB338
PrL	-	+	-	-
LO	-	+	+	-
MO	-	+	-	-
VO	-	+	-	+
M1	+	+	+	-
M2	-	+	+	+
AI	+	+	+	+
GI	+	+	+	-
ACC	+	+	+	-
S1	+	+	+	+
S2	-	+	+	-
Piriform	-	+	-	-
DLS (rostral)	-	+	-	+
DMS (rostral)	-	-	+	+
VLS (rostral)	+	+	+	+
VMS (rostral)	-	+	+	+
NAc core (rostral)	+	+	+	+
NAc shell (rostral)	-	+	+	+
DLS (intermediate)	+	+	+	+
DMS (intermediate)	-	+	+	+
VLS (intermediate)	+	+	+	-
VMS (intermediate)	+	+	+	+
BNST	+	+	+	+
IPAC	+	+	+	+
GPe	+	+	+	+
VP	+	+	+	+
LPO	+	+	+	+
T. Str.	+	+	+	-
CeA	+	+	+	+
STN	+	+	+	+
pSTN	+	+	+	+
VTA	+	+	+	+
SNc	+	+	+	+
SNR	+	+	+	+
SC	+	+	+	+
Rt	+	+	+	+
DR	+	+	+	+
PPN	+	+	+	+
PBN	+	+	+	+
LC	+	+	+	+
LCN	+	+	+	+

Table 5.2: A comparison of areas projecting to RRF dopaminergic neurons. The presence (+) or absence (-) of RV-eGFP⁺ input neurons was recorded but no attempt was made to further qualitatively score these from low to high number/density. This table can be compared to Figure 5.5. Note, the input neurons detected within the VTA and SNc were almost exclusively non-dopaminergic. ACC, anterior cingulate cortex; AI, agranular insular cortex; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DMS, dorsomedial striatum; DLS, dorsolateral striatum; DR, dorsal raphe; GI, granular insular cortex; GPe, external globus pallidus; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LC, locus coeruleus; LCN, lateral cerebellar nucleus; LO, lateral orbital cortex; LPO, lateral preoptic area; MO, medial orbital cortex; M1, primary motor cortex; M2, secondary motor cortex; NAc, nucleus accumbens; PBN, parabrachial nucleus; PPN, pedunculopontine nucleus; PrL, prelimbic cortex; pSTN, parastubthalamic nucleus; Rt, reticular formation; SC, superior colliculus; STN, subthalamic nucleus; S1, primary sensory cortex; S2, secondary sensory cortex; T. Str., tail of the striatum; VLS, ventrolateral striatum; VMS, ventromedial striatum; VO, ventral orbital cortex; VP, ventral pallidum.

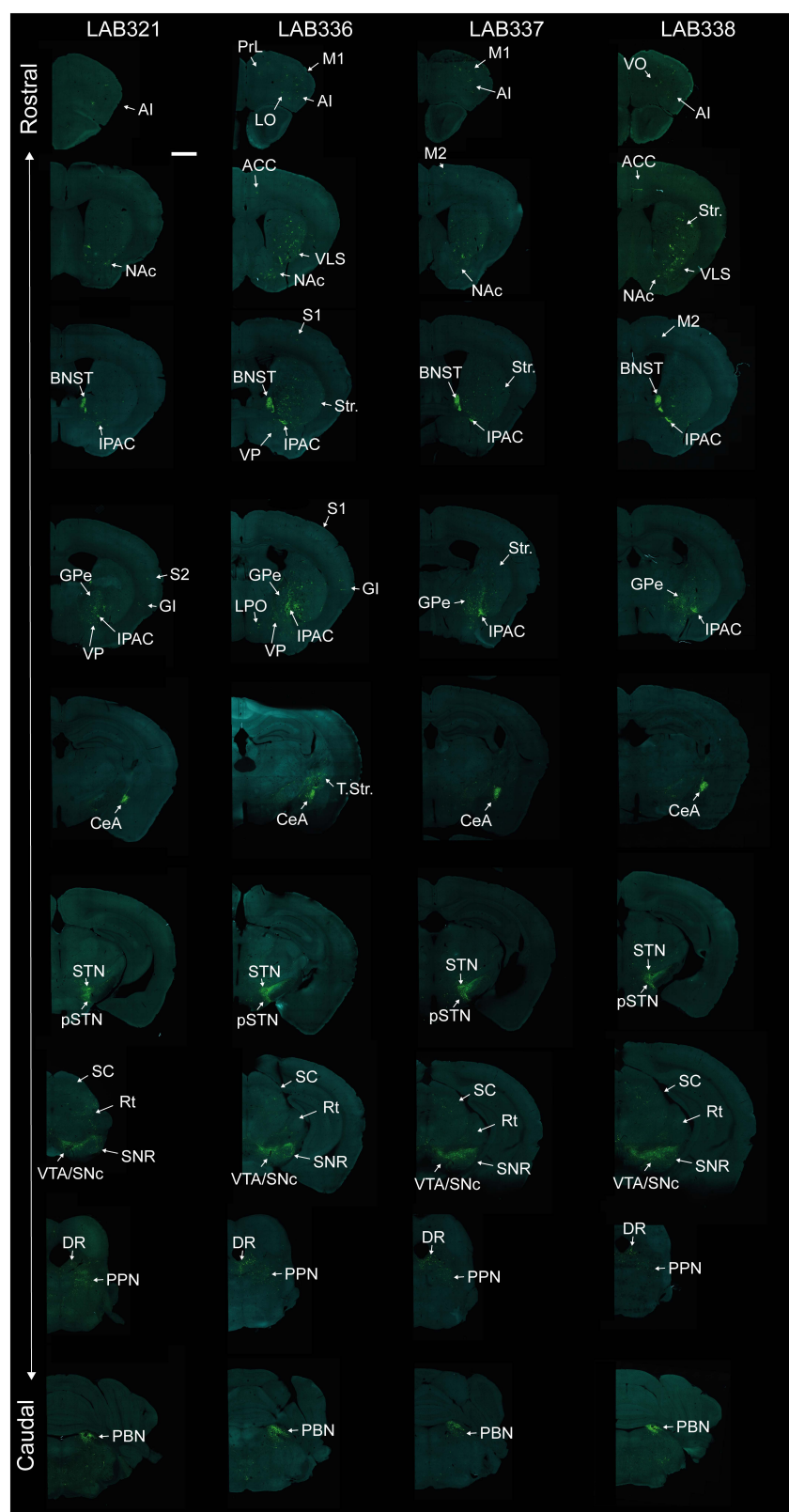


Figure 5.5: A comparison of the inputs to RRF dopaminergic neurons in four mice. Representative sections containing RV-eGFP⁺ neurons (green) were selected along the rostrocaudal axis for each mouse. Note, some green cells in these confocal micrographs are ectopically-transduced glial cells (see Figure 5.4), particularly in the striatum. For guidance, areas in which RV-eGFP⁺ neurons were observed are labelled. Note, there were no V5⁺ neurons in sections containing rostral midbrain dopaminergic neurons (third row from bottom), and thus all RV-eGFP⁺ neurons are input neurons and not starter neurons. For definitions of brain regions, see legend in Table 5.2. Scale bar, 1 mm.

5.4.3 Objectively defining the regions innervating RRF dopaminergic neurons

As mentioned above, the starter neurons in mouse LAB338 were almost exclusively restricted to the RRF. In the following Sections, the areas projecting to RRF dopaminergic neurons in this mouse will be examined. Importantly, regions containing transsynaptically-labelled input neurons were objectively delineated using a selection of molecular markers. The brain regions examined were non-exhaustive, however the main areas containing input neurons are presented.

5.4.3.1 Striatum and nucleus accumbens

SNC and VTA dopaminergic neurons receive direct inputs from many spiny projection neurons (SPNs) in the striatum (Watabe-Uchida et al., 2012; Menegas et al., 2015). In the present study, there were inputs from putative SPNs (Figure 5.6D) to RRF dopaminergic neurons (Figure 5.5 and 5.6). The striatum can be parcellated into the functionally-distinct striosomal (also known as patches) and matrix compartments (Smith et al., 2016), with low density of calbindin-1 immunoreactivity providing a marker of striosomes (mouse: Tajima and Fukuda, 2013; rat and non-human primate: Gerfen et al., 1985). Input neurons were observed in the striatum, in both its ventral (Figure 5.6A) and dorsal aspects (Figure 5.6B). Within the striatum, input neurons were located in both striosomes and the matrix (Figure 5.6Aii and Bii–Biv).

In addition to the direct inputs from the striatum, RRF dopaminergic neurons received inputs from neurons in the nucleus accumbens (NAc) (Figure 5.6Aiii). Typically, the NAc can be subdivided into the core and shell. As well as delineating the striosomal and matrix compartments of the striatum (see above), calbindin-1 immunoreactivity can aid in defining the NAc as it is densely expressed in the core and weakly expressed in the underlying shell (rat: Garas, 2015; human: Prensa et al., 2003). Therefore, it provides an objective means for differentiating these two regions. I found that this dichotomy held true in the mouse (Figure 5.6Ai) and therefore it was used to define the boundary between the core and shell. No clear difference in calbindin-1 immunoreactivity between the NAc core and dorsal striatum was observed, thus this border was drawn semi-arbitrarily, guided

by Paxinos and Franklin (2013). In the NAc, neurons providing inputs to RRF dopaminergic neurons were observed in both the core and shell (ventral and lateral; Figure 5.6Aiii). However, these input neurons were sparse and there were very few (often < 5) transsynaptically-labelled neurons per section examined. In a more caudal section, a cluster of input neurons were observed in the ventrolateral striatum (VLS) (Figure 5.6C); immunoreactivity for somatostatin in this section serves only as a background signal and does not delineate any brain regions per se. Transsynaptically-labelled input neurons located in the dorsal striatum and NAc were putative SPNs, based on their densely spiny dendrites (Inokawa et al., 2010; Figure 5.6D).

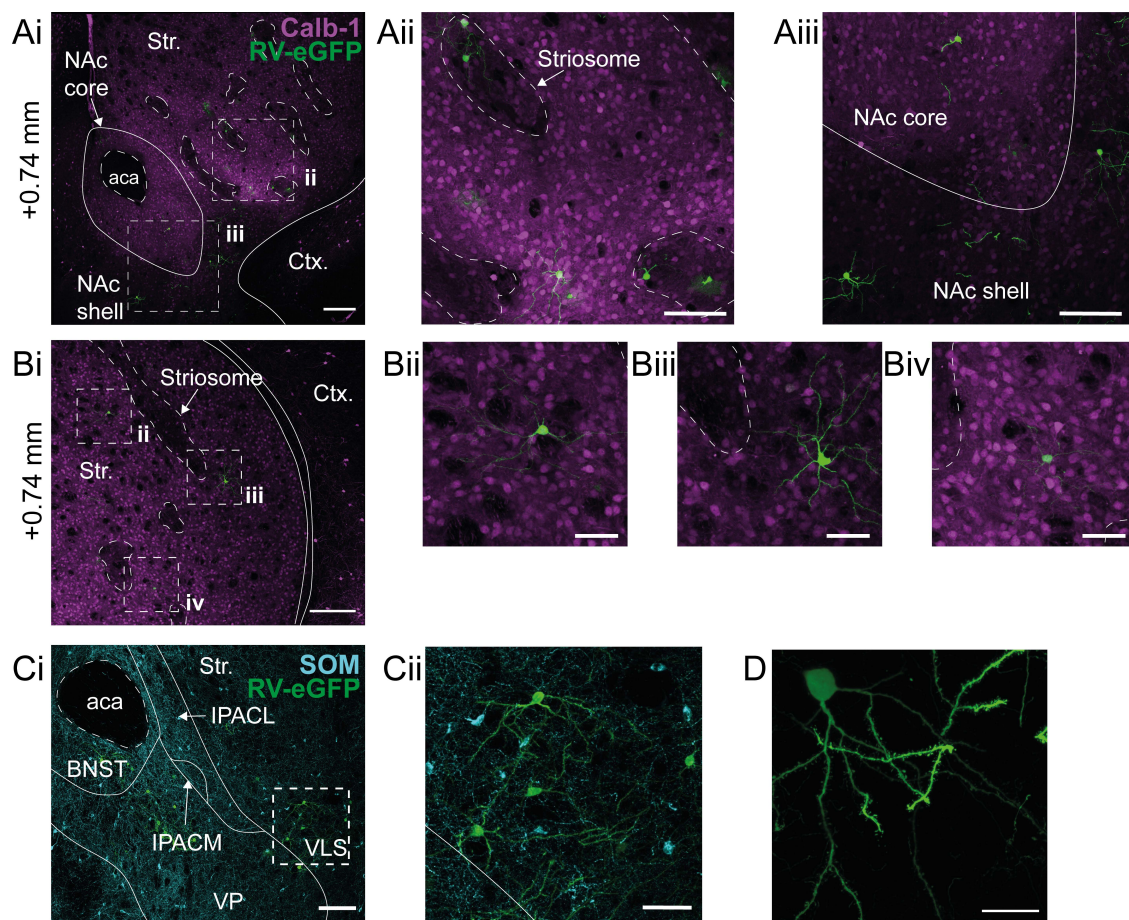


Figure 5.6: Sparse striatal and accumbal inputs to RRF dopaminergic neurons. **A–B**, Calbindin-1 (Calb-1) immunoreactivity delineated the border between nucleus accumbens (NAc) core and shell, and areas of weak calbindin-1 immunoreactivity delineated striosomes in the striatum. RV-eGFP⁺ neurons were located in both the striosome and matrix compartments of the ventral aspects of the dorsal striatum (**Aii**) and in the NAc core and shell (**Aiii**). RV-eGFP⁺ neurons were located in the matrix compartments of the dorsal striatum (**Bii–Biv**); note, there were also examples of neurons in striosomes in the dorsal striatum (not shown). Scale bars, 200 μ m (**Ai**, **Bi**), 100 μ m (**Aii**, **Aiii**) and 50 μ m (**Bii–Biv**). **C**, RV-eGFP⁺ neurons were observed in the ventrolateral striatum (VLS). In this case, somatostatin (SOM) immunoreactivity was included for perspective. Scale bars, 200 μ m (**i**) and 50 μ m (**ii**). **D**, RV-eGFP⁺ neurons in the striatum had densely spiny dendrites, indicating they were spiny projection neurons (SPNs). aca, anterior commissure, anterior part; Ctx., cortex; Str., striatum. Note, **A** and **B** are the same coronal section.

5.4.3.2 *Bed nucleus of the stria terminalis*

The BNST is part of the extended amygdala and is central to sustaining anxiety-like states (Davis et al., 2010). Here, transsynaptically-labelled neurons within the BNST were tightly clustered in the anterior lateral division of the BNST (BNST-l_d; Figure 5.7), also known as the oval nucleus (Ju and Swanson, 1989). I examined the expression of somatostatin in the BNST as it is known that the BNST-l_d is enriched for this neuropeptide (Nguyen et al., 2016; Ye and Veinante, 2019). Thus, I was able to delineate the borders of the BNST-l_d, particularly at its more caudal aspect where somatostatin immunoreactivity is dense (Figure 5.7C).

Ventral to this oval cluster, there was another smaller cluster of neurons within the posterior division of the lateral BNST (BNST-l_p). In contrast, the ventral BNST (BNST-v) was devoid of RV-eGFP⁺ neurons (Figure 5.7C). In sections containing the most rostral aspects of the BNST (forward of +0.26 mm from Bregma), very few transsynaptically-labelled neurons were observed (Figure 5.7A, B). However, it was more difficult to define this part of the BNST because its somatostatin immunoreactivity was continuous with the adjacent striatum and rostral IPAC (Figure 5.7A, B). Thus, the borders between these brain regions were drawn based on the Paxinos and Franklin (2013) atlas.

In addition to the clusters of neurons within the BNST-l_d/l_p, there were also neurons in the rostral part of the lateral IPAC (IPACL) (Figure 5.7B, C) and at a more rostral position (Figure 5.7B). Although it was challenging to delineate the IPAC at this rostral level, the fact that the cluster of RV-eGFP⁺ neurons were directly below and lateral to the anterior commissure strongly suggests these neurons are within the IPACL (Paxinos and Franklin, 2013). Overall, this shows that RRF dopaminergic neurons receive input from many neurons within the anterior part of the lateral BNST.

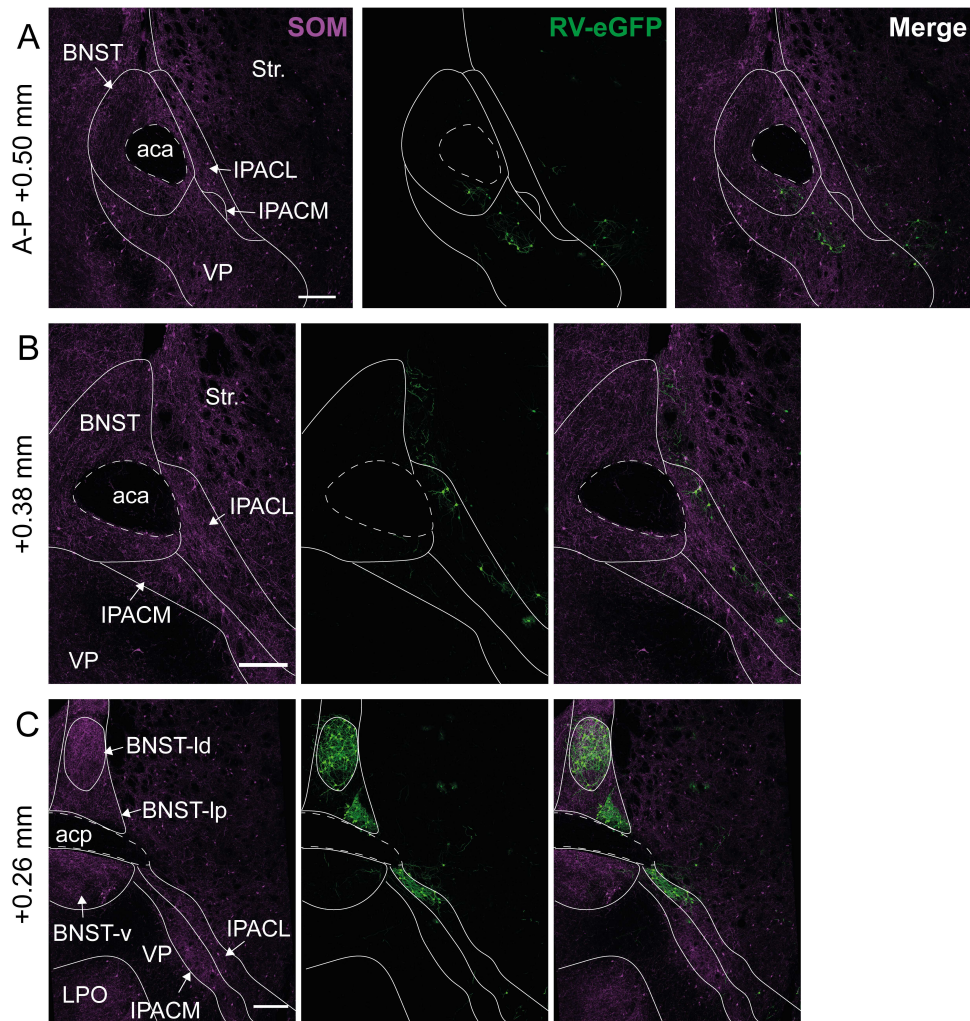


Figure 5.7: RRF dopaminergic neurons receive inputs from the lateral BNST and rostral IPACL. **A–C**, Somatostatin (SOM) immunoreactivity delineated anatomical landmarks (**A**, **B**) and the anterior lateral division of the BNST (BNST-lid; **C**). Between +0.38 and +0.50 mm from Bregma, RV-eGFP⁺ neurons were detected in the BNST, VP and ventral striatum (**A**, **B**) and in the lateral interstitial nucleus of the posterior limb of the anterior commissure (IPACL; **B**, **C**). More caudally, RV-eGFP⁺ neurons were observed in the BNST-lid and posterior part of the lateral BNST (BNST-lp), and in the underlying IPACL (**C**). Scale bars, 200 μ m. acp, anterior commissure, posterior part; BNST-v, bed nucleus of the stria terminalis, ventral division; IPACM, medial interstitial nucleus of the posterior limb of the anterior commissure; LPO, lateral preoptic area; VP, ventral pallidum.

5.4.3.3 Interstitial nucleus of the posterior limb of the anterior commissure

In Chapter 4, I showed that the IPAC receives dense innervation from RRF dopaminergic neurons.

Here, I demonstrate that many neurons in the IPAC send direct projections to RRF dopaminergic neurons (Figure 5.8), specifically the caudal aspects of the lateral IPAC (IPACL; Figure 5.8C) and the posterior IPAC (Figure 5.8D and 5.9A). Additionally, some RV-eGFP⁺ input neurons were located in the most rostral aspect of the IPACL (Figure 5.7C). As in Chapter 4, μ opioid receptor (MOR)

immunoreactivity proved useful for distinguishing the borders of the IPAC at its intermediate and caudal levels, particularly the dorsal border it shares with the striatum (Figure 5.8; Tanaka et al., 2019). The ventral border with the ventral pallidum (VP) (Figure 5.8A–C) or extended amygdala (EA) (Figure 5.8C, D) was less easy to define with MOR immunoreactivity alone, so should be considered more arbitrary. However, most labelled input neurons within the IPAC were within the dorsal area of dense MOR immunoreactivity and thus it was clear that they were within the IPAC.

In the most caudal aspect of the IPAC, transsynaptically-traced input neurons were also detected, just above the CeA (Figure 5.9A). The IPAC contains numerous Forkhead box protein P2 (FoxP2)-expressing neurons, whereas in the underlying CeA only a minority of neurons express FoxP2, which aided in defining the border between the two structures at this level. Moreover, when examining serial sections it was clear that the input neurons in the IPAC and CeA were discontinuous (Figure 5.9A) and represented two distinct pools of input neurons. Thus, I was confident in my distinction of these two adjoining regions.

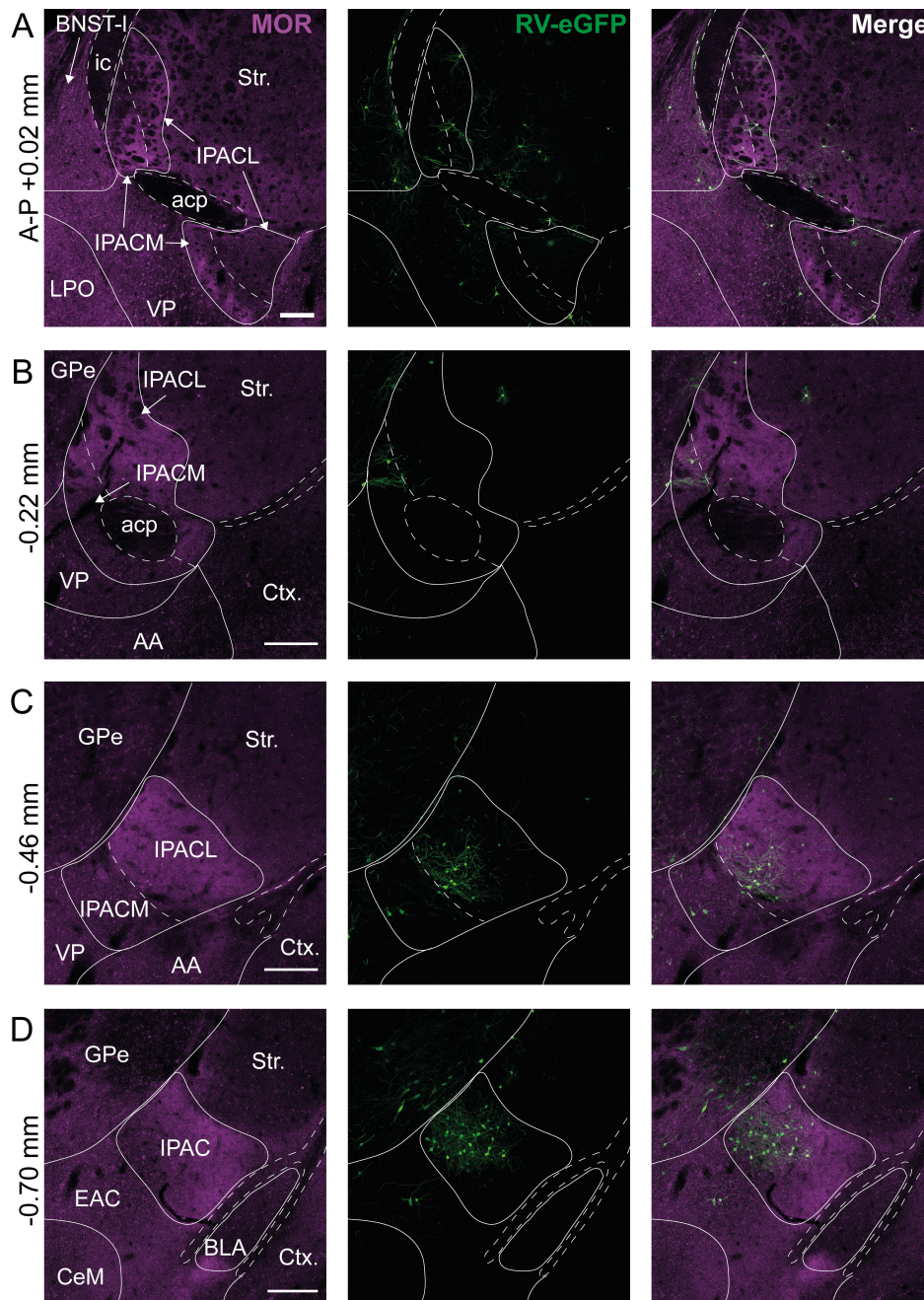


Figure 5.8: The IPAC sends direct inputs to RRF dopaminergic neurons. **A–D**, μ opioid receptor (MOR) was densely expressed in the IPAC, differentiating it from the overlying striatum. At intermediate levels (**A**, **B**), RV-eGFP⁺ neurons were sparse, both in the lateral IPAC (IPACL) and medial IPAC (IPACM). At caudal levels, many RV-eGFP⁺ neurons were within the IPACL (**C**) and the posterior IPAC (**D**). Scale bars, 200 μ m. AA, anterior amygdaloid area; A-P, anterior-posterior distance relative to Bregma; BLA, basolateral amygdala; CeM, central amygdala, medial part; EAC, extended amygdala, central part; GPe, external globus pallidus; ic, internal capsule.

5.4.3.4 Central amygdala

The amygdala is a highly complex collection of nuclei important for the processing of emotions (Janak and Tye, 2015). The CeA is involved in controlling behavioural and physiological responses to

emotionally-relevant stimuli by integrating sensory input via its connections with other nuclei of the amygdala and the brainstem/cortex (Gilpin et al., 2015). It is comprised of different subnuclei, the lateral CeA (CeL), the medial CeA (CeM) and the capsular division of the CeA (CeC) (Figure 5.9). The CeA is located between the caudal striatum and other aspects of the extended amygdala, making it a challenge to objectively define. To confidently define the boundary of the CeA, two differentially-expressed molecular markers were selected. FoxP2 is expressed by many striatal neurons, whereas it is rarely expressed by neurons in the CeA (Campbell et al., 2009). Thus, FoxP2 expression was a useful marker for delineating the dorsal border between the striatum and the CeA (Figure 5.9). Additionally, somatostatin is densely expressed in the neuropil of the CeA (McCullough et al., 2018; Bartonjo and Lundy, 2020), thus providing a further molecular marker for objectively defining this part of the amygdala (Figure 5.9). Here, it was demonstrated that the CeA sends direct projections to RRF dopaminergic neuron (Figure 5.9), specifically its lateral (Figure 5.9A–C) and capsular divisions (Figure 5.9B). The CeM contained considerably fewer input neurons (Figure 5.9A, B). Notably, the underlying extended amygdala and the adjacent basolateral amygdala (BLA) were devoid of transsynaptically-labelled neurons, suggesting these regions do not innervate RRF dopaminergic neurons.

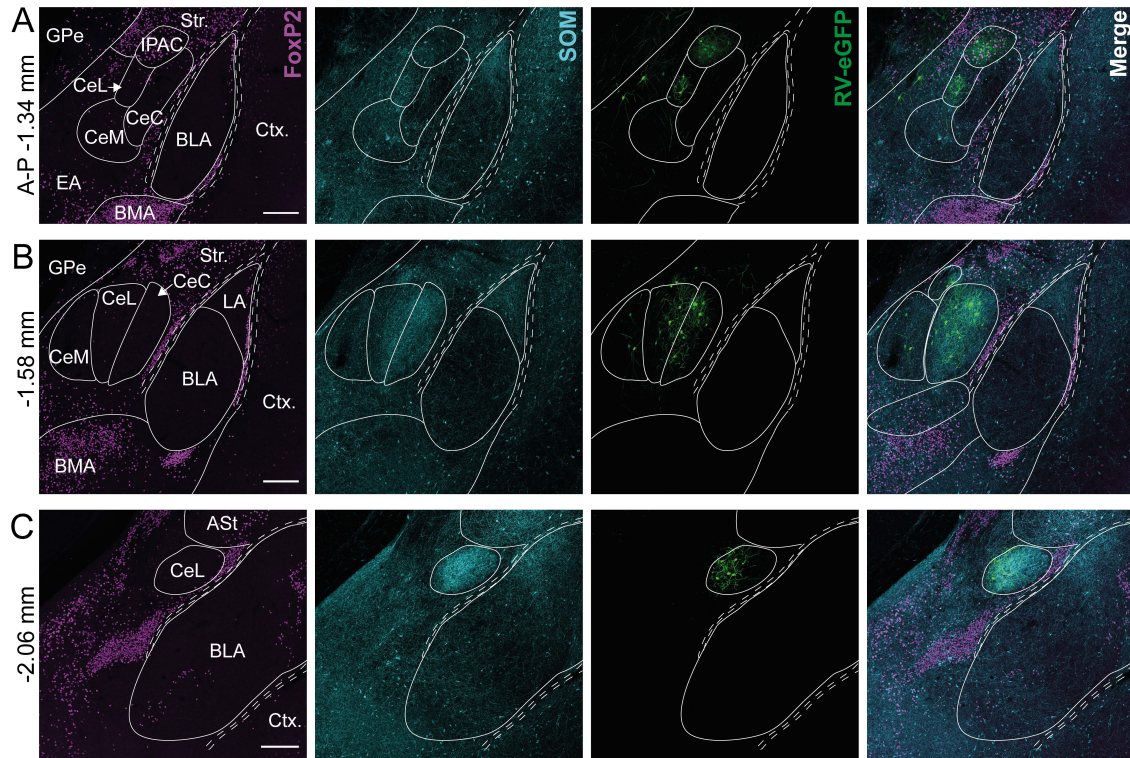


Figure 5.9: RRF dopaminergic neurons receive inputs from the CeA. **A–C**, Neurons expressing forkhead box protein P2 (FoxP2) were lacking in the central amygdala (CeA) but prevalent in the striatum, differentiating these two regions. Within the CeA at intermediate (**B**) and caudal levels (**C**), the dense expression of somatostatin (SOM) in the neuropil permitted its ventral border to be determined. RV-eGFP⁺ neurons were observed throughout the CeA, mainly in its lateral (**A–C**) and capsular divisions (**B**). Additionally, a cluster of RV-eGFP⁺ neurons were observed in the most caudal aspect of the IPAC, just above the CeA (**A**). Scale bars, 200 μ m. AST, amygdalostratial transition area; BMA, basomedial amygdala; CeC, central amygdala, capsular division; CeL, central amygdala, lateral division; CeM, central amygdala, medial division; EA, extended amygdala; LA, lateral amygdala.

5.4.3.5 Subthalamic nucleus and parsubthalamic nucleus

In addition to its ‘classical’ output to the internal globus pallidus (GPi)/substantia nigra pars reticulata (SNr) as part of the indirect pathway, the subthalamic nucleus (STN) directly projects to SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012). In the present study, I show that RRF dopaminergic neurons receive direct input from STN and parsubthalamic nucleus (pSTN) neurons (Figure 5.10). The STN consists of densely-packed neurons, many of which express the transcription factor FoxP2 (mouse: Campbell et al., 2009; rat: Abdi et al., 2015). Here, FoxP2 immunoreactivity was used to define the STN so it could be determined whether RV-eGFP-expressing neurons were within the borders of this nucleus or in adjacent brain regions (Figure 5.10). Differentiating the STN and pSTN is non-trivial, however neurons of the pSTN

extend ventrally around the tip of the cerebral peduncle (Figure 5.10). Caudally, the pSTN is larger and constitutes most of the subthalamus (approx -2.50 mm from Bregma; Figure 5.10C).

Transsynaptically-labelled input neurons were sparsely distributed in the medial aspects of the rostral STN and in the abutting pSTN (Figure 5.10A, B). In more caudal sections containing the largest aspect of the pSTN, many transsynaptically-labelled input neurons were detected (Figure 5.10C). Also, there were some direct input neurons detected in the overlying zona incerta (ZI) (Figure 5.10B, C). Overall, neurons of both the STN and pSTN directly innervate RRF dopaminergic neurons, though qualitatively it appears that more of this innervation arises from the pSTN.

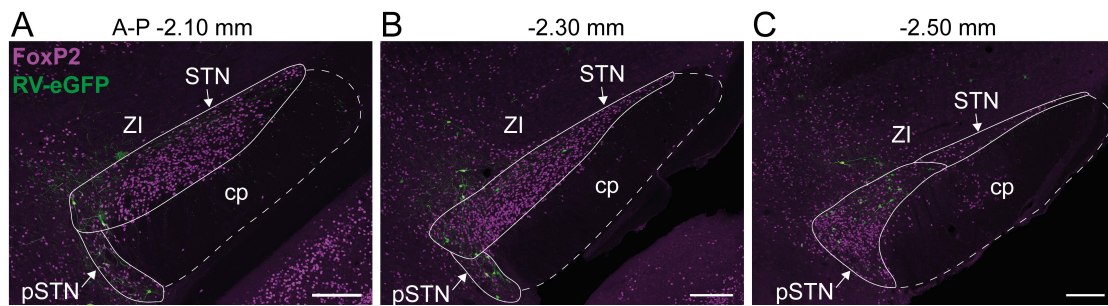


Figure 5.10: The STN and pSTN directly innervate RRF dopaminergic neurons. A–C, An oval-shaped area of densely-packed FoxP2-expressing neurons defined the borders of the STN and pSTN. RV-eGFP⁺ neurons were observed in medial aspects of the STN and in the pSTN. Scale bars, 200 μm. cp, cerebral peduncle; ZI, zona incerta.

5.4.3.6 Parabrachial nucleus and locus coeruleus

Previous work with classical retrograde tracers demonstrated that locus coeruleus (LC) noradrenergic neurons project to the RRF in the rat (Mejías-Aponte et al., 2009). Here, the use of a dopaminergic-neuron-selective retrograde tracing method provided no evidence for direct inputs from LC noradrenergic neurons to RRF dopaminergic neurons in mice (Figure 5.11). The LC was easily identifiable as the noradrenergic neurons within the structure expressed TH. The few transsynaptically-labelled neurons within the LC were observed to be immunonegative for TH, indicating they were not noradrenergic (Figure 5.11C). In the adjacent parabrachial nucleus (PBN), however, there were numerous transsynaptically-traced input neurons in both the medial PBN (PBN-m) and the lateral PBN (PBN-l) (Figure 5.11A, B). The superior cerebellar peduncle passes

through the PBN, permitting the PBN-m and PBN-l to be delineated.

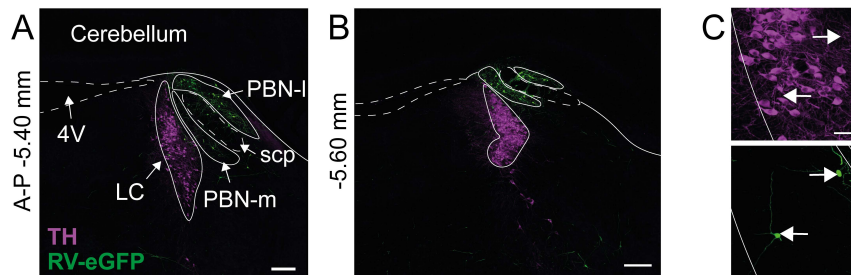


Figure 5.11: RRF dopaminergic neurons receive direct inputs from the PBN neurons but not from LC noradrenergic neurons. **A–C**, Tyrosine hydroxylase (TH) immunoreactivity defined the borders of the LC. Two serial sections (**A**, **B**) were sampled and RV-eGFP⁺ neurons were observed in the PBN in both its medial (PBN-M) and lateral aspects (PBN-l). Closer examination of the LC in **A** determined that the RV-EGFP⁺ neurons therein were not noradrenergic (white arrows; **C**). Scale bars 200 μ m (**A**, **B**) and 50 μ m (**C**). scp, superior cerebellar peduncle; 4V, fourth ventricle.

To summarise, RRF dopaminergic neurons receive inputs from a diverse range of brain regions. Although no attempt has been made to quantify input neurons in terms of absolute number or density, this close examination suggests dense inputs from the BNST, IPAC and CeA, compared with sparse inputs from the striatum and NAc. In future, it will be crucial that the brain-wide inputs to dopaminergic neurons of the RRF are rigorously quantified in order that these observations can be fully supported. Moreover, brain-wide quantification of transsynaptically-traced input neurons will allow the number and pattern to be compared to previous work examining the inputs to SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012).

5.5 Discussion

Monosynaptic tracing from SNc and VTA dopaminergic neurons has revealed that they receive distinct patterns of inputs. Regions of the basal ganglia such as the dorsal striatum, GPe, STN and SNr project densely to SNc dopaminergic neurons, whereas the NAc, dorsal raphe and lateral hypothalamus are the densest source of inputs to VTA dopaminergic neurons (Watabe-Uchida et al., 2012). Here, I used a modified rabies virus-based approach to trace the monosynaptic inputs to RRF dopaminergic neurons and qualitatively examined the pattern of brain-wide inputs. Firstly, it was demonstrated using rigorous stereological quantification that the starter neurons from which

inputs were traced were overwhelmingly dopaminergic and mostly restricted to the RRF. Dopaminergic neurons in the RRF received many inputs from the BNST, CeA and IPAC, whereas there were comparatively fewer inputs from the striatum and NAc. This low level of input from the striatum and NAc sets the RRF apart from the SNc and VTA, which receive many inputs from these areas (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015; Menegas et al., 2015).

5.5.1 Starter neurons were mostly restricted to the RRF

From the outset, it was important to ensure that viral transduction strategy was well targeted to the RRF. Significant numbers of starter neurons in the SNc and/or VTA would preclude any conclusions about the particular pattern of inputs to RRF dopaminergic neurons. The strategy developed was successful to this end, with 69–99% of starters being within the RRF of four mice. This targeting efficiency was comparable to previous work mapping the inputs to SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012). However, the variability in targeting efficiency was suboptimal, with transduction very well targeted in two mice (88 and 99% in the RRF) but comparatively less well targeted in the other two mice (69 and 74% in the RRF). Thus, to reduce variability it may be desirable to inject further mice with the aim of achieving a targeting efficiency of > 85% in 2–3 more mice.

5.5.2 Brain-wide monosynaptic inputs to RRF dopaminergic neurons

When examined in a purely binary manner (i.e. presence or not of ≥ 1 input neuron(s)), the brain regions projecting to RRF dopaminergic neurons in the present study are broadly similar to previous reports describing the input areas of the SNc and VTA (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015). As discussed above, there did seem to be very few input neuron labelled in the striatum and NAc in the present work, which is in contrast to the pattern described for SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012). In future, it will be important to quantify the number and density of inputs in order to permit more robust comparisons. The aim

of this comparison would be to outline similarities and differences between the inputs to RRF, SNc and VTA dopaminergic neurons.

However, even with quantification, comparing the results of the present study with previous work (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015) should be approached with caution. Previous work mapping the inputs to SNc and VTA dopaminergic neurons adopted an approach using two AAVs for the initial transduction of dopaminergic neurons to generate starter neurons (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015). Briefly, the TVA and RG proteins were encoded by two separate AAVs, injected simultaneously, whereas in the present study a single AAV approach was used (Ährlund-Richter et al., 2019). A recent study demonstrated that the efficiency of transsynaptic tracing when using two AAVs is variable and depends on the concentrations used (Lavin et al., 2020). Moreover, in the same study it was determined that a single AAV system (as used in the present study, but a different construct: AAV1-syn-FLEX-splitTVA-EGFP-B19G) resulted in fewer transsynaptically labelled neurons than when two AAVs were used. Thus, it seems that transsynaptic labelling of input neurons critically depends on experimental conditions. In future, it may be insufficient to simply compare to previous work in which a different virus strategy was used. Instead, a more robust approach would be to target the virus injection to the RRF, SNc or VTA in separate cohorts of mice with the single AAV approach (Ährlund-Richter et al., 2019) and then directly compare their patterns of inputs, thereby closely matching the experimental conditions.

5.5.3 Objectively defining the regions innervating RRF dopaminergic neurons

Although reference atlases provide a reliable framework for navigating brain regions in histological experiments, using objective criteria for delineating brain regions in tissue sections is necessary because adjacent brain regions are often continuous with one another (i.e. not separated by fibre tracts or ventricles). Ideally, brain regions within a tissue section would be fully aligned in all axes perpendicular to the plane in which sections are cut. However, this is often not the case and it can be

challenging to match tissue sections with those of the references atlases. With this in mind, the present study used molecular markers to delineate brain regions of interest.

5.5.3.1 *Striatum and nucleus accumbens*

Using calbindin-1 immunoreactivity, it was possible to distinguish the core and shell subdivisions of the NAc (rat: Garas, 2015; human: Prensa et al., 2003) and show that these regions directly project to RRF dopaminergic neurons. This is in line with previous work in the rat using the classical retrograde tracer FluorogoldTM injected into the RRF, which demonstrated projections from the NAc core and shell (Zahm et al., 2011). Interestingly, while I observed a very sparse distribution of transsynaptically-traced neurons in the NAc (often < 5 neurons/section), previous work noted that the innervation of the RRF from the NAc is dense (Zahm et al., 2011). In this previous report, the retrograde tracer would have been taken up by all efferents, irrespective of the cell type(s) they make synapses with in the RRF. Also, there is evidence that FluorogoldTM is taken up by fibres of passage (Dado et al., 1990), therefore potentially labelling neurons outside of the injection site. Thus, one inference is that much input from the NAc to the RRF forms synapses with non-dopaminergic neurons. Alternatively, the transsynaptic spread of the RV-eGFP may capture fewer inputs than Fluorogold, leading to an under-representation of the accumbal input.

In addition to inputs from the NAc, I observed occasional input neurons in the dorsal striatum. In the striatum, these neurons were found to be in both the striosomal and matrix compartments, suggesting there was no exclusive innervation of RRF dopaminergic neurons by either compartment. In the ventral aspects of the striatum, there were often clusters of transsynaptically-labelled neurons, demonstrating that both the dorsal and ventral striatum innervate RRF dopaminergic neurons. The sparsity of dorsal striatal input neurons is similar to previous observations with a classical retrograde tracer (Zahm et al., 2011). The inputs to RRF dopaminergic neurons from the NAc and striatum seen in the present study reflect previous work with classical anterograde tracers, which described fibre and terminal labelling with the RRF following tracer deposits in the NAc (rat: Nauta et al., 1978;

Usuda et al., 1998) and ventral striatum (non-human primate: Haber et al., 1990). The sparsity of input neurons in the striatum and NAc in the present study may reflect the fact that NAc (Xia et al., 2011) and striatal SPNs target mainly non-dopaminergic midbrain neurons (Chuhma et al., 2011). Similarly, previous work found no evidence for synaptic contacts between striatal projection neurons and RRF neurons (von Krosigk et al., 1992), a paucity which again is reflected by the sparse labelling of striatal input neurons in the present study.

What does the low number of striatal/accumbal inputs to RRF dopaminergic neurons suggest about their function? Firstly, it is important to be clear that a sparse population of input neurons does not necessarily equal a weak input. It could be that axons of projection neurons from the striatum and NAc are highly arborised within the RRF and therefore affect the function of many dopaminergic neurons (a divergent projection). If, however, the striatal and accumbal input to RRF dopaminergic neurons is weak, this has implications for the possible behavioural roles of these neurons. Previous modelling work has suggested that striatal connections to midbrain dopaminergic neurons are important for encoding the prediction error signal (Suri, 2002), which is supported by more recent anatomical (Watabe-Uchida et al., 2012), electrophysiological and behavioural data (Yang et al., 2018). Based on the observed low numbers of striatal/accumbal inputs to RRF dopaminergic neurons observed in the present study, it is interesting to speculate that these neurons may not be as centrally embedded in the circuitry of reward prediction/motivated behaviours as their counterparts in the SNc and VTA.

5.5.3.2 Bed nucleus of the stria terminalis, central amygdala and interstitial nucleus of the posterior limb of the anterior commissure

In the present study, three structures that stood out in terms of the density of input neurons were the BNST, specifically its dorsal and lateral divisions, the CeA, and the IPAC. The anterior lateral BNST was easily identifiable as it is separated from the adjacent striatum by the internal capsule and has dense somatostatin immunoreactivity, wherein most transsynaptically-labelled neurons were

located. The CeA was delineated based on a paucity of FoxP2-expressing neurons compared with the overlying striatum (Campbell et al., 2009) and dense immunoreactivity for somatostatin (McCullough et al., 2018; Bartonjo and Lundy, 2020). Thus, I was confident in my delineation of these two nuclei of the amygdala. The density of input neurons in these two structures is in keeping with previous work in the rat and mouse using classical retrograde and anterograde tracers (Zahm et al., 2011; Dabrowska et al., 2016; Bruzsik et al., 2021). For the first time, my study demonstrates that this BNST/CeA input to the RRF targets dopaminergic neurons. In contrast to the dense innervation of RRF dopaminergic neurons by the CeA and dorsolateral BNST, structures of the medial amygdala (medial BNST, basomedial amygdala and medial extended amygdala) contained no input neurons. Again, this is in keeping with previous reports with classical tracers in the rat which detected no fibre labelling in the RRF following anterograde tracer injections in the structures of the medial amygdala (Canteras et al., 1995).

Rostral of the CeA is the IPAC, which is a structure considered to be part of the extended amygdala (Alheid et al., 1999) but which shares neurochemical and connectivity features of the striatum (rat: Shammah-Lagnado et al., 2001). Thus, it can be conceptualised as a transition area between the striatum and extended amygdala. As demonstrated by others (Tanaka et al., 2019) and in Chapter 4, dense immunoreactivity for MOR delineates the dorsal border of the IPAC. Based on this, the present study demonstrated a dense projection from the IPAC to RRF dopaminergic neurons, particularly from the IPACL. The present work confirms that the IPAC innervates the RRF (rat: Shammah-Lagnado et al., 1999; Shammah-Lagnado et al., 2001; Zahm et al., 2011) and demonstrates for the first time this innervation directly targets dopaminergic neurons.

If future quantification experiments confirm the qualitative observation that the BNST and CeA are indeed amongst the densest source of inputs to RRF dopaminergic neurons, it is interesting to consider the functional significance of this circuitry. A very simplified conceptualisation of the BNST and CeA considers them to be key nodes in translating the neural processing of emotional stimuli and states into behavioural output (Janak and Tye, 2015). The data in the present study

demonstrate that RRF dopaminergic neurons are downstream of these nuclei and are thus positioned to facilitate behavioural output related to emotional processing. Relevant to this, it was recently demonstrated that neurons in the RRF (putatively identified) of the rat play a role in representing aversive outcomes and threat (Moaddab and McDannald, 2021). My anatomical data may help guide future studies investigating the behavioural significance of RRF dopaminergic neurons and their inputs from the BNST and CeA.

Much less is known about the role of the IPAC in behaviour, though a recent study suggested the activity of neurons in this region is related to the processing of innate and learned disgust in response to aversive tastants (Tanaka et al., 2019). In future, it would be interesting to further explore the behavioural role of this brain region and its connections with RRF dopaminergic neurons.

5.5.3.3 *Subthalamic nucleus and paraventricular nucleus*

A particularly novel finding of the present work was a direct projection from the STN to RRF dopaminergic neurons. Anterograde tracing experiments in rats did not describe axonal projections from the STN to the RRF (Ricardo, 1980; Kita and Kitai, 1987; Groenewegen and Berendse, 1990), suggesting these two areas are not connected. Although one previous report in the rat noted fibres running through the RRF, it was concluded these were not sites of termination due to their smooth appearance (Groenewegen and Berendse, 1990). Here, it is clearly demonstrated that RRF dopaminergic neurons are indeed innervated by STN neurons. The disconnect between the results of the present study and previous work with classical tracers is reminiscent of another study in which a rabies virus-based approach demonstrated that the STN directly innervates SNc and VTA dopaminergic neurons (Watabe-Uchida et al., 2012). In this study, the many STN neurons innervated SNc and VTA dopaminergic neurons, despite previous literature suggesting very little innervation (Kita and Kitai, 1987; Groenewegen and Berendse, 1990).

It is difficult to reconcile why the results of the present study are not in keeping with previous work in the rat (Ricardo, 1980; Kita and Kitai, 1987; Groenewegen and Berendse, 1990), however

one consideration is the definition of the STN. I detected transsynaptically-labelled neurons specifically in the medial STN. In previous work with classical anterograde tracers, the tracer deposit was often targeted to the lateral part of the STN (Ricardo, 1980; Kita and Kitai, 1987; Groenewegen and Berendse, 1990), meaning that the projections of the medial STN were not mapped. Moving to the pSTN, one previous study in the rat described anterograde projections and putative termination sites within the RRF (Goto and Swanson, 2004), which aligns with the data shown in the present study. Moreover, retrograde tracing from the RRF labels input neurons in the pSTN in the rat (Zahm et al., 2011). Here, I confirm that this projection from the medial STN and pSTN forms a direct connection to RRF dopaminergic neurons.

5.5.3.4 *Parabrachial nucleus and locus coeruleus*

The PBN is part of the rostral hindbrain, situated between the midbrain and the pons. It is a complex nucleus that receives sensory input and relays it to the forebrain (reviewed in: Palmiter, 2018). In the rat, some PBN neurons process nociceptive information (Gauriau and Bernard, 2002) and relay this to SNc dopaminergic neurons (Coizet et al., 2010). In addition to innervating the SNc, both the medial and lateral PBN project to the RRF (Saper and Loewy, 1980; Zahm et al., 2011). Here, I show that, as is the case for dopaminergic neurons of the SNc and VTA (Watabe-Uchida et al., 2012), dopaminergic neurons of the RRF receive direct inputs from the PBN. In light of previous work demonstrating that the PBN relays nociceptive information to SNc dopaminergic neurons, causing them to pause (Coizet et al., 2010), it is interesting to speculate that perhaps the same holds true for its connection with RRF neurons. Like their counterparts in SNc, RRF dopaminergic neurons pause in response to foot shocks (Chapter 6). However, future functional studies are required to evidence this.

Noradrenergic neurons project to the VTA in the rat (Phillipson, 1979) and in most cases form non-synaptic appositions in the vicinity of dopaminergic dendrites, with only a minority (< 10%) of contacts being synaptic in nature (Liprando et al., 2004). In the rat, retrograde tracing studies have also determined that the RRF receives noradrenergic innervation, mainly from the A1, A2 and LC

nuclei (Mejías-Aponte et al., 2009). In this previous study, it was noted that retrograde tracer injections targeted to the RRF labelled significantly more neurons in noradrenergic regions than injections targeted to the SNc or VTA. However, it is unclear whether this innervation targets dopaminergic or non-dopaminergic neurons in the RRF. SNc and VTA dopaminergic neurons receive direct inputs from the LC (Watabe-Uchida et al., 2012) but it is unclear whether these are noradrenergic neurons. Thus, the nature of the LC-midbrain direct connection is unknown. The LC is adjacent to the PBN and is easily distinguished as a dense column of TH-expressing somata, which allowed me to delineate this nucleus in the present study. I observed that RRF dopaminergic neurons receive little direct input from the LC, as only a small number of input neurons were detected. Moreover, the small input that was detected was not from TH neurons in the LC, suggesting the LC input to RRF dopaminergic neurons is not noradrenergic. However, other noradrenergic cell groups, such as A1 and A2, were not investigated, and thus these other cell groups may provide direct noradrenergic inputs to the RRF dopaminergic neurons.

This lack of noradrenergic input to the RRF from the LC would suggest that the input to the RRF detected in previous work (Mejías-Aponte et al., 2009) represented innervation of non-dopaminergic neurons. In future, it will be important to characterise the direct inputs to non-dopaminergic neurons in the RRF, principally GABAergic neurons (rat: Nair-Roberts et al., 2008) and glutamatergic neurons (rat: Yamaguchi et al., 2013). It is important to also consider the possibility that the lack of noradrenergic neurons labelled could be due to a methodological issue. In short, it is possible that the non-synaptic nature of LC innervation of dopaminergic neurons (Liprando et al., 2004) precludes the transsynaptic spread of the RV-eGFP from the starter cells to the direct input neurons. This idea has been postulated to explain why, in some studies, few dopaminergic neurons are transsynaptically labelled following input tracing from striatal (Wall et al., 2013), accumbal neurons (Li et al., 2018) and amygdala neurons (Fu et al., 2012), despite the well documented dopaminergic innervation of these regions (Björklund and Dunnett, 2007). However, there is no direct evidence for this explanation. Indeed, the mechanism of transsynaptic

spread of the rabies virus is not understood in detail. It is highly important that these unknowns are investigated in future, because if the transsynaptic spread of the rabies virus is selective for certain types of innervation, then this has implications for data interpretation.

5.5.4 Conclusions and future direction

This study outlines the brain-wide monosynaptic inputs to RRF dopaminergic neurons for the first time. One of the main findings of this study is that RRF dopaminergic neurons receive comparatively fewer inputs from the striatum and NAc than SNc and VTA neurons (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015; Menegas et al., 2015). The densest cluster of input neurons observed in the present study were in the BNST, CeA and IPAC, which is in line with previous observations in the rat with a classical retrograde tracer (Zahm et al., 2011).

When compared to the anterograde tracing results in Chapter 4, some interesting relationships emerge. The results of Chapter 4 and the present Chapter show that RRF dopaminergic neurons do not project to the BNST or CeA but do receive direct inputs from these areas. This suggests the connectivity between RRF neurons and the extended amygdala is not reciprocal. On the other hand, the dense projection from RRF dopaminergic neurons to the IPAC is reciprocated by direct inputs. In future, the reciprocity, or lack thereof, of these connections can be studied in more detail using input-output circuit mapping techniques (Beier et al., 2015).

It is key to underline that in future it will be crucial that the qualitative observations made in the present study are supported by quantification. There are two main aims of this next stage. Firstly, it is important to normalise the number of input neurons to the number of starter cells, in order that the pattern of inputs can be compared across subjects. Secondly, quantification will determine the relative contribution of each input area, allowing the number and density of inputs from different brain regions to be directly compared.

Chapter 6

Electrophysiological properties of RRF neurons

6.1 Introduction

In vivo during anaesthesia, SNc (Brown et al., 2009; Farassat et al., 2019) and VTA (Floresco et al., 2001; Ungless et al., 2004; Brischoux et al., 2009) dopaminergic neurons tonically discharge at 1–10 spikes/s in the mouse/rat in three firing modes: irregular, regular and bursting. From early work in the rat, it was observed that most dopaminergic neurons fire in an irregular pattern (Tepper et al., 1995), a proportion of which display a propensity to fire some of their action potentials in bursts of 3–8 spikes (Grace and Bunney, 1983, 1984b). A smaller population of dopaminergic neurons fire in a regular, pacemaker pattern (Paladini and Tepper, 1999). Intrinsic properties (Grace and Onn, 1989) and afferent inputs (Chergui et al., 1994; Floresco et al., 2003; Lodge and Grace, 2006) determine the firing properties of midbrain dopaminergic neurons in the rat, dictating how they engage during ongoing behaviour.

There is a paucity of information regarding the spontaneous firing properties of RRF dopaminergic neurons *in vivo*, and thus one aim of this Chapter was to begin addressing this gap. It is of interest to consider RRF dopaminergic neurons as a distinct cell group because previous

research shows that the location of dopaminergic neurons within the midbrain is a determinant of their firing properties. For example, VTA dopaminergic neurons fire more of their action potentials in bursts than SNc neurons in the rat (Grenhoff et al., 1988; Marinelli and White, 2000). Moreover, dopaminergic neurons in the medial VTA that project to the prefrontal cortex fire at a higher rate and with more bursts than other dopaminergic neuron subpopulations in the mouse (Lammel et al., 2008) and rat (Chiodo et al., 1984); within the SNc, lateral dopaminergic neurons more often fire in bursts than their medial counterparts (Farassat et al., 2019). Recordings made putatively in the RRF of behaving monkeys indicate that the firing properties of this group of dopaminergic neurons are within the ranges seen for SNc and VTA dopaminergic neurons (Fiorillo et al., 2013b). To date, however, the *in vivo* firing properties of *identified* RRF dopaminergic neurons have not been characterised.

In addition to heterogeneous spontaneous firing properties, the firing dynamics of dopaminergic neurons in response to salient stimuli vary between subpopulations. In the anaesthetised rat, some have reported that the majority of identified SNc dopaminergic neurons do not respond to electrical stimulation of the hindpaw (putatively aversive stimulus), and those that do respond are suppressed (Brown et al., 2009). Others have reported that the same stimulus suppresses the firing of most presumed dopaminergic neurons in the SNc of the rat (Coizet et al., 2006, 2010). The majority of identified VTA dopaminergic neurons change their firing rate in response to noxious stimuli in a manner that is location dependent in the anaesthetised rat (Ungless et al., 2004; Brischoux et al., 2009). Noxious stimuli suppress the firing rate of some identified lateral VTA dopaminergic neurons (Ungless et al., 2004; Brischoux et al., 2009), whereas some ventral neurons are activated (Brischoux et al., 2009). This functional distinction in the anaesthetised preparation is also evident during an aversive conditioning task in awake behaving mice (de Jong et al., 2019), in which the axon terminal activity of lateral VTA dopaminergic neurons is suppressed by aversive stimuli, whereas axon terminal activity of their ventral counterparts is increased. An important and hitherto unanswered research question is whether and how identified RRF dopaminergic neurons

respond to aversive stimuli.

6.2 Aims

- To characterise the spontaneous *in vivo* firing properties of RRF dopaminergic neurons during cortical slow-wave activity (SWA).
- To characterise whether and how RRF dopaminergic neurons respond to noxious electrical stimulation of the hindpaw.

6.3 Methods

Adult C57Bl/6J and DAT-Cre mice were used for experiments described in this results Chapter. DAT-Cre mice were stereotactically-injected with either AAV2-Ef1a-DIO-hChR2(E123T/T159C)-eYFP or AAV2-CAG-Flex-ArchT-GFP (UNC Vector Core) ~4 weeks before recording (see Materials and Methods Section 2.3 for more details). All electrophysiological recordings were made during urethane anaesthesia (see Materials and Methods Section 2.5 for more details). In some experiments with opsin-injected mice, the spontaneous firing properties, response to noxious electrical stimulation of the hindpaw and response to optogenetic manipulation were assessed in the same dopaminergic neurons. This Chapter considers the spontaneous firing properties and the response to noxious stimulation; the optogenetic manipulation of midbrain dopamine neurons is considered in Chapter 7. For recordings of spontaneous firing properties, 14 male mice (11 C57Bl/6J and 3 opsin-injected DAT-Cre) and 7 female mice (all opsin-injected DAT-Cre) were used, aged 2–4 months. For recordings of firing properties in response to a noxious stimulus, 13 male (9 C57Bl/6J and 4 opsin-injected DAT-Cre) and 8 female (all opsin-injected DAT-Cre) mice were used, aged 2–5 months. These two sets of mice are not mutually exclusive, as some recordings of spontaneous firing properties and firing properties in response to a noxious stimulus were made in the same mice.

All analyses in this Chapter were carried out on data from neurons that were juxtacellularly labelled during recording and subsequently histochemically identified as either midbrain dopaminergic (TH positive), or non-dopaminergic (TH negative). The coronal schematics with locations of recorded and labelled neurons were generated in Adobe Illustrator (version 23.1), guided by Paxinos and Franklin (2013). All data expressed as mean (standard deviation (SD)) or mean $\pm$ standard error of the mean (SEM) where appropriate for normally distributed data, and median (Q1–Q3) for non-normally distributed data, where Q1–Q3 is the interquartile range.

6.4 Results

6.4.1 *Spontaneous firing properties of identified dopaminergic neurons are similar in the RRF, SNc and VTA*

SNc and VTA dopaminergic neurons exhibit both tonic and burst firing modes *in vivo* during anaesthesia (rat: Grace and Bunney, 1980; Grace and Bunney, 1983; Ungless et al., 2004; Brown et al., 2009), yet there is not a comparable description of these properties for RRF neurons. I aimed to record the single-unit spiking activity of RRF dopaminergic neurons (Figure 6.1A) and label them using the juxtacellular method (Pinault, 1996) to permit *post-hoc* neurochemical identification (Figure 6.1A, right) and anatomical localisation (Figure 6.1B). The majority of recorded and juxtacellularly-identified dopaminergic neurons were in the RRF (n = 20 neurons from 14 mice; anterior-posterior distance from Bregma (A-P) range: -3.80 to -4.20 mm). Additionally, some were located in the SNc (n = 8 neurons from 8 mice; A-P range: -3.15 to -3.90 mm) and VTA (n = 3 neurons from 2 mice; A-P range: -3.20 to -3.60 mm) cell groups (Figure 6.1B), providing useful comparators for describing the spontaneous firing properties of RRF dopaminergic neurons.

Previous research shows that brain state fluctuations between SWA and activated can impact on the firing rate and burst activity of SNc and VTA dopaminergic neurons in the rat (Walczak and Błasiak, 2017). Therefore, I chose to analyse the firing activity of dopaminergic neurons during

robust cortical SWA only; there were not enough recordings of dopaminergic neurons during activated brain state to allow for meaningful analyses during this brain state. In the mouse, it is difficult to sustain activated brain state whilst maintaining sufficient depth of anaesthesia. The firing rate of identified dopaminergic neurons was typically between 2 and 10 spikes/s, and the biphasic action potential duration > 0.9 ms (example dopaminergic unit in Figure 6.1A).

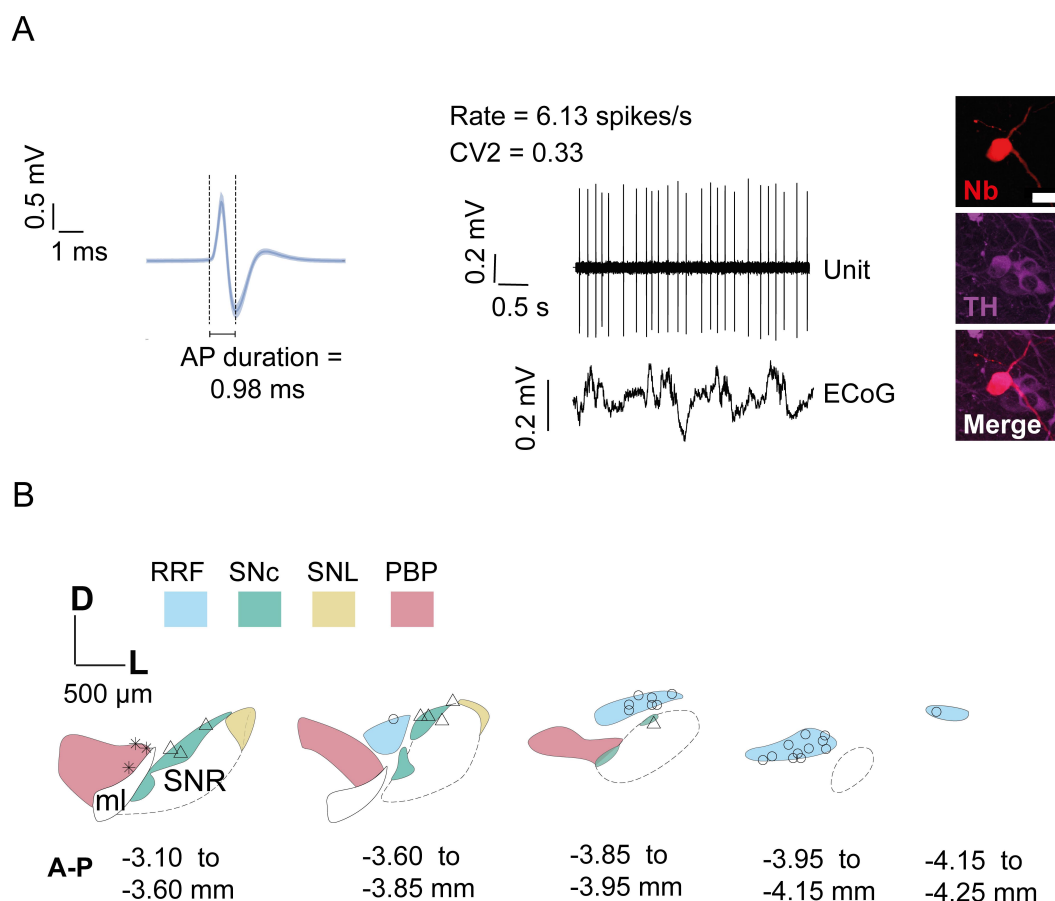


Figure 6.1: Spontaneous *in vivo* spiking activity of identified midbrain dopaminergic neurons in urethane-anaesthetised mice during robust cortical slow-wave activity (SWA). **A**, Example single-unit average action potential (AP) waveform (*left*) and spiking activity (*middle*) of a dopaminergic neuron during ECoG-verified SWA (*middle*, bottom panel). The single unit was juxtacellularly labelled with Neurobiotin, revealed using posthoc histochemistry and confocal microscopy (*right*, top panel). Immunoreactivity for tyrosine hydroxylase (TH; *right*, middle panel) identified the recorded and labelled neuron as dopaminergic (*right*, bottom panel). Scale bar, 20 μ m. CV2, a measure of the coefficient of variation. **B**, The location of identified dopaminergic neurons (i.e. TH positive) in the midbrain was mapped. Coronal midbrain schematics show the approximate location of neurobiotin (Nb)-labelled dopaminergic neurons. Location key for labelled neurons: circles, RRF; triangles, SNc; asterisks, VTA. ml, medial lemniscus; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticulata.

As a population, identified dopaminergic neurons in the RRF had firing properties typical of SNc and VTA dopaminergic neurons, in that their mean firing rate and biphasic action potential duration was $\sim$ 6 spikes/s and $\sim$ 1.2 ms, respectively (Figure 6.2A, B) (Ungless and Grace, 2012).

However, some identified dopaminergic RRF neurons had properties that fell outside of these putative bounds: 2/20 neurons had firing rates of > 10 spikes/s, and 3/20 neurons had biphasic action potential durations of < 1.1 ms (Figure 6.2A, B). Neither the mean firing rate nor biphasic action potential duration of identified dopaminergic neurons in the RRF differed significantly from SNc or VTA neurons (Figure 6.2A, B). The lack of statistical difference between groups should be interpreted cautiously as the power of the analysis was lower (0.05) than desired (0.8) due to a low number of neurons in the VTA group.

To determine the firing dynamics of the spiking data for each identified dopaminergic neuron, I used the CV2 measure (Holt et al., 1996) to assess firing regularity and the robust gaussian surprise (RGS) method (Ko et al., 2012) to assess the burst propensity. CV2 was chosen as opposed to CV because it is less sensitive to the slow variations in firing rate seen *in vivo*. Similarly, the RGS method for burst detection was chosen as it is less sensitive to differences in background firing rate. Some RRF dopaminergic neurons fired regularly (low CV2) and others in a less regular (high CV2) pattern (Figure 6.2C). Similarly, there was a range of burst firing propensities: some neurons fired no action potentials in bursts (4/20 neurons), others fired up to $\sim 25\%$ of their action potentials in bursts (Figure 6.2D). On average, identified RRF dopaminergic neurons had similar firing variability and burst propensity to SNc and VTA dopaminergic neurons (Figure 6.2D, E). For the dopaminergic RRF neurons that did fire some of their action potentials in bursts, there was considerable variation in the average intra-burst firing frequency for each identified neuron, but this was not significantly different from SNc or VTA neurons (Figure 6.2E). Again, the lack of statistical difference between groups should be interpreted cautiously as the power of the analysis was lower than desired (low number of neurons in the VTA group).

Although the firing properties of circumscribed midbrain dopaminergic neuron cell groups were similar, I conducted analyses to determine whether there was any correlation between neuron location (both in the A-P and medial-lateral (M-L) axes) and firing properties (Figure 6.3). This correlation analysis was to determine whether anatomical location, independent of cell group, was

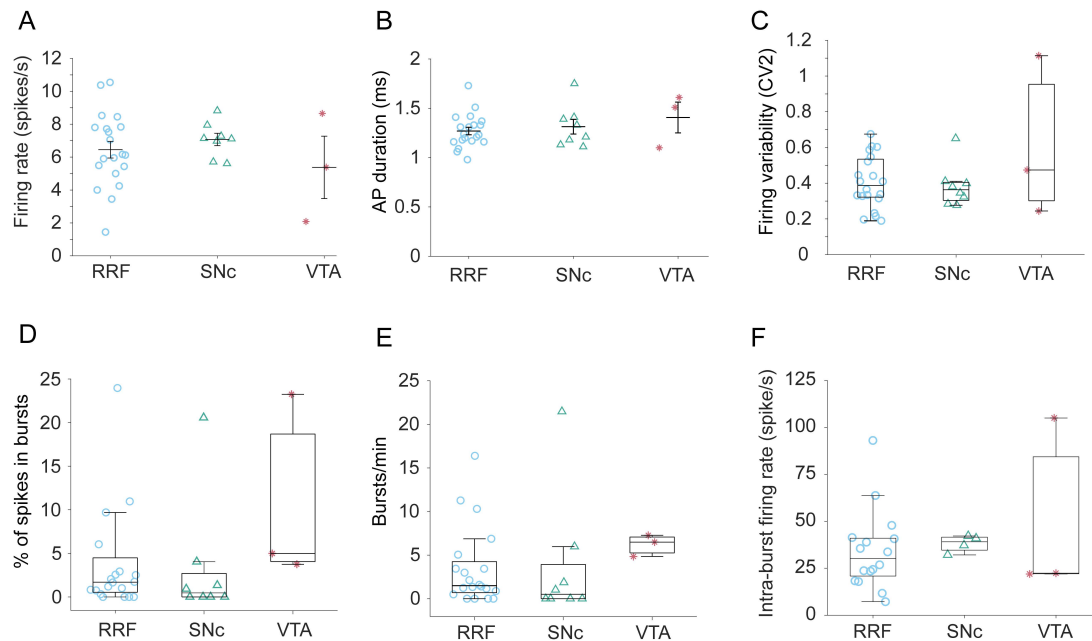


Figure 6.2: The spontaneous firing properties of identified RRF ($n = 20$), SNc ($n = 8$) and VTA ($n = 3$) dopaminergic neurons are similar. **A**, Firing rate (RRF: 6.45 ± 0.51 spikes/s; SNc: 7.07 ± 0.38 spikes/s; VTA: 5.38 ± 1.90 spikes/s; One-way ANOVA: no effect, $p = 0.50$). **B**, Action potential (action potential (AP)) duration (RRF: 1.27 ± 0.04 ms; SNc: 1.31 ± 0.07 ms; VTA: 1.41 ± 0.16 ms; One-way ANOVA: no effect, $p = 0.49$). **C**, Firing variability (RRF: 0.39 (0.32–0.54); SNc: 0.36 (0.28–0.41); VTA: 0.47 (0.24–1.11); Kruskal-Wallis on ranks: no effect, $p = 0.73$). **D**, Percentage of spikes in bursts (RRF: 1.69 (0.43–5.26); SNc: 0.46 (0.00–3.37); VTA: 4.99 (3.75–23.26); Kruskal-Wallis on ranks: no effect, $p = 0.10$). **E**, Bursts per minute (RRF: 1.50 (0.59–4.65); SNc: 0.51 (0.00–4.95); VTA: 6.49 (4.84–7.27); Kruskal-Wallis on ranks: no effect, $p = 0.14$). **F**, Intra-burst firing rate (RRF: 30.30 (19.60–41.21); SNc: 39.07 (33.39–41.89); VTA: 22.40 (21.98–104.98); Kruskal-Wallis on ranks: no effect, $p = 0.57$). Data presented as mean $\pm$ SEM or median (interquartile range).

related to firing properties. This was done in case binary statistical analyses may miss subtle gradients across the dopaminergic midbrain. In most cases, there was no relationship between the firing properties of midbrain dopaminergic neurons and their A-P or M-L location, except in the case of firing variability (CV2) vs A-P location. For this relationship, more rostral neurons tended to have a higher firing variability (Figure 6.3C). Parameters of burst firing were not statistically related to anatomical location (Figure 6.3E–J), suggesting that the significant relationship between firing variability and A-P location was not driven by burst firing.

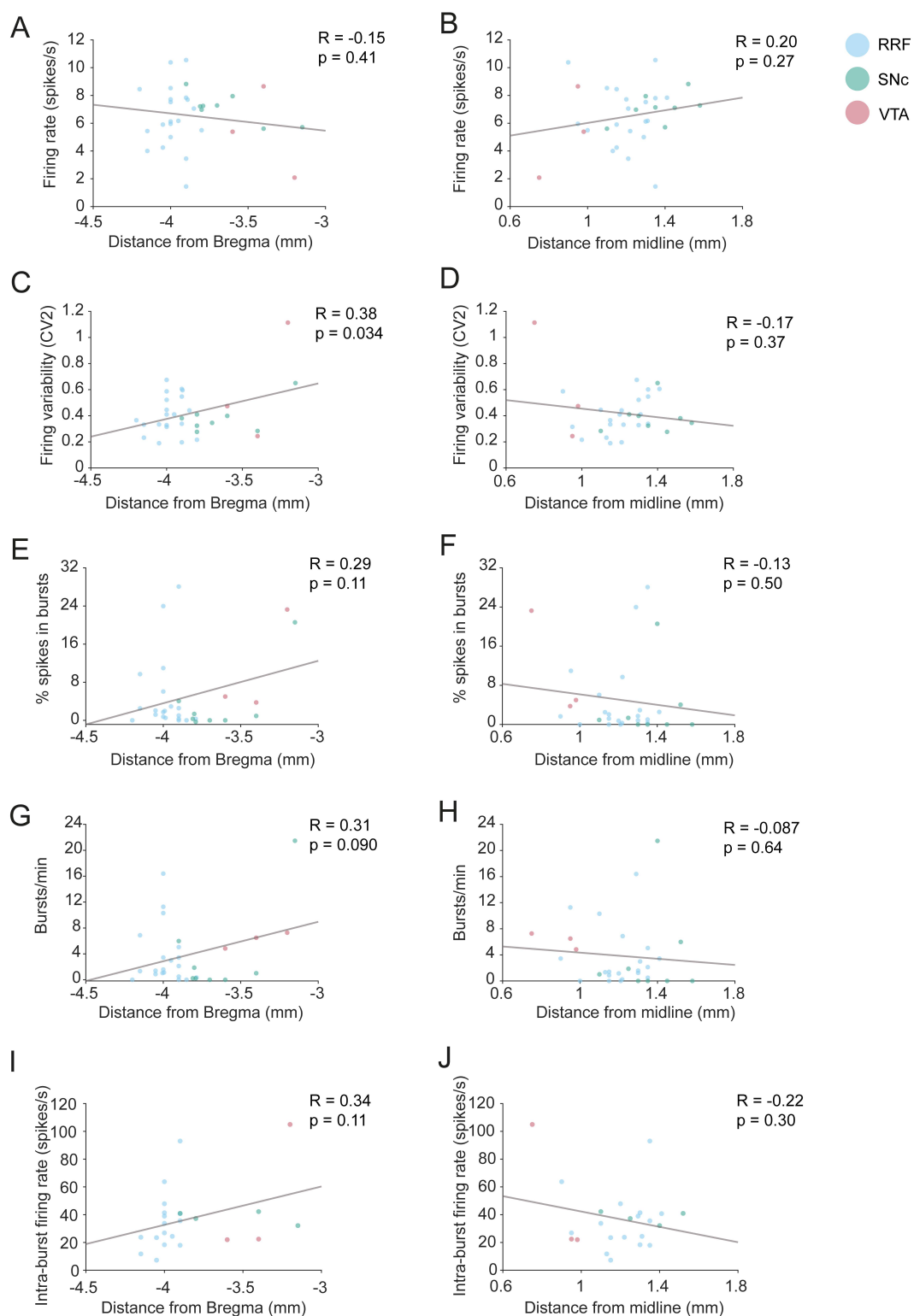


Figure 6.3: The relationship between the spontaneous firing properties of midbrain dopaminergic neurons and their anatomical location. **A, B**, Firing rate was not correlated to A-P (**A**) or (**B**) location. **C, D**, Firing variability and anatomical location are positively correlated in the A-P (**C**) but not M-L (**D**) axis. **E-J**, Burst firing parameters are not correlated to A-P (**E, G, I**) or M-L (**F, H, J**) location. Insets show Pearson's correlation coefficient (R) and its significance value (p). The grey line through the data represents a linear fit of the data.

6.4.2 Most RRF and SNc dopaminergic neurons display suppression of firing followed by rebound activation in response to electrical stimulation of the hindpaw

Noxious stimuli are represented by a phasic decrease in the firing rate of some SNc dopaminergic neurons in the rat (Coizet et al., 2006; Brown et al., 2009; Coizet et al., 2010), whereas the effect on VTA dopaminergic neurons is more heterogeneous, with the firing rate of some neurons increasing (Brischoux et al., 2009) and others decreasing (Ungless et al., 2004; Brischoux et al., 2009). Because the effect of noxious stimuli on RRF dopaminergic neuron firing has not been described, I recorded the single-unit spiking activity of these neurons in urethane-anaesthetised mice to determine whether and how an electrical stimulus applied to the hindpaw influenced their firing properties (Figure 6.4A). The efficacy of this hindpaw stimulus was determined by the presence of a stimulation artefact in the local field potential (LFP) (Figure 6.4B), as has been previously described in the rat (Brown et al., 2009). Many identified dopaminergic neurons in both the RRF and SNc briefly and consistently paused their firing in response to noxious stimulation of the hindpaw, as is shown by the single neuron example raster and corresponding PSTH graphs in Figure 6.4C and D. Recorded and juxtacellularly-labelled dopaminergic neurons were located throughout the RRF (n = 15 from 11 mice; A-P range: -3.85 mm to -4.20 mm), as well as a smaller number in the SNc (n = 6 from 5 mice; A-P range: -3.40 mm to -3.85 mm) (Figure 6.4E).

The temporal dynamics of RRF dopaminergic neuron firing in response to noxious stimulation of the hindpaw were multiphasic. Initially, the firing rate of almost all RRF dopaminergic neurons (14 of 15) was suppressed by the stimulus, resulting from a pause in activity lasting 220.38 (18.15) ms (single example: Figure 6.4C; all RRF neurons: Figure 6.5A-C) with an onset latency of 30 ms (interquartile range (IQR) = 30–35 ms). This short-latency pause was typically followed by a rebound activation phase in which the firing rate increased compared to baseline. This pattern of activity change was present in most RRF dopaminergic neurons (12 of 15 neurons).

Dopaminergic neurons of the SNc had a similar response profile: a short-latency (32.50ms (IQR =

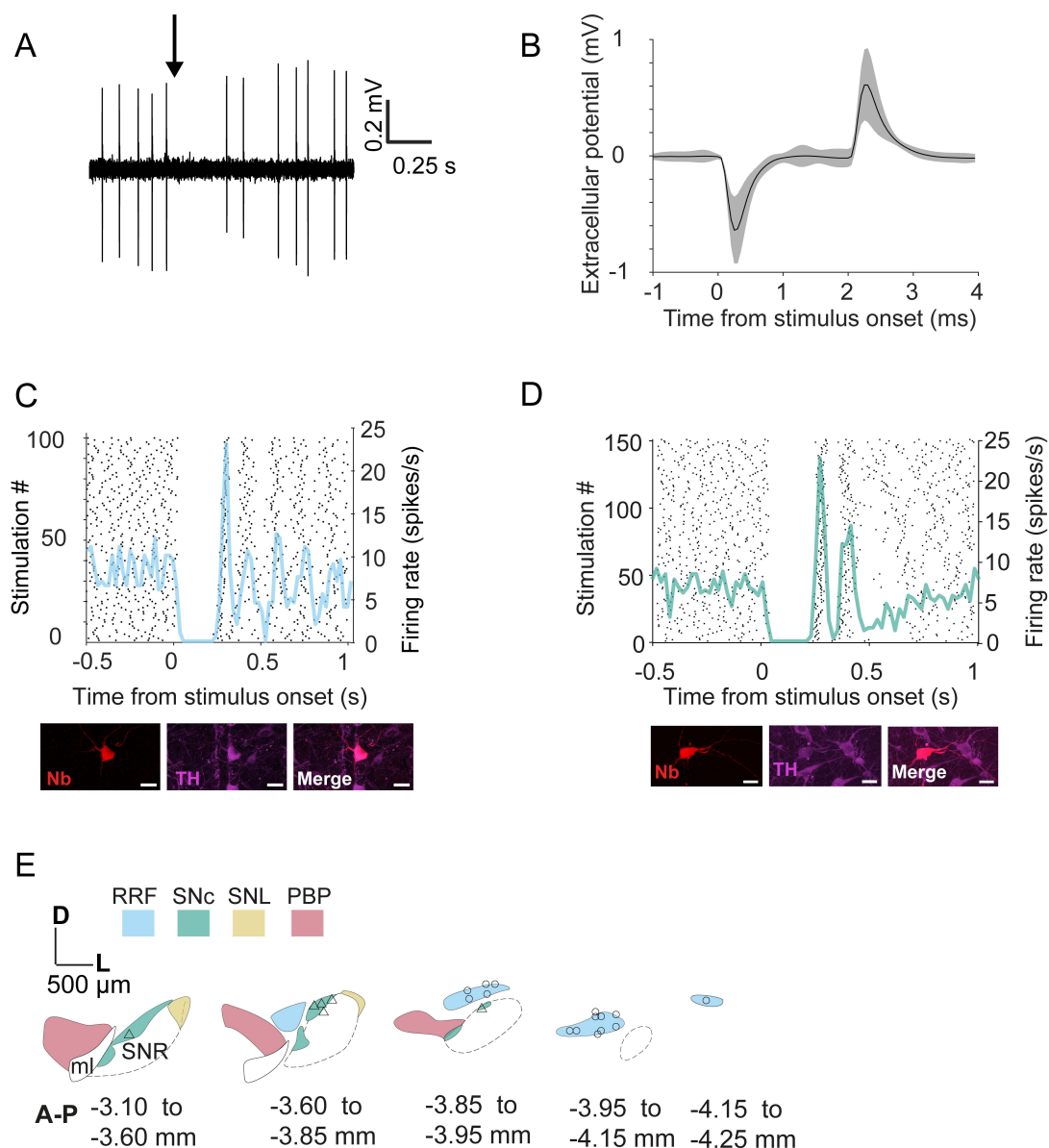


Figure 6.4: Individual responses of identified RRF and SNc dopaminergic neurons to noxious electrical stimulation of the hindpaw in urethane-anaesthetised mice. **A**, Application of a brief (2 ms) electrical stimulus (onset shown by black arrow) to the hindpaw induced a short pause in firing activity. **B**, A brief (~ 2 ms) response in the midbrain LFP recording following application of the electrical stimulus to the hindpaw indicated stimulus efficacy. **C**, **D**, Raster plots overlaid with the trial-averaged PSTH for an example identified RRF (**C**) and SNc (**D**) dopaminergic neurons show the response profile: a pause in activity proceeded by a rebound activation phase. The recorded neurons were labelled (Neurobiotin positive) and identified as dopaminergic (TH positive), as shown in the confocal micrographs below the plots. **E**, Midbrain coronal section schematics indicating the approximate locations of the recorded and labelled dopaminergic (TH positive) neurons. Location key for labelled neurons: circles, RRF; triangles, SNc. ml, medial lemniscus; Nb, neurobiotin; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticulata.

30–35 ms) pause (240 (44.27) ms), followed by rebound activation (Figure 6.5D–F). Whereas the RRF dopaminergic neurons displayed a single rebound peak in the population response, SNc neurons had a ‘double peak’ in the rebound phase (Figure 6.5D). The onset and duration of the pause in activity

following stimulation were not significantly different between RRF and SNc dopaminergic neurons (onset: Mann-Whitney U test, $p = 0.93$; pause: Student's t -test, two-tailed p -value = 0.54). All identified dopaminergic SNc neurons displayed short-latency suppression followed by rebound activation, as is shown by the heat plot in Figure 6.5E.

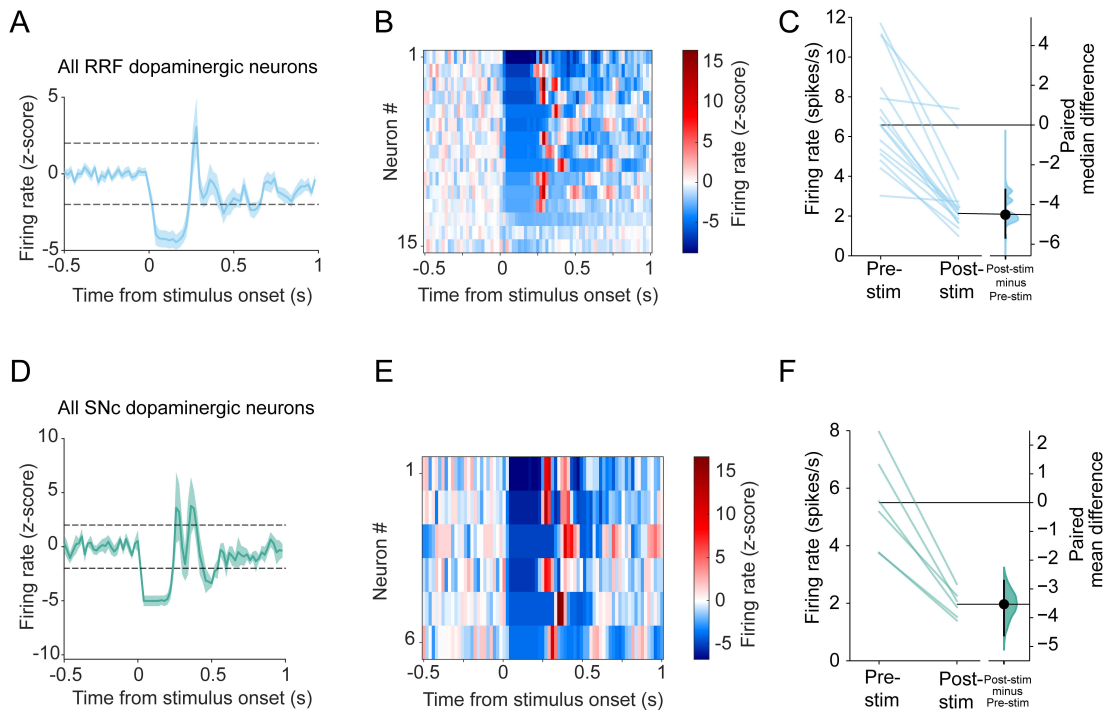


Figure 6.5: Population responses of identified RRF ($n = 15$) and SNc ($n = 6$) dopaminergic neurons to noxious electrical stimulation of the hindpaw. **A, D,** Populations of identified dopaminergic neurons were pooled, and the normalised firing rate PSTHs of all identified RRF (**A**) and SNc (**D**) neurons showed a significant drop in firing rate followed by a rebound activation phase in response to noxious stimulation. **B, E,** Heat maps of normalised trial-averaged firing rates of all RRF (**B**) and SNc (**E**) neurons, ordered from strongest to weakest short-latency suppression of firing rate (top to bottom). **C, F,** Gardner-Altman estimation plots of firing rate compared between the pre-stimulation baseline period (-500 to 0 ms) and the post-stimulation suppression period (0 to 150 ms) for RRF (**C**) and SNc (**F**) dopaminergic neurons. The floating axes on the right of each graph is the paired mean/median difference (effect size) plotted as a bootstrap sampling distribution (RRF, **C**: -4.51 [95% confidence interval (CI): -5.67, -3.25]; SNc, **F**: -3.53 [95% CI: -4.62, -2.71]).

As reported above, most RRF dopaminergic neurons responded to noxious stimulation of the hindpaw with suppression followed by rebound activation ($n = 12$ of 15; Figure 6.6A), which prevails in the average data of all ($n = 15$) dopaminergic RRF neurons (Figure 6.5A). However, even within this majority subpopulation there was marked heterogeneity in the temporal dynamics of the response, with some neurons showing more pronounced time-locked firing in a oscillatory pattern (Figure 6.6B) than others (Figure 6.6C) during rebound. Moreover, the firing rate of a small subpopulation of RRF dopaminergic neurons ($n = 2$) was suppressed by the noxious stimulus but

did *not* display significant rebound activation upon resumption of firing following the pause in activity (Figure 6.6D, E). One of these neurons was slow to recover, not resuming baseline firing until ~ 500 ms post-stimulation (Figure 6.6D). One RRF dopaminergic neuron did not respond to the noxious stimulus (Figure 6.6F). This lack of response could not be explained by lack of stimulus efficacy, as there was a stimulation artefact in the LFP (Figure 6.6F, far right plot), and there was also a neuron recorded in the pedunculopontine nucleus (PPN) of the same animal that responded positively to the paw stimulus (see Section 6.4.4 below).

When dopaminergic neurons transiently increase their rate, such as in response to unpredicted reward, it is usually the result of a short burst of action potentials, typically < 500 ms in duration (mouse: Cohen et al., 2012; rat: Grace and Bunney, 1984b; Mirenowicz and Schultz, 1994). In this experiment, however, the increase in firing rate during rebound was the result of single spikes that occurred with a very consistent latency across trials. Across dopaminergic neurons that displayed a suppression of firing followed by rebound activation (both RRF and SNc), the coefficient of variation (CV) for the rebound delay was low (0.08 (0.04)): this indicates that the delay between the onset of noxious stimulation and the onset of rebound firing is precisely-timed across trials within a neuron. The result of this consistent rebound latency is an apparent ‘excitation’ in the PSTHs. To further illustrate this, I calculated the occurrence of bursting activity in the baseline period before the onset of noxious stimulation (-500 to 0 ms) and compared this to the occurrence of bursting activity during the rebound period (0 to 300 ms after the onset of rebound firing). There was no increase in bursting during the rebound period; there was in fact a lower propensity for burst firing during the rebound period compared with the baseline period (Figure 6.7A). This observation was reflected by an increase in the average interspike interval (ISI) in the rebound period compared with the pre-stimulation period (Figure 6.7B). In combination with the low CV of the rebound latency, this demonstrates that the firing rate increase during the rebound period was not as a result of burst firing. Rather, the resumption of firing following the pause in neuronal activity occurs with a very consistent latency across trials, leading to an apparent ‘excitation’ upon rebound.

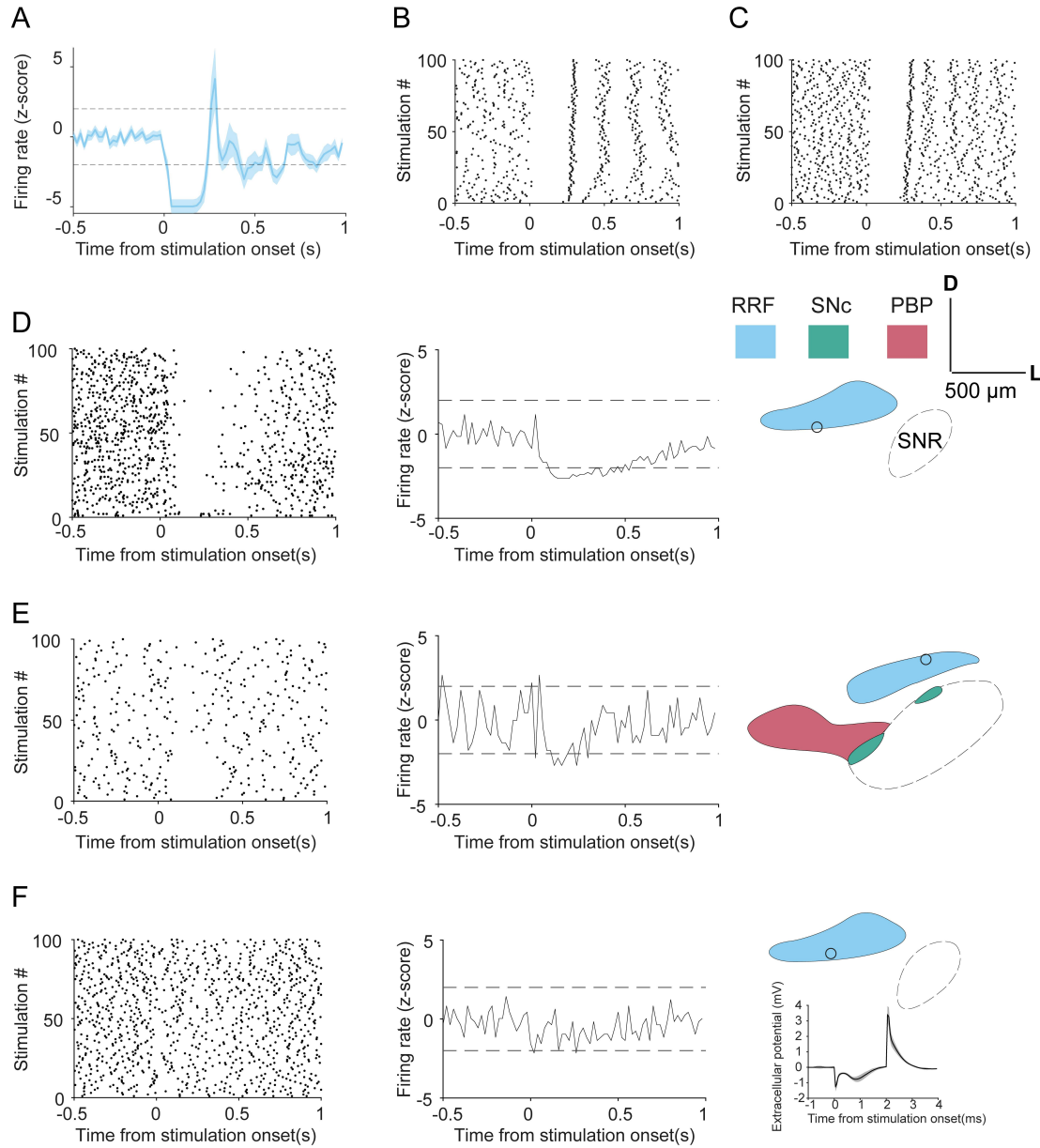


Figure 6.6: Heterogeneity in response types and profiles of identified RRF dopaminergic neurons following noxious electrical stimulation of the hindpaw. **A**, **B**, **C**, Normalised firing rate PSTH showing most RRF neurons (n = 12) had a suppression-rebound activation response type (**A**). Raster plots illustrate the heterogeneity in the temporal dynamics within this population often manifested as differences in the oscillatory pattern following rebound, with some neurons exhibiting much stronger oscillations (**B**) than others (**C**). **D**, **E**, Raster plots and normalised firing rate PSTHs of identified RRF dopaminergic neurons exhibiting suppression without rebound activation following noxious stimulation. **F**, An unresponsive neuron. Efficacy of hindpaw stimulus confirmed by the presence of an LFP response (inset graph, *right*). Right-hand side schematics indicate approximate locations of neurons in **D**, **E**, **F**.

Given that for most RRF and SNc dopaminergic neurons the suppression after the noxious stimulus is followed by a rebound of opposite sign (i.e. activation), it may be that these two phases of the response are mechanistically linked (Tateno and Robinson, 2011). Correlation analyses did not show any significant relationship between duration of suppression, change in firing rate at rebound,

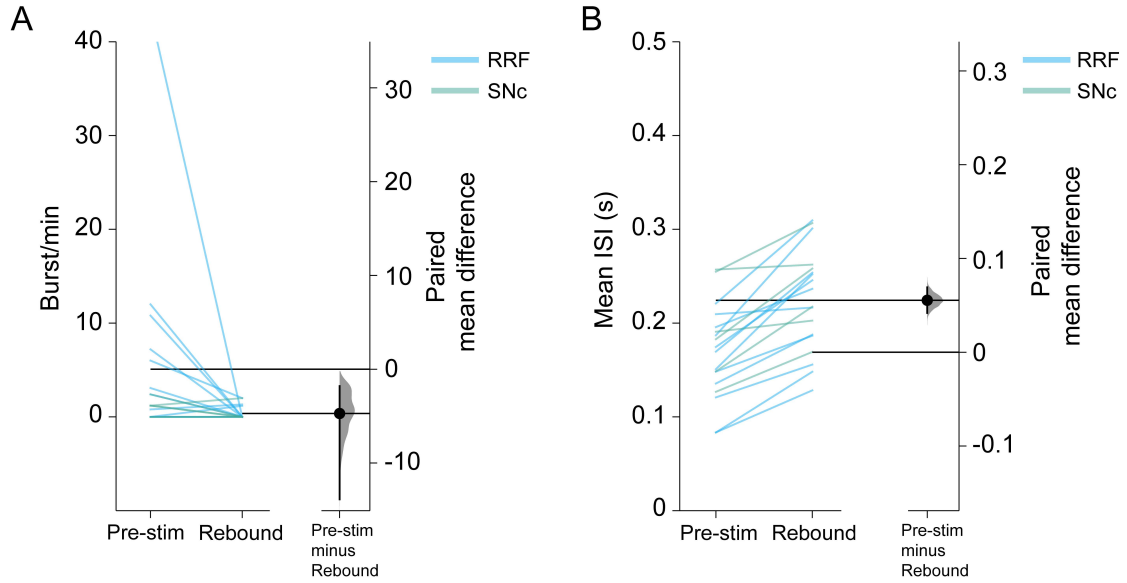


Figure 6.7: Decreased burst firing and increased interspike interval (ISI) during rebound for identified RRF ($n = 12$) and SNc ($n = 6$) dopaminergic neurons. Neurons included responded to noxious electrical stimulation of the hindpaw with suppression followed by rebound activation. Data represented in Gardner-Altman estimation plots, with the mean difference (effect size) between pre-stimulation and rebound periods displayed on the floating axes on the right-hand side as a bootstrap sampling distribution. **A**, Bursts/min were lower in the rebound period than during the pre-stimulation period. The paired mean difference between pre-stimulation and rebound is -4.72 bursts/min [95% confidence interval (CI): $-13.87, -1.80$]. **B**, The mean ISI was higher in the rebound period than during the pre-stimulation period. The paired mean difference between pre-stimulation and rebound is 0.06 s [95% CI: $0.04, 0.07$]

or baseline firing rate (Figure 6.8). Previous work has shown that anatomical location is a predictor of suppression level in midbrain dopaminergic neurons during an aversive learning task (Fiorillo et al., 2013b); however, I did not find any relationship between firing dynamics in response to the noxious stimulus and anatomical location in the A-P (Figure 6.9A, C) or M-L (Figure 6.9B, D) axes.

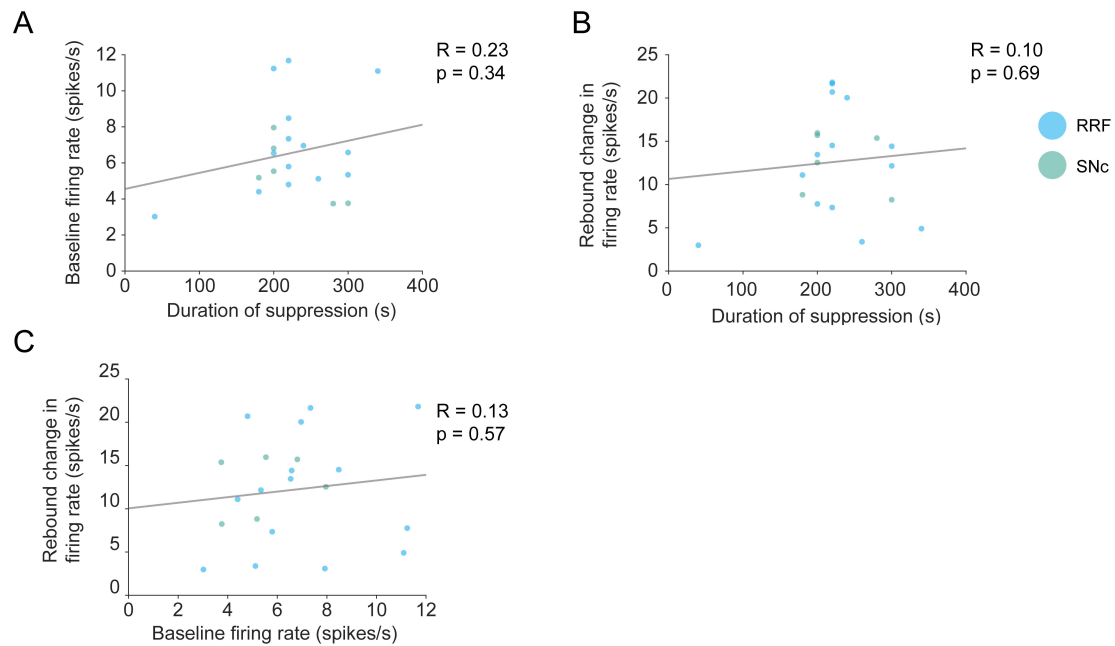


Figure 6.8: Firing dynamics of identified RRF and SNc dopaminergic neurons in response to noxious electrical stimulation of the hindpaw are not inter-related. **A, B**, Duration of suppression is not significantly correlated with baseline (pre-stimulation) firing rate (**A**) or the change in firing rate at the peak of the rebound period (**B**). **C**, Baseline firing rate is not significantly correlated with the change in firing rate at the peak of the rebound period. Insets show Pearson's correlation coefficient (R) and its significance value (p). The grey line through the data represents a linear fit of the data.

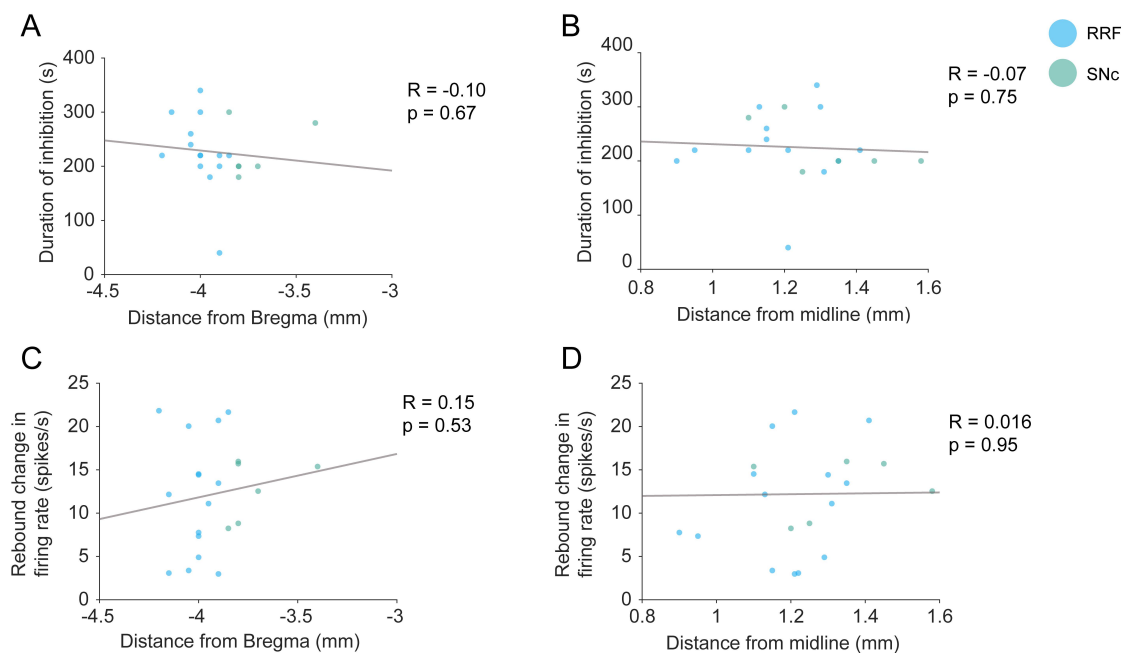


Figure 6.9: Firing dynamics of identified RRF and SNc dopaminergic neurons in response to noxious electrical stimulation of the hindpaw are not related to anatomical location. **A, B**, Duration of suppression was not significantly correlated with anatomical location in the anterior-posterior (**A**) or medial-lateral axis (**B**). **C, D**, Change in firing rate at the peak of the rebound period was not significantly correlated with anatomical location in the anterior-posterior (**C**) or medial-lateral axis (**D**). Insets show Pearson's correlation coefficient (R) and its significance value (p). The grey line through the data represents a linear fit of the data.

6.4.3 *Non-dopaminergic neurons in the RRF and caudal SNc have diverse responses to electrical stimulation of the hindpaw*

In Chapter 3, I demonstrated that intermingled amongst RRF dopaminergic (i.e. TH positive) neurons are other neurons that are non-dopaminergic, some of which were putatively GABAergic. In addition to this previous anatomical identification of non-dopaminergic neurons, I recorded the single-unit firing properties of a small number of non-dopaminergic neurons ($n = 5$) in the RRF/caudal SNc (Figure 6.10). The response dynamics of these neurons in response to noxious electrical stimulation of the hindpaw were diverse: two neurons did not respond (Figure 6.10A, B); one neuron displayed long-latency activation (Figure 6.10C); one neuron displayed short-latency activation (Figure 6.10D), and one neuron displayed short-latency suppression (Figure 6.10E). Most of these identified non-dopaminergic neurons were in the RRF (4/5 neurons; Figure 6.10B-E), and one was located in the caudal SNc (Figure 6.10A). Interestingly, although the noxious stimulus did not phasically modulate the firing rate of the neuron in Figure 6.10A, the firing rate across trials did increase (first ten trials vs last ten trials: Student's t -test, $p = 7.53 \times 10^{-10}$), suggesting some potentiation of firing rate by the stimulus. Overall, the response profile of most (4 of 5; Figure 6.10A-D) identified non-dopaminergic neurons was distinct from identified dopaminergic neurons (Figure 6.5 and 6.6). One neuron (Figure 6.10E) displayed a short-latency suppression of firing rate following the stimulation, in a manner similar to that of identified dopaminergic neurons (Figure 6.5 and 6.6).

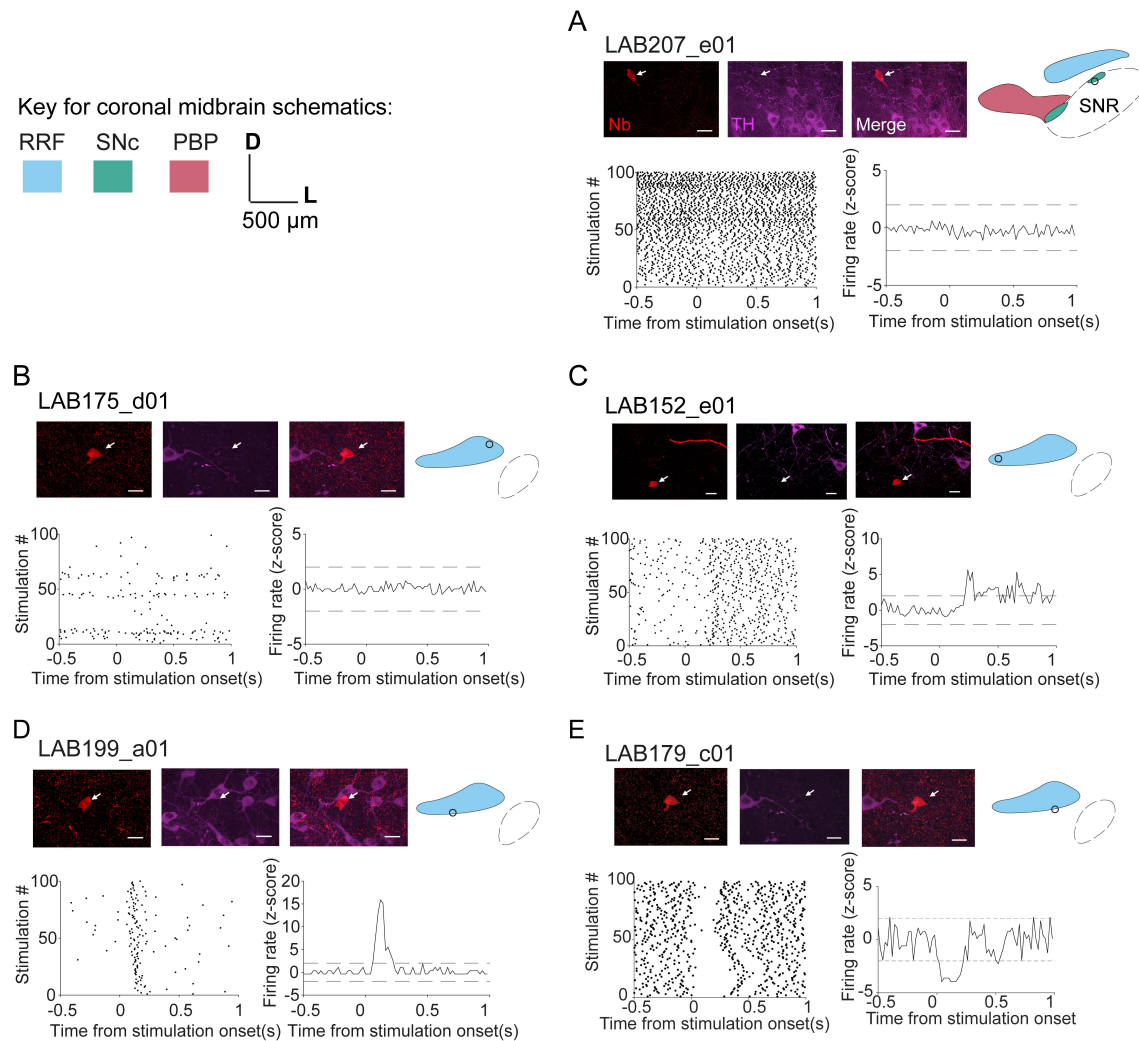


Figure 6.10: Diverse responses of identified non-dopaminergic midbrain neurons to noxious electrical stimulation of the hindpaw. **A, B**, Normalised firing rate PSTHs showing that two neurons, one in the caudal SNc (**A**) and one in the RRF (**B**), were not responsive to the noxious stimulation. **C**, RRF neuron that responded to the noxious stimulation with a long-latency activation. **D**, RRF neuron that responded to the noxious stimulation with a short-latency activation. **E**, A neuron in the RRF that responded to the noxious stimulation with a short-latency suppression. Inset schematics represent approximate locations of recorded and labelled neurons. Confocal micrographs show that the neurobiotin (Nb)-labelled neurons corresponding to the recordings were negative for tyrosine hydroxylase (TH) and therefore were identified as non-dopaminergic. Scale bars, 20 μ m.

During SWA brain state, the spontaneous firing properties of the identified non-dopaminergic neurons were diverse (Table 6.1). One of the electrophysiological criteria used to identify dopaminergic neurons putatively is an AP duration (threshold-to-trough) of > 1.1 ms (Ungless and Grace, 2012). Two of the identified non-dopaminergic neurons had AP durations of > 1.1 ms, one of which (Figure 6.10E) responded to electrical stimulation of the hindpaw similarly to identified dopaminergic neurons (Figure 6.5). Note, the spontaneous firing properties for cell 'LAB199_a01'

were not listed as there was not enough baseline SWA activity recorded to give meaningful values. However, it should be noted that the biphasic AP duration of this neuron was within the range used to identify dopaminergic neurons and, interestingly, the noxious stimulus activated the neuron.

Cell ID	Response Type	AP duration (ms)	Firing rate (spikes/s)	CV2	% spikes in burts	Burst/min
LAB207_e01	Non-responsive	0.62	2.6	0.69	26.0	8.4
LAB175_d01	Non-responsive	0.91	0.37	0.99	25	2.0
LAB152_e01	Long-latency excitation	0.75	0.94	0.74	25.9	3.0
LAB199_a01	Short-latency excitation	1.2	—	—	—	—
LAB179_c01	Short-latency inhibition	1.4	5.8	0.47	7.8	8.3

Table 6.1: A summary of the spontaneous firing properties of the identified non-dopaminergic neurons shown in Figure 6.10. Response type was assigned to each neuron based on its temporal profile in response to noxious electrical stimulation of the hindpaw. Note, there are no values for the spontaneous firing properties of LAB199_a01, except for action potential (AP) duration, because there was not enough firing data recorded during robust cortical slow-wave activity to permit analyses. CV2, firing variability.

6.4.4 A cholinergic neuron in the pedunculopontine nucleus was phasically activated in response to electrical stimulation of the hindpaw

During recording experiments, some electrode penetrations went through the RRF and into the PPN below. A generous putative criterion to identify dopaminergic neurons was a biphasic AP width of ≥ 0.9 ms and thus these were the focus of recording experiments. However, occasionally neurons with AP widths < 0.9 ms were recorded and labelled, a majority of which were in the RRF/caudal SNc (see previous Section 6.4.3), and one of which was in the PPN (Figure 6.11).

Juxtacellular labelling and *post-hoc* immunohistochemistry revealed that the recorded PPN neuron was cholinergic, as it was immunopositive for choline acetyltransferase (ChAT) (Figure 6.11A). The PPN was defined by a cluster of ChAT-positive neurons in the rostral brainstem (Mesulam et al., 1983), the more rostral portion of which lies below the RRF, wherein the labelled neuron was located (Figure 6.11B). This neuron fired slowly (1.62 spikes/s) in the pre-stimulation period and exhibited short-latency (20 ms) phasic activation following noxious electrical stimulation of the hindpaw (Figure 6.11C, D).

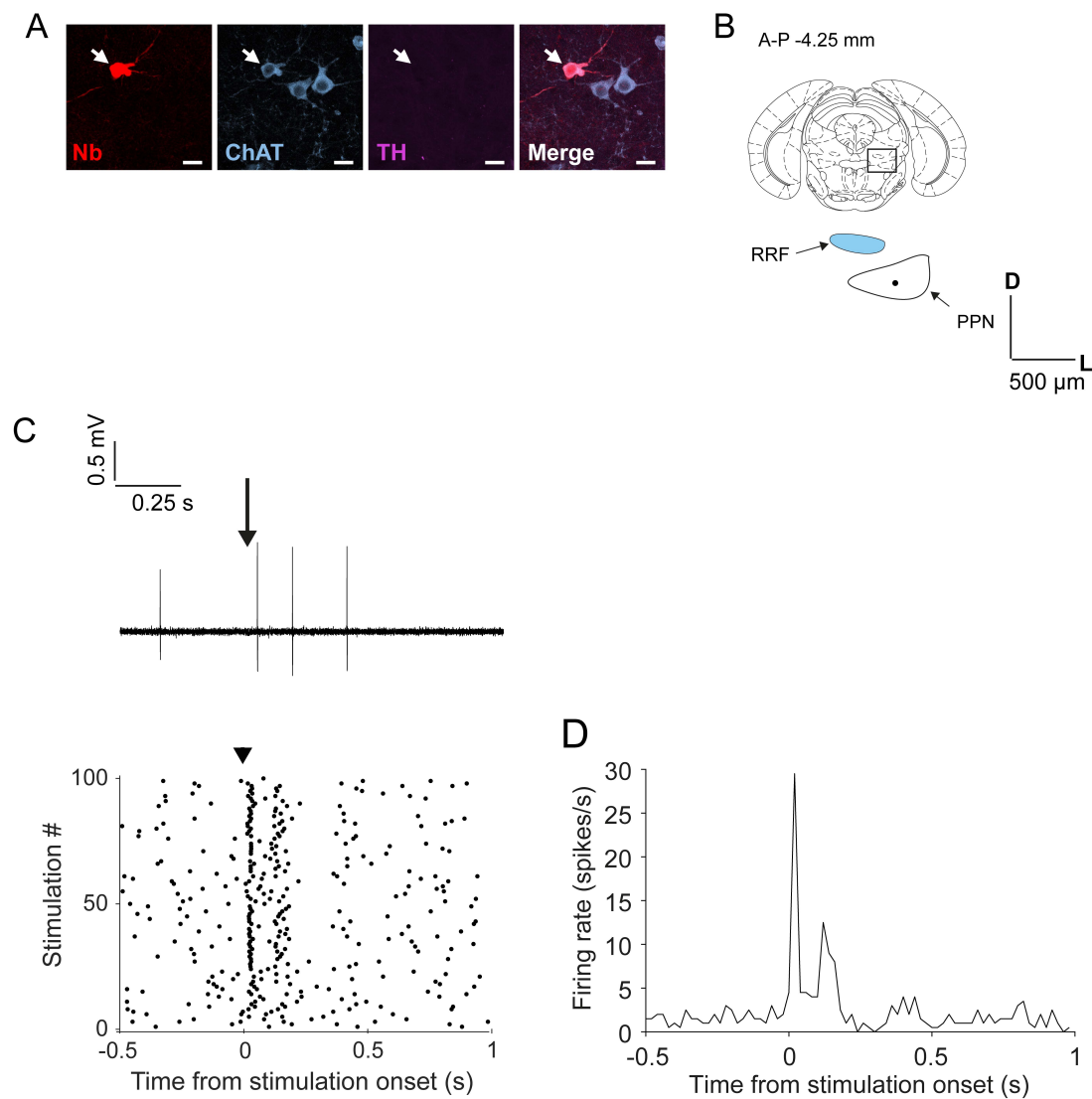


Figure 6.11: A cholinergic neuron in the pedunculopontine nucleus (PPN) is activated by noxious electrical stimulation of the hindpaw. **A, B,** The neurobiotin(Nb)-labelled neuron was immunopositive for choline acetyltransferase (ChAT; **A**) and located in the PPN (**B**, black dot). The scale bar is for the RRF/PPN schematic. **C, D,** Raw recording trace plot (**C**, top), raster plot (**C**, bottom) and firing rate PSTH (**D**) showing a short-latency activation in response to the noxious stimulation. A-P, anterior-posterior distance from Bregma.

6.5 Discussion

In this Chapter, I identify the firing properties of RRF dopaminergic neurons *in vivo* during anaesthesia, both spontaneous firing during the SWA brain state and firing responses to noxious electrical stimulation of the hindpaw. RRF dopaminergic neurons fire spontaneously with properties similar to SNc and VTA neurons, in terms of rate and pattern. Although the firing rate dynamics of identified dopaminergic RRF neurons were somewhat heterogeneous in response to a

noxious stimulus, an initial suppression prevailed in nearly all neurons.

6.5.1 *Spontaneous firing properties of identified dopaminergic neurons are similar in RRF, SNc and VTA*

During SWA, RRF dopaminergic neurons fired slowly (< 10 spikes/s) in an irregular pattern with varying degrees of burstiness, similar to SNc and VTA dopaminergic neurons. The two main electrophysiological criteria used to identify SNc and VTA dopaminergic neurons putatively are a firing rate of < 10 spikes/s and a biphasic action potential duration of > 1.1 ms (Grace and Bunney, 1983, 1984a; Ungless and Grace, 2012), matching most RRF dopaminergic neurons in this study. Moreover, the slow firing rate and wide AP duration of identified RRF dopaminergic neurons in this study are in line with previous reports that described the firing properties of putative RRF dopaminergic neurons in awake monkeys (Schultz, 1986; Ljungberg et al., 1992; Fiorillo et al., 2013b). Ungless et al. (2004) determined that a threshold-to-trough AP waveform width of > 1.1 ms distinguishes dopaminergic neurons from non-dopaminergic neurons in rats. Interestingly, I found that some non-dopaminergic neurons in the RRF had a threshold-to-trough AP width of > 1.1 ms, suggesting this criterion is not a reliable method for putative identification of dopaminergic neurons in the RRF, at least in the mouse. Whether this is the case for SNc and VTA neurons is unclear, as few identified non-dopaminergic neurons were recorded in these cell groups in the present study. Crucially, the identified non-dopaminergic RRF neurons with AP widths of > 1.1 ms in this study would have been included as false positives if cell identification had been incomplete (i.e. only based on electrophysiological properties). Additionally, there was a group of identified **dopaminergic** RRF neurons that had spontaneous firing rates and AP durations *outside* the electrophysiological criteria used to putatively identify dopaminergic neurons. Altogether, although these groups of outlier neurons represent a small proportion of the overall population, they illustrate the importance of using a technique to unambiguously identify the type of neuron recorded (i.e. TH positive or negative in this study). This allows for confident reporting of noteworthy exceptions where they would have otherwise been overlooked.

Some midbrain dopaminergic neurons have a propensity to fire in bursts, typically defined as a cluster of action potentials with reduced ISIs, beginning at < 80 ms and widening until the burst ends when the ISI is > 160 ms (Grace and Bunney, 1984b; Ko et al., 2012). Most often, this bursting is described in the context of reward signalling (mouse: Zweifel et al., 2009; rat: Hyland et al., 2002; non-human primate: Schultz, 2007) but is also observed spontaneously during anaesthesia in rodents. Identified dopaminergic neurons (RRF, SNc and VTA) in this study fired only a small percentage of their action potentials in bursts during SWA, which was three-fold lower than has been observed previously for SNc neurons in the same preparation (Pristerà et al., 2015). Notably, most SNc dopaminergic neurons in the present study were recorded in the caudal midbrain. In contrast, previous observations (Pristerà et al., 2015) of higher bursting activity in SNc neurons were at more rostral locations (most caudal neuron A-P -3.5 mm), pointing towards a relationship between anatomical location and burst propensity. The finding in the present study that there is a negative correlation between A-P location and firing variability (CV2) supports this idea, but such a relationship did not exist for burst parameters.

In this study, the burst propensity in identified VTA and SNc dopaminergic neurons was not significantly different, contrasting with previous work showing that VTA dopaminergic neurons fire more of their spikes in bursts than SNc neurons in the rat (Grenhoff et al., 1988; Marinelli and White, 2000). However, the low number of VTA neurons recorded in the present study means the power of the statistical comparison was weak, and more VTA dopaminergic neurons would need to be recorded to make robust statistical comparisons between the VTA and SNc groups.

6.5.2 Most RRF and SNc dopaminergic neurons display suppression followed by rebound activation in response to electrical stimulation of the hindpaw

The most common function ascribed to SNc and VTA dopaminergic neurons is the encoding of a reward prediction error (RPE) signal (mouse: Eshel et al., 2016; non-human primate: Mirenowicz and Schultz, 1994; Schultz et al., 1997). In this model, stimuli of positive motivational value elicit

a phasic burst of action potentials, whereas stimuli of negative motivational value suppress firing rate. Consistent with this, aversive stimuli suppress the firing rate of some dopaminergic neurons in the SNc and VTA (mouse: Cohen et al., 2012; Tan et al., 2012; Matsumoto et al., 2016; rat: Ungless et al., 2004; Brischoux et al., 2009; Brown et al., 2009). However, some dopaminergic neurons in the ventromedial VTA (mouse: Tan et al., 2012; rat: Brischoux et al., 2009) and dorsolateral SNc (non-human primate: Matsumoto and Hikosaka, 2009) are excited by aversive stimuli, pointing towards a role for these neurons that is more consistent with motivational salience (Bromberg-Martin et al., 2010).

Previous reports in the rat have shown that most identified dopaminergic neurons in the lateral VTA pause their firing in response to noxious electrical (Brischoux et al., 2009) and pinch (Ungless et al., 2004) stimuli. In this study, most RRF dopaminergic neurons respond similarly, with a phasic decrease in firing rate in response to the noxious stimulus. My findings suggest that firing rate changes in dopaminergic neurons of the RRF are consistent with the reward theory of dopaminergic neuron function (Schultz et al., 1997), in that the suppression of firing following a noxious stimulus fits with the value-coding function of dopaminergic neurons (Matsumoto et al., 2016). Whether or not this firing rate suppression also leads to a reduction of dopamine efflux in target structures is a question for future research. Unconditioned aversive stimuli transiently reduce the extracellular dopamine concentration in the nucleus accumbens (NAc) shell of the rat (Roitman et al., 2008). This observation is in line with the reduced firing rate of some VTA dopaminergic neurons (Ungless et al., 2004; Brischoux et al., 2009), known to project heavily to the NAc (Breton et al., 2019). This linear relationship may exist between RRF dopaminergic neuron firing and dopamine release in target structures. However, recent studies have highlighted that such linear relationships between dopaminergic neuron firing dynamics and dopamine release cannot always be presumed (Mohebi et al., 2019), and dopamine release may be shaped at the level of axonal release sites (Threlfell et al., 2012; Berke, 2018; Mohebi et al., 2019).

In this study, all identified dopaminergic SNc neurons were initially suppressed by a noxious

electrical hindpaw stimulus, followed by rebound activation. This responsiveness contrasts with previous findings in the rat using an identical stimulus regime, in which the stimulus inhibited less than 20% of identified SNc dopaminergic neurons (Brown et al., 2009). All identified dopaminergic SNc neurons I recorded and tested for responsiveness to electrical stimulation of the hindpaw were in mice in the caudal SNc, whereas Brown et al. (2009) recorded all neurons in rats, and most of them were within the more rostral SNc. Thus, a combination of species difference and A-P location may explain the discrepancy between the present study and previous findings. The results reported by Coizet and colleagues (Coizet et al., 2006, 2010), in which the noxious electrical hindpaw stimulus suppressed the majority of *presumed* SNc dopaminergic neurons in the rat, are more in line with my findings.

Representation of motivational salience is a function ascribed to subpopulations of SNc and VTA neurons that phasically increase their firing rate in response to aversive stimuli (mouse: Tang et al., 2020; rat: Brischoux et al., 2009; non-human primate: Matsumoto and Hikosaka, 2009; reviewed in: Bromberg-Martin et al., 2010). No identified RRF dopaminergic neurons were phasically activated by noxious electrical stimulation of the hindpaw, suggesting that this subpopulation of dopaminergic neurons do not generally encode a salience signal. However, absence of evidence for RRF dopaminergic neurons that are activated in response to a noxious stimulus should not be inferred to mean evidence of absence. It is possible a group of salience-encoding neurons do exist that were not sampled in this study. Moreover, differences in the stimulus parameters (i.e. single stimulation vs trains of repeated stimulation) make it difficult to reconcile variation in results across studies.

Although the present data are the first description of *identified* RRF dopaminergic neuron firing properties, previous work in anaesthetised monkeys (Schultz and Romo, 1987) outlined heterogeneity in responses of *presumed* dopaminergic RRF neurons, with some neurons suppressed and others activated by a noxious pinch stimulus. It is interesting to speculate that some of the presumed dopaminergic neurons in Schultz and Romo (1987) may, as I have shown in this study,

have actually been non-dopaminergic (i.e. non-dopaminergic neurons with electrophysiological properties similar to those of dopaminergic neurons). Thus, if I had not unequivocally identified the recorded neurons, I too would have seen heterogeneity in the response to a noxious stimulus. Note, the discrepancy between my data and Schultz and Romo (1987) could be because the effect of an electrical stimulus (the present study) on the firing of midbrain dopaminergic neurons is different to that of a pinch stimulus (Schultz and Romo, 1987).

RRF dopaminergic neurons responded to electrical stimulation of the hindpaw with similar temporal dynamics to SNc neurons, within the range described in previous studies of dopaminergic neurons in the mouse (Tan et al., 2012) and rat (Tsai et al., 1980; Coizet et al., 2006; Brown et al., 2009; Coizet et al., 2010). Previous studies reported that SNc dopaminergic neurons exhibit a short-latency suppression followed by rebound activation in the rat (Hommer and Bunney, 1980; Coizet et al., 2006), and I show for the first time that this response profile is also common to RRF neurons. An increased inhibitory drive is the most likely reason dopaminergic neurons pause their firing in response to electrical stimulation of the hindpaw, as opposed to reduced excitatory drive. Between 50 and 70% of inputs to dopaminergic SNc neurons are GABAergic in the rat (Bolam and Smith, 1990; Henny et al., 2012), and even when disfacilitated, such as in *ex vivo* slices, they fire at a slow, regular rate (rat: Overton and Clark, 1997) that is inhibited by phasic GABA_A receptor activation (Lobb et al., 2010).

In the rat, feedforward inhibition driven by the superior colliculus (Comoli et al., 2003; Coizet et al., 2006) and parabrachial nucleus (PBN) (Coizet et al., 2010) suppresses the firing of SNc dopaminergic neurons in response to noxious stimuli. Beyond these two input regions, SNc (Watabe-Uchida et al., 2012)—and VTA (Watabe-Uchida et al., 2012; Beier et al., 2015)—dopaminergic neurons receive monosynaptic inputs from a diverse network of brain regions that may also be candidates for inducing a pause in firing. In Chapter 5, I demonstrated that RRF dopaminergic neurons, like the SNc (Comoli et al., 2003; Coizet et al., 2006, 2010), also receives direct inputs from the superior colliculus and PBN. Thus, these regions may relay nociceptive

information to RRF dopaminergic neurons.

In the present study, the increased firing rate that occurs when dopaminergic neurons rebound is due to a precisely timed resumption of spiking activity, as has been previously demonstrated for SNc neurons (Coizet et al., 2006), and not as a result of a burst of action potentials. The rebound firing likely occurs as a result of precisely timed intracellular homeostatic mechanisms that mean the trial-by-trial rebound latency is relatively invariant, such as via hyperpolarisation-activated cyclic nucleotide-gated channel (HCN) channels (mouse: Neuhoff et al., 2002; non-human primate: Fiorillo et al., 2013a). If this temporal precision is common across a population of dopaminergic neurons and leads to synchronous activation following a pause, this may have implications for the phasic release of dopamine in striatum and subsequent modulation of pathways involved in behavioural processing. This has been recently conceptualised in a ‘domain-overlap model’ (Liu et al., 2021), which postulates that synchronous activation of midbrain dopaminergic neurons is required to overwhelm reuptake mechanisms and activate distal receptors on striatal neurons, thereby modulating behavioural processing. Future experiments in which multiple single units are recorded in the same animal are needed to determine whether such synchronous activation occurs *in vivo*.

Interestingly, although SNc dopaminergic neurons showed a ‘double-peak’ of rebound activation, RRF neurons tended to exhibit rebound activation during a single period. The double-peak effect seen in SNc dopaminergic neurons is due to the firing rate of the neurons oscillating between activation and suppression, a result that has been shown previously for both SNc (rat: Hommer and Bunney, 1980; Coizet et al., 2006) and VTA neurons (Tan et al., 2012). Although this pronounced oscillation occurred in some dopaminergic RRF neurons, the population is less entrained than SNc neurons.

6.5.3 *Non-dopaminergic neurons in the RRF and caudal SNc have diverse responses to electrical stimulation of the hindpaw*

There are glutamatergic and GABAergic neurons intermingled with dopaminergic neurons within the RRF (Chapter 3; rat: Nair-Roberts et al., 2008; Yamaguchi et al., 2013), making them likely candidate cell types for the small population of identified non-dopaminergic neurons in the present Chapter. Unfortunately, lack of somatic immunohistochemical markers for glutamatergic/GABAergic neurons precluded the unequivocal identification of the neurotransmitter phenotype of these neurons. However, a population of presumed GABAergic neurons was identified (Chapter 3).

In the rat, glutamatergic neurons in the midbrain predominately express vesicular-glutamate transporter 2 (VGluT2) and are found in the VTA (Yamaguchi et al., 2007), SNc and RRF (Yamaguchi et al., 2013). The *in vivo* firing rate of identified vGluT2 neurons in the VTA ranges from < 1 spike/s to > 30 spikes/s, and some respond to an aversive stimulus with a phasic increase in firing rate (Root et al., 2018). In the present study, a noxious stimulus phasically activated one identified non-dopaminergic neuron in the RRF. Additionally, one identified non-dopaminergic neuron in this study showed an increased baseline firing rate in response to repeated noxious stimulation of the hindpaw, which is an effect seen in identified vGluT2 neurons of the VTA in response to consecutive aversive stimuli (Root et al., 2018). Although I did not further neurochemically define the non-dopaminergic neurons in the present study, it seems unlikely that they were GABAergic neurons. The identified non-dopaminergic neurons in this study fired at relatively slow rates, whereas it is known that midbrain GABAergic neurons fire at rates of > 15 spikes/s in the mouse (Cohen et al., 2012) and rat (Steffensen et al., 1998). It is, therefore, more likely that the identified non-dopaminergic neurons recorded in this study were glutamatergic than GABAergic, or a different cell type altogether.

Although some of the recorded and identified non-dopaminergic neurons had firing rates and

biphasic AP widths that were distinct from those used to identify dopaminergic neurons putatively, there were some in the RRF that had overlapping characteristics. Thus, these neurons could have been mistakenly classified as dopaminergic if cell type identification was incomplete, indicating that putative criteria are insufficient to capture all dopaminergic neurons in the RRF. Identified non-dopaminergic neurons with the putative electrophysiological characteristics of dopaminergic neurons are observed in the VTA (Ungless et al., 2004; Ungless and Grace, 2012) but not the SNc (Brown et al., 2009) of rats, whereas in mice they are observed in both VTA and SNc (Kaufmann, 2017).

A previous study demonstrated that some non-dopaminergic neurons within the VTA have electrophysiological characteristics similar to dopaminergic neurons and respond to a noxious stimulus with phasic activation (Ungless et al., 2004). Similarly, there was one identified non-dopaminergic neuron in the RRF with a wide (> 1.1 ms) biphasic AP duration (i.e. dopaminergic-like) that was phasically activated by the noxious stimulus. Conversely, there was an identified non-dopaminergic neuron in the RRF that was phasically suppressed by the noxious stimulus, in a manner similar to identified dopaminergic neurons. This overlap in electrophysiological characteristics between dopaminergic and non-dopaminergic neurons exemplifies the importance of unequivocal cell type identification, and although mismatch between putative and actual identity is rare, such occurrences have the potential to skew interpretations.

6.5.4 A cholinergic neuron in the pedunculopontine nucleus is phasically activated in response to electrical stimulation of the hindpaw

Cholinergic neurons of the PPN innervate SNc (Watabe-Uchida et al., 2012) and VTA (mouse: Watabe-Uchida et al., 2012; rat: Dautan et al., 2016) dopaminergic neurons, and there is evidence that the PPN innervates the RRF (mouse: Chapter 5; rat: Zahm et al., 2011). A noxious stimulus phasically activated the identified cholinergic PPN neuron in the present study, which is in line with previous findings in the rat (Petzold et al., 2015). Activation of cholinergic PPN neurons is known to have

diverse modulatory effects on the firing activity of dopaminergic neurons (Dautan et al., 2016) and it would be interesting to consider how the temporal dynamics of the responses to noxious stimulation interplay between these structures in future work. However, whether or not the recorded neuron in this study has axon terminals in the dopaminergic midbrain is unknown, and any such conclusions cannot be directly assessed.

In conclusion, the results of this Chapter have shown that identified RRF dopaminergic neurons have spontaneous firing properties broadly similar to other midbrain dopaminergic neuron subgroups. Most dopaminergic neurons described in this Chapter were in the caudal midbrain (RRF and caudal SNc) and had generally low propensity for spontaneous bursting and low firing variability (CV2) compared to previous reports of dopaminergic neurons in the more rostral midbrain. To determine quantitatively whether this is the case, the firing properties of identified dopaminergic neurons in more rostral midbrain regions could be compared directly to RRF/caudal SNc neurons in the same preparation in future work. Nearly all RRF dopaminergic neurons represent a noxious somatosensory stimulus with a phasic pause in firing, indicating that RRF dopaminergic neurons are equipped to represent aversive stimuli, and thus value.

Chapter 7

An optogenetic strategy to explore the effect of physiologically-relevant pauses in midbrain dopaminergic firing on place preference behaviours

7.1 Introduction

An animal's behaviour is strongly governed by learned associations. The ability to associate an action or environmental stimulus with a reliable positive (rewarding) or negative (aversive) outcome is critical for adaptive behaviour. Some midbrain dopaminergic neurons phasically increase their firing rate when an unexpected reward is received (mouse: Eshel et al., [2016](#); non-human primate: Mirenowicz and Schultz, [1994](#); Pan, [2005](#)) and pause their firing when an expected reward is omitted (mouse: Cohen et al., [2012](#); non-human primate: Hollerman and Schultz, [1998](#)), the temporal dynamics of which are thought to underpin associative learning. These temporal dynamics are conceptualised in the reward prediction error (RPE) framework (Schultz et al., [1997](#)). The prevailing view is that a phasic increase in the firing of midbrain dopaminergic neurons strengthens

associations and reinforces behaviour (Tsai et al., 2009; Zweifel et al., 2009), whereas a pause in firing has the opposite effect (rat: Chang et al., 2016). These firing dynamics suggest that midbrain dopaminergic neurons are a possible neural substrate for reinforcement (Pavlov, 1927; Rescorla and Wagner, 1972) and temporal-difference learning (Sutton, 1988; Sutton and Barto, 1998).

Consistent with this conceptualisation, rewarding stimuli induce brief activation and mildly aversive electric shocks or air puffs induce brief pauses in the activity of some dopaminergic neurons in the SNc (Lerner et al., 2015) and VTA (Cohen et al., 2012; Matsumoto et al., 2016) of awake mice, respectively. These responses occur in a manner that suggests some dopaminergic neurons encode the value of stimuli in a continuous dimension (however, see Fiorillo, 2013). This pause in activity in response to mildly aversive electric shocks or air puffs is also observed in awake (Milevskiy and Morales, 2011) and anaesthetised rats (Ungless, 2004; Brischoux et al., 2009; Brown et al., 2009), and in awake (Mirenowicz and Schultz, 1996; Matsumoto and Hikosaka, 2009; Fiorillo et al., 2013c) and anaesthetised non-human primates (Schultz and Romo, 1987). Accordingly, dopamine release in the nucleus accumbens (NAc) medial shell (Roitman et al., 2008) and bed nucleus of the stria terminalis (BNST) of rats (Park et al., 2012) is suppressed following the delivery of aversive bitter tastants. Although the most common response of midbrain dopaminergic neurons to aversive stimuli is a suppression of activity, there are also subpopulations that increase their activity. Within the midbrain, lateral SNc and medial VTA neurons are activated by foot shocks, whereas their counterparts in the medial SNc and lateral VTA display the opposite response profile (mouse: Lerner et al., 2015; Steinberg et al., 2020; rat: Ungless, 2004; Brischoux et al., 2009; non-human primate: Matsumoto and Hikosaka, 2009). Similar diversity is seen in dopamine release profiles in the NAc; in the core, release is suppressed during presentation of a fear conditioned stimulus in the rat, whereas release in the shell is elevated (Badrinarayan et al., 2012).

Optogenetically inhibiting VTA dopaminergic neurons induces conditioned place aversion (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019), suggesting that pausing dopaminergic neuron firing serves as a negative reinforcement signal during

associative learning. However, the parameters used to optogenetically inhibit midbrain dopaminergic neurons in these studies were ‘supra-physiological’ (i.e. laser pulses of seconds in duration), meaning it is unclear whether brief pauses in firing (250–500 ms), as seen in anaesthetised and awake animals (rat: Brischoux et al., 2009; mouse: Brischoux et al., 2009; Matsumoto et al., 2016 and Chapter 6), are of causative importance for mediating aversive behaviours. Even in experiments that had the express aim of optogenetically mimicking negative RPEs during associative learning, midbrain dopaminergic neurons were inhibited for 2 s (rat: Chang et al., 2016). This dissociation between physiological responses and optogenetic manipulation parameters leads to difficulty in reconciling results across experiments. It also raises the issue of whether naturalistic pauses in firing are of causative importance for mediating aversive behaviours. It should be noted, however, that during fear conditioning experiments in rats the firing activity of VTA dopaminergic neurons (Mileykovskiy and Morales, 2011) and the release of dopamine in the NAc core (Badrinarayan et al., 2012) is suppressed for the duration of the conditioned stimulus (3–10 s). Thus, suppressing the firing of midbrain dopaminergic neurons for several seconds may be physiological in certain behavioural contexts.

It is unclear how midbrain dopaminergic neurons transduced with an inhibitory opsin respond to these long (seconds) periods of optogenetic manipulation *in vivo*. Yellow laser pulses can suppress the firing of midbrain dopaminergic neurons transduced with archaerhodopsin (Arch) (Danjo et al., 2014) or *Natronomonas pharaonis* halorhodopsin (NpHR) (Salinas-Hernández et al., 2018) for up to 1 s. It is important to characterise the firing dynamics in response to the laser as they cannot always be presumed to reflect the digital nature of the stimulus parameters. For example, laser offset induces sharp rebound activation in NpHR-transduced neurons following a 1 s laser pulse (Salinas-Hernández et al., 2018), an effect not observed following inhibition of Arch-transduced neurons (Danjo et al., 2014). This rebound effect may be explained by the different biophysical properties of NpHR and Arch (Raimondo et al., 2012). Given there is a paucity of data regarding the effect of optogenetic manipulations on the firing of midbrain dopaminergic neurons, the first aim of

this Chapter was to determine the firing dynamics of archaerhodopsin T (ArchT)-transduced midbrain dopaminergic neurons in response to short (250 ms) and longer (2–30 s) laser pulses. Thus, any behavioural effects seen in optogenetic place preference experiments could be related to these firing dynamics.

In Chapter 6, I demonstrated that RRF, SNc and VTA dopaminergic neurons briefly (~220 ms) pause their firing in response to an electrical foot shock in mice. Reflecting on this data, I hypothesised that optogenetically mimicking this pause in RRF dopaminergic neurons would be aversive in awake, behaving mice. In pilot experiments in which RRF dopaminergic neurons were transduced with ArchT, optogenetically manipulating these neurons did not induce place aversion in an unbiased conditioned place preference (CPP) paradigm (data not shown). However, inspection of the data revealed it was likely that the mice had developed a preference for the white chamber of the setup (i.e. a bias), potentially masking any effect of the optogenetic manipulation. As a result, a biased CPP paradigm was implemented (Cunningham et al., 2006) in which the optogenetic manipulation was applied in the preferred chamber (i.e. white chamber). However, rather than again targeting RRF dopaminergic neurons for optogenetic manipulation, I decided to target VTA dopaminergic neurons. This was done because the baseline preference I observed for one of the chambers is atypical (Tsai et al., 2009; Lobo et al., 2010; Soares-Cunha et al., 2020), thus it was deemed particularly important to establish a ‘positive’ control. Targeting manipulations to VTA dopaminergic neurons was done in light of previous work demonstrating the aversive effect of optogenetically inhibiting these neurons in place preference paradigms (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019).

7.2 Aims

- To optogenetically mimic the pause in firing observed in dopaminergic neurons following a noxious stimulus.

- To determine whether brief optogenetic inhibition of VTA dopaminergic neurons is sufficient to induce place aversion.

7.3 Methods

7.3.1 Electrophysiological recordings during optogenetic manipulation

For anaesthetised electrophysiological recordings, DAT-Cre mice (2 males and 3 females) were unilaterally (right hemisphere) injected with 330 μ l of AAV2-CAG-FLEX-ArchT-green fluorescent protein (GFP) (stereotaxic coordinates: A-P -4.00 mm, M-L +1.20 mm, D-V -3.20 mm) to allow for selective transduction of midbrain dopaminergic neurons (see Materials and Methods Section 2.3 for more details). Recordings were performed 4–10 weeks after injection to allow time for viral transduction. For electrophysiological experiments, a 200 μ m diameter optic fibre was inserted above the midbrain and a recording pipette lowered beneath it at an angle of 10° in the anterior direction (Figure 7.1A; see Materials and Methods Section 2.5 and 2.5.5 for more details). Dopaminergic neurons were recorded during optogenetic manipulation (5 mW laser power, unless otherwise stated), and in some cases recorded neurons were juxtacellularly labelled for *post hoc* localisation and neurochemical identification in perfusion-fixed tissue (Figure 7.1B).

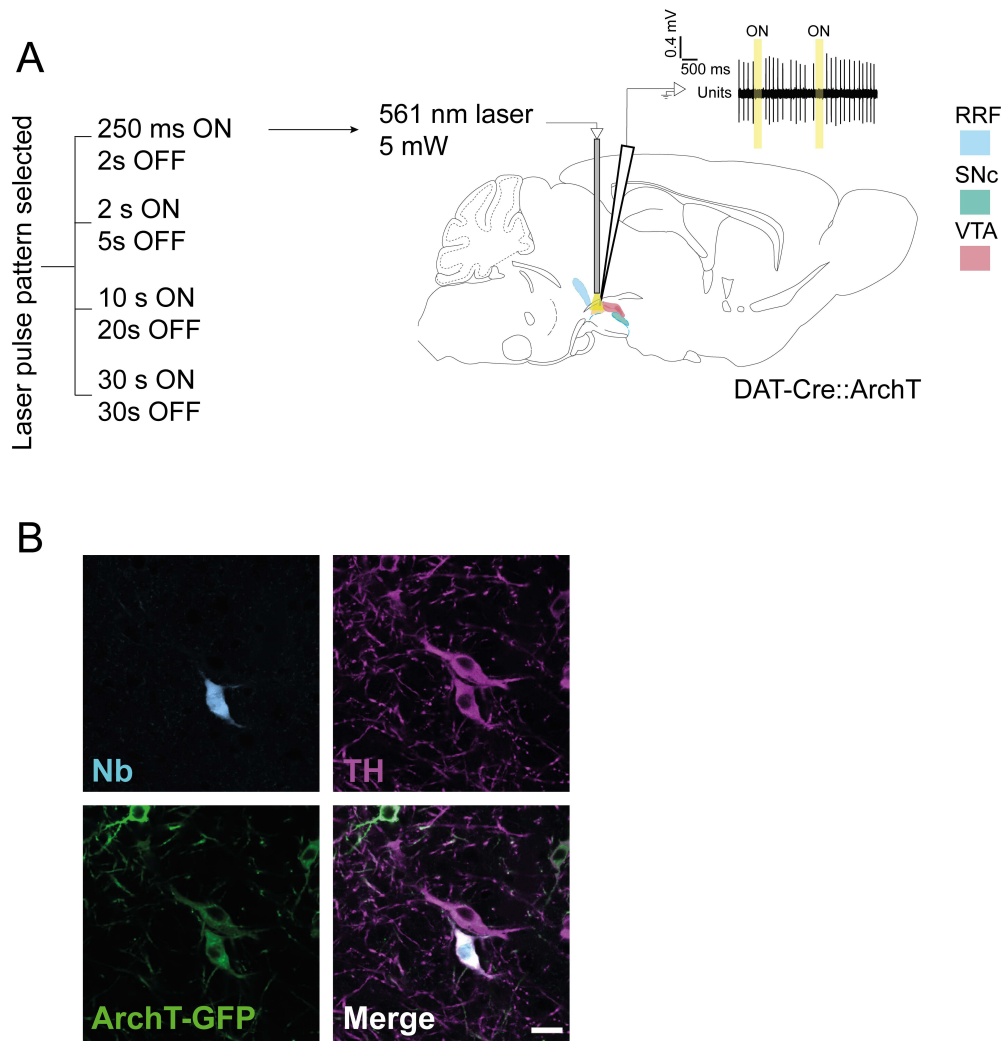


Figure 7.1: Experimental setup: electrophysiological verification of the effects of local light delivery on the firing of midbrain dopaminergic neurons selectively transduced with ArchT-GFP. **A**, Single-unit recordings of midbrain dopaminergic neurons were performed during delivery of laser pulses in ArchT-injected DAT-Cre mice (DAT-Cre::ArchT). Separate recordings were made for each of the laser pulse patterns, with some neurons being recorded during all four patterns. **B**, Following recording, some neurons were juxtacellularly labelled with Neurobiotin (Nb; top left) and their immunohistochemical identity and anatomical location determined. Localisation of tyrosine hydroxylase (TH; top right) and green fluorescent protein (GFP; bottom left) confirmed the neuron was dopaminergic and expressed ArchT. These juxtacellularly-labelled neurons were designated ‘juxta-identified’ so as to differentiate them from those that were not labelled but were suppressed by local light delivery, designated ‘opto-identified’. Scale bar, 20 μ m.

7.3.2 Behavioural experiments

For CPP and real-time place preference (RTPP) control experiments, naive C57Bl/6J mice were used ($n = 7$ (1 male and 6 females) and $n = 9$ (7 males and 2 females), respectively). For the optogenetic behavioural experiments, mice in the GFP control group were injected with AAV2-CAG-FLEX-GFP (250 ms pulse experiments: $n = 6$, 3 males and 3 females; 2 s pulse experiments: $n = 3$, 1 male and 2 females) and mice in the ArchT test group were injected with AAV2-CAG-FLEX-ArchT-GFP (250 ms

pulse experiments: $n = 7$, 2 males and 5 females; 2 s pulse experiments: $n = 4$, 1 male and 3 females). Following this, mice were implanted bilaterally with 200 μm diameter optic fibres (10° angle in the mediolateral axis) positioned above the VTA in both ArchT test and GFP control groups. These mice were subjected to both CPP and RTPP behavioural paradigms (see Materials and Methods Section 2.6 for more details). Data are reported as mean (standard deviation (SD)), mean $\pm$ standard error of the mean (SEM) or median (interquartile range (IQR) = Q_1 – Q_3) unless otherwise stated.

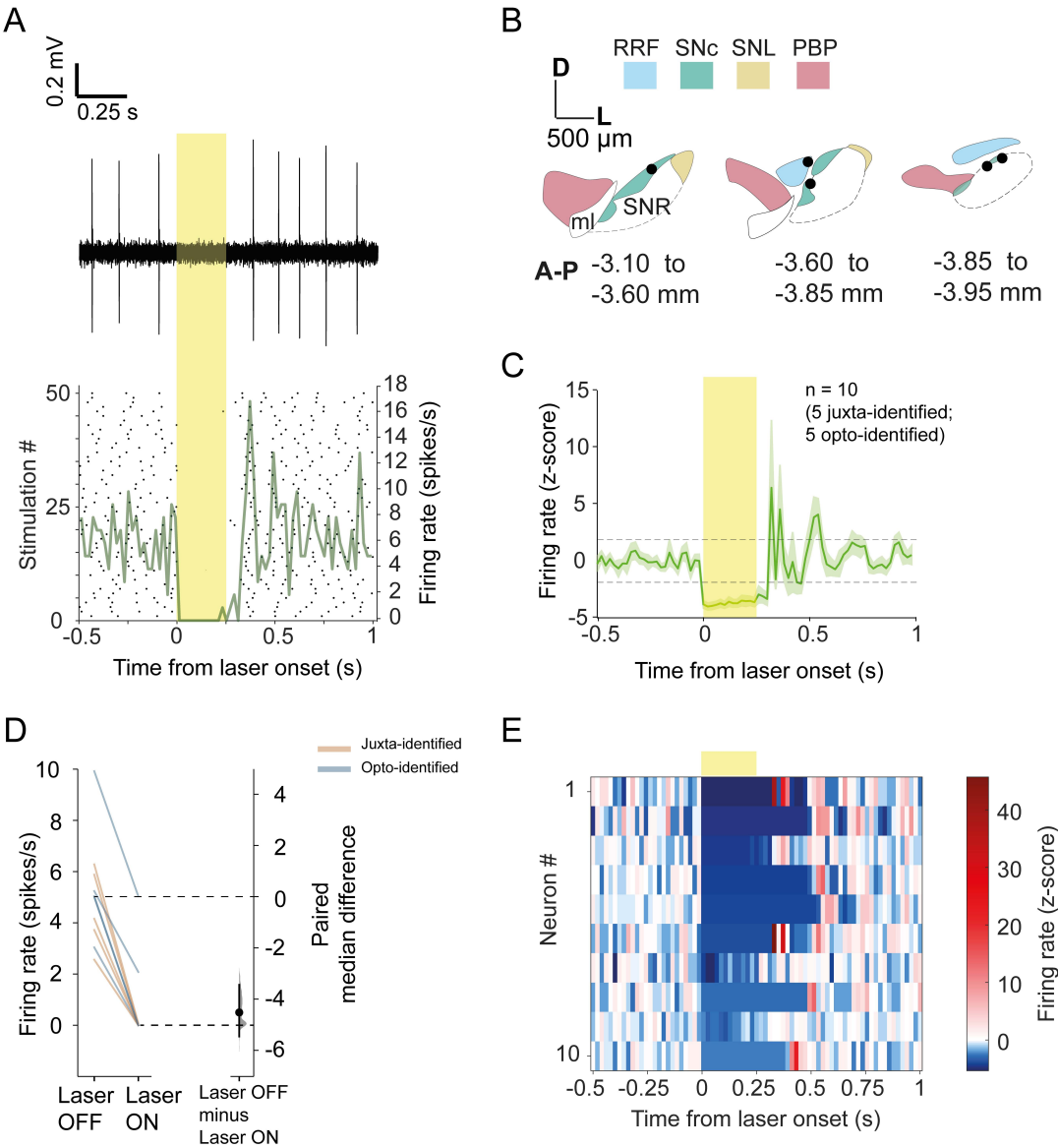
7.4 Results

As I demonstrated in Chapter 6, noxious electrical stimulation applied to the hindpaw of anaesthetised mice induces a pause in the firing of midbrain dopaminergic neurons lasting ~ 220 ms. The primary aim of work in this Chapter was to determine whether optogenetically mimicking this pause is sufficient to induce place aversion. Previous work has demonstrated that optogenetic inhibition of VTA (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019) and SNc (Ilango et al., 2014) dopaminergic neurons induces place aversion. In these studies, the duration of the laser pulses was in the order of seconds (up to 30 s), based on a RTPP experimental design in which the laser was ON for the entire time the mouse was in the laser-paired chamber. Thus, suppressing the firing of midbrain dopaminergic neurons with physiologically-relevant laser parameters has not been assessed in place preference paradigms. Firstly, I wanted to electrophysiologically validate the effects of laser parameters to be used for behavioural experiments.

7.4.1 Electrophysiological experiments: brief (250 ms), physiologically-relevant laser pulses reliably suppresses the firing of midbrain dopaminergic neurons

Applying brief (250 ms) laser pulses every 2 s reliably suppressed the firing of responsive (i.e. ArchT-transduced) midbrain dopaminergic neurons (Figure 7.2A). Half of the recorded neurons (5 of 10) were successfully labelled using the juxtacellular method and found to be dopaminergic and

located in the RRF ($n = 1$) or SNc ($n = 4$) (Figure 7.2B). On average, the firing rate of responsive dopaminergic neurons (juxta-identified and opto-identified) was suppressed for the duration of the laser pulse, followed by rebound firing at laser offset (Figure 7.2C). Most responsive neurons (8/10) were completely silenced by the 250 ms laser pulses and the remaining neurons displayed a significant reduction in firing rate during the laser ON period as compared to baseline (Figure 7.2D, E). Although the reduction in firing rate during the laser ON period was fairly consistent across neurons, the firing dynamics after laser offset were more heterogeneous (Figure 7.2E). The mean duration of suppression following laser onset was 432.2 (100) ms, meaning that resumption of firing did not occur for ~ 182 ms after laser offset. It is clear that the temporal control over the firing was not as precise as desired, in that ideally the firing rate would resume within tens of milliseconds of the laser pulse ending.

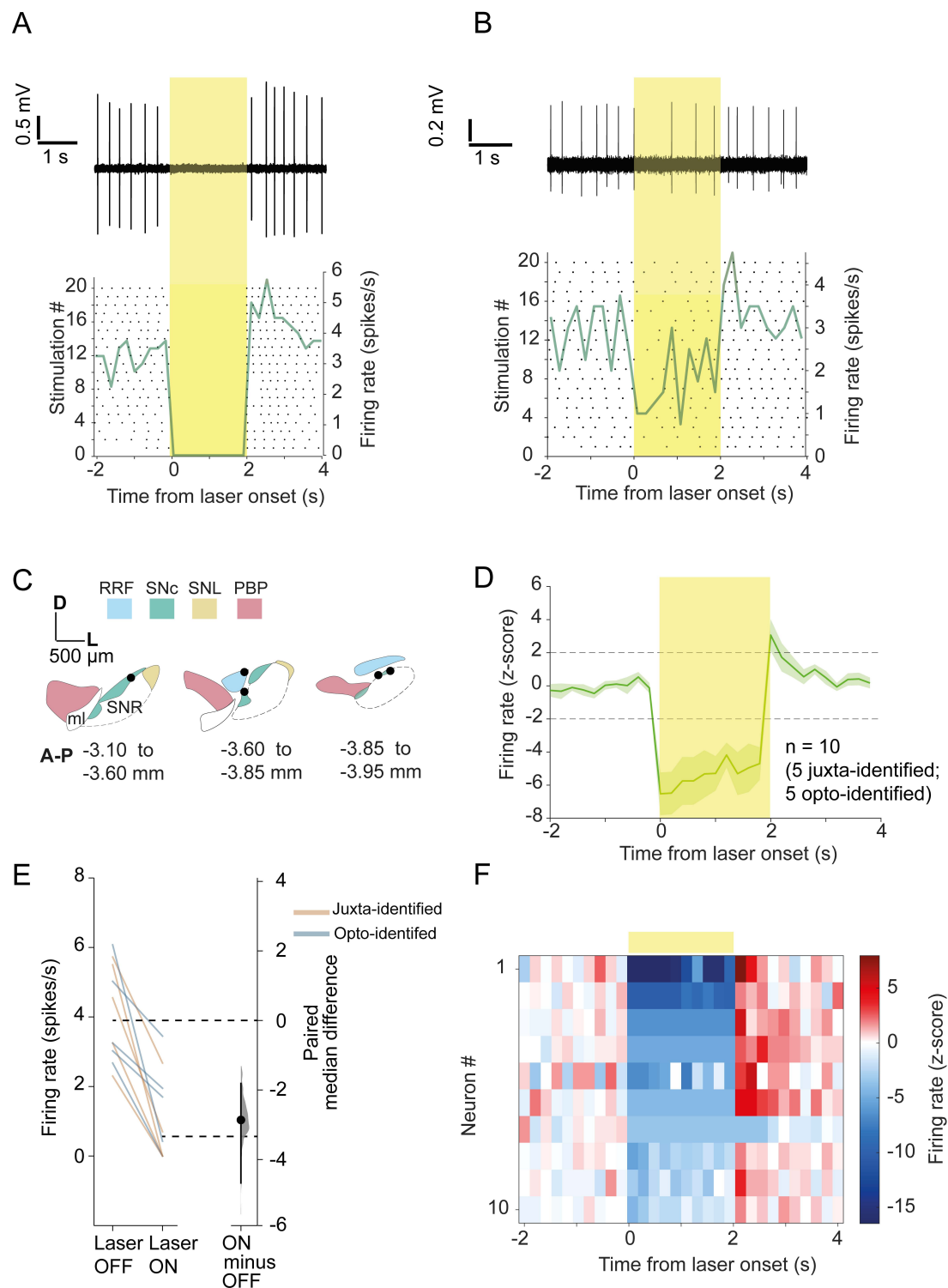


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Figure 7.2: 250 ms laser pulses silenced most ArchT-transduced midbrain dopaminergic neurons. **A**, Example single-unit spiking activity of a dopaminergic neuron (top) that was silenced during light delivery (one trial example). The neuron was effectively silenced across trials, as shown by the overlaid raster and peri-stimulus histogram (PSTH) (bottom). **B**, Coronal mouse midbrain schematics showing the locations of juxta-identified dopaminergic neurons (i.e. TH and ArchT-GFP expressing; filled black circles) that were recorded during 250 ms laser pulses ($n = 5$ neurons). **C**, The firing activity of juxta-identified ($n = 5$) and opto-identified ($n = 5$) dopaminergic neurons was pooled and normalised. PSTHs show that the firing rate was transiently and significantly suppressed by local light delivery, followed by multiphasic rebound activation after laser offset. Dashed lines are positioned at z-scores ± 1.96 . **D**, The firing rate of responsive neurons was strongly suppressed during laser ON. The mean firing rates in the laser OFF (-500–0 ms) and laser ON (0–250 ms) periods are plotted on the left axes for each neuron in **C**; each paired set of observations is connected by a line. The horizontal dashed lines depict the median of the laser OFF (top) and laser ON groups (bottom). On the right axes, the paired median differences are plotted as a bootstrap resampling distribution. The median difference is depicted as a filled black circle with 95% confidence intervals (CIs) indicated by the ends of the vertical bars (Laser ON minus Laser OFF: -4.52 [95% CI: -5.46, -3.44]). $N = 10$ neurons. **E**, A heat map of the trial-averaged normalised firing rates of all neurons in **C** and **D**. The shaded yellow area in **A**, **C** and **E** represents the laser ON period. A-P, anterior-posterior distance from Bregma; D, dorsal; L, lateral; ml, medial lemniscus; PBP, parabrachial pigmented nucleus of the VTA; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticulata.

7.4.2 Electrophysiological experiments: longer laser pulses less reliably suppress the firing of midbrain dopaminergic neurons

When I was designing the optogenetic experiments, it was foreseeable that inducing brief pauses in the firing of midbrain dopaminergic neurons would be insufficient to drive place aversion. This scenario would require experiments with longer pauses to potentially reveal a behavioural effect, in order to ascertain that pauses in firing were indeed insufficient to induce place aversion. This rationale is based on previous work in which long duration (seconds) laser pulses were shown to induce place aversion (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019). In the previous work, many of the place preference paradigms were based on RTPP, in that the animal could move freely from the laser ON chamber to the laser OFF chamber. This meant that the laser pulse duration was not defined by the experimenter but rather by how long the mouse spent in the laser ON chamber at a time, with a maximum pulse duration of 30 s (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Krol et al., 2019). Before carrying out such experiments in the present study, it was deemed crucial to electrophysiologically verify the effects of laser pulses longer than 250 ms. In light of previous work (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019), I selected three ‘long’ pulse durations of 2, 10 and 30 s for electrophysiological validation.



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Figure 7.3: 2 s laser pulses silenced or suppressed ArchT-transduced midbrain dopaminergic neurons. **A, B,** Example single-unit spiking activity of silenced (**A**) and suppressed (**B**) midbrain dopaminergic neurons during light delivery in one trial (top), along with raster plots overlaid with trial-averaged PSTH graphs (bottom). **C,** Coronal mouse midbrain schematics showing the locations of juxta-identified dopaminergic neurons (i.e. TH and ArchT expressing; filled black circles) that were recorded during 2 s local light delivery ($n = 5$ neurons). **D,** Juxta-identified ($n = 5$) and opto-identified ($n = 5$) dopaminergic neurons were pooled and their firing activity normalised. PSTHs show that the firing rate was transiently and significantly suppressed by local light delivery, followed by brief rebound activation after laser offset. Dashed lines are positioned at z -scores ± 1.96 . **E,** The firing rate of responsive neurons was strongly suppressed during laser ON. The mean firing rates in the laser OFF (-2 – 0 s) and laser ON (0 – 2 s) periods are plotted on the left axes for neurons in **D** (Laser ON minus Laser OFF: -2.86 [95% CI: -4.68 , -1.81]). $N = 10$ neurons. **F,** Heat map of trial-averaged normalised firing rates of all neurons in **D** and **E**. The shaded yellow area in **A, B, D** and **F** represents the laser ON period.

Delivery of 2 s laser pulses resulted in two main response types: silencing (Figure 7.3A) or suppression of firing (Figure 7.3B). Of the ten recorded neurons, five were juxta-identified (Figure 7.3C) and five were opto-identified. As a population, the firing of dopaminergic neurons was strongly suppressed by the laser followed by rebound activation at laser offset (Figure 7.3D). However, the strength of this suppression waned towards the end of the laser ON period, perhaps reflecting a weakening of the opsin-dependent suppression. The effect of 2 s laser pulses on the firing of dopaminergic neurons was fairly heterogeneous, with some neurons silenced ($n = 4$) and others suppressed ($n = 6$) to varying degrees (Figure 7.3E). Following laser offset, firing resumed with a median delay of 52.5 ms (IQR = 17.5–240 ms), and for most neurons there was a rebound increase in firing rate after laser offset (Figure 7.3F).

The observation that some responsive neurons were suppressed but not fully silenced by 2 s laser pulses could be because the locally-delivered light was not powerful enough (Figure 7.3B, E, F). To determine if this was the case, the laser power was increased from 5 to 10 mW. The response to 5 (Figure 7.4A) and 10 mW (Figure 7.4B) laser pulses was broadly similar: increasing the laser power did not lead to more neurons being silenced. Note, Figure 7.3D and E are replicated in Figure 7.4A for easier side-by-side comparison to 10 mW laser power (Figure 7.4B). A subgroup of these neurons were recorded during both 5 and 10 mW laser pulses (20 simulations at 5 mW, then 10–20 simulations at 10 mW), allowing the response profile to be compared in a within-neuron manner. This analysis demonstrated that increasing the laser power did lead to a stronger suppression of firing (Figure

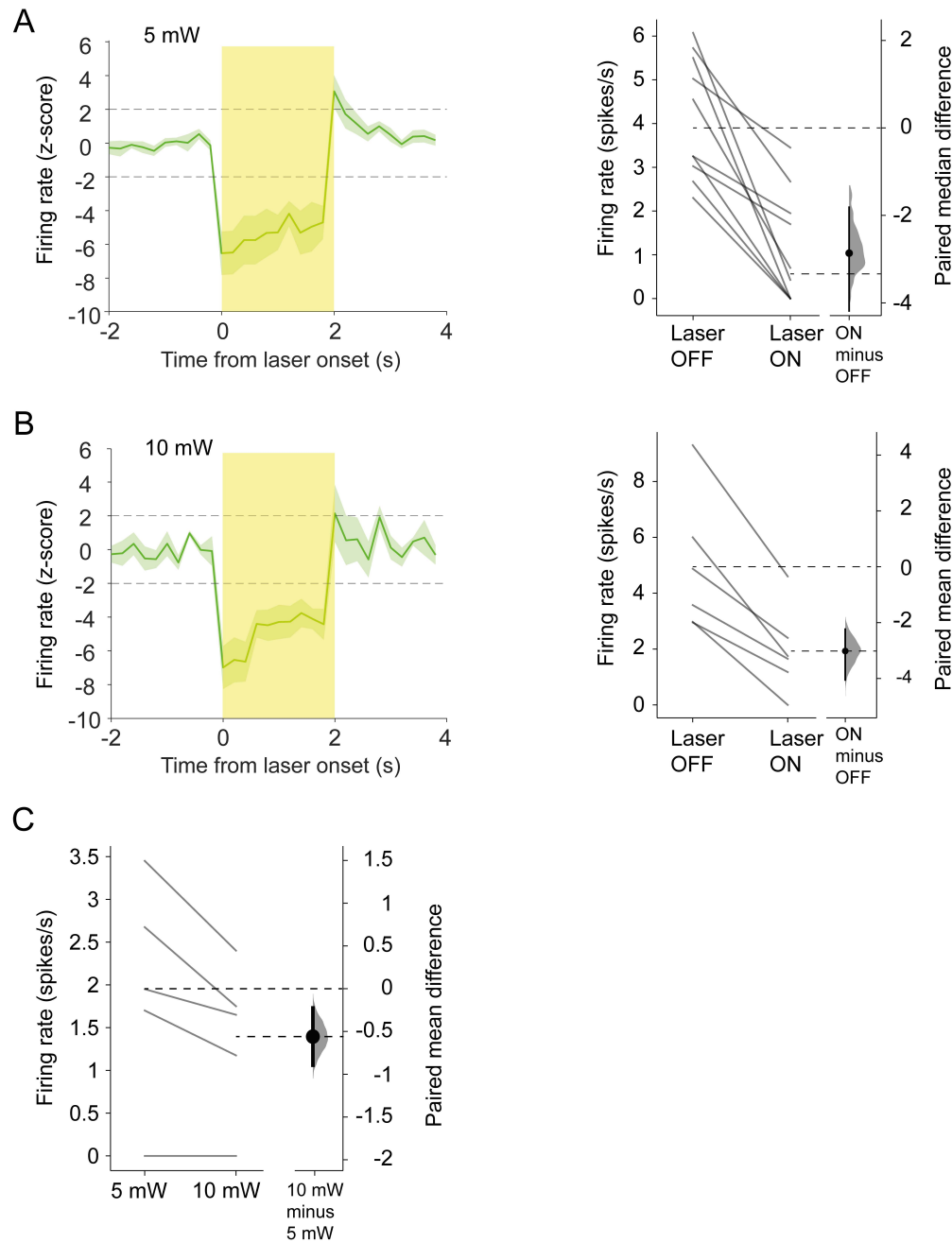


Figure 7.4: Comparison of midbrain dopaminergic firing during 5 and 10 mW laser pulses. **A, B, Left:** Responsive dopaminergic neurons were pooled and their firing activity normalised. PSTHs show that the firing rate was transiently and significantly suppressed by local light delivery at 5 (**A**; $n = 10$ neurons) and 10 mW (**B**; $n = 6$ neurons), followed by brief rebound activation. Dashed lines are positioned at z -scores ± 1.96 . **Right:** The firing rate of responsive neurons was suppressed during laser ON. On the left axes, the mean firing rates in the laser OFF (-2-0 s) and laser ON (0-2 s) periods are plotted for neurons in corresponding PSTH graphs (**A**, Laser ON minus Laser OFF: -2.86 [95% CI: -4.68, -1.81]; **B**, Laser ON minus Laser OFF: -3.02 [95% CI: -4.05, -2.23]). **C,** Responsive dopaminergic neurons were more strongly suppressed by 10 mW compared with 5 mW laser power ($n = 4$). On the left axes, the mean firing rates in the laser ON (0-2 s) period are plotted for 5 and 10 mW laser power (10 mW minus 5 mW: -0.56 [95% CI: -0.90, -0.23]). The shaded yellow area in **A** and **B** represents the laser ON period.

7.4C), though no additional neurons were fully silenced by 10 mW pulses. I was reluctant to use even higher laser power due to the concern that this would lead to substantial tissue heating and potential

opsin-independent effects on firing activity, particularly with longer pulses (Stujenske et al., 2015).

Only three neurons were recorded during 10 s laser pulses (Figure 7.5), all of which were juxta-identified (Figure 7.5A). Similar to the response to 2 laser pulses, delivering 10s laser pulses silenced two neurons (Figure 7.5B, C, E) and suppressed another (Figure 7.5D, E). In the case of the suppressed neuron (Figure 7.5D), firing activity was initially silenced during the laser ON period before resuming at a slow rate. Following laser offset, two neurons displayed a significant increase in firing rate during the rebound period (Figure 7.5B, C), and one neuron resumed firing at a similar rate to baseline in the rebound period (Figure 7.5D).

The final pulse duration used was 30 s, chosen in line with the maximum laser pulse duration used in previous RTPP studies in which VTA dopaminergic neurons were optogenetically inhibited (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Krol et al., 2019). Applying 30 s laser pulses silenced (Figure 7.6A) or suppressed (Figure 7.6B) the firing of most responsive neurons. When the firing activity of the neurons was pooled, the average response profile was a significant suppression of firing rate throughout the laser pulse followed by a significant increase in firing immediately after laser offset (Figure 7.6C–E). Of these recorded neurons ($n = 6$), 4 were juxta-identified and found to be located in the SNc and rostral RRF (Figure 7.6F).

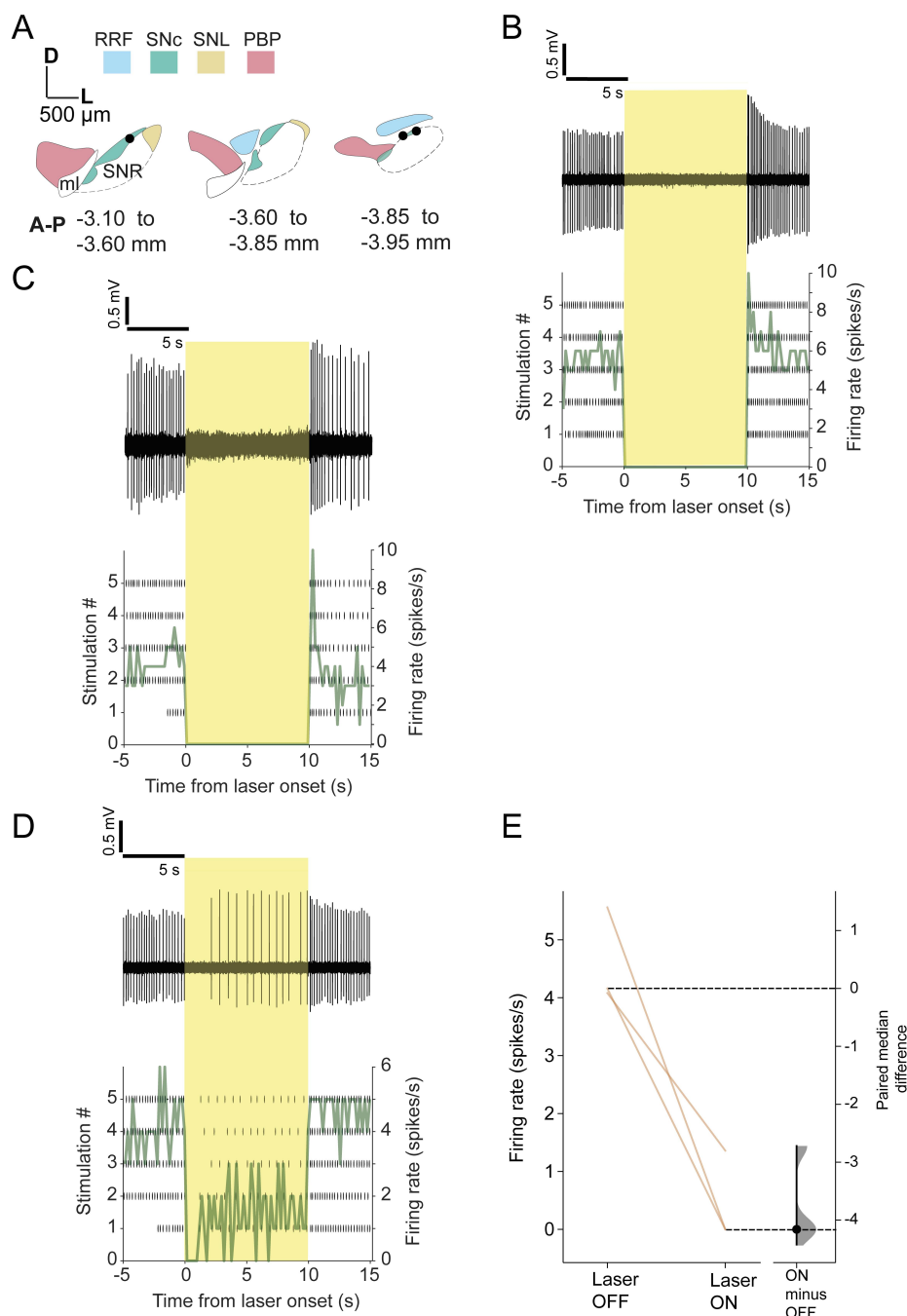


Figure 7.5: Single-unit spiking activity of three ArchT-transduced midbrain dopaminergic neurons in response to 10 s laser pulses. **A**, Coronal mouse midbrain schematics showing the locations of the juxta-identified dopaminergic neurons (i.e. TH and ArchT expressing; $n = 3$ neurons). All neurons in **B–D** were juxtacellularly-labelled. **B–D**, Example spiking activity (top), and raster plots overlaid with trial-averaged PSTH graphs (bottom). These were the response profiles of two silenced (**B, C**) and one suppressed (**D**) dopaminergic neuron. Note: the first trial of **C** and **D** (bottom row of each raster) shows a missing period before the first laser pulse was applied. This is because the light was triggered less than 5 s after the start of the recording file, hence some pre-stimulation spikes were missed for the first trial. **E**, The firing rate of responsive neurons was strongly suppressed during laser ON. The mean firing rates in the laser OFF (–5–0 s) and laser ON (0–10 s) periods are plotted on the left axes for neurons in **B–D** (Laser ON minus Laser OFF: -4.16 [95% CI: -5.56 , -2.72]). The shaded yellow area in **B, C** and **D** represents the laser ON period.

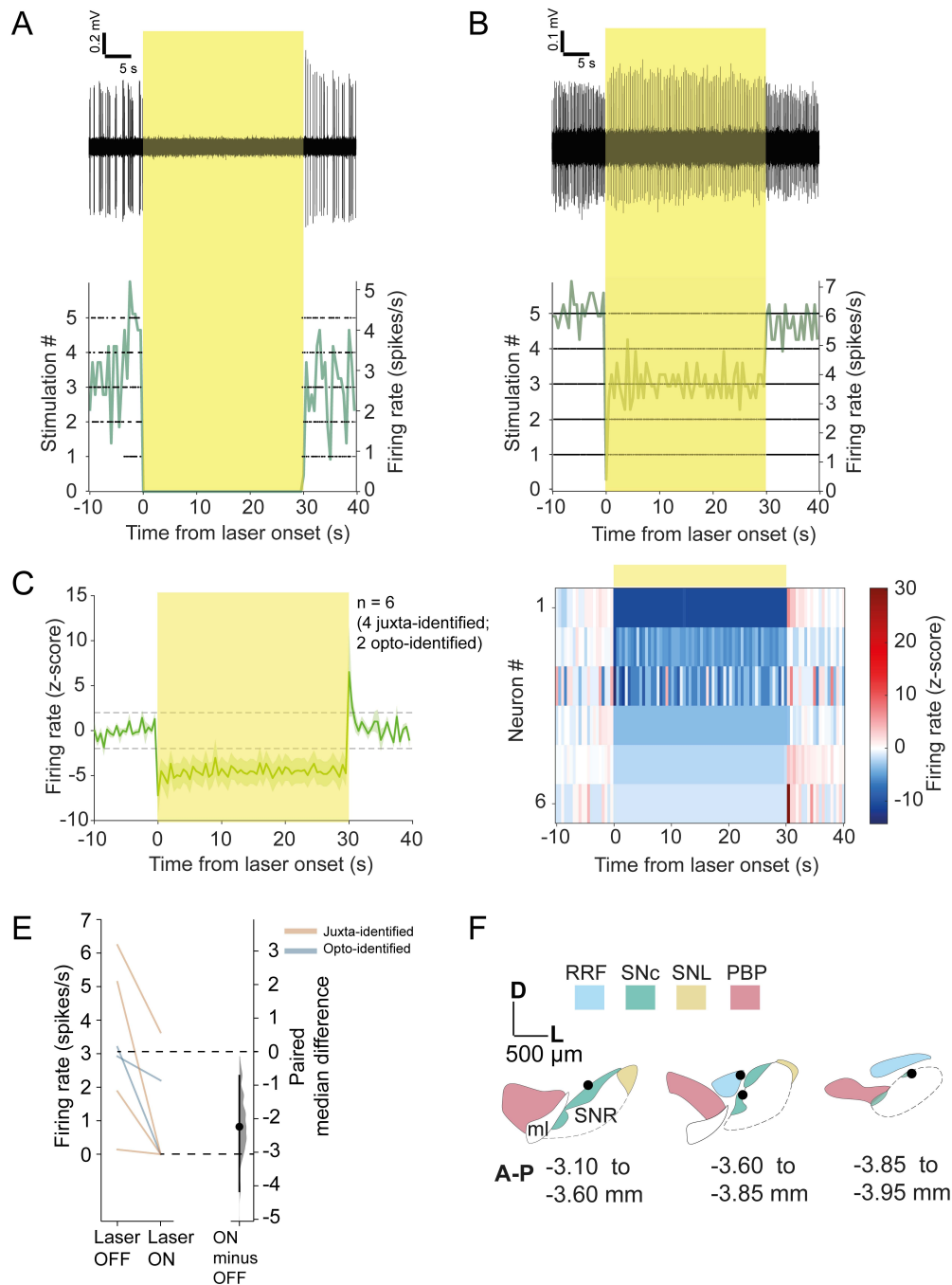


Figure 7.6: The firing rate of most ArchT-transduced midbrain dopaminergic neurons was silenced or suppressed by 30 s laser pulses. **A, B,** Example single-unit spiking activity in one trial (top) and raster plots overlaid with trial-averaged PSTHs (bottom) of midbrain dopaminergic neurons displaying time-locked silencing (**E**) or suppression of firing (**F**) during local light delivery. Note: the first trial of **A** (bottom row of raster) shows a missing period before the first laser pulse was applied. This is because the light was triggered less than 10 s after the start of the recording file, hence some pre-stimulation spikes were missed for the first trial. **C,** PSTH of the normalised firing rate of juxta-identified (n = 4) and opto-identified (n = 2) dopaminergic neurons. The firing rate was transiently and significantly suppressed by local light delivery, followed by brief rebound activation at laser offset. Dashed lines are positioned at z-scores ± 1.96 . **D** A heat map of trial-averaged normalised firing rates of all neurons in **C**. **E,** The firing rate of responsive neurons was suppressed during laser ON. The mean firing rates in the laser OFF (-10–0 s) and laser ON (0–30 s) periods are plotted on the left axes for neurons in **C** and **D** (Laser ON minus Laser OFF: -2.26 [95% CI: $-4.47, -0.72$]). N = 6 neurons. **F,** Coronal mouse midbrain schematics showing the locations of juxta-identified (i.e. TH and ArchT expressing; filled black circles) that were silenced or suppressed by 30 s laser pulses (n = 4). The shaded yellow area in **A–D** represents the laser ON period.

In addition to these ‘expected’ response profiles, there were also neurons with very different response dynamics. One slow-firing dopaminergic neuron was silenced by the laser but did not resume firing until ~ 15 s after laser offset (Figure 7.7A). This neuron was juxta-identified and found to be located in the SNc (Figure 7.7A). A different neuron was briefly (1 s) silenced by the laser, before displaying an elevated firing rate for the rest of the 30 s pulse, followed by a brief increase in firing rate at rebound after laser offset (Figure 7.7B).

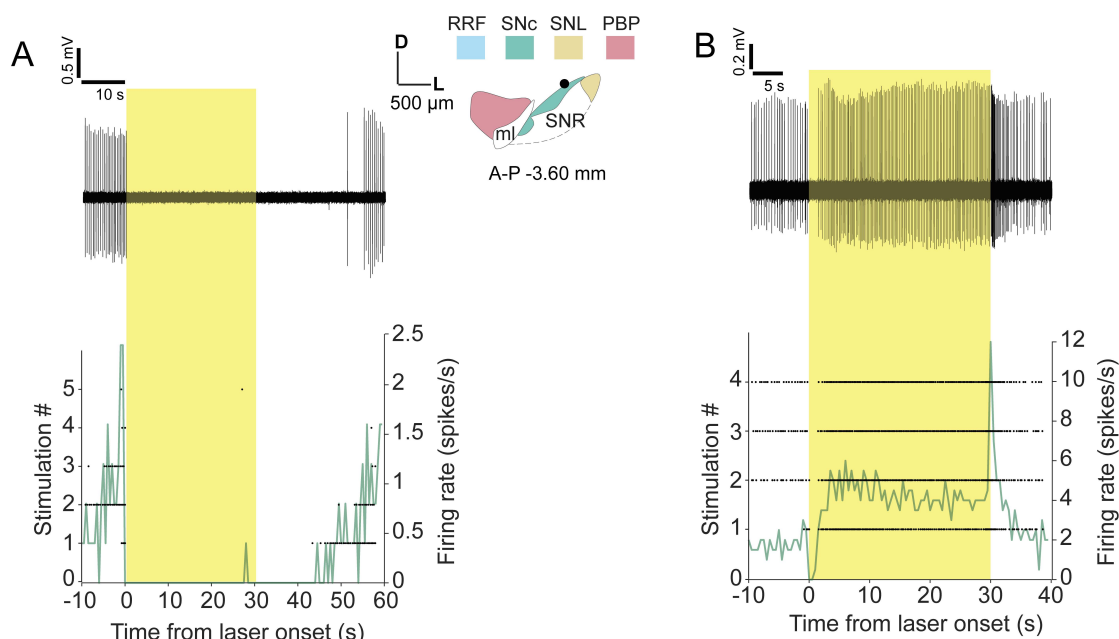


Figure 7.7: Some neurons displayed atypical responses to 30 s laser pulses. Example single-unit spiking activity in one trial (top) and raster plots overlaid with trial-averaged PSTHs (middle) during local light delivery. **A**, This juxta-identified neuron (coronal schematic, bottom) was slow to recover firing after the laser ON period. **B**, This opto-identified neuron was briefly silenced by the laser pulse, before displaying an elevated firing rate, followed by a sharp increase in firing rate after laser offset. Note: the first trial of **A** and **B** (bottom row of raster) shows a missing period before the first laser pulse was applied. This is because the light was triggered less than 10 s after the start of the recording file, hence some pre-stimulation spikes were missed for the first trial. The yellow shaded area represents the laser ON period.

The atypical elevated firing rate displayed by the neuron in Figure 7.7B during the laser ON period could be explained by opsin-independent effects such as tissue heating (Stujenske et al., 2015). It was difficult to infer opsin-independent effects for this neuron because the initial period of silencing would suggest it was transduced with ArchT. Another neuron that did not respond to the laser—and was thus considered to be not transduced with ArchT—did not display an elevation in firing rate following laser onset (Figure 7.8). This neuron was putatively dopaminergic as it had a threshold-to-trough action potential (AP) waveform of 1.24 ms and a baseline firing rate of 4.2

spikes/s (Ungless and Grace, 2012). Overall, atypical responses to the optogenetic manipulation were uncommon, with only two recorded neurons (Figure 7.7) displaying aberrant responses. Nevertheless, further recordings of transduced and non-transduced neurons during local light delivery are needed to strengthen this conclusion.

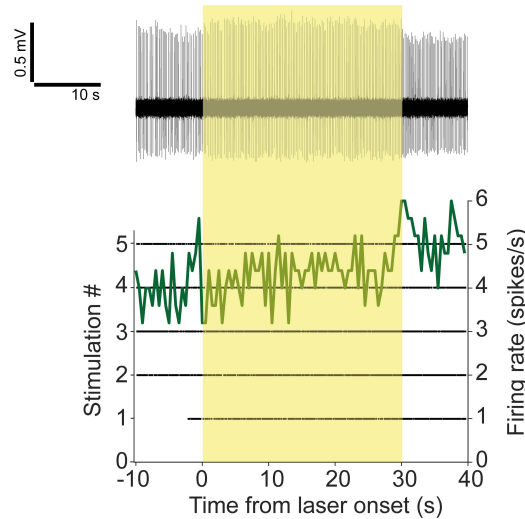


Figure 7.8: The firing activity of a single non-responsive neuron during 30 s laser pulses. Example single-unit spiking activity in one trial (top) and raster plot overlaid with the trial-averaged PSTH (bottom) during local light delivery. Note: the first trial (bottom row of raster) shows a missing period before the first laser pulse was applied. This is because the light was triggered less than 10 s after the start of the recording file, hence some pre-stimulation spikes were missed for the first trial. The yellow shaded area represents the laser ON period.

In summary, the electrophysiological recording experiments demonstrated that brief (250–500 ms), physiologically-relevant pauses could be induced in ArchT-transduced midbrain dopaminergic neurons. Additionally, the firing rate of responsive neurons was suppressed by longer laser pulses (2, 10 and 30 s). However, 30 s laser pulses induced aberrant activity in a subset of recorded neurons, suggesting a dissociation between stimulus parameters and electrophysiological responses in some neurons.

7.4.3 Place preference behaviour

Following the electrophysiology experiments, I next sought to determine whether optogenetically inhibiting VTA dopaminergic neurons affects place preference behaviours. Specifically, the sufficiency of the optogenetic manipulation to induce behavioural conditioning and contingent learning was assessed using the CPP paradigm and RTPP, respectively.

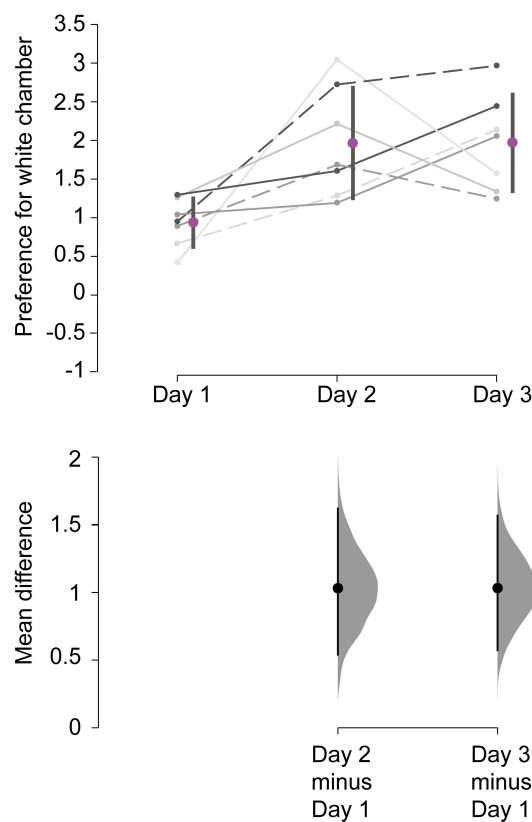
7.4.3.1 Mice have a baseline bias in a conditioned place preference paradigm

The typical experimental design for CPP experiments involves one pre-test session to establish baseline preference, two conditioning sessions, and a final post-test session to establish whether the optogenetic manipulation induces a change in place preference (Tsai et al., 2009; see Materials and Methods Section 2.6.2). If mice do not have a pre-test baseline preference for either conditioning chamber (unbias), then they are subject to an unbiased CPP protocol in which the delivery of laser pulses is paired with either chamber in a counterbalanced manner. If, however, mice do have a baseline preference for one chamber (a bias), then a biased protocol is carried out in which, depending on the experimental hypothesis, the stimulus is applied in the preferred or non-preferred chamber. For example, if the hypothesis is that the conditioned stimulus will induce place aversion (as in the present study), then the stimulus is applied in the preferred chamber to determine whether the hypothesised aversive quality of the stimulus can reverse this baseline preference (Prus et al., 2009).

In some early pilot optogenetic experiments (data not shown), I noticed that mice developed a place preference for the white chamber despite having no significant preference for either chamber during the pre-test session. This preference during the post-test session was irrespective of the chamber in which the optogenetic stimulus was delivered during conditioning. Assessing these data, it was hypothesised that the mice had developed a preference for the white chamber after repeated exposure to the behavioural setup. Initially it was suspected that uneven illumination within the CPP apparatus may have resulted in the observed bias because mice are innately averse to brightly illuminated environments. However, efforts to balance illumination across the chambers did not alter the baseline preference for the white chamber (see Materials and Methods Section 2.6.2).

To test the hypothesis that mice develop a preference for the white chamber, I carried out repeated pre-testing in a cohort of naive wild-type mice (Figure 7.9). As had been suggested in the pilot study, mice developed a preference for the white chamber after repeated exposure to the behavioural setup.

On the first pre-test day, mice did not generally have a preference for the white chamber (preference score: 0.94 ± 0.12). However, on subsequent pre-test days 2 and 3, the mice developed a preference for the white chamber. By the third day, the median difference was 1.10 (95% CI: 1.78, 0.31), meaning that on average the mice spent over twice as much time in the white chamber as they did in the black chamber. There was little change in preference for the white chamber between days 2 and 3, indicating the preference was relatively stable after two days. In light of these data, I decided to implement the biased CPP protocol with three days of pre-testing prior to the conditioning sessions (see Figure 2.3 in Materials and Methods). If the design had involved only one pre-test day then there could have been an interaction between the changing preference of the mice and the optogenetic manipulation, confounding the interpretation of results. However, by measuring preference on three pre-test days the baseline preference of the mice could be factored into the experimental design, reducing any such interaction.



(Figure legend on next page)

Figure 7.9: Mice develop a baseline preference for one (white) chamber in the CPP paradigm. The preference for the white chamber was calculated as the time spent in the white chamber divided by the time spent in the black chamber; a preference value of greater than 1 indicates more time spent in the white chamber, whereas a value of less than 1 indicates more time spent in the black chamber. On the upper axes, each line represents the change in preference for the white chamber for a mouse over the three sessions ($n = 7$ mice); the ends of the thick vertical bars represent the SD and the magenta dot represents the mean group preference for each time point. On the lower axes, mean differences are plotted as bootstrap resampling distributions (Day 2 minus Day 1: 1.03 [95% CI: 0.54, 1.62]; Day 3 minus Day 1: 1.03 [95% CI: 0.57, 1.57]). The median differences are represented by the filled black circles, and the 95% CI by the ends of the black bars above and below.

7.4.3.2 Optogenetically suppressing the firing of VTA dopaminergic neurons does not induce conditioned place aversion

The aim of the optogenetic behavioural experiments was to target viral transduction to VTA dopaminergic neurons. Although many neurons in the VTA were transduced, there was also spread to medial (and in some cases lateral) SNc in most mice (Figure 7.10A). This means that although optic fibres were targeted to the VTA, the spread of viral transduction and delivery of light probably resulted in some SNc dopaminergic neurons being inhibited during behavioural experiments in the ArchT test group. For brevity, the anatomical target will be referred to as 'VTA' in the following sections, but it does not mean this was the exclusive target of optogenetic manipulations. The midbrain of each mouse in the GFP control and ArchT test groups was inspected and the transduction of dopaminergic neurons with GFP (Figure 7.10B) or ArchT (Figure 7.10C) was confirmed using immunohistochemistry.

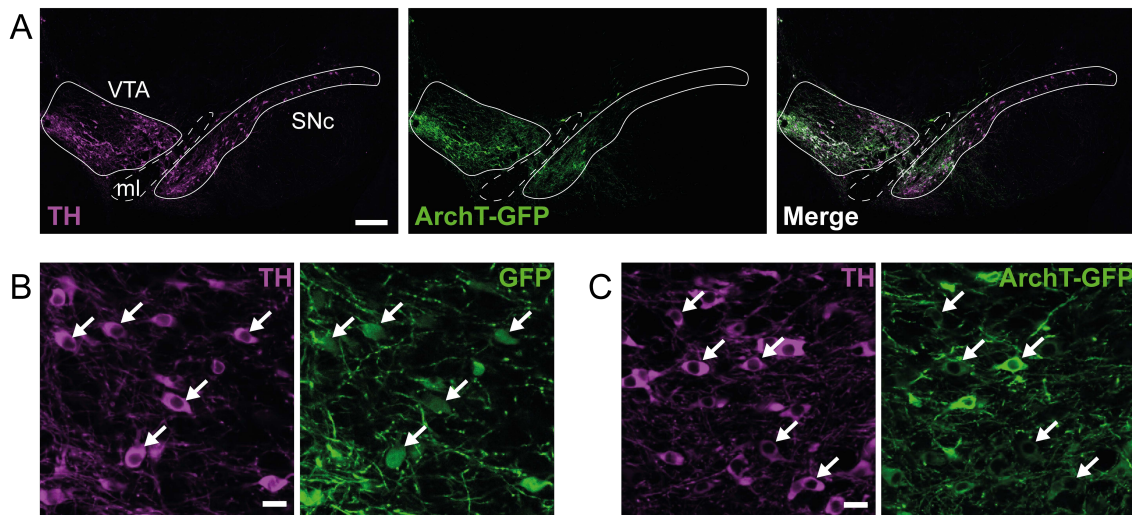


Figure 7.10: GFP and ArchT transduction in midbrain dopaminergic neurons. **A**, Spread of ArchT transduction. Although most ArchT-transduced neurons were located in the VTA, there was spread to SNc. Scale bar, 200 μm . **B**, **C**, Confocal micrographs of the VTA. Transduction with GFP (**B**) and ArchT (**C**) was mostly limited to dopaminergic neurons. Scale bars, 20 μm . ml, medial lemniscus.

As in the experiment with wild-type mice (Figure 7.9), the majority of mice in both the GFP control and ArchT test groups developed a preference for the white chamber after repeated pre-test sessions (Figure 7.11A). The optogenetic manipulation was applied on the conditioning day when the mouse was in the white chamber (biased CPP) because the hypothesis was that optogenetically inhibiting VTA dopaminergic neurons would be aversive. Working against the baseline preference of the mice in this way maximises the window for seeing an effect of the optogenetic manipulation (Bozarth, 1987). After behavioural testing, the preference for the conditioned chamber (white) was calculated by dividing the amount of time spent in the conditioned chamber in the post-test session by the amount of time spent there in the final pre-test session; a preference value of 1 indicates there was no change in the amount of time spent in the conditioned chamber, whereas a value of >1 indicates more time spent in the conditioned chamber in the post-test session and vice versa. The GFP control and ArchT test groups spent similar amounts of time in the conditioned chamber in the pre-test and post-test sessions (Figure 7.11B), meaning that brief (250 ms laser pulses every 3–7 s, 30 min conditioning session; see Materials and Methods Section 2.6.2) optogenetic inhibition of dopaminergic neurons did not change the preference for the white chamber.

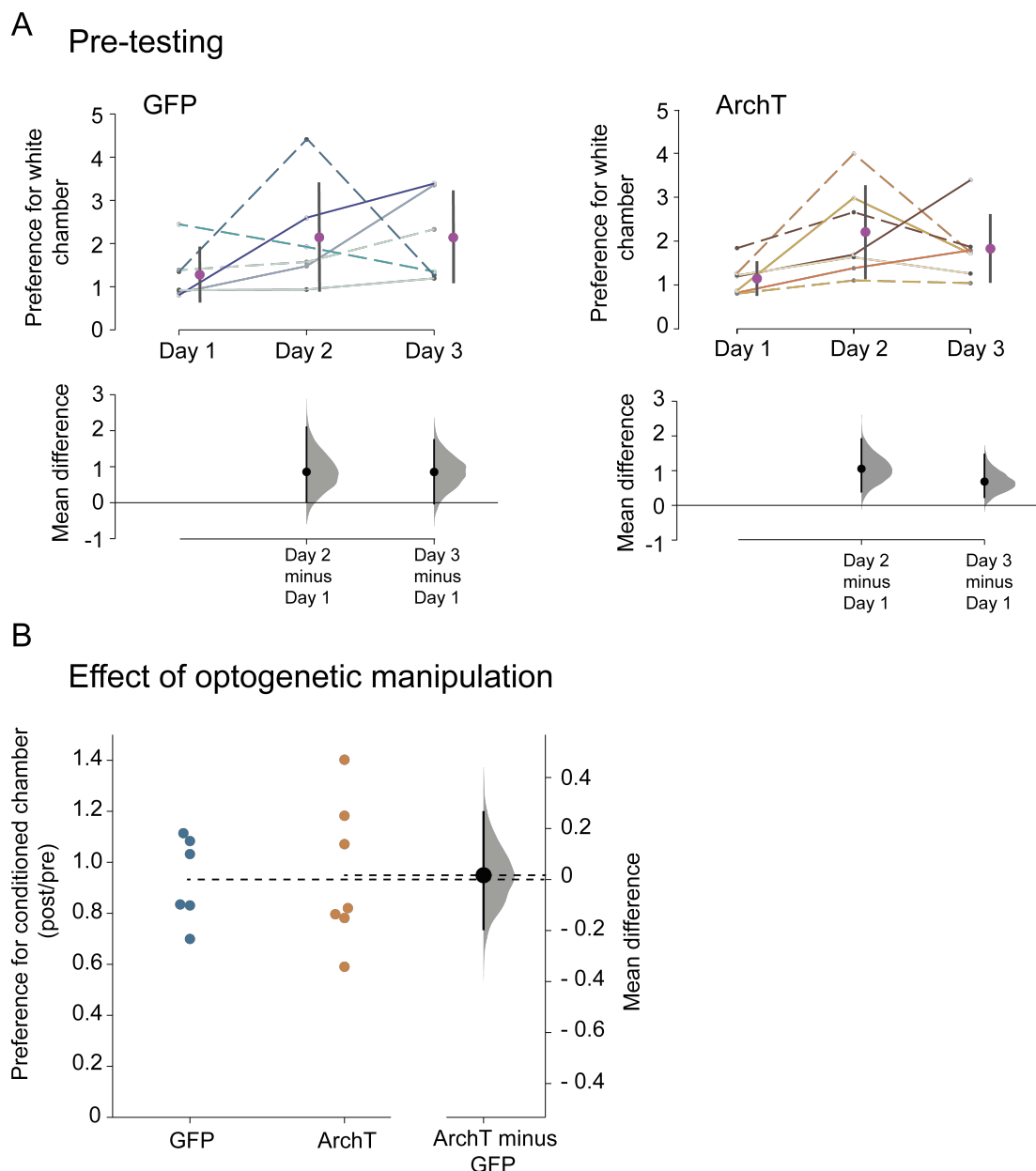


Figure 7.11: Briefly (250 ms) suppressing the firing of VTA dopaminergic neurons does not affect CPP behaviour. **A**, Both the GFP control ($n = 6$ mice; left) and ArchT test groups ($n = 7$ mice; right) developed a preference for the white chamber during pre-testing (GFP, Day 2 minus Day 1: 0.86 [95% CI: 0.02, 2.1], Day 3 minus Day 1: 0.85 [95% CI: -0.02, 1.74]; ArchT, Day 2 minus Day 1: 1.06 [95% CI: 0.3, 1.91], Day 3 minus Day 1: 0.69 [95% CI: 0.24, 1.47]). **B**, Optogenetically suppressing the firing of VTA dopaminergic neurons does not change the preference for the conditioned chamber (ArchT minus GFP: 0.02 [95% CI: -0.19, 0.26]).

Previous work has demonstrated that suppressing the firing of VTA dopaminergic neurons with longer pulses (seconds in duration) induces conditioned place aversion (Tan et al., 2012; Krol et al., 2019). To determine whether longer pulses would induce a behavioural effect not seen with the shorter (250 ms) pulses, I carried out the same protocol as in Figure 7.11 but with 2 s laser pulses (2 s pulse every 3–7 s, 40 min per conditioning session; see Materials and Methods Section 2.6.2). The

mice used for the experiments with 2 s laser pulses were a subset of mice used for the experiments with 250 ms laser pulses (Figure 7.11). After the 250 ms pulse experiments, there was a ‘washout’ period of 3 weeks before commencing experiments with 2 s pulses in the CPP paradigm (see Figure 2.4 in Materials and Methods Section 2.6.4).

During pre-testing, mice did develop a slight preference for the white chamber (Figure 7.12A) but this was less stable and pronounced than in the previous experiment (Figure 7.11A), and one mouse in fact developed a slight preference for the black chamber by Day 3 (Figure 7.12A). Nevertheless, a bias CPP protocol was implemented, with the laser pulses applied in whichever chamber the mouse spent most time in during the last day of pre-testing. Although there was a trend towards a reduced preference for the conditioned chamber in the ArchT group (Figure 7.12), the smaller group sizes in this experiment precluded any robust comparisons between the GFP control and ArchT test groups. This dataset will need to be expanded in future.

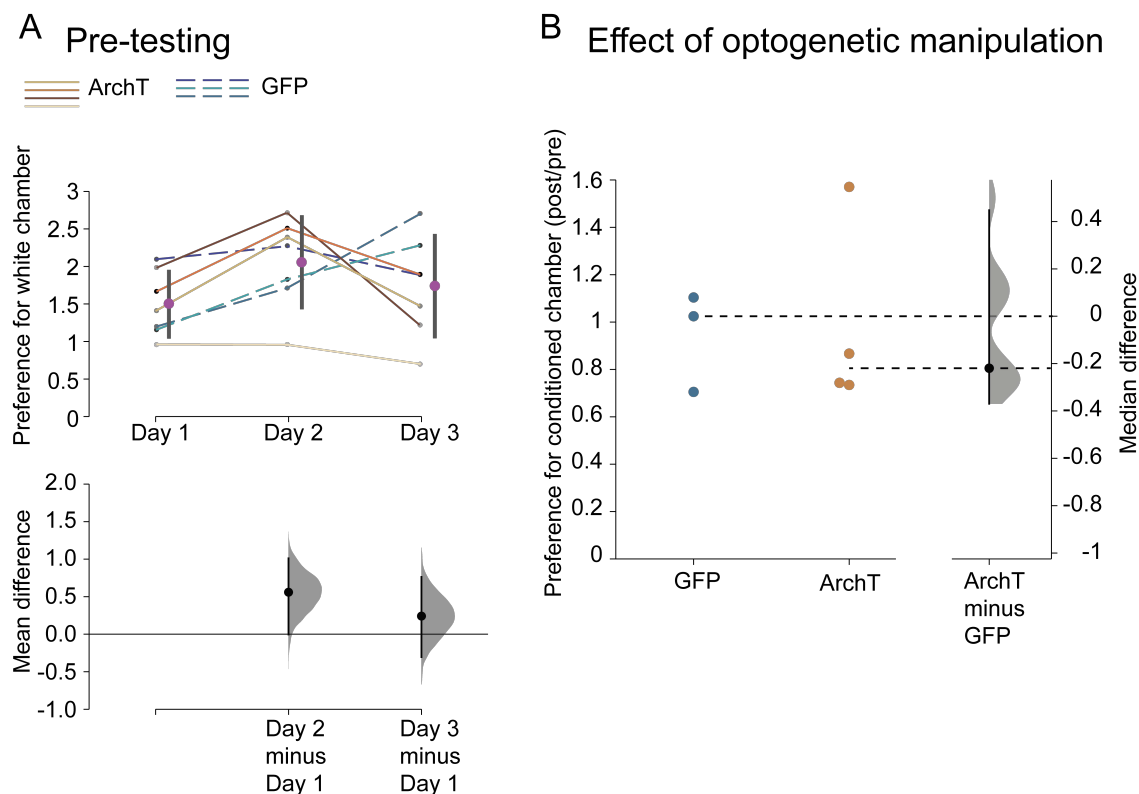


Figure 7.12: Suppressing the firing of VTA dopaminergic neurons for 2 s does not affect CPP behaviour. **A**, Most (6 of 7 mice) mice in the GFP control ($n = 3$ mice; dashed lines) and ArchT test groups ($n = 4$ mice; solid lines) developed a preference for the white chamber during pre-testing. One mouse in the ArchT group developed a preference for the black chamber (lowest, light grey line) on Day 3 (Day 2 minus Day 1: 0.56 [95% CI: 0, 1.01], Day 3 minus Day 1: 0.24 [95% CI: -0.3, 0.76]). **B**, Optogenetically suppressing the firing of VTA dopaminergic neurons does not change the preference for the conditioned chamber (ArchT minus GFP: -0.22 [95% CI: -0.37, 0.45]).

7.4.3.3 Optogenetically suppressing the firing of VTA dopaminergic neurons does not induce real-time place aversion

Pairing optogenetic suppression of VTA dopaminergic neurons with one of the chambers in the CPP protocol assessed whether pausing the firing of these neurons is sufficient to serve as an negative (aversive) reinforcement learning signal. Using a real-time place preference (RTPP) experimental design, I next sought to determine whether these pauses were sufficient to induce contingent learning. In the RTPP paradigm, the mouse could choose to leave the laser-paired context if the optogenetic manipulation was aversive. Before commencing optogenetic experiments, a cohort of naive wild-type mice were exposed to the RTPP apparatus for 15 min to determine if there was a side preference. The apparatus was an open-field arena divided into two equal sized

areas with identical features (white walls and floor) using a dividing wall through which the mouse could pass. In this experiment, some mice displayed a preference for one side of the open field, whereas others displayed no strong preference (Figure 7.13A). Overall, however, there was no consistent preference for one side compared with the other. It was therefore determined that counterbalancing would be sufficient to counteract the potential skewing effect any innate preference would have on the data.

Similar to the experiment with naive wild-type mice, a cohort of GFP control and ArchT test mice were exposed to the open field. However, in this experiment one side of the open field was paired with the delivery of laser pulses (counterbalanced across cohort). When a mouse entered the laser ON side, light delivery commenced according to the user-defined parameters; when the mouse exited the laser ON side, the light delivery was stopped. Similar to the CPP experiment, brief, physiologically-relevant laser pulses (250 ms pulses every 3–7 s; 15 min session) did not induce place aversion in the RTPP behavioural paradigm. Both the GFP control and ArchT test groups spent a similar amount of time in the laser ON and laser OFF sides (Figure 7.13B). If optogenetically inhibiting VTA dopaminergic neurons was perceived as aversive, then mice may seek to avoid the delivery of laser pulses by escaping to an environment in which the manipulation ceases (i.e. the laser OFF side of the open field). However, on entering the laser ON side and receiving the optogenetic manipulation, the mice in the ArchT test group were no more likely to U-turn (i.e. escape) than when they entered the laser OFF side (Figure 7.13C). In addition to these measures of aversion, the locomotor activity of GFP and ArchT mice did not differ when in the laser ON side (Figure 7.13D).

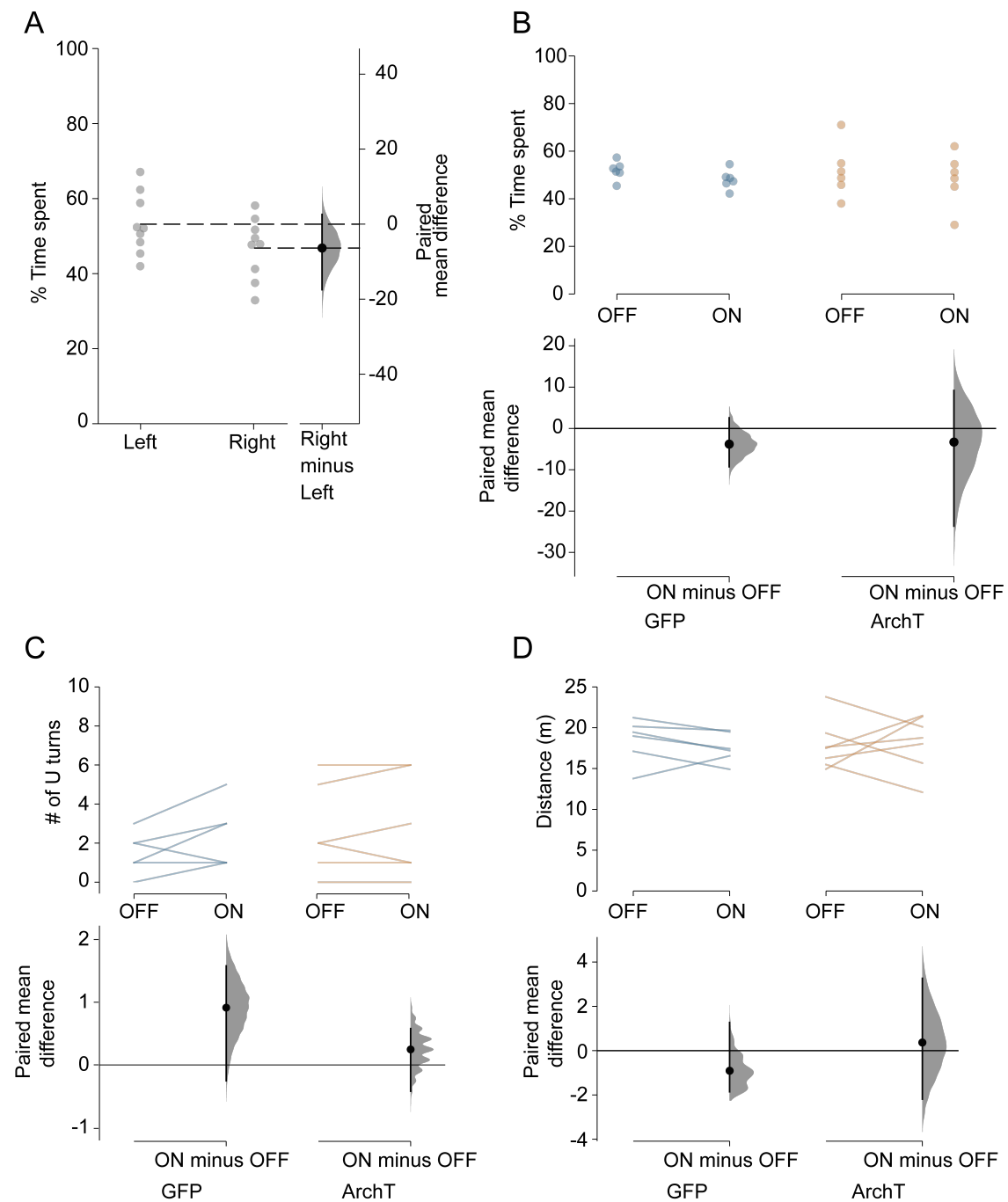


Figure 7.13: Briefly suppressing (250 ms pulses every 3–7 s) the firing of VTA dopaminergic neurons does not affect RTPP behaviour. **A**, Naive wild-type mice ($n = 9$) spend similar amounts of time in the left and right sides of the open field in a 15 minute session (Right minus Left: -6.37 [95% CI: $-17.49, 2.59$]). **B**, The GFP control (left; $n = 6$ mice) and ArchT test groups (right; $n = 7$ mice) spent similar amounts of time in the laser ON and laser OFF sides of the open field. The amount of time spent in the laser ON and laser OFF sides is plotted on the upper axes. On the lower axes, the paired mean differences are plotted as bootstrap resampling distributions. The mean differences are depicted as filled circles with 95% CIs indicated by the ends of the vertical bars (GFP, laser ON minus laser OFF: -3.79 [95% CI: $-9.27, 2.59$]; ArchT, laser ON minus laser OFF: -3.30 [95% CI: $-23.64, 9.21$]). **C**, The optogenetic manipulation did not cause mice to display escape behaviour (U-turn). A U-turn was defined as when a mouse left the respective chamber ≤ 1 s after entering (GFP, laser ON minus laser OFF: 0.83 [95% CI: $-0.33, 1.5$]; ArchT, laser ON minus laser OFF: 0.17 [95% CI: $-0.5, 0.5$]; $n = 6$ mice per group). **D**, The optogenetic manipulation did not affect overall locomotor activity (GFP, laser ON minus laser OFF: -0.91 [95% CI: $-1.86, 1.27$]; ArchT, laser ON minus laser OFF: 0.37 [95% CI: $-2.19, 3.27$]). $N = 6$ GFP and 7 ArchT mice. For **C** and **D**, each line represents one mouse.

Although the overall distance travelled by mice in the GFP control and ArchT test groups during the RTPP session was similar (Figure 7.13D), it was possible that the optogenetic inhibition of dopaminergic neurons would have a transient effect on movement that was not detected in general locomotor activity. To assess this, an accelerometer was attached to a subgroup of mice during the RTPP experiment. In this small subgroup, the optogenetic manipulation did not transiently alter ongoing movement in either the GFP control (Figure 7.14A) or ArchT test groups (Figure 7.14B).

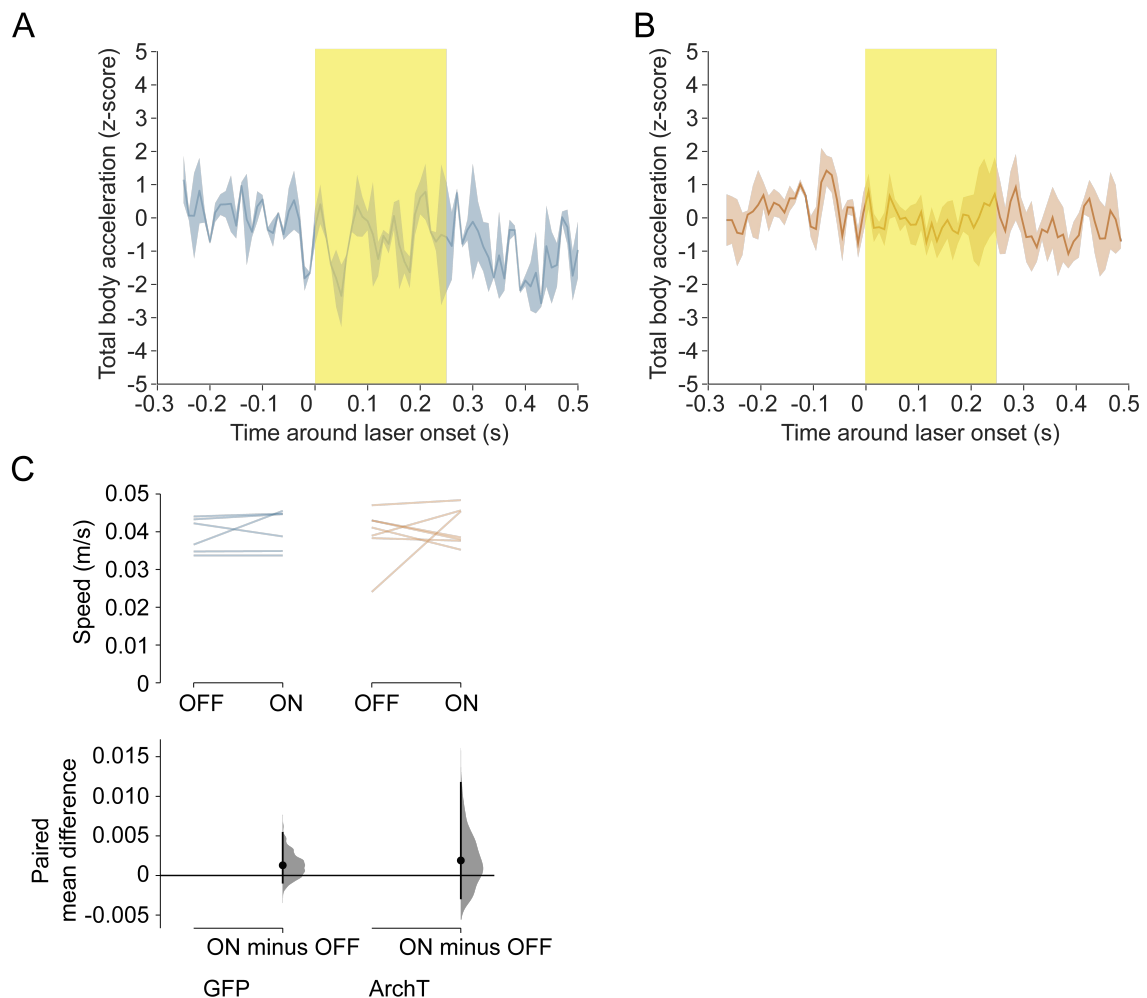


Figure 7.14: Briefly suppressing the firing of VTA dopaminergic neurons does not induce transient changes in ongoing movement. **A, B,** Delivering laser pulses did not induce transient changes in the ongoing activity of mice (**A:** *GFP*, $n = 2$ mice; **B:** *ArchT*, $n = 3$ mice). Data presented as mean $\pm$ SEM. The shaded yellow area in **A** and **B** represents the laser ON period. **C,** The average speed during the RTPP session was similar in the GFP control and ArchT test groups (*GFP*, laser ON minus laser OFF: 0.0013 [95% CI: -0.0009, 0.0054]; *ArchT*, laser ON minus laser OFF: 0.0019 [95% CI: -0.0029, 0.012]; $n = 6$ and 7 mice, respectively).

Four weeks after RTPP experiments in which 250 ms laser pulses were applied, a subset of mice were taken forward for experiments with longer laser pulses (see Figure 2.4 in Materials and Methods

Section 2.6.4). Similar to the CPP experiment above, applying longer laser pulses (2 s pulse every 3–7 s; 20 min session) did not result in a pronounced effect in the ArchT test group (Figure 7.15): both the GFP control and ArchT test groups spent similar amounts of time in the ON and OFF chambers. However, as was the case for the CPP experiment (Figure 7.14), the groups sizes were small for the longer pulse experiments, which precluded any meaningful comparisons between the GFP control and ArchT test groups.

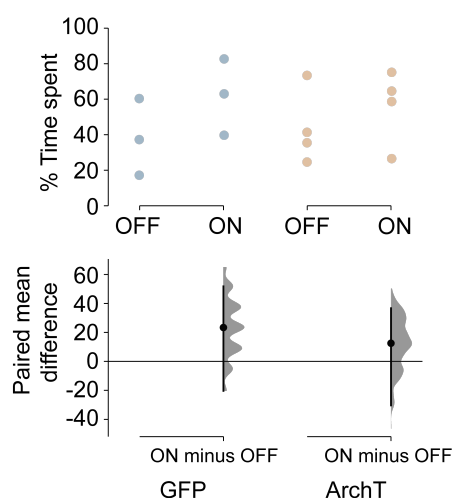


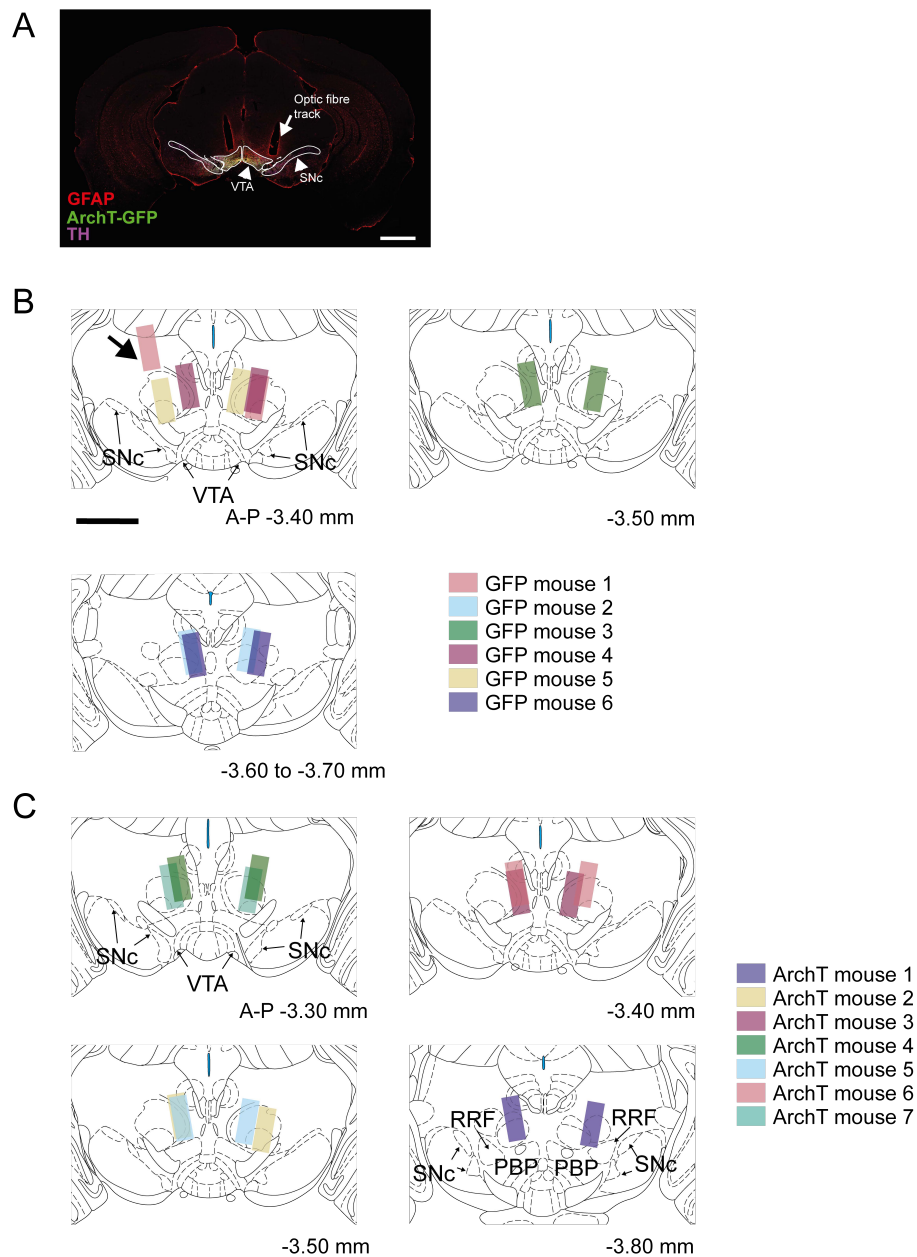
Figure 7.15: Suppressing the firing of VTA dopaminergic neurons for 2 s does not affect RTTPP behaviour. The GFP control (left; $n = 3$ mice) and ArchT test groups (right; $n = 4$ mice) spent similar amounts of time in the laser ON and laser OFF sides of the open field (GFP, laser ON minus laser OFF: -3.79 [95% CI: 2.59, -9.27]; ArchT, laser ON minus laser OFF: -3.30 [95% CI: 9.21, -23.64]).

Overall, the behavioural results of this Chapter have shown that pausing the firing of midbrain dopaminergic neurons in the VTA and SNc for 250–500 ms does not induce any change in place preference behaviour in either a bias CPP protocol or in a RTTPP paradigm. Increasing the duration of this pause to ~ 2 s does not result in any clear effect on place preference behaviour, although the group sizes for this set of experiments need to be expanded in order to draw a firm conclusion.

7.4.4 Optic fibre placement

The placement of the optic fibre above the VTA was confirmed for each mouse by examining the midbrain tissue *post hoc* and determining the location of the fibre tip using glial fibrillary acidic protein (GFAP), a marker for astrocytes (Figure 7.16A). Most fibres were located above the VTA in

experiments with brief (250 ms) laser pulses (Figure 7.16B), except one fibre in the left hemisphere of a mouse in the GFP control group which was dorsal compared with the other fibre placements (Figure 7.16B, arrow). Some mice from the experiments with brief (250 ms) laser pulses (Figure 7.11 and 7.13) were also used for experiments with longer (2 s) laser pulses (Figure 7.12 and 7.15); the location of the fibre tips for these experiments are shown in a separate figure for clarity (Figure 7.17).



(Figure legend on next page)

Figure 7.16: Placement of optic fibre above the VTA: brief (250 ms) pulse experiments. **A**, A confocal micrograph showing an example of the fibre tip placement (GFAP) above ArchT-transduced dopaminergic neurons. Dashed line is the medial lemniscus. **B, C**, Mouse midbrain coronal schematics showing the locations of fibre tips for mice in the GFP control (**B**) and ArchT test group (**C**). The arrow on the top right schematic indicates the location of the misplaced fibre tip for a mouse in the GFP control group. Note, some of the mice represented in this Figure were also used for the experiments with longer (2 s) laser pulses (Figure 7.17). A-P, anterior-posterior distance from Bregma; scale bar, 1 mm.

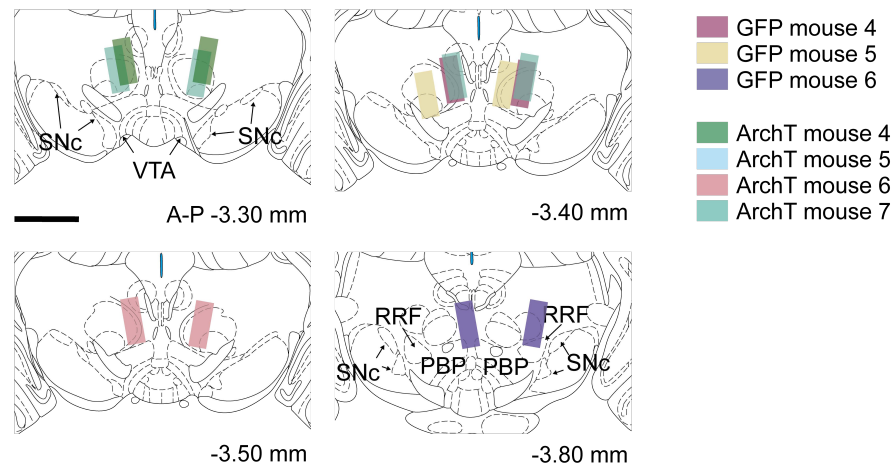


Figure 7.17: Placement of optic fibre above the VTA: longer (2 s) pulse experiments. Mouse midbrain coronal schematics showing the locations of fibre tips for all mice used in behavioural experiments. Note, all the mice represented in this Figure were also used for the experiments with brief (250 ms) laser pulses (Figure 7.16B). Scale bar, 1 mm.

7.5 Discussion

In this Chapter, I outline the results of an optogenetic strategy designed to mimic the brief pauses observed in the firing of midbrain dopaminergic neurons following a noxious stimulus (see Chapter 6). Although data in the previous results Chapter were recordings of RRF and SNc dopaminergic neurons, I decided to optogenetically manipulate VTA dopaminergic neurons in behaving mice for two main reasons. Firstly, there is an established literature demonstrating that optogenetically inhibiting VTA dopaminergic neurons induces place aversion (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019). Secondly, following from this, it was deemed important to establish a ‘positive control’ before embarking on completely novel behavioural experiments in which RRF dopaminergic neurons are manipulated.

7.5.1 *Electrophysiological experiments: brief (250 ms), physiologically-relevant laser pulses reliably suppresses the firing of midbrain dopaminergic neurons*

In Chapter 6 it was demonstrated that midbrain dopaminergic neurons in the RRF and SNc briefly (~220 ms) pause their firing following a noxious stimulus. This led to the first aim of this Chapter, which was to mimic this pause using an optogenetic approach. Most ArchT-transduced dopaminergic neurons were rapidly silenced at laser onset and remained silenced for the whole duration of the 250 ms pulse. After laser offset, some neurons exhibited a rebound increase in firing (as compared to baseline). In some cases, neurons did not resume firing until hundreds of milliseconds after laser offset, leading to a suppression of firing that lasted approximately 400 ms on average. This was not of major concern, as this period of suppression was within the range of pauses seen in Chapter 6.

In Chapter 6, the pause in firing of midbrain dopaminergic neurons in response to a noxious stimulus was followed immediately by a brief rebound activation phase, much like the response profile around delivery of laser pulses in the optogenetic experiments in the current Chapter. In a previous study, efforts were made to reduce the activation of midbrain dopaminergic neurons at rebound by ramping down the laser power towards the end of the pulse, in order that neurons resumed firing at a pre-stimulation rate (Salinas-Hernández et al., 2018). Although such an approach was not adopted in the present study, the similar firing activity profiles of dopaminergic neurons in response to a noxious and optogenetic stimulus suggests that rebound activation is physiological. However, such a rebound may represent a confound when interpreting behavioural results, as will be discussed below.

The main behavioural aim of this Chapter was to implement a physiologically-relevant optogenetic strategy to determine if brief pauses in the firing of VTA dopaminergic neurons were sufficient to induce place aversion. Although the results from the electrophysiological recordings broadly meet the first part of this aim, it is important to reflect on potential shortcomings. Previous work has shown that aversive stimuli evoke heterogeneous responses in the activity of VTA dopaminergic neurons; in some studies, lateral VTA dopaminergic neurons sharply decreased their

firing rate in anaesthetised rats (Ungless, 2004; Brischoux et al., 2009) and awake mice (Matsumoto et al., 2016), whereas their medial counterparts increased their firing rate (Brischoux et al., 2009). Moreover, it has been demonstrated that some VTA dopaminergic neurons increase their firing rate in response to foot shocks during fear conditioning (Tang et al., 2020). Importantly, the response direction of these neurons may vary depending on whether mice are in a high or low reward context (Matsumoto et al., 2016). This diversity in electrophysiological responses to aversive stimuli is in marked contrast to the optogenetic manipulation strategy implemented in the current Chapter, in that brief laser pulses uniformly decreased the firing rate of all transduced neurons through activation of the ArchT opsin. Thus, changes in the downstream release of dopamine in response to the manipulation are potentially not representative of the heterogeneity observed in response to aversive stimuli. For example, the activity of VTA dopaminergic terminals in the NAc core, lateral shell and dorsomedial shell decrease in response to foot shocks in calcium imaging experiments, whereas terminals in the NAc ventromedial shell increase their activity (de Jong et al., 2019). In light of previous work such as this, it is important to be cognisant of functional diversity when interpreting results from optogenetic manipulations.

The behavioural results of this Chapter focus on optogenetically manipulating VTA dopaminergic neurons. However, all of the dopaminergic neurons—for which anatomical location could be determined—recorded during optogenetic manipulation were located within the SNc and the RRF. It would be important to record from VTA neurons in the future, so that there is better alignment between the location of the *in vivo* electrophysiological recordings in the anaesthetised experiments and the optic fibres in the behavioural experiments. Although the heterogeneous membrane properties of midbrain dopaminergic neurons (Neuhoff et al., 2002; Lammel et al., 2008) could result in different electrophysiological responses to ArchT activation in different subpopulations, there is reason to believe that VTA dopaminergic neurons would respond in a similar way to RRF and SNc neurons. In a previous study, Arch-transduced (albeit, Arch, not ArchT) dopaminergic neurons were reliably suppressed by 500 ms laser pulses *in vivo* (5 mW output power,

as in the current Chapter; Danjo et al., 2014).

7.5.2 *Electrophysiological experiments: longer laser pulses less reliably suppress the firing of midbrain dopaminergic neurons*

Optogenetically inhibiting VTA dopaminergic neurons with laser pulses of supra-physiological duration induces place aversion in various permutations of a RTPP behavioural setup (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016; Krol et al., 2019). In these place preference paradigms, the laser was illuminated for the entire time the mouse was in the conditioned chamber, only being turned off when it exited the chamber. The maximum duration of a single laser pulse was 30 s in most cases (Tan et al., 2012; Danjo et al., 2014; Krol et al., 2019), but in some cases the laser was illuminated as long as the animal was in the conditioned chamber with no upper bound set (Ilango et al., 2014; Berndt et al., 2016). Though it is difficult to ascertain the average time an animal spent in the conditioned chamber (and thus the average pulse duration), it can be presumed it was in the order of seconds (based on my own observations in the RTPP study in the current Chapter). However, the electrophysiological response of midbrain dopaminergic neurons to long laser pulses was not determined in previous studies.

In case inducing brief, physiologically-relevant pauses in VTA dopaminergic neuron firing did not induce place aversion, I wanted to apply longer laser pulses during behaviour to potentially achieve a ‘positive control’. Any resulting behavioural effect would occur as a function of the suppressed firing rate and its dynamics. As I have demonstrated with the shorter (250 ms) pulses, these dynamics can not be presumed to precisely align with the optogenetic manipulation parameters in a digital manner. It was therefore imperative that the electrophysiological response of ArchT-transduced neurons to longer laser pulses was determined. Most responsive midbrain dopaminergic neurons were reliably silenced or suppressed by 2 and 10 s laser pulses, though a low number of neurons recorded during 10 s pulses ($n = 3$) meant it was difficult to infer anything at the population level.

When 30 s laser pulses were applied, the aberrant responses of a minority of neurons would

suggest that the temporal dynamics of firing in response to long laser pulses cannot always be assumed to closely reflect the stimulus parameters, possibly confounding the interpretation of behavioural results. One ArchT-transduced neuron did not recover spontaneous firing after laser offset, suggesting the prolonged extrusion of protons by ArchT prevented efficient membrane repolarisation in this neuron (Figure 7.7A). This type of response profile is reflected in a similar experiment from a previous study, in which ArchT-transduced SNc dopaminergic neurons were slow to recover their spontaneous firing rate after 5 s laser pulses (35 mW) *in vivo* (Da Silva et al., 2018).

In the present study, another neuron was initially silenced followed by an elevated firing rate throughout the rest of the laser pulse, with a sharp rebound increase in firing at laser offset (Figure 7.7B). Although the neuron was not juxtacellularly-labelled to unequivocally determine its neurochemical identity (i.e. whether it was dopaminergic and expressed ArchT), the fact it was silenced at laser onset with short latency suggests it was indeed an ArchT-transduced neuron. Moreover, the sharp increase in firing rate at laser offset is suggestive of a homeostatic response to membrane hyperpolarisation following ArchT activation. Therefore, the reason for the elevated firing rate is unclear. Somatodendritic release of dopamine from midbrain dopaminergic neurons regulates the excitability of neighbouring dopaminergic neurons by inducing inhibitory currents via D₂ autoreceptors (Beckstead et al., 2004). It is therefore plausible that optogenetically inhibiting the activity of ArchT-transduced dopaminergic neurons suppresses somatodendritic release of dopamine, thereby reducing lateral inhibition and increasing the firing rate of non-transduced neurons. Although the initial period of ArchT-mediated silencing would seem inconsistent with such a mechanism, it is possible that the rapid onset of inhibition following ArchT activation is then surmounted by the excitatory effect of reduced D₂ autoreceptor activation. However, this proposed mechanism has not been determined experimentally.

It is unknown how manipulating the firing of midbrain dopaminergic neurons for such long periods affects downstream circuits. Could reducing tonic dopamine release in target structures for

prolonged periods lead to aberrant changes in downstream circuitry that induce place aversion independent of dopaminergic signalling per se? A recent study demonstrated that brief vs prolonged optogenetic activation of D₂ receptor-expressing spiny projection neurons (SPNs) resulted in conditioned place preference and aversion, respectively, mediated by divergent effects on downstream target structures (Soares-Cunha et al., 2020). Although no such disconnect has been demonstrated for the midbrain dopaminergic system, this study highlights the importance of carefully considering the impact of optogenetic manipulations on downstream circuit dynamics and behaviour. Similar studies in the context of optogenetically suppressing midbrain dopaminergic neurons are warranted in order to understand any behavioural effects more clearly.

The above considerations were the main motivation for initially using optogenetic manipulation parameters that were naturalistic. Secondly, there was a concern that longer laser pulses would induce tissue heating, potentially disrupting the firing of neurons near the fibre tip independent of ArchT activation. Indeed, 30 s laser (532 nm) pulses at 5 mW induce tissue heating and a subsequent increase in the firing of prefrontal cortex (PFC) neurons *in vivo* in naive, wild-type mice (Stujenske et al., 2015). This may explain why one ArchT-transduced neuron in this Chapter displayed an elevated firing rate during 30 s laser pulses, as a result of an opsin-independent physiological process. However, the longer wavelength of light (561 nm) used in this Chapter is predicted to induce less tissue heating (Stujenske et al., 2015), which may explain why aberrant electrophysiological activity was uncommon. Indeed, the firing rate of one presumed non-ArchT-transduced dopaminergic neuron was unaffected by 30 s laser pulses in the present study. However, it would be prudent to assess the effect of the optogenetic manipulation parameters used in this Chapter on non-transduced midbrain dopaminergic neurons in wild-type mice without the confound of stimulating nearby ArchT-expressing neurons.

7.5.3 Place preference behaviour: mice have a baseline bias in a conditioned place preference paradigm

The conditioned place preference test is routinely used in rodents to assess the motivational properties of abused drugs. The drug (i.e. the unconditioned stimulus) is experienced in a certain environment (i.e. the conditioned stimulus) and if the experience is rewarding/aversive the animal will form a positive/negative association with the environment and spend more/less time there on subsequent exposure (Cunningham et al., 2006). More recently, this behavioural paradigm has been used to assess the motivational effect of optogenetically stimulating discrete neuronal populations (Tsai et al., 2009; Lobo et al., 2010; Maze et al., 2010; Zhang et al., 2015).

In this Chapter, most mice developed a preference for the white chamber during pre-testing. This was surprising because in other studies using a similar experimental design, most mice did not have a pre-test preference (Tsai et al., 2009; Lobo et al., 2010; Maze et al., 2010; Soares-Cunha et al., 2020). However, there were subtle differences in the visual and tactile properties of the conditioning chambers in these previous studies compared with the present study that may explain why I observed a pre-test preference for the white chamber. As in most CPP experiments (Cunningham et al., 2006), the tactile nature of the floor of the two conditioning chambers was distinct, and therefore a preference for the mesh floor in the white chamber may explain the pre-test preference. In a previous study, mice preferred a chamber with a mesh floor than a chamber with a grid floor (Cunningham et al., 2003), like in the present study. However, the grid floor in the previous study had very thick bars (6.4 mm, compared to 3 mm in the present study) that were widely spaced (13 mm compared with 5 mm in the present study). It is therefore unclear whether the observed preference for a mesh floor (Cunningham et al., 2003) was driven by the tactile properties of the mesh per se, or by an aversion to thick, widely spaced bars in the other chamber.

The expression of a pre-test preference for the white chamber could potentially confound the interpretation of results following conditioning. Some have argued that the effect of an unconditioned stimulus on CPP behaviour is state-dependent, in that a baseline preference for an

environment affects the direction of behavioural change induced by a drug in mice (Heinrichs and Martinez, 1986) and rats (Schenk et al., 1985; Swerdlow et al., 2003), as a result of an interaction between the drug effect and an unknown motivational property of the environment. However, others have demonstrated that an initial preference does not influence the effect of a drug on CPP behaviour in rats (Hinson et al., 1991; Cunningham et al., 2003). In the present Chapter, it was hypothesised that optogenetically inhibiting VTA dopaminergic neurons would induce conditioned place aversion (CPA), hence to maximise the chance of seeing an aversive effect, the delivery of laser pulses was paired with the innately preferred chamber. Moreover, this prevented any floor effect that could occur if the delivery of laser pulses was paired with the least preferred chamber. Reversing a baseline preference is possible with drugs that have an aversive effect (Hinson et al., 1991), thus it was assumed that any aversive effect of optogenetically inhibiting VTA dopaminergic neurons would emerge in the present study.

7.5.4 Place preference behaviour: optogenetically suppressing the firing of VTA dopaminergic neurons does not induce conditioned place aversion or real-time place aversion

It was clearly demonstrated that brief (250 ms) yellow laser pulses reliably suppress the firing of ArchT-transduced midbrain dopaminergic neurons in a manner reflective of the firing dynamics observed in response to a noxious stimulus in Chapter 6, and elsewhere (Ungless, 2004; Brischoux et al., 2009; Tan et al., 2012). However, in awake, freely-moving mice this optogenetic approach did not induce CPA or real-time place aversion (RTPA), despite previous research demonstrating that suppressing the firing of VTA dopaminergic neurons for prolonged periods induces CPA (Tan et al., 2012; Danjo et al., 2014) and RTPA (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016).

Some experiments suggest that brief pauses in the firing of VTA dopaminergic neurons signal a negative prediction error, which drives learning and thereby suppresses associated behaviour (Chang et al., 2016, 2018). Why, then, do brief pauses not suppress preference behaviour in the present study? There are several methodological and biological avenues to consider when reflecting

on this question. Firstly, it is possible that the number of VTA dopaminergic neurons transduced with ArchT was insufficient to drive a behavioural effect. In future it will be important to quantify the proportion of VTA dopaminergic neurons transduced in order to determine whether this is a probable explanation. Moreover, although the targeting of the optic fibres above the midbrain was determined histologically, it was not determined whether the application of laser pulses led to the suppression of dopaminergic neuron firing. An important future experiment will be to combine the optogenetic manipulation with electrophysiological recordings of midbrain dopaminergic neurons in awake mice. Alternatively, acute anaesthetised recordings could be carried out after behavioural experiments in mice implanted with optic fibres.

Firstly, aversive stimuli briefly pause the firing of VTA dopaminergic neurons (Tan et al., 2012; Matsumoto et al., 2016), which in turn is thought to reduce dopamine release in the NAc (Roitman et al., 2008) and subsequently increase the activity of D₂ receptor-expressing spiny projection neurons (D₂-SPNs) (Lee et al., 2021), promoting aversive learning (Hikida et al., 2010; Danjo et al., 2014). Pausing dopamine release in the NAc activates protein kinase A (PKA) (Lee et al., 2021) and enlarges dendritic spines on D₂-SPNs (Iino et al., 2020) as part of the synaptic plasticity process. Critically, this plasticity depends on prior activation of the glutamatergic input to the dendritic spines, such as occurs when cortical afferents to the striatum are activated by salient sensory events (Fisher et al., 2017). This tight temporal coupling between the dynamics of midbrain dopaminergic neuron activity and glutamatergic input to the striatum may not occur in the CPP paradigm because optogenetic inhibition of dopaminergic neurons occurs effectively at random. Thus, corticostriatal circuits may not be effectively strengthened to promote aversive learning because these random pauses in dopaminergic activity are not necessarily preceded by activation of glutamatergic input to D₂-SPNs. Behaviourally, this means the animal may be unable to determine what predicts the pause in dopaminergic activity and thereby learning fails to take place. Considering the RTPP experiments, a similar issue with temporal coupling may have occurred. That is, although entering the laser-paired area resulted in the delivery of laser pulses, it may have been ambiguous to the

animal that this was associated with that context (cue) because the pulses were delivered repeatedly until the animal exited the laser-paired area. A less ambiguous experimental design would be to deliver a single laser pulse at the moment of entering the laser-paired context. In this design, the optogenetic manipulation is associated specifically with entering the laser-paired environment and may therefore drive the mouse to avoid entering the environment in future if the manipulation is perceived as aversive. On the other hand, repeatedly delivering laser pulses may lead to associations with multiple aspects of the laser-paired environment and prevent any clear avoidance strategy being manifest.

In addition to a biological/behavioural explanation, it is also possible that no behavioural effect was observed due to a methodological factor. As discussed above in Section 7.5.3, some motivational property/properties of the CPP apparatus led to the expression of a pre-test preference for the white chamber. It is possible that this bias was caused by such a strong preference that any aversive learning occurring as a result of the optogenetic manipulation was insufficient to reverse it. However, Danjo et al., 2014 showed that constant optogenetic inhibition of VTA dopaminergic neurons in a strongly preferred ($\sim 80\%$ of time spent in a pre-test session) dark chamber robustly reduced the amount of time mice spent in the chamber on the post-test day. This would suggest that strong baseline preferences—even to a dark, innately preferred (Bourin and Hascoët, 2003) environment—are reversible with manipulations that induce aversive conditioning. Similarly, the aversive effect of D-amphetamine reverses the baseline preference when paired with the most preferred chamber (Hinson et al., 1991).

Brief, physiologically-relevant pauses in VTA dopaminergic neuron firing did not lead to mice avoiding the laser ON-paired chamber in the RTPP paradigm, suggesting that briefly inhibiting this population of neurons is not perceived as aversive. The RTPP paradigm assesses whether the manipulation induces contingent learning, in that the mouse can choose to avoid the delivery of laser pulses by leaving the laser ON chamber. In previous work (Tan et al., 2012; Danjo et al., 2014; Berndt et al., 2016), the distinction between the laser ON and laser OFF chambers was clear because

the laser was applied near-constantly until the mouse exited the laser ON chamber. In the present study a 250 ms pulse was applied every 3 to 7 seconds, meaning it may not have been perceptually clear that exiting the laser ON chamber resulted in the cessation of optogenetic manipulations.

In addition to probing potential aversive effects of optogenetically inhibiting VTA dopaminergic neurons, accelerometers were attached to a small subset of mice in order to investigate if there was any effect on ongoing movement. This is in light of emerging evidence that suggests the activity of VTA (Dodson et al., 2016; Hughes et al., 2019) and SNc dopaminergic neurons (Dodson et al., 2016; Howe and Dombeck, 2016; Da Silva et al., 2018) is associated with movement. Optogenetically inhibiting VTA dopaminergic neurons for brief (250–500 ms) periods did not cause any transient changes in ongoing movement, which is similar to previous research in which SNc neurons were inhibited (Da Silva et al., 2018). However, Da Silva et al. (2018) did find that inhibiting SNc dopaminergic neurons prevented movement initiation. In future, it would be interesting to assess whether this is also the case when VTA neurons are inhibited.

In order to determine whether longer, supraphysiological pauses in VTA dopaminergic neuron firing could induce CPA (as has been previously shown (Tan et al., 2012; Danjo et al., 2014; Ilango et al., 2014; Berndt et al., 2016)), I chose 2 s laser pulses, delivered every 3 to 7 seconds. Optogenetic inhibition of VTA dopaminergic neurons with pulses of similar duration suppresses rats' responses to a reward-predicting cue in an associative learning task (2 s pulses; Chang et al., 2018) and also impairs fear extinction learning (3 s pulses; Salinas-Hernández et al., 2018), indicating that these parameters are sufficient to affect associative learning. Although it could be reasonably argued that inhibiting these neurons for 2 s is supra-physiological, it should be noted that some of VTA dopaminergic neurons do display a suppression of firing lasting up to 6 s during the presentation of a foot shock-predicting cue in rats (Mileykovskiy and Morales, 2011). In the present study, the low number of animals in the GFP control and ArchT test groups precluded any robust quantitative analysis of the effect on CPP and RTPP behaviours. In future, it will be important to achieve a positive control experiment in which an unconditioned stimulus (optogenetic or otherwise) is

shown to reverse the baseline pre-test preference for a given chamber, thereby demonstrating that it is possible to detect aversion with the experimental setup used in this study.

The tentative conclusion from the present data is that brief optogenetic inhibition of VTA dopaminergic neurons does not induce aversive conditioning. However, an important future experiment will be to increase the number of conditioning sessions in order to determine whether multiple days of conditioning are needed for an association to be formed, and to expand the number of animals in the experiments with 2 s laser pulses.

Chapter 8

General Discussion

8.1 Summary

The overarching aim of this thesis was to functionally characterise a relatively overlooked group of midbrain dopaminergic neurons in the retrorubral field, in terms of their molecular makeup, connectivity and electrophysiological activity *in vivo*. The axonal projections and direct inputs of RRF dopaminergic neurons (connectivity) were assessed using modern techniques that permitted precise anatomical characterisation. Immunohistochemistry experiments revealed that the molecular markers expressed by RRF dopaminergic neurons overlap with those expressed by SNc and VTA neurons. The use of dopaminergic-neuron-selective methods ensured that the anterograde tracing mapped only the outputs of dopaminergic neurons, advancing on previous studies that used non-selective anterograde tracers. Similarly, the use of a modified rabies virus-based retrograde tracing method mapped direct inputs to RRF dopaminergic neurons, whereas previous studies had the caveat that the input neurons to the RRF were traced irrespective of the cell types they formed synapses with in the RRF. Together, this allowed the precise nature of RRF dopaminergic neuron connectivity to be elucidated. For electrophysiological recording experiments, extracellular single-cell recording followed by juxtacellular labelling of individual neurons allowed the precise anatomical location and neurochemical phenotype of recorded neurons to be determined. Because

RRF dopaminergic neurons are in close proximity to neighbouring SNc neurons, this level of precision was required in order to confidently assess the electrophysiological activity of these neurons specifically.

Based on the results of the electrophysiology experiments, a set of behavioural experiments were designed to assess whether optogenetically inhibiting RRF dopaminergic neurons was sufficient to induce place aversion. As part of this, great importance was placed on electrophysiologically validating the optogenetic manipulation parameters, as it is crucial that the neural dynamics underlying any behavioural effect are accurately characterised and not presumed to reflect manipulation parameters. Although the behavioural experiments involving the manipulation of RRF dopaminergic neurons were not completed, initial pilot experiments involving the inhibition of VTA neurons were conducted as a ‘positive control’. Overall, this provided a basis for future work characterising the behavioural roles of RRF dopaminergic neurons.

8.1.1 Chapter 3—Molecular markers of RRF dopaminergic neurons

In the first description of catecholaminergic neurons (now known to be dopaminergic) in the midbrain (Dahlström and Fuxe, 1964), it was noted that the borders between the RRF, SNc and VTA are not clear because the neurons in each region are spatially contiguous. Since these early studies, functional characterisation of midbrain dopaminergic neurons has allowed for more objective delineations of the regional boundaries. In the mouse, dopaminergic neurons of the rostral RRF and caudal SNc are abutting, at around -3.80 to -3.95 mm with respect to Bregma. Therefore, the first challenge of my D.Phil. project was to define the boundaries of the rostral RRF in order that it could be confidently distinguished from the neighbouring caudal SNc. Gene expression profiling studies have determined that the molecular signatures of SNc and VTA dopaminergic neurons are distinct (Chung et al., 2005; Greene et al., 2005; Poulin et al., 2014; Brichta et al., 2015). Although there are studies that describe molecular markers of RRF neurons, to date there has not been a comprehensive characterisation of their makeup, particularly in the mouse.

In Chapter 3, I determined that the pattern of molecular marker expression in RRF dopaminergic neurons shares characteristics of both SNc and VTA neurons. The transcription factors SRY-Box Transcription Factor 6 (Sox6) and orthodenticle homolog 2 (Otx2) are key to the development of SNc and VTA dopaminergic neurons (Panman et al., 2014), respectively, and are expressed into adulthood. This thesis research shows that the dichotomous expression of these transcription factors holds true for RRF dopaminergic neurons, which often express Sox6 but rarely express Otx2. Thus, RRF neurons share more in common with SNc than VTA neurons when considering these proteins. However, unlike SNc dopaminergic neurons, many neurons in the RRF express calretinin and calbindin-1 but few express aldehyde dehydrogenase 1a1 (Aldh1a1). Taken together with the expression pattern of Sox6 and Otx2, RRF dopaminergic neurons share features of both SNc and VTA neurons in terms of their molecular makeup.

From this molecular marker characterisation, I was able to show that a combination of calbindin-1 and Aldh1a1 immunoreactivity provide an objective means of delineating the border between the caudal SNc (Aldh1a1^{high}, calbindin-1^{low}) and rostral RRF (Aldh1a1^{low}, calbindin-1^{high}). Though the delineation of the RRF using these markers is by no means perfect, they provide an objective set of criteria that allow for a more confident definition of the RRF boundaries. This stage of the project was crucial in moving forward to characterise the connectivity and electrophysiological properties of RRF dopaminergic neurons.

8.1.2 Chapters 4 and 5—Anterograde projections and monosynaptic inputs of RRF dopaminergic neurons

Collectively, the aim of work in Chapters 4 and 5 was to characterise the connectivity of RRF dopaminergic neurons, in terms of their axonal projections and direct inputs. To achieve this, I leveraged genetic tools that permit selective access to dopaminergic neurons. Filling RRF dopaminergic neurons with a GFP reporter protein revealed the brain-wide axonal projections of these neurons; principal targets of RRF dopaminergic neurons included, in order of projection density: ventrolateral striatum (VLS), lateral interstitial nucleus of the posterior limb of the anterior

commissure (IPACL), dorsolateral striatum (DLS) and olfactory tubercle (OT). This is summarised in Figure 8.1. This work confirmed some previous findings using classical tracers (rat: Deutch et al., 1988; non-human primate: François et al., 1999), but this is the first report of a dense projection to the IPACL. Moreover, I found no evidence of axonal projections to the bed nucleus of the stria terminalis (BNST) or central amygdala (CeA), counter to previous work in the rat (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002).

Moving to direct inputs, a modified rabies virus-based approach permitted tracing of neurons that provide monosynaptic inputs to RRF dopaminergic neurons. Using this method, it was revealed that dense clusters of neurons in the BNST, CeA and interstitial nucleus of the posterior limb of the anterior commissure (IPAC) send direct inputs to RRF dopaminergic neurons, along with less prevalent input neurons located in a multitude of brain regions, including the external globus pallidus (GPe), VLS, nucleus accumbens (NAc) and subthalamic nucleus (STN). The efferent and afferent connectivity seemed to be reciprocal for some brain regions (Figure 8.1). In future, examining whether projection target-defined subpopulations of RRF dopaminergic neurons (e.g. IPAC-projecting) receive a larger proportion of their inputs from the same region will reveal whether precise reciprocity is a common feature of RRF dopaminergic neuron circuitry.

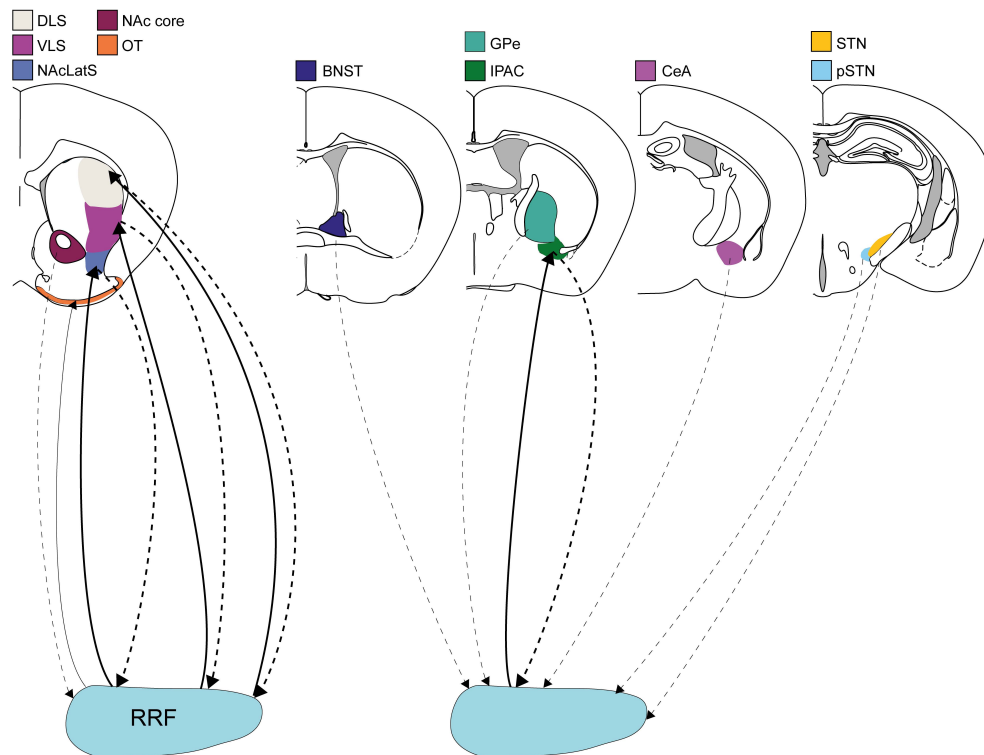


Figure 8.1: A summary of RRF dopaminergic neuron connectivity elucidated in this research. Solid lines indicate regions receiving axonal projections from RRF dopaminergic neurons; dashed lines indicate regions that send monosynaptic inputs to RRF dopaminergic neurons. Connections that were reciprocal are in **bold**: dorsolateral striatum (DLS), nucleus accumbens (NAc) lateral shell (NAcLatS), ventrolateral striatum (VLS), interstitial nucleus of the posterior limb of the anterior commissure (IPAC). Areas with non-reciprocal connectivity: bed nucleus of the stria terminalis (BNST), central amygdala (CeA), external globus pallidus (GPe), NAc core, olfactory tubercle (OT), paraventricular nucleus (pSTN) and subthalamic nucleus (STN). Note, this illustration of output and input regions is not exhaustive and some ‘minor’ projections are left out for simplicity. See Chapters 4 and 5 for a full description.

8.1.3 Chapter 6—Electrophysiological properties of RRF neurons

Prior to the work presented in this thesis, there were no studies describing the firing properties of identified RRF dopaminergic neurons. By using the juxtacellular recording and labelling method, I was able to differentiate RRF, SNc and VTA neurons and unequivocally determine whether they were dopaminergic using histochemistry. The spontaneous firing properties of RRF dopaminergic neurons in mice under urethane anaesthesia were broadly similar to those of SNc and VTA dopaminergic neurons. Using the same recording/labelling method, I demonstrated that RRF dopaminergic neurons briefly pause (~220 ms) their firing following foot shocks applied to the hindpaw, indicating they are equipped to represent aversive stimuli with changes in their firing rate. This electrophysiological response to foot shock was also observed in SNc dopaminergic neurons.

Overall, RRF and SNc dopaminergic neurons had similar firing properties *in vivo*, reflective of the similarity between SNc and VTA dopaminergic neurons (Ungless and Grace, 2012).

8.1.4 Chapter 7—Briefly pausing the firing of VTA dopaminergic neurons does not induce place aversion

The work in Chapter 6, demonstrated that RRF dopaminergic neurons pause their firing following an electrical shock applied to the hindpaw. This led to the main aim of Chapter 7, which was to optogenetically mimic this pause in firing and determine whether it was sufficient to mediate aversive behaviours. Previous work has established that optogenetically inhibiting SNc (Ilango et al., 2014) and VTA dopaminergic neurons induces place aversion (Tan et al., 2012; Danjo et al., 2014; Berndt et al., 2016; Krol et al., 2019). However, in this previous work the duration of optogenetic manipulation was in the order of seconds, which is non-physiological. In order to test whether brief, physiologically-relevant pauses in dopaminergic neuron firing were sufficient to induce place aversion, I first electrophysiologically-validated that the optogenetic approach by demonstrating that brief laser pulses could pause the firing of archaerhodopsin T (ArchT)-transduced dopaminergic neurons. The dynamics of this pause were similar to those observed following application of a foot shock to the hindpaw, demonstrating the physiological relevance of the optogenetic strategy adopted.

Before moving on to behavioural experiments in which RRF dopaminergic neurons were optogenetically manipulated, I first carried out experiments with optic fibres targeting the VTA. This is because there is an established literature regarding the role of VTA dopaminergic neurons in mediating aversive behaviours, so it was deemed a ‘positive control’ experiment. Briefly pausing the firing of VTA dopaminergic neurons did not induce place aversion, suggesting the brief pauses in firing observed in dopaminergic neurons in response to aversive stimuli are not of causative importance in mediating the behaviours that were tested.

8.2 Theoretical and technical considerations

8.2.1 *What distinguishes RRF dopaminergic neurons from those of the SNc and VTA?*

In research, functional distinctions are often made between regions of interest. This is evident in the literature surrounding the midbrain dopaminergic system, wherein the dopaminergic neurons of the SNc and VTA are often compared and ascribed distinct functions. In the context of this thesis, it leads one to ask what—if anything—distinguishes the dopaminergic neurons of the RRF from those in the neighbouring SNc and VTA? A group of neurons can be defined by their connectivity (inputs and outputs), molecular makeup and physiological properties (Zeng and Sanes, 2017). Below, these aspects will be considered for RRF dopaminergic neurons.

The anterograde projections of RRF dopaminergic neurons shared features of both the nigrostriatal pathway (SNc → dorsal striatum) and mesolimbic pathway (VTA → ventral striatum/olfactory tubercle/amygdala) pathways, in that RRF neurons projected to the dorsal and ventrolateral striatum. However, it could be concluded that the projection pattern was more similar to that of VTA dopaminergic neurons, given that RRF projections are prominent in the ventral striatum, olfactory tubercle and IPAC. In terms of the brain regions sending direct inputs to RRF dopaminergic neurons, there was much overlap with previous reports of the inputs to SNc and VTA neurons (Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015; Menegas et al., 2015). However, qualitative observations suggested a larger proportion of inputs to dopaminergic neurons in the RRF arise from neurons in the extended amygdala and IPAC, whereas for SNc and VTA neurons the largest proportion of inputs arise from the striatum. In terms of electrophysiological properties, the spontaneous firing activity and response to a foot shock stimulus were broadly similar across midbrain dopaminergic subregions. Although the tentative conclusion from the data in this thesis is that the RRF has distinct and overlapping characteristics, it is important to reflect on what is the most insightful way to describe the dopaminergic midbrain.

A more objective and data-driven approach to classifying midbrain dopaminergic neurons is to

use unbiased methods for functional profiling. For example, the elegant single-cell gene expression profiling study by Poulin et al., 2014 demonstrated that, based on a distinct molecular barcode, midbrain dopaminergic neurons could be divided into five main groups based on principal component analysis. The anatomical distribution of these groups revealed distinct heterogeneity within the VTA, as well as an overlap in expression profile of lateral VTA and dorsal SNc neurons, pointing towards a less clear cut distinction between the VTA and SNc. Although RRF dopaminergic neurons were represented in some of these molecularly-distinct subgroups, a thorough examination has not been carried out. In future, a similar study incorporating the RRF in more detail would help to determine whether the RRF belongs to one or more of these five groups, or perhaps whether some RRF dopaminergic neurons have a molecular profile distinct from those of VTA and SNc neurons. The merits of using unbiased methods for defining clusters of midbrain dopaminergic neurons is highlighted by recent work demonstrating that the molecularly-defined subgroups outlined by Poulin et al. (2014) have distinct axonal projection patterns (Poulin et al., 2018). It will be interesting to see if this is also true for molecularly-distinct subsets of RRF dopaminergic neurons.

8.2.2 The genetic tools used to define the axonal projections and monosynaptic inputs of RRF dopaminergic neurons

Previous work with classical anterograde tracers has mapped the axonal projections of RRF neurons irrespective of cell type, whereas studies in this thesis used DAT-Cre mice and an AAV encoding a Cre-dependent fluorescent reporter to selectively trace the axons of dopaminergic neurons. Although this genetic approach has distinct advantages over classical anterograde tracers, it is important to reflect on the limits of this technique.

Filling RRF dopaminergic neurons with green fluorescent protein (GFP) revealed dense, highly branched and varicose axon fibres in the striatum, olfactory tubercle and IPAC. These features of the innervation suggest they are sites of termination and are presumed therefore to be sites of dopamine release. However, there are two main caveats to consider; one caveat is a general issue with

anterograde tracing and the second caveat is unique to dopaminergic/neuromodulatory projections.

The first caveat is that the resolution limit of light microscopy precludes the examination of synaptic contacts. Only electron microscopy and electrophysiological recordings can confirm a synaptic contact between two neurons and therefore any presumptions about connectivity based on the observation of varicosities and axonal arborisation is imprecise. Tools that disambiguate synaptic sites from axon fibres are necessary to determine the location of synaptic contacts. To this end, progress has been made recently with the development of Cre-dependent AAVs that express one fluorescent protein (e.g. GFP) throughout the axon of transduced neurons and another fluorescent protein (e.g. mRuby) which is tagged to synaptophysin, thereby labelling putative presynaptic sites (Li et al., 2010; Lerner et al., 2015). An example of such a tool is AAV-hSyn-FLEX-mGFP-2A-Synaptophysin-mRuby. However, studies of the rat hippocampus (Mishchenko et al., 2010) and cat visual cortex (Costa and Martin, 2011) have demonstrated that only around $\sim 30\%$ of appositions between pre- and post-synaptic neurons represent true synaptic contact, therefore the labelling of synaptophysin with a fluorescent protein likely gives an over-representation of connectivity. In future, this tool could be used to map the putative presynaptic terminals of RRF dopaminergic neurons, bearing in mind that the number/density of labelled terminals may be an overestimation of connectivity. A recent advancement has been the development of the GFP reconstitution across synaptic partners (GRASP) technique (Kim et al., 2011; Feng et al., 2014). This method involves the virus-mediated expression of non-fluorescent GFP fragments to the pre- and post-synaptic membranes of circumscribed neuron populations. The GFP fragments reconstitute when they are in close contact at the synaptic cleft, and only then does the protein fluoresce, revealing the site of synaptic contact. However, if one is interested in the brain-wide projections of a population of neurons, this method is not suitable as it requires both the pre- and post-synaptic neuron to be transduced. Moreover, much dopaminergic transmission occurs outside of classical synapses (Moss and Bolam, 2008) and thus utility of the GRASP method may be limited.

The second caveat that is unique to dopaminergic neurons (and perhaps neuromodulatory neurons more generally) is that the release of dopamine is not limited to classical synapses. The striatum is the main input nucleus of the basal ganglia and receives dense dopaminergic innervation. Much of the glutamatergic input of the striatum arises from the cortex and thalamus (Frotscher et al., 1981; Moss and Bolam, 2008), which is modulated by dopaminergic innervation from the midbrain (Reynolds et al., 2001). The action of dopamine is mostly outside of classical synapses, as only around 9% of cortical and thalamic terminals in the striatum are associated with a dopaminergic synapse (Moss and Bolam, 2008). Therefore, determining the sites of release and sites of dopamine action is non-trivial. It is proposed that the action of dopamine occurs through ‘volume transmission’ (Agnati et al., 1995), because the majority of dopamine receptors are distal to dopaminergic axons at extrasynaptic sites (Yung et al., 1995; Caillé et al., 1996), and therefore rely on the diffusion of dopamine away from release sites. In light of the mostly non-synaptic nature of dopamine secretion in the striatum, varicosities are often a proxy for sites of release. However, recent data suggest only ~30% of these are active release sites (Liu et al., 2018), and thus the correlation of varicosities with putative release sites in Chapter 4 may not be entirely valid. However, the sheer density of axonal arborisation observed for dopaminergic neurons (Matsuda et al., 2009; Aransay et al., 2015) suggests that even with only ~30% of varicosities being equipped to release dopamine, the influence of dopamine in the striatum is massive.

The main advantage of using the modified rabies-virus based approach for mapping the inputs to RRF dopaminergic neurons is the cell type-selectivity. Only dopaminergic neurons are transduced with the helper virus and modified rabies virus, ensuring that the retrogradely-labelled neurons are specifically inputs to dopaminergic neurons and not other, non-dopaminergic neurons in the RRF (see Materials and Methods Figure 2.1 and Chapter 5 details). However, the drawbacks of this technique should be considered as they have implications for the way the data are interpreted. The exact mechanism by which the rabies virus is spread from the starter neurons to the input neurons is unknown. Despite this, results are often framed in terms of ‘monosynaptic

inputs', despite little direct evidence the retrograde transfer of rabies occurs at synapses. However, some functional studies have given evidence that the retrogradely-labelled presynaptic neurons are in direct contact with the starter neurons. For example, a study in which a single pyramidal neuron in visual cortex was transduced (rendering it a starter neuron) determined that the orientation tuning of the starter neuron and the retrogradely-labelled presynaptic partner neurons was closely aligned (Wertz et al., 2015), suggesting direct connectivity. Similarly, paired electrophysiological recordings of cortical starter neurons and retrogradely-labelled input neurons in the slice preparation have provided evidence of a monosynaptic relationship (Wickersham et al., 2007). However, it is not known whether these observations can be generalised to other circuits because few studies implementing the modified rabies virus system include functional experiments.

In contrast to other work (Wickersham et al., 2007; Wertz et al., 2015), some studies have shown a disconnect between anatomical and electrophysiological data. In the barrel cortex, somatostatin and parvalbumin (PV)-expressing interneurons receive a quantitatively similar number of inputs from the ventral posteromedial nucleus of the thalamus (VPM) when mapped using the modified rabies virus method (Wall et al., 2016). However, the size of the excitatory post-synaptic potential (EPSP) in somatostatin interneurons in response to optogenetic stimulation of thalamocortical efferents in the cortex is 20-fold lower than for PV interneurons (Cruikshank et al., 2010). This stark contrast between the anatomical (Wall et al., 2016) and electrophysiological data (Cruikshank et al., 2010) seems to suggest that the modified rabies-virus technique is either more selective for VPM synapses on somatostatin interneurons or is retrogradely transported by a non-synaptic mechanism. It is also possible that VPM input to somatostatin interneurons is convergent, whereas input to PV interneurons is divergent, which could manifest anatomically as seen by Wall et al. (2016). However, unless the synapses between VPM and somatostatin interneurons are inherently weak or extrinsically dampened, this would not explain the electrophysiological observations (Cruikshank et al., 2010). Therefore, it is possible that the 'trans-synaptic' spread of rabies from starter neurons to input neurons is not synaptic, or only partially synaptic; it may be that

rabies-containing viral capsids are released from dendrites and taken up by proximal axons outside of synaptic contacts. Even if this is the case, it is unlikely the viral capsids can diffuse far in the extracellular space, therefore it is still likely that the axons of the retrogradely-labelled input neurons are at least proximal to the starter neurons.

The passage of rabies virus from the starter neuron to presynaptic input neurons is presumed to be non-selective, passing efficiently to any input neuron. However, a number of studies refute this assumption. Studies with classical tracers in rats clearly demonstrate that the prelimbic cortex (Fallon, 1981; Swanson, 1982; Chandler et al., 2013) and striatum (Fallon and Moore, 1978; Fallon et al., 1978) receive dopaminergic input, and that the prelimbic cortex also receives noradrenergic input from the locus coeruleus (LC) (Hoover and Vertes, 2007). (However, unpublished observations in the mouse (Dr Paul Dodson and Dr Anna-Kristin Kaufmann) from studies with the retrograde tracer cholera toxin subunit B (CTB) found little dopaminergic innervation of the frontal cortex, suggesting a difference between species or based on the type of retrograde tracer used (previous work in the rat used fluorescent tracers, such as FluoroRuby).) Retrograde tracing of inputs to neurons in the prelimbic cortex using the modified rabies virus-based method does not label dopaminergic or noradrenergic input neurons (DeNardo et al., 2015; Cruz et al., 2021). Similarly, tracing from spiny projection neurons (SPNs) in the dorsal striatum does not label many input neurons in the midbrain in some studies (Wall et al., 2013), though in other studies more midbrain inputs are labelled (Guo et al., 2015). This suggests that the rabies virus is not as efficiently transferred to neuromodulatory input neurons as it is to other types of input neurons. This may be because neuromodulatory axon terminals do not make classical synapses with postsynaptic neurons (Descarries et al., 1996) and are thus prohibitively far for efficient transfer of rabies viral capsids. In short, the uptake of rabies and subsequent labelling of input neurons might be cell type selective. However, future work is needed to establish whether this is the case.

As outlined above, there are several drawbacks to the genetic approaches used to trace the anterograde projections and retrograde inputs of RRF dopaminergic neurons. Even with these

drawbacks in mind, the fact that these tools have the potential to exclusively trace the connectivity of dopaminergic neurons and not nearby non-dopaminergic neurons is of huge benefit over classical tracers. In the past, classical anterograde tracing had to be followed up with retrograde tracing to confirm the dopaminergic contribution of an anterogradely-labelled projection, whereas using a dopaminergic-neuron-selective method—as in Chapter 4—bypasses the need for such a followup. Moreover, the brain-wide distribution of dopaminergic efferents and their fine-scale innervation patterns are revealed using cell type-selective methods.

8.2.3 *Species differences*

The mouse was chosen as a model organism because some of the techniques used in this thesis required genetic access to dopaminergic neurons, which is more easily achieved in mice (i.e. DAT-Cre mice; Bäckman et al., 2006). Although DAT-Cre and TH-Cre rats are now available (Brown et al., 2013; Liu et al., 2016), much literature in the dopamine field is based on the mouse as a model organism, particularly circuit-mapping studies. Thus, in order that the work in this thesis was comparable to previous literature, the mouse was chosen. However, much early work in the dopamine field in the ‘pre-genetic era’ involving rodents chose the rat as a model organism. Although the trend away from the rat and towards using the mouse was understandable given the earlier advent of genetically-modified mouse lines (Thomas and Capecchi, 1987), it is important to be mindful that the rat is often better placed for neurological studies, particularly those involving complex behaviours (Iannaccone and Jacob, 2009).

The majority of the literature surrounding the RRF involved the rat or non-human primate as a model organism. Though ethical and practical constraints make routine use of the non-human primate a challenge for basic research, it is important to reflect on how species differences may impact on the comparison of my results with the work of others. One novel finding in the present work was the lack of axonal projections of RRF dopaminergic neurons in the BNST or CeA, despite previous work with the classical retrograde tracer wheat germ agglutinin conjugated to horseradish

peroxidase (WGA-HRP) demonstrating dopaminergic projections from the RRF to these structures (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002). When using a modern tracing tool, such as in Chapter 4, it is tempting to assume that disagreements with earlier work using classical tracers is underlain by their caveats, such as polysynaptic spread (Goshgarian and Buttry, 2014; Sillitoe, 2016). However, it is important to consider whether the difference in species used in my work (mouse) and previous work (rat; Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002) may explain the discrepant findings. To date, there have been few studies directly comparing mice and rats in tracing studies, meaning it is difficult to conclude whether species differences are an important factor. A recent study observed denser retrogradely-labelling of BNST neurons in mice compared with rats following injection of a retrograde tracer in the VTA (Kaufling et al., 2017), suggesting connectivity differences between mice and rats do exist. However, no studies have been conducted comparing the anterograde projections or retrograde inputs of dopaminergic neurons in mice and rats, leaving open the question regarding species differences.

8.3 Future direction

In Chapters 3, 4, 5, 6 and 7, I have suggested extensions to the completed studies, for example, increasing sample sizes (Chapter 4 and 7) and undertaking quantitative analyses of outputs (Chapter 4) and inputs (Chapter 5). Here, I discuss some further studies I believe are warranted given the results outlined in the work of this thesis.

8.3.1 *Can RRF dopaminergic neurons be more objectively defined?*

Subdividing the midbrain dopaminergic system into the RRF, SNc and VTA (Dahlström and Fuxe, 1964) is a useful basis for functional studies of these neurons. However, as discussed above, a more unbiased—and perhaps more informative—method for classifying dopaminergic neurons is single-cell profiling of their molecular makeup followed by principal component analysis in order to objectively group neurons based on their combinatorial expression of markers (Poulin et al., 2014;

La Manno et al., 2016). Each subgroup is defined by the presence and absence of a circumscribed set of expressed genes. Previous work focused mainly on SNc and VTA when mapping the distribution of the molecularly-distinct subgroups within the midbrain, so in future it will be useful to determine how RRF dopaminergic neurons fit into this schema. There were some clues from previous work regarding RRF dopaminergic neurons. For example, a small subset of dopaminergic neurons in the RRF express *Aldh1a1*, *Sox6* and Neuron Derived Neurotrophic Factor (NDNF) but lack calbindin-1 (Poulin et al., 2014). However, a wholesale mapping of these subtypes in the RRF is yet to be done. This could be achieved with immunohistochemistry for the various markers described in previous work (Poulin et al., 2014; La Manno et al., 2016) and stereological counting to determine the distribution of molecularly-distinct subtypes in the RRF. A more unbiased examination would be to repeat the single-cell gene expression profiling study ensuring that RRF dopaminergic neurons are well represented in the pool of neurons taken forward for analysis. In previous work, it is unclear if the RRF was included when the brain tissue was dissected. This experiment would then determine whether the same subgroups emerge from the principal components analysis, or whether including RRF dopaminergic neurons gives rise to other subgroups. Importantly, the distribution of these molecularly-distinct subgroups would also need to be examined with immunohistochemistry.

8.3.2 Do the axonal projection targets of midbrain dopaminergic subgroups overlap?

In Chapter 4, one of the main observations was that the axonal projection pattern of RRF dopaminergic neurons shared features of both the nigrostriatal and mesolimbic pathways. This leads to the question, do the axonal arbors of RRF dopaminergic neurons overlaps with those of SNc/VTA dopaminergic neurons? And, if so, are these overlapping projections functionally distinct? In the literature, there is evidence that different subpopulations of SNc and VTA dopaminergic neurons have distinct yet overlapping axonal projections (Beier et al., 2015; Poulin et al., 2018). On the other hand, some studies suggest that midbrain dopaminergic neurons have largely segregated projection targets (Lerner et al., 2015). Retrograde tracer injections targeted to, for example, the

dorsal striatum result in retrogradely-labelled neurons in both the SNc and VTA (Lammel et al., 2008; Farassat et al., 2019). However, the relatively bulky deposits required for retrograde tracing preclude any fine-scale analysis determining whether different subpopulations of midbrain dopaminergic neurons innervate distinct or overlapping microdomains. In the context of the present study, the lateral shell of the NAc receives dopaminergic input from the SNc, VTA (Lammel et al., 2008) and RRF (Chapter 4). In future, it will be interesting to explore whether the projections from these different regions of the midbrain are distinct or overlapping.

To investigate how overlapping or distinct the projections of midbrain dopaminergic neuron subpopulations are, a similar anterograde tracing method to the one implemented in Chapter 4 may be useful. To directly compare RRF, SNc and VTA dopaminergic projections in the same mouse, each region could be separately injected with a Cre-dependent AAV encoding a different colour fluorescent reporter protein (e.g. GFP, RFP and BFP) in DAT-Cre mice. In this way, the axonal projections could be examined to determine whether they overlap or are mostly distinct.

8.3.3 How does the firing of RRF dopaminergic neurons relate to behaviour?

Clues to the behavioural role of RRF dopaminergic neurons were offered in Chapter 6, where it was shown that they pause their firing in response to foot shock (putatively aversive) in anaesthetised mice. However, the dynamics of firing in response to behaviourally-relevant stimuli may be different in awake mice. A good starting point for future experiments would be to record from identified RRF dopaminergic neurons in the awake mouse during Pavlovian conditioning, involving both conditioned aversive and rewarding stimuli (e.g. Cohen et al., 2012; Matsumoto et al., 2016). In these experiments, it would be important to have confidence that the recorded single-unit activity was from RRF dopaminergic neurons and not nearby SNc or VTA neurons. This could be achieved in two ways. One option would be to carry out head-fixed awake extracellular recordings followed by juxtacellular labelling (Dodson et al., 2016; Coddington and Dudman, 2018), which would permit *post hoc* neurochemical identification and anatomical localisation of recorded neurons. A second

option would be to transduce RRF dopaminergic neurons with channelrhodopsin-2 (ChR2) in DAT-Cre mice and ‘opto-tag’ recorded neurons to confirm their dopaminergic identity. After this, the restriction of ChR2 expression to the RRF could be confirmed *post hoc*, and thus it could be determined the electrophysiological recordings were from RRF dopaminergic neurons. The first option has the advantage of offering maximum spatial resolution; the second option has the advantage of being achievable in freely-moving mice.

In freely-moving animals, the behavioural experiment most aligned with the anaesthetised experiments I carried out in Chapter 6 would be fear conditioning. In these experiments, a brief foot shock is applied to the mouse via the metal bars of the chamber floor, either unpredicted or predicted by a sensory cue. In a recent study, most putative dopaminergic neurons in the lateral VTA responded to foot shock with an increase in firing rate (Tang et al., 2020), in contrast to previous work in the anaesthetised (Brischoux et al., 2009; Mejias-Aponte et al., 2015) and head-fixed awake rat (Mileykovskiy and Morales, 2011). This suggests that the results of anaesthetised and awake electrophysiological recording experiments can diverge, which demonstrates why recordings of RRF dopaminergic neurons in awake, behaving mice are necessary. That said, one should be aware that the electrophysiological properties of dopaminergic neurons are insufficient for their identification, particularly in the VTA (Ungless, 2004; Mejias-Aponte et al., 2015). Hence, it is possible that some putative dopaminergic neurons recorded in freely-moving fear conditioning experiments were in fact non-dopaminergic (Tang et al., 2020).

8.4 Concluding statement

As a whole, I have demonstrated that the functional properties of RRF dopaminergic neurons are distinct but partly overlapping with respect to neighbouring SNc and VTA dopaminergic neurons, in terms of molecular makeup, connectivity and electrophysiological activity. I conclude that RRF dopaminergic neurons are equipped to fulfil distinct and specialised roles in the brain as compared to their midbrain counterparts.

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