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Title:
Monosynaptic Tracing of Cerebellar Nuclei Projections to the Ventral Tegmental Area and Substantia Nigra pars compacta

SCHOOL OF PHYSIOLOGY, PHARMACOLOGY AND NEUROSCIENCE

Monosynaptic Tracing of Cerebellar Nuclei Projections to the Ventral Tegmental Area and Substantia Nigra pars compacta

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Master of Science by Research in the Faculty of Life Sciences.

Monday 28th March, 2022

Word Count: 13389

Abstract

The cerebellum has traditionally been associated with motor control but there is increasing evidence which suggests a role in reward processing. Recent experiments have shown the existence of a monosynaptic connection from the DCN to the VTA, a major dopaminergic area in the mid-brain and a part of the brain's reward system. Optogenetic activation of this connection has been shown to produce place preference and, conversely, the inhibition led to place aversion and abnormal social behaviour, suggesting that this connection modulates reward and social behaviours. Another major dopaminergic area of the brain is the SNc. Activation of dopamine neurons in this area leads to a similar level of place preference as activation of dopamine neurons in the VTA, suggesting a similar role in reward-processing. Both the VTA and SNc have been shown to receive a monosynaptic projection from the DCN. All three of these areas display a large amount of heterogeneity, so a detailed mapping is required before the functional role can be determined. Therefore, the objective of this study is to identify which cerebellar nuclei are sending projections to the mid-brain and what neuronal sub-types in the VTA and SNc are receiving them. To do this we used a deletion mutant rabies virus (CVS-N2c(ΔG)) to infect neurons in the VTA or SNc and transsynaptically label the pre-synaptic partners to these neurons. For cell type specific tracing FLEXed virus constructs and C57BL/6J-DAT$^{\mathrm{IRES}}$Cre mice were used to limit the initial infection. Evidence of a contralateral connection from all DCN was observed and although it was hypothesised that there would be a larger connection to dopaminergic compared to GABA or glutamatergic neurons, we did not observe evidence of this. This project lays the foundation for future work to investigate the function of this connection via photostimulation during reward-based tasks.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Katie Marquand, Monday 28th March, 2022

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Notation and Acronyms

AAV	:	Adeno-associated Virus
ADHD	:	Attention-Deficit Hyperactivity Disorder
BFP	:	Blue Fluorescent Protein
DAT	:	Dopamine Transporter
DCN	:	Deep Cerebellar Nuclei
EnvA	:	Avian virus envelope protein
GABA	:	Gamma-aminobutyric acid
GFP	:	Green Fluorescent Protein
int	:	interposed nucleus
lat	:	lateral (dentate) nucleus
med	:	medial (fastigial) nucleus
NA	:	Nucleus Accumbens
PBS	:	Phosphate Buffered Saline
PFA	:	Paraformaldehyde
PFC	:	Prefrontal Cortex
RL	:	Reinforcement Learning
RN	:	Red Nucleus
RPE	:	Reward Prediction Error
SN	:	Substantia nigra
SNc	:	Substantia nigra pars compacta
SNr	:	Substantia nigra pars reticulata
TH	:	Tyrosine Hydroxylase
VTA	:	Ventral Tegmental Area

WT-VTA	:	experiment targeting the VTA in wild-type mice
WT-SNC	:	experiment targeting the SNc in wild-type mice
DAT-VTA	:	experiment targeting the VTA in DAT-Cre mice
DAT-SNC	:	experiment targeting the SNc in DAT-Cre mice

Acknowledgements

Firstly, thank you to M. Strom and T. Margrie from the Sainsbury Wellcome Centre, UCL, who generously gifted all viruses used and without whom this project would not be possible. Thank you also to the Wolfson Bioimaging Facility along with BrisSynBio for their support and assistance with all imaging taken place during the course of this study. Next I would like to extend my thanks to the members of the Chadderton and Dodson labs, with particular acknowledgement to R. Avvisati, S. Bright, and P. Chadderton for their constant support, teaching, and endless patience. Lastly, I would like to thank my friends and family for their motivation and proof reading skills.

Chapter 1

Introduction

This chapter first provides a discussion of related work that helps to motivate and give the wider context surrounding the project. Secondly, a description of why this area is important to investigate is given, along with the challenges associated. Finally, the experimental plan and hypothesis is stated and key objectives identified.

1.1 Motivation

1.1.1 Reward-based Learning

Classical conditioning [1] depends on the development of association between events, whereas operant conditioning [2] [3] involves learning from consequences of behaviour. Both of these learning paradigms rely on reward to drive learning. In Pavlov's conditioning experiment [1], food acted as a reward and the dogs involved in the experiments learnt to expect this reward when presented with the stimuli after being repeatedly presented with the reward together with the stimuli. Similarly, Skinner's rats [4] learnt that their behaviour, pressing a lever, led to food, a reward, so they quickly learned to press the lever when presented with a similar environment. Rewards are crucial for driving a process known as reinforcement learning (RL). This type of learning involves predicting the value of future rewards and taking the appropriate action to maximise this reward. To do this requires a way to assign weight to reward-indicating stimuli [5] (like the stimuli for Pavolv's dogs), and a way to determine if a change in the environment (like the presentation of food after taking an action in Skinner's experiment) or a completely novel environment has led to a reward better or worse than expected [6]. This difference between the reward received and the expected reward is termed the reward prediction error (RPE) and is used as a learning signal to update the expectation. Rescorla and Wagner [6] formulated this idea mathematically in the Rescorla-Wagner rule which suggests that the strength of association between a stimuli and its associated reward is updated in proportion to the RPE. In this way, the

prediction error drives learning. Numerous observations of animal behaviour seem to show that some sort of RL is happening in the brain, and recordings of neural activity during rewarding behaviours also supports this idea [7] [8]. Olds and Milner [9] made significant discoveries when they implanted electrodes into the brains of rats and allowed animals to press a lever. Instead of giving food or another physical reward to encourage lever presses the reward was direct electrical stimulation of the brain. This revealed that when the electrode was planted in certain areas of the brain, the result was the same for if they were receiving a food reward [4]; the rats learnt to repeatedly press the lever to receive stimulation. This suggests that there are particular brain areas which give some internal reward when stimulated, and are therefore part of the brain's reward system which mediates rewarding experiences. Some areas, particularly those situated on major dopaminergic pathways, showed a particularly strong effect such that the rat preferred to receive direct stimulation over physical rewards such as food or socialisation [10].

It was soon recognised that it was the dopamine neurons that were being activated during this type of stimulation. When rats were administered with the dopamine antagonist pimozide they were slower to learn associations between the lever press and a reward [11], which suggests a decreased value of reward when dopamine is blocked. Dopamine antagonists have also been shown to reduce the rewarding qualities of drugs like amphetamines [12] [13], further supporting a role for dopamine in reward and addiction. Originally it was thought that dopamine was responsible for the overall experience of pleasure [14], however, over time, and with new evidence, it has come to be understood that dopamine is involved in encoding the reward prediction error.

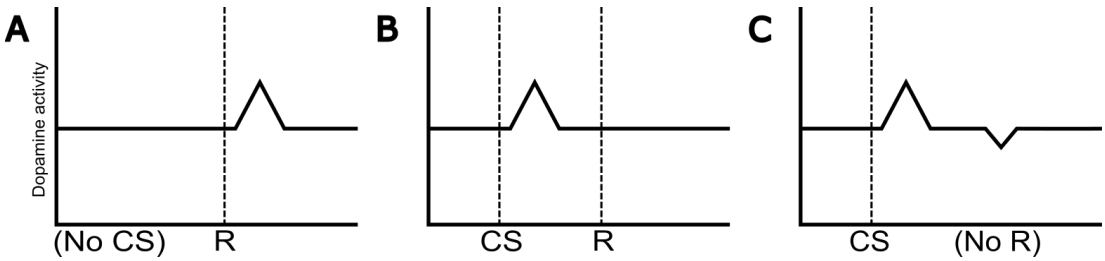

Figure 1.1: Dopamine responds according to an error in reward prediction.
(A) Reward (R) administered although no reward is predicted at the time, thus giving a positive reward error. Dopamine neurons are activated due to this unpredicted reward. (B) Conditioned stimulus (CS) indicates future reward and a reward is given as expected, hence no reward error. Dopamine activity increases activity at onset of stimulus but does not respond to expected reward. (C) Conditioned stimulus indicates future reward, however, no reward is given, leading to a negative reward error. Dopamine responds to stimulus onset and activity is depressed at the time when the reward would have occurred. Figures adapted from results set out in Schultz et al. (1997) [15].

The neural activity of dopamine neurons correlates closely to the RPE term in RL algorithms. Electrophysiological recordings in non-human primates show this relationship [15]. In this experiment the animal has previously learnt the association between some stimulus and a reward

after 1 second of the stimulus onset. Figure 1.1 gives a simplified representation of the results of this experiment. In the first case (Figure 1.1 A) the stimulus is not shown but the reward is received, there is an increase in dopamine activity after the reward. Secondly, if the stimulus is shown and then the reward is received (Figure 1.1 B), there is a dopamine increase after the stimulus onset but not after the reward. Finally, if the stimulus is shown but there is no reward received (Figure 1.1 C), there is a positive change in dopamine activity after the stimulus, but a negative response after the time the reward would be expected. This directly correlates with the reward prediction error. When a reward is received but not expected, there is a positive error, when a reward is expected but not received, there is a negative error, and when a reward is expected and received there is no error. This suggests the dopamine signal is encoding the *expectation* of reward and the strength of the dopamine response allows different weightings to be associated with different reward cues. When the reward deviates from what is predicted, the dopamine neurons also deviate from their baseline response, thus updating future reward predictions.

1.1.2 Structure and Function of the Ventral Tegmental Area

The mid-brain structure Schultz and colleagues recorded from was the ventral tegmental area (VTA), one of the main dopaminergic areas of the brain [15]. The VTA is primarily associated with a variety of cognitive and emotional processes involving motivation [16], reinforcement [17], working memory [18], addiction [19], and aversion [20]. Due to the large population of dopamine cells the most prominent function of the VTA is its role in the reward system [21]. There are two major dopaminergic pathways (Figure 1.2 A) that include the VTA; the first is the mesolimbic pathway which connects the VTA to the nucleus accumbens. When we experience a reward the dopamine neurons in the VTA send projections, i.e. send information in the form of action potentials via axons that extend to distant brain regions, to the nucleus accumbens, increasing the dopamine levels in this area. Rodents which have learnt to press a lever to receive an addictive drug reward stop performing this behaviour when the mesolimbic pathway has been disrupted [22]. This suggests that the dopaminergic connection between the VTA and nucleus accumbens plays an important role in the development of addiction. The second pathway is the mesocortical pathway, which connects the VTA to the prefrontal cortex. Although the role of this connection is less established than the mesolimbic pathway, it is thought to have a role in motivation and reward. The varied areas which receive projections from the VTA dopamine neurons suggest that dopamine is important for normal cognitive function and so disruption results in various disorders [23]. For example, elevated levels of dopamine activity has been seen in disorders such as schizophrenia [24], and abnormalities in the mesocortical pathway also has been linked to psychoses [25]. On the other hand, low dopamine activity in the VTA is associated with attention-deficit hyperactivity disorder (ADHD) [26] [27].

Although dopamine neurons compose around 55-65% of the VTA, it also contains about 35% GABAergic neurons, and around 2-3% glutamatergic neurons which help to regulate the

activity of dopamine [28]. Therefore, these other types of neurons may also have a role in reward processing. For example, the GABAergic neurons in the VTA inhibit dopamine neuron activity and there are many addictive drugs that affect these GABA neurons. Opioids inhibit the GABAergic neurons which indirectly affects the dopamine levels, causing them to increase because those GABAergic neurons are no longer regulating the dopamine neuron activity [29]. This could lead to a sustained RPE despite negative consequences of these drugs and so GABA neurons are instrumental in drug addiction. It has also been shown, in mice, that these GABA neurons also have a persistent firing rate during the delay period between a stimulus signalling a reward and the reward itself [30]. This could mean that GABA cells are encoding information about expectation of reward, and indeed the signal is not affected by whether or not you receive a reward. In contrast to the function of GABAergic neurons, the activation of glutamatergic neurons in the VTA excites dopamine neurons. Photostimulation of these glutamatergic neurons has been shown to cause conditioned place preference [31], and indicates that there could be a micro-circuitry within the VTA which integrates the large amount of information received from other areas.

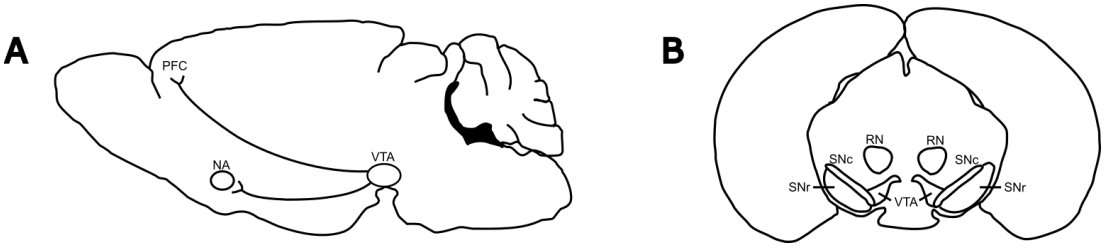

Figure 1.2: Connectivity and structure of the mid-brain dopaminergic areas.
(A) The two major dopaminergic pathways. Mesolimbic pathway connecting the VTA to the nucleus accumbens (NA) and the mesocortical pathway connecting the VTA to the prefrontal cortex (PFC). (B) Cortical slice of the mid-brain. Abbreviations: RN, red nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area. Figures adapted from the Paxinos and Franklin Mouse Brain Atlas [32].

1.1.3 Structure and Function of the Substantia Nigra

The substantia nigra is the other major dopaminergic area of the brain and is located adjacent to the VTA. The substantia nigra itself can be split anatomically and functionally into two distinct structures, namely the substantia nigra pars compacta (SNc) and the substantia pars reticulata (SNr). While the latter is made up of a large population of GABAergic neurons, the SNc is densely packed with dopamine neurons which makes it more interesting in terms of this thesis. These regions are also part of a group of structures known as the basal ganglia.

The basal ganglia is made up of a series of interconnected brain structures which form a network associated with a variety of cognitive [33], emotional [34], and movement related [35] functions. The most well-researched and well-known function of this network is that of facilitating movement. The network is thought to be involved in choosing physical actions which will

lead to positive outcomes and avoiding those which result in negative consequences. One popular hypothesis is that the basal ganglia facilitates desired movements and inhibits unwanted movements, leading to smooth and fluid motion while avoiding jerky involuntary movements [36]. Evidence of this role can be seen when looking at cases of damage to the basal ganglia, such as in Parkinson's disease [37]. This disease arises from the deterioration of dopamine neurons in the SNc and symptoms present as involuntary tremors, slow movement, and rigidity. This suggests that the SNc is crucial for facilitating smooth movement. Patients with Parkinson's disease also struggle with both motor and perceptual timing tasks, but this can be improved with dopaminergic medication [38], which suggests a role for the SNc dopamine neurons in temporal processing. Indeed, an experiment where participants were tasked to reproduce both a short and long time interval showed SNc activation during timing for both intervals [39]. This contradicts earlier hypotheses which suggested that the basal ganglia mediated the timing of long intervals only and the cerebellum was responsible for short intervals.

In addition to movement disorders such as Parkinson's disease, due to the large population of dopamine neurons, the SNc is associated with reward learning, much like the VTA [40]. A study involving optogenetically stimulating dopamine neurons in either the SNc or VTA of mice revealed that stimulation in both areas resulted in similar levels of place preference [41]. Conversely, when inhibiting these dopamine neurons the mice were place averse. This shows that the SNc is similarly rewarding compared to the VTA. This means that by stimulating either of these brain areas the reward the mice receive is sufficient to cause place preference in both cases. If the SNc was less rewarding than the VTA we might see a difference in the extent of the place preference, with SNc stimulated mice not showing place preference behaviour as strongly. Research on addiction, aversion, and reward tend to focus on what role dopamine neurons in the VTA play. It is important that we also include the role of the SNc in these discussions as this region clearly has a lot of functionality in common with the VTA.

1.1.4 Structure and Function of the Cerebellar Nuclei

Another region with a well established link to reinforcement learning is the deep cerebellar nuclei (DCN). The DCN consists of four separate nuclei which contain the only output cells of the cerebellar cortex: the dentate nucleus which is the largest and most lateral, the fastigial nucleus which is the most medial, and, in mammalian species the the emboliform and globose nuclei are often grouped together to form the interposed nuclei, however in rodent species the emboliform and globose nuclei are termed anterior and posterior divisions of nucleus interpositus, respectively. Following the convention set out in the Paxinos and Franklin Mouse Brain Atlas [32] this thesis will henceforth refer to these nuclei as the lateral (lat), interpositus (int) or medial (med) nucleus. Figure 1.3 A shows the location of the deep cerebellar nuclei in relation to the VTA. The neurons located in these nuclei can be split into three sub-categories: excitatory neurons, 50-60%, which project to a variety of targets, inhibitory neurons, 30-35%, which project exclusively to the inferior olive, and local inhibitory interneurons, <10% [42]. These

neuron types are distributed throughout the DCN, but each nucleus is distinct in both location and function. The cerebellum has a well established role in motor control and motor learning, and each nucleus contributes to this. The lateral nucleus is thought to regulate fine-control of voluntary movements [43], the interposed nucleus sends information to the red nucleus in the mid-brain which is associated with modulating limb muscle stretch reflexes [44], and the medial nucleus is linked with ocular motor control [45]. Alongside these motor functions, it is becoming increasingly clear that the cerebellum is also involved in cognitive processes and non-motor behaviour such as social processing [46], emotion [47], and reward processing [48]. Moreover, abnormalities in the cerebellum are associated with symptoms of non-motor conditions including autism spectrum disorder [49], addiction [50] and other impairments [51] [52]. The lateral nucleus has been found to be involved in regulating language [53], the interposed nucleus is required in delayed Pavlovian conditioning [54], and abnormalities in the medial nucleus have been observed in patients with autism spectrum disorder [55].

The cerebellum has also been implicated as a prediction tool, being involved in creating and storing internal models of the motor system in order to give predictions about the state of the system and the sensory inputs [56]. Movement coordination tasks rely on this system to control motor commands of one effector depending on the predicted state of another. For example, a movement experiment in humans containing two parts; firstly an arm movement, and secondly a periodic thumb press, showed that when these two movements temporally overlapped the thumb press was controlled by an estimate of the state of the arm [57]. This was shown due to increased activity in the cerebellum during state-dependent control which was not present when the thumb press relied only on time-dependent control. Since the cerebellum could be thought of as a predictive machine in this context it could also be involved in computing reward prediction or other types of predictions in the brain.

Figure 1.3 B gives a representation of the inputs received by the cerebellum. The main input to the cerebellum reaches the Purkinje cells either directly via the climbing fibres from the inferior olivary complex or via the mossy fibres from a number of other nuclei. Classical theories [58] [59] [60] suggest that these projections from the inferior olive help to instruct cerebellar learning using a reinforcement learning error rule. These models suggest that when expected sensory feedback after a movement does not match the actual feedback this is signalled by climbing fibre activity. Since the climbing fibres make many synaptic connections with Purkinje cells, this results in complex spikes which represent the error signal. These complex spikes induce changes at parallel fibre-Purkinje cell synapses though plasticity mechanisms that alter the simple spike response to mossy fibre inputs. This results in a learnt simple spike response from Purkinje cells which is sent to other areas of the brain via the DCN and reflected in modified behaviour. This key circuit is present throughout the cerebellar cortex and presumably the computation it is performing is also similar throughout, even if the function is very different, i.e. limb movements versus language regulation. Therefore, the functional differences between different areas of the cerebellar cortex are thought to be due to the differences in input and

output connectivity [61]. The cerebellar cortex can be split into zones in which the Purkinje cells are receiving climbing fibre input from a specific region of the inferior olive and sending output to a specific region in the cerebellar nuclei [62]. These zones can further be broken down into microzones within which all Purkinje cells receive input from climbing-fibres with similar receptive fields, i.e. have received sensory input from a specific limb [63]. This modular organisation has an important implications for function and how the cerebellar nuclei integrate information to perform various functions. Therefore it is important to investigate the distinct cerebellar nuclei and their connections separately as they are a part of this modular system.

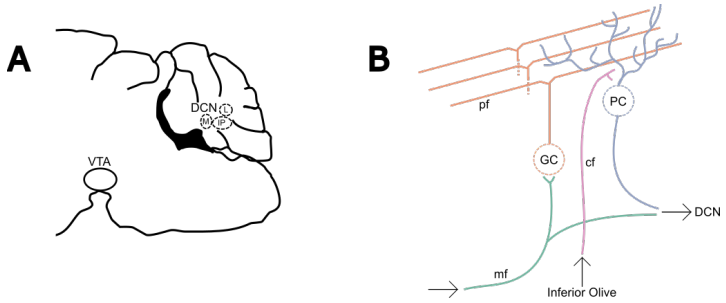

Figure 1.3: Connectivity of the deep cerebellar nuclei.
(A) Sagittal slice of the cerebellum. Abbreviations: DCN, deep cerebellar nuclei; L, lateral nucleus; IP, interposed nucleus; M, medial nucleus; VTA, ventral tegmental area. Figure adapted from the Paxinos and Franklin Mouse Brain Atlas [32]. (B) Circuit diagram of the connections received by the cerebellum. Abbreviations: pf, parallel fibre; PC, Purkinje cell; GC, granule cell; cf, climbing fibre; mf, mossy fibre.

There is evidence that climbing fibre responses signal more than just motor errors and thus might contribute to learning in other non-motor situations including temporal-difference [64], and reward prediction [65]. This suggests that the cerebellum might support a wide range of behaviours including reward-motivated behaviour by using an alternative learning rule. For example, recordings from granule cells show three different types of response during Pavlovian reward tasks: reward responding, omitted reward responding, and reward anticipation [66]. Unexpected reward led to an increased response in the reward responding cells, reminiscent of dopamine cells in the VTA increasing their response following an unexpected reward.

A role for the cerebellum in modulating the reward circuits of the brain has become increasingly probable due to the identification of a monosynaptic connection between the cerebellum and the VTA. Carta et al. [67] performed two sets of experiments, optogenetic activation and inhibition, to show the presence of a functional connection between the VTA and cerebellum. Behavioural tests show that optogenetic activation of this connection was sufficient to produce long term place preference, demonstrating that this pathway is rewarding. Inhibition of this connection also leads to abnormal social behaviour by abolishing social preference, suggesting that this connection is required for normal social behaviour as well. This connection has been further investigated by a tracing experiment [68]. Using the rabies virus to label monosynaptic connections, this paper aimed to elucidate the difference between inputs to the VTA and SNc

and showed that both of these dopaminergic mid-brain areas received projections from the lateral cerebellar nucleus. Since the cerebellum has a close association with timing operations in the range of milliseconds [69] it has been hypothesised that the connection to the VTA could be providing information about timing. In fact, in reinforcement learning tasks which require precise timing of motor actions, complex spikes encode RPE differently than when precise timing is not required to receive the reward [48]. The similarities between the VTA and the SNc in reward based tasks and the fact that they both have been shown to have a connection to the cerebellum suggests that the CN may provide information to both of these areas to facilitate a wide range of cognitive and emotional processes. The association between the SNc and movement also indicates a connection to the cerebellum. All three areas are thought to play a role in addiction [50] [19] [70], and other social impairments, therefore mapping the connections from the cerebellum to these areas could reveal pathways relevant to these disorders.

In order to reveal more information about these connections and their function in relation to RPE, a more thorough mapping of the connection is needed. Due to the range of functions associated with each individual cerebellar nucleus it is crucial we map exactly which nuclei are projecting to the mid-brain areas to infer possible functions for the connection. Similarly, since the VTA has considerable heterogeneity in terms of the neurochemical sub-types, it is important to reveal which neurons are receiving this projection: dopaminergic, glutamatergic, GABAergic, or some combination. After creating a clear mapping of these connections the function in relation to RPE can be looked at more closely by performing experiments manipulating the connection during reinforcement learning tasks.

1.1.5 Connectomics

Connectomics involves mapping connections between different areas of the brain at the level of synapses. This allows us to create a circuit diagram which can then be used for further investigation and analysis. Attempts at the complete reconstruction of wiring diagrams for small insects, and invertebrates are underway [71] [72] [73], and scientists have even been successful in creating a representation of over 25,000 neurons and their connections in the central brain of adult drosophila [74]. This connectome has provided data to create an incredibly detailed atlas of a nervous system which can then be used to generate new hypotheses and novel experiments. Mapping a full connectome of the mouse brain would be a much larger task but would provide data to lay the foundation for future circuit based studies. Creating this mapping in a healthy brain would also allow us to compare brain wiring in healthy mice to the brain wiring in mouse models of disorders, possibly leading towards therapies which target the root cause.

In particular, this thesis focuses on a small subsection of the mouse connectome, mapping connections from the cerebellum to the mid-brain, specifically the VTA and SNc. There is large heterogeneity in both ends of these connections. The cerebellum has been shown to contain reward-suppressed and reward-activated microzones which encode RPE signals differently to

each other [75]. This means it is important to know which microzone is sending information to the VTA and SNc as they represent different information. Similarly, there are many different types of neuron present in the VTA: GABAergic, glutamatergic, and dopaminergic, each with different roles in the reward system [76] [28]. When these dopaminergic or non-dopaminergic neurons are optogenetically stimulated both types can produce reward related behaviours independently from each other. This means it is important to understand which neurochemical sub-types are receiving projections from the DCN or if different nuclei project to different neuron types.

1.2 Proposed Experiments

This thesis will build on the previous work that has revealed a monosynaptic connection between the cerebellar nuclei and the VTA [67] [68]. We will focus on the connectome and topography of this connection, by investigating the specific areas and cell types involved. Along with the VTA, we will also consider the SNc, which seems to have a similar role to the VTA in the reward system and thus might have a similar connection from the cerebellum. We will approach this task using the retrograde monosynaptic tracing techniques introduced by Wickersham et al. [77] in which a modified rabies virus is used to infect postsynaptic starter cells. The rabies virus then spreads to the cell's presynaptic partners, clearly labelling the neurons which send direct projections to the starter cells.

Usually, rabies virus will replicate and spread across multiple synapses, making it impossible to distiguish which neurons are directly connected and which are indirectly connected to the starter population. To solve this problem, the virus is modified so that the 'G' gene, encoding the envelope glycoprotein required for transsynaptic spread, is removed, thus limiting the virus to replicate only in initially infected cells. The glycoprotein can be replaced with the coding sequence for enhanced green fluorescent protein (EGFP), or another fluorescent marker, so that as the virus replicates in the initially infected cell it produces sufficient fluorescence to brightly label the infected cells. In order to genetically target specific cells for infection the rabies virus is pseudotyped with an avian virus envelope protein (EnvA) which can only infect cells which also express the EnvA binding partner, TVA. The target population of cells must be engineered to express TVA as it is not naturally found in mammals, only birds. In order to do this, the target cells are infected with adeno-associated (AAV) helper viruses, via injection, which deliver the TVA and also the deleted glycoprotein gene. After some time the cells expressing TVA are then infected with the modified rabies virus. Due to the glycoprotein gene being supplied in trans with these intitially infected cells the rabies virus can use this to spread one step to the presynaptic neurons. The virus is unable to spread any further as the 'G' protein is no longer present. This gives us a reliable method of labelling cells that are monosynaptically connected to the starter population, and a way to genetically select which cells are part of that starter population.

It is even possible to target specific neuronal sub-types by using transgenic mice in combination with a FLEXed version of the AAV helper viruses [78] [79]. In our tracing experiments we will target dopaminergic neurons by using DAT-Cre mice, that is mice which express Cre recombinase in dopaminergic neurons. The FLEXed virus means that the sequence of interest, for example the gene for expressing TVA, is flanked by two sets of different lox sites. The Cre proteins bind to the lox sites and cause a series of recombinations to take place resulting in the gene of interest being inverted. In one orientation the gene will be expressed but when inverted it will not be expressed. This means that if the virus has the TVA expression turned off originally, when the DAT-Cre mouse is infected with this virus the dopamine cells expressing Cre recombinase will invert the sequence effectively turning TVA expression on. In our experiments we will use FLEXed versions of the AAV helper viruses to specifically target dopamine neurons with our rabies virus infection. We will also use the non-FLEXed versions of the same viruses in wild-type mice which do not express Cre recombinase to target all cells.

This monosynaptic tracing method has become a standard for neuronal circuit mapping and can be used in a variety of contexts. For example, this method has been used to delineate two parallel motor pathways responsible for controlling whisker movement in mice [80], and has also previously been used to investigate the differences between inputs to the dopamine neurons of the VTA and SNc by producing a whole-brain mapping of these inputs [68].

1.3 Objectives and Challenges

The high-level objective of this project is to investigate which regions of the CN provide projections to the VTA and the SNc in order to begin to understand the topography of this monosynaptic projection. We also want to investigate which neuronal sub-types in the midbrain receive these projections. More specifically, the concrete aims are as follows:

1. Accurately label a population of starter cells in the VTA and SNc using the modified rabies tracing techniques described above.
2. Perform fluorescence microscopy in perfusion fixed brain slices to identify cells in the cerebellum which have been labelled by the virus and are thus pre-synaptically connected to the starter cells.
3. Identify which regions of the DCN provide projections to the VTA and SNc.
4. Identify which neuronal sub-types are receiving the projections in the VTA and SNc.
5. Calculate, analyse, and compare convergence indices to determine the relative connectivity.

We will do this for both wild-type mice, to target all cell types, and DAT-Cre mice, to target specifically dopamine neurons.

The main challenge of performing these tracing experiments is perfecting the surgical technique, consistently targeting the correct brain region, and injecting an appropriate volume of virus for the experiments to work. The red nucleus is a structure of the mid-brain located laterally to the VTA and is well known to have a role in motor coordination, particularly in controlling the limb muscles [44]. The cerebellar nuclei provide inputs to the red nucleus, including a large projection from the interposed nucleus [81]. This means that a retrograde tracing experiment with starter cells located in the red nucleus will result in labelled neurons seen in the CN. For this reason we must be careful not to infect any cells in the red nucleus with the modified rabies virus, otherwise it will be impossible to distinguish if the signal in the cerebellum is due to a connection to the VTA or the already established connection to the red nucleus.

Hypothesis

The lateral and interposed nuclei provide projections to both the SNc and VTA. This projection converges to all neuronal sub-types, GABAergic, glutamatergic and dopaminergic but there will be a larger convergence to dopamine neurons.

Chapter 2

Materials and Methods

2.1 Animal Maintenance and Breeding

A total of 10 adult (10-22 weeks) mice were used during the course of these experiments. Two rounds of experiments took place, the first involved 3 nNos-Cre (C57BL/6J-nNosCre; nNosCre +/+; JAX: 017526) mice as wild-types targeting all different neurochemical subtypes and 4 DAT-Cre (C57BL/6J-DAT^{IRES}Cre; DATCre +/-; JAX:006660) mice expressing Cre-recombinase in dopamine neurons for cell type specific targeting. The second experiments used 3 DAT-Cre mice only. Mice were sourced from the in-house breeding facility and Charles River. Mice were housed on a 12-hour light/dark cycle, maintained at a constant temperature, and provided with sufficient environmental enrichment. All procedures were approved by the University of Bristol Faculty of Life Sciences and Faculty of Science Research Ethics Committee and performed in compliance with Animals (Scientific Procedures) Act 1986 (UK).

2.2 Stereotaxic Surgeries

Prior to surgery mice were aneasthetised via isoflurane inhalation (3% induction, 1.5-2% maintenance with oxygen). To manage pain, analgesics were administered subcutaneously before the procedure (0.3mg/ml buprenorphine diluted 1:10 in sterile saline so animals received 0.03mg buprenorphine in a 0.1ml solution). Mice were secured on a stereotaxic frame (Model 963, David Kopf Instruments [82]), eyes protected with ophthalmic gel (Lubrithal, Alcon) and their body temperature was measured and maintained at 37-38°C using a closed-loop heating pad.

Craniotomies and injections were made to the right hemisphere of the mid-brain either in the Ventral Tegmental Area (VTA) or the substantia nigra pars compacta (SNc) using coordinates based on the Paxinos and Franklin Mouse Brain Atlas [32]. For experiments targeting the VTA the coordinates used to drill craniotomies and make injections were -3.00mm anterior-posterior,

-0.50mm medial-lateral relative to bregma and -4.3mm dorsal-ventral relative to the dura. For experiments targeting the SNc the coordinates were -3.00mm anterior-posterior, -1.50mm medial-lateral relative to bregma and -4.3mm dorsal-ventral relative to the dura. Long-shaft pulled glass pipettes were frontfilled with the virus, lowered to the injection coordinates, and left in place for 1 minute before delivering the virus at a rate of 75nl/min. After the injection is complete, the pipettes were left in place for another 10 minutes and then removed slowly to reduce flux.

On day 1 of the experiment mice were injected with a mixture of two AAV helper viruses, mixed in a 1:1 ratio using a vortex mixture. For dopamine specific tracing, DAT-Cre mice recieved injections of approximately 200nl of flexed AAV virus constructs; AAV1-syn-Flex-H2bG-N2cG and AAV1-syn-Flex-nucEBFP-TVA for the first experiment and AAV1-syn-Flex-H2bG-N2cG and AAV2/8-Ef1a-Flex-GT-EGFP-TVA in the second experiment. Wild-type nNos-Cre mice recieved injections of approximately 75nl of the non-flexed versions of these viruses; AAV1-Ef1a-cre-off-H2BG-N2cG and AAV1-EF1a-cre-off-EGFP-TVA. Following the procedure, craniotomy incisions were closed using vetbond tissue adhesive and mice were removed from isoflurane, given a subcutaneous sterile saline injection to rehydrate, and, once able to self-right, transferred to a small animal recovery chamber maintained at 36.5°C. During recovery and for three days post surgery mice were provided with a nutritionally fortified water gel, Diet® Gel Recovery (ClearH$_2$0, Portland, ME, USA [83]). Once the animal's posture and gait had returned to normal, they were returned to their home cage and monitored closely for the following 48 hours.

At least 14 days after the AAV helper virus injections, the same volume (200nl for DAT-Cre mice or 75nl for wild-type mice) of modified rabies virus (CVS-N2c(ΔG)-mCherry(EnvA) [84]) was delivered using the same coordinates.

All viruses were gifted by the Margrie group (Sainsbury Wellcome Centre for Neural Circuits and Behaviour; UCL) and stored at -80°C.

2.3 Tissue Fixation and Preparation

At least 14 days after the rabies injection, transcardial perfusions take place. Mice were first anaesthetised via isoflurane inhalation (5%) and then given a lethal dose of pentobarbital (>100mg/kg) via intraperitoneal injection. Perfusions are performed with approximately 100ml phosphate buffered saline (PBS) followed by 100ml 4% paraformaldehyde (PFA). Brains were extracted, post-fixed overnight submerged in 4% PFA and stored in a refrigerator at 4°C. 50μm thick coronal sections of the cerebellum (between -5.20mm and -7.60mm from bregma) and the mid-brain (between -4.70mm and -2.50mm from bregma) were cut using a vibratome (Leica). Every fourth slice (ie. slices 200μm apart) were mounted on slides with a gelvatol-based mounting medium and coverslipped for imaging and analysis. Remaining slices were submerged

in PBS-azide and kept refrigerated.

All stereotaxic surgeries and transcardial perfusions were performed by Dr Riccardo Avvisati and observed by the author. All subsequent slicing and imaging was performed solely by the author.

2.4 Microscopy and Image Analysis

All microscopy data was obtained using Wolfson Bioimaging Facility equipment. Widefield imaging was performed using a Leica DMI6000 inverted epifluorescence microscope and associated Leica Application Suite X (LAS X) software. Slices were imaged under blue light (λ450-490) for GFP expression associated with AAV helper viruses, UV light (λ340-380) for BFP expression associated with AAV helper viruses, and green light (λ515-560) for mCherry expression associated with rabies virus. Images obtained were processed using the Fiji image processing software and outlines and labels of regions of interest were added manually.

2.5 Statistical Analysis

For starter cell distribution analysis, cell counts of GFP^+ cells were taken from each 50μm thick slice from the mid-brain using in built methods from Fiji image processing software. Regions of interest were added manually to get cell counts for each area. The x and y coordinates of each cell was extracted and the locations were manually mapped onto a reference image based on Paxinos and Franklin Mouse Brian Atlas [32]. A confidence ellipses of the distribution was plotted using 1 standard deviation in both the x and y directions as the radius.

For starter cell distribution analysis in the anterior-posterior direction, centre of gravity was calculated as $\sum$(number of GFP^+ neurons $\times$ coordinate from bregma) / $\sum$ number of GFP^+ neurons.

For rabies traced neuron analysis, a two-tailed Student T-test was used for single comparisons and all values were reported as 95% confidence intervals of the mean. For multiple comparison analysis, we use a two-way analysis of variance (ANOVA) test followed by a post-hoc Tukey's test to determine where significant differences identified by ANOVA lie. An alpha level of 0.10 is used throughout the statistical analysis. Due to our small sample size, which increases the standard errors, we accept a larger alpha value. Where applicable we also highlight differences that are significant using the alpha level of 0.05, which is the scientific standard.

All graphs were generated using the Seaborn data visualisation library in Python. T-tests were calculated using standard functions of the SciPy scientific computing library in Python. ANOVA and Tukey's test were performed using the Pingouin statistical package in Python.

Chapter 3

Results

This chapter describes questions we set out to answer, how we performed each stage of the experiment, and the key experimental results. Any statistical analysis and it's implications are described as well as a discussion of the reliability and quality of results.

3.1 Visualisation of neurons infected with AAV and rabies viruses in VTA and SNc

The purpose of this study was to identify the monosynaptic connections from the cerebellum to the VTA and the SNc. Specifically, we aimed to identify which cerebellar nuclei were connected to these mid-brain areas and which neurochemical sub-types receive these projections. In order to achieve these aims, we used a rabies virus monosynaptic tracing technique described by Wickersham et al. [77] in four experimental setups. In the first case, we used wild-type mice to target all neuronal sub-types in the VTA, we will refer to this case as WT-VTA (number of mice = 1). Secondly, we used wild-type mice to target all neuronal sub-types in the SNc (WT-SNC, n=2), next we used DAT-Cre mice to target dopamine neurons in the VTA (DAT-VTA, n=1), and finally we used DAT-Cre mice to target dopamine neurons in the SNc (DAT-SNC, n=2).

For the monosynaptic tracing to work the rabies virus is modified in two ways. Firstly, there must be a way to specify and limit initial infection. To do this the rabies virus is pseudotyped with the avian envelope protein (EnvA) which can only infect cells which also express the EnvA binding partner, TVA. TVA does not occur naturally in mammals, so the target starter population must be engineered to express it, providing a way to specify which cells you want to target. Secondly, the rabies virus spreads in a retrograde direction across synapses, but this spread needs to be limited to moving across one synapse only in order to infect cells which are directly connected to the starter population. To achieve this monosynaptic spread, the rabies envelope glycoprotein, which is necessary for movement across synapses, is removed.

The target starter population is engineered to express this glycoprotein separately so the rabies virus infecting those cells is able to move across one synapse only. In the modified virus, the glycoprotein is replaced, in our case, with a gene encoding the fluorescent marker mCherry so the cells can be visualised under a fluorescent microscope.

The TVA and the rabies envelope glycoprotein required for this method to work are delivered to the starter cell population via two AAV viruses. In the experiments using wild-type mice, these AAV viruses were non-flexed and could infect all neuronal sub-types in the target area (Figure 3.1 A, Figure 3.2 A). However, for more specific targeting of dopamine neurons we used DAT-Cre mice, which express Cre recombinase in dopamine neurons. These transgenic mice in combination with flexed versions of the AAV viruses means that the TVA and rabies glycoprotein is only expressed in dopamine neurons, and in turn the modified rabies virus can only infect these dopamine neurons (Figure 3.3 A, Figure 3.4 A).

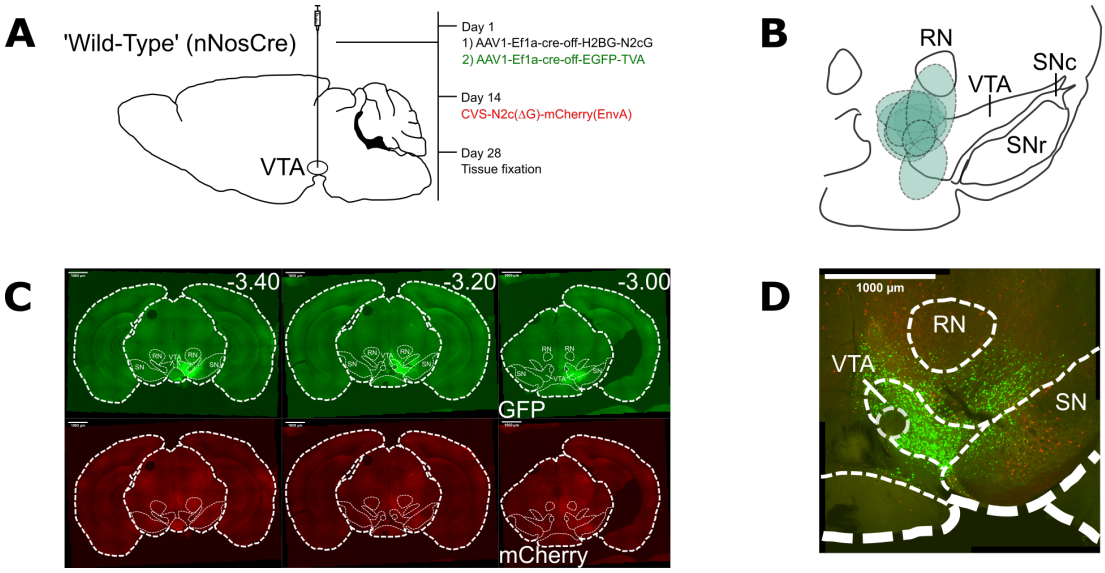

Figure 3.1: Overview of monosynaptic tracing methodology in wild-type mice targeting the VTA

(A) Experimental design for delivery of non-flexed AAV helper viruses and rabies virus to all cells of the VTA. (B) Coronal section of the mid-brain representing the starter cell positions. Each confidence ellipses represents distribution of GFP^+ cells of one 50μm slice (number of slices = 10). The ellipse is centred at the centre of mass of starter cells and the radius is calculated as one standard deviation in the medial-lateral and dorsal-ventral directions separately. (C) Representational images of AAV-TVA (EGFP) and rabies (mCherry) labelling in VTA. Scale bars represent 1000μm. (D) Higher magnification view of GFP and mCherry labelled cells in VTA (Bregma, -3.20mm).

In our initial experiments, the AAV virus used to deliver TVA to DAT-Cre mice contained the gene to express enhanced blue fluorescent protein (EBFP) (Figure 3.3 A). Coronal sections of the mid-brain were imaged under ultraviolet light to identify starter cells expressing EBFP and an example is shown in Figure 3.3 E. However, during imaging it was clear that the cells did not have a bright fluorescence as expected. The cerebellum was also imaged under green light

for mCherry expression representing rabies labelling due to a monosynaptic connection. This showed zero to minimal rabies labelling which lead us to believe there may have been a problem with the AAV helper virus. This problem was not only with the level of fluorescence of the EBFP but also with the efficiency of the rabies virus initially infecting the starter population of cells. To solve these problems we acquired a new AAV virus which instead encoded the fluorescent marker enhanced green fluorescent protein (EGFP) and the results obtained from the 4 DAT-Cre mice infected with the original AAV viruses were disregarded.

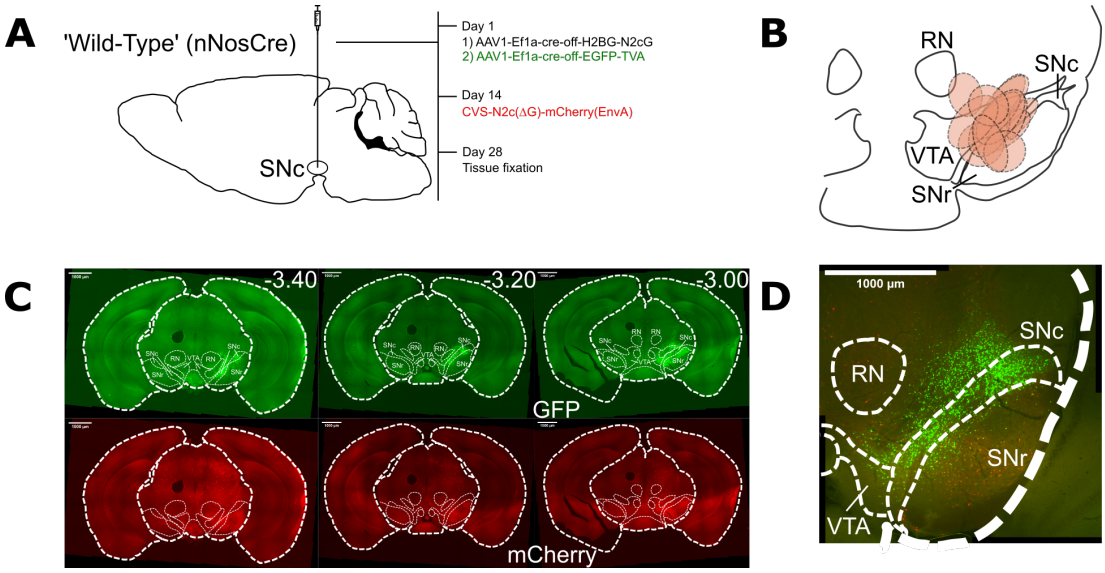

Figure 3.2: Overview of monosynaptic tracing methodology in wild-type mice targeting the SNc

(A) Experimental design for delivery of non-flexed AAV helper viruses and rabies virus to all cells of the SNc. (B) Coronal section of the mid-brain representing the starter cell positions. Each confidence ellipses represents distribution of GFP^+ cells of one 50μm slice (number of slices = 14). The ellipse is centred at the centre of mass of starter cells and the radius is calculated as one standard deviation in the medial-lateral and dorsal-ventral directions separately. (C) Representational images of AAV-TVA (EGFP) and rabies (mCherry) labelling in SNc. Scale bars represent 1000μm. (D) Higher magnification view of GFP and mCherry labelled cells in SNc (Bregma, -3.20mm).

For the remainder of the experiments all mice were infected with AAV viruses encoding EGFP and so coronal slices of the mid-brain were imaged under both blue (for GFP expression) and green (for mCherry expression) light to identify starter cells (Figures 3.1, 3.2, 3.3, 3.4 C). There was strong GFP labelling observed in close proximity to the injection site, however the mCherry labelling throughout the mid-brain was fairly weak in comparison. We expected that the mCherry labelling in the mid-brain would be much stronger and that the starter cell population would be defined as cells which co-express both GFP and mCherry. These would be the cells which were infected with the AAV helper viruses and the rabies virus via injection. Cells which express GFP only would be those infected with the AAV viruses but not initially infected with the rabies virus and so they would not contribute to any rabies virus expression

seen elsewhere in the brain. Conversely, expression of mCherry only suggests that those are neurons that had been transsynaptically labelled. Further away from the injection site there are cells which express mCherry and not GFP, which suggest that there has been some successful transsynaptic labelling within the mid-brain. However, the extent of mCherry labelling was weaker than expected.

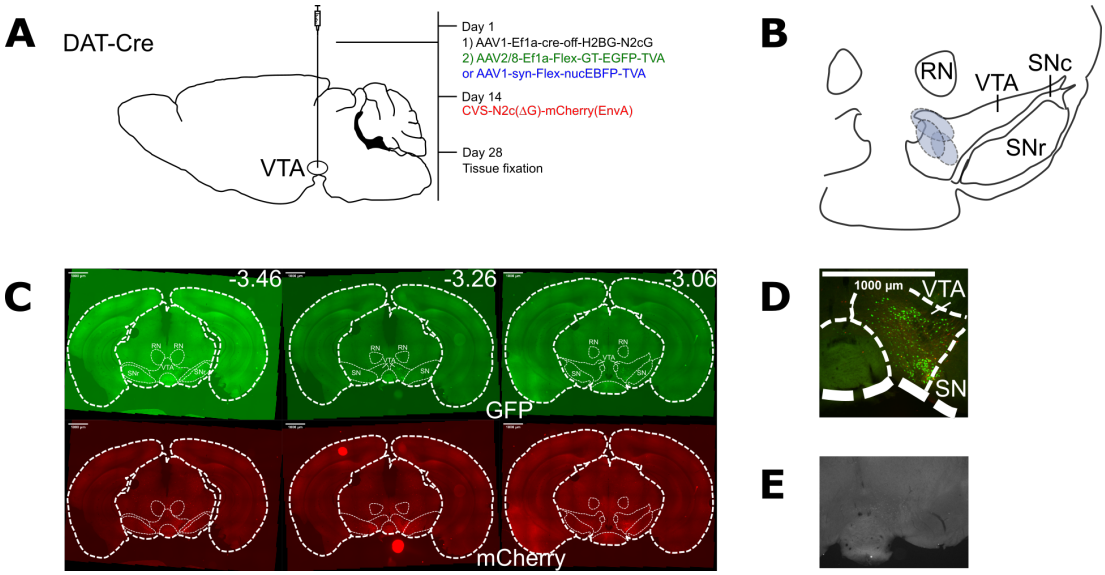

Figure 3.3: Overview of monosynaptic tracing methodology in DAT-Cre mice targeting the VTA

(A) Experimental design for delivery of flexed AAV helper viruses and rabies virus to dopamine cells of the VTA. (B) Coronal section of the mid-brain representing the starter cell positions. Each confidence ellipses represents the distribution of GFP^+ cells of one 50μm slice (number of slices = 3). The ellipse is centred at the centre of mass of starter cells and the radius is calculated as one standard deviation in the medial-lateral and dorsal-ventral directions separately. (C) Representational images of AAV-TVA (EGFP) and rabies (mCherry) labelling in VTA. Scale bars represent 1000μm. (D) Higher magnification view of GFP and mCherry labelled cells in VTA (Bregma, -3.26mm). (E) Higher magnification (x10 magnification) view of the VTA imaged under UV light to show any BFP labelled cells (Bregma, -3.26mm). There is very minimal fluorescence seen.

Figures 3.1, 3.2, 3.3, and 3.4 D show a medium magnification image of the injection site. The lack of brightly labelled red cells in contrast to the bright green cells is made clear in these images, and there is little to no overlap between the two. As cells must have received both the AAV helper viruses and the rabies virus in order for the monosynaptic tracing to take place, and there is evidence of transsynaptic labelling in the mid-brain, it is likely that the modified rabies virus was present in a proportion of the cells expressing GFP despite the lack of mCherry signal. Due to this, we define our starter cells as all GFP expressing cells. This results in an over estimation of the number of starter cells since not all of those GFP^+ cells are also initially infected with rabies virus, but assuming that the ratio of GFP expressing cells to actual starter cells (ie. cells infected with both AAV and rabies viruses via injection) is the same for all

experiments this over estimation has little effect on results.

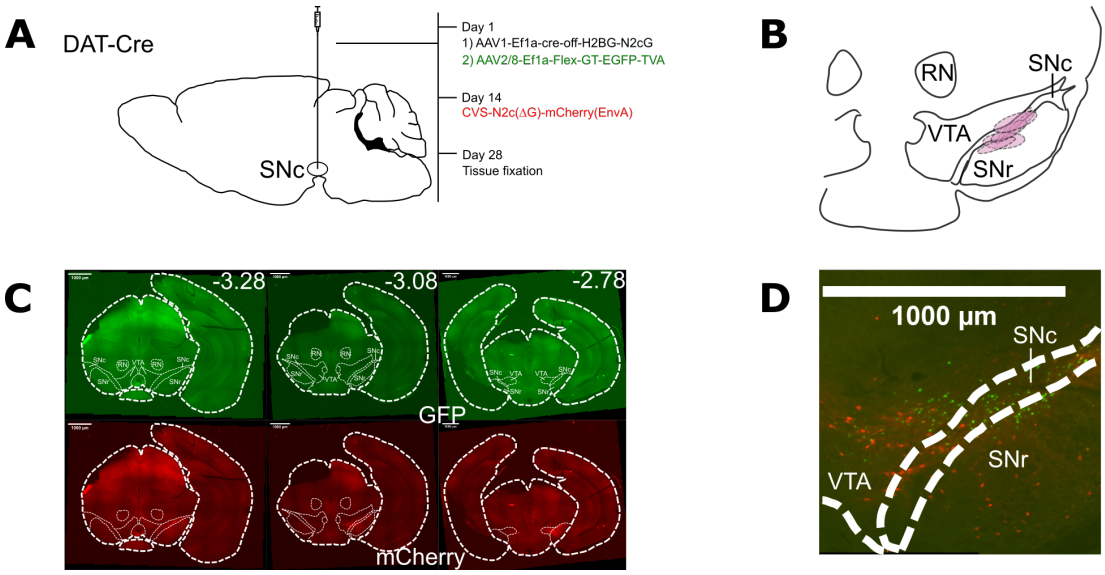

Figure 3.4: Overview of monosynaptic tracing methodology in DAT-Cre mice targeting the SNc (A) Experimental design for delivery of flexed AAV helper viruses and rabies virus to dopamine cells of the SNc. (B) Coronal section of the mid-brain representing the starter cell positions. Each confidence ellipses represents the distribution of GFP^+ cells of one 50μm slice (number of slices = 6). The ellipse is centred at the centre of mass of starter cells and the radius is calculated as one standard deviation in the medial-lateral and dorsal-ventral directions separately. (C) Representational images of AAV-TVA (EGFP) and rabies (mCherry) labelling in SNc. Scale bars represent 1000μm. (D) Higher magnification view of GFP and mCherry labelled cells in SNc (Bregma, -3.08mm).

3.1.1 Is GFP expression limited to the target structures?

It is important that the starter neuron population is contained to the target area so we can be sure that any connections traced to the cerebellum have originated from the VTA or SNc. This is especially important because the VTA and SNc are located adjacent to each other and we want to distinguish the connections between each of these areas. The red nucleus is also located adjacent to the VTA and has a well known role in motor function, receiving a large projection from the interposed nucleus of the cerebellum. If a large proportion of our starter cells are located in the red nucleus it will be impossible to determine if any mCherry expression in the cerebellum, especially in the interposed nuclei, is due to the red nucleus connection, or because of a connection to the VTA or SNc.

Evidence that GFP expression is not limited to target structures in wild-type mice

Figure 3.1 B gives a representation of the starter cell distribution in the medial-lateral and dorsal-ventral directions when targeting the VTA in wild-type mice. Each oval represents the

confidence ellipses for one coronal slice of the mid-brain, with the radius of the ellipse showing one standard deviation in each direction. There is a large overlap of these confidence ellipses over the VTA showing that there were a large proportion of cells located in that area. However, in the dorsal-ventral direction there is a large spread, with starter cells encroaching on the red nucleus.

Figure 3.2 B shows a similar image representing the spread of starter cells when targeting the SNc in wild-type mice. The cluster of ellipses is positioned more laterally than the starter cells in the VTA which is to be expected as the injection coordinates used to target the SNc were more lateral. The distribution of starter cells seems to be quite even in both directions but since the SNc is a small and thin area this spread could have a big effect especially since it appears the starter cells are located in the VTA as well.

Since the target areas are small (especially the SNc) and adjacent to each other it is virtually impossible to limit the expression of GFP to only one of these structures. Also, the confidence ellipses are taken from each slice and collapsed to a 2D representation so we are losing some information about the shape and size of the VTA or SNc at that particular slice. Although this method does not give a completely accurate representation of the distribution we can see that the starter cells do spread outside of their target area.

In order to fill in the gaps about the 3D distribution of starter cells we next considered the anterior-posterior axis. As you move through the brain in this direction the VTA and SNc change shape and size. The VTA, for example, is present in the brain approximately between -3.88mm and -2.70mm from bregma. Similarly, the SNc is seen in the brain approximately between -3.88mm and -2.46mm from bregma ([32]). We can see in Figure 3.5 A that both wild-type cases have similarly large distributions which extend beyond the presence of their target structure in both directions. This results in GFP expression in cells which are not part of the target structures but still may contribute to transsynaptically labelling cells. Also plotted in Figure 3.5 A is the centre of mass of the starter population (represented by a black circle). We expect this centre of mass to be at -3.00mm from bregma because that was the anterior-posterior coordinate of our injections. Both wild-type cases have their centre of mass within 0.5mm of this target coordinate which shows that our injections were consistent and precise.

Next, we wanted to establish to what extent the non-target area starter cells would have an effect on transsynaptically labelled cells in the cerebellar nuclei. The red nucleus has a well established projection from the interposed cerebellar nuclei so we especially want to avoid starter cells in this area. Another problem is starter cells meant for the VTA ending up in the SNc and vice versa. The surrounding areas of the VTA pose less of a problem as they do not have connections from the cerebellum. The percentage of total starter neurons located in each of these areas: VTA, SN, red nucleus (RN), or other areas was calculated and displayed in Figure 3.5 B. Both wild-type mice cases have a large proportion of GFP expressing starter neurons located in 'other' regions outside of the target (53.3% for WT-VTA, and 47.8% for WT-SNC).

This could largely be due to the anterior-posterior distribution, with a large amount of starter cells located beyond the boundaries of the target area in this direction. However, these cells are not likely to effect the results as they are not likely to have connections to the cerebellum. Both wild-type cases also have a small proportion of starter cells located in the RN (0.6% for WT-VTA and 2.0% for WT-SNC). Although these are small percentages and are not likely to have a huge impact on results, since there is a well known connection from the lateral cerebellum we will need to consider this.

In the case of WT-VTA there is also a small proportion (2.8%) of starter cells located in the SN, which includes both the SNc and the SNr. Similarly to the red nucleus this needs to be taken into account when looking at results but is such a small percentage that it is unlikely to have any effect. For the WT-SNC experiment, however, 9.3% of starter neurons were located in the VTA. Since the SNc is a small area located adjacent to the VTA it is virtually impossible to restrict infection to just this area without spreading to the VTA. Although this is to be expected, the fact that there are cells in the VTA which are contributing to rabies labelled cells in the cerebellum will be taken into account when discussing results.

Overall, for the wild-type experiments the GFP expression had a large spread and was not contained to the target structures. Representational images can be seen in Figure 3.5 C and D. Since our definition of starter cells gives a large overestimation for the amount of cells contributing to transsynaptic labelling, there is likely also a large over estimation of the spread of these starter cells. There are starter cells located in the red nucleus and the VTA or SNc (opposite to the target structure) which need to be considered when analysing the monosynaptic connection but these are in such small proportions that it is unlikely to have a significant effect on results. If we were to redo these experiments these problems could be minimised by reducing the volume of viral injections.

Evidence that GFP expression is limited to target structures in DAT-Cre mice

Figure 3.3 B gives a representation of the starter cell distribution in the medial-lateral and dorsal-ventral directions when targeting the VTA in DAT-Cre mice. Each oval represents the confidence ellipses for one coronal slice of the mid-brain, with the radius of the ellipse showing one standard deviation in each direction. The ellipses are all located in the VTA, with very minimal spread to outside this target structure.

Figure 3.4 B shows a similar image representing the spread of starter cells when targeting the SNc in DAT-Cre mice. The cluster of ellipses is located more laterally than the starter cells in the VTA which is to be expected since the injection coordinates used to target the SNc were more lateral. The direction of the spread seems to follow the SNc's thin and diagonal structure with minimal overlap to other structures.

Both of these DAT-Cre cases appear to have a much smaller medial-lateral and dorsal-ventral

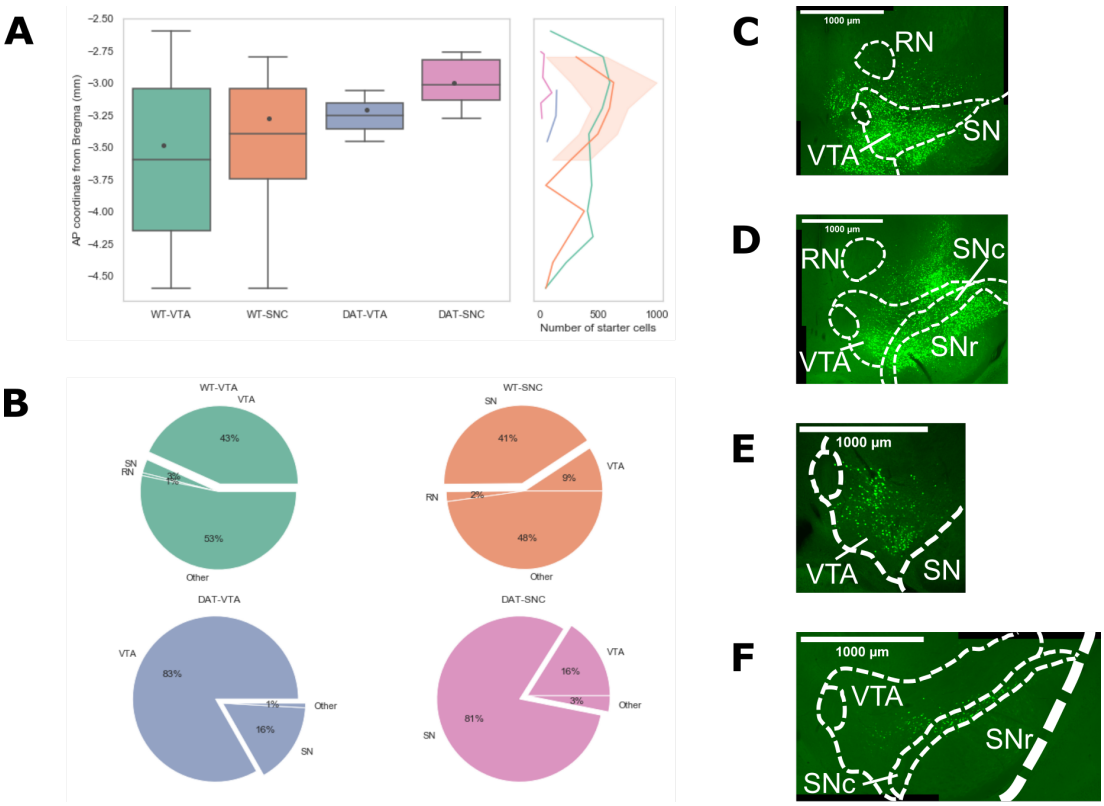

Figure 3.5: Starter neuron distributions in VTA and SNc
(A) (Left) The anterior-posterior extent of GFP$^+$ starter cell labelling. The point plotted (circle) indicates the centre of gravity of the starter cell population. Center line of boxplot shows the median distance from bregma of the slices containing starter cells. Boxes extend from the 25th to 75th percentile. Whiskers indicate the smallest and largest bregma distance of slices containing starter cells. (Right) Number of starter cells plotted as the mean cell count for each 50μm slice at each distance from bregma. Shaded regions represent standard error of the mean. (B) Percentage of starter cells found in different areas of the mid-brain (total number of GFP$^+$ cells: WT-VTA, n=3742; WT-SNC, n=5162; DAT-VTA, n=340; DAT-SNC, n=193). (C-F) Higher magnification images of GFP labelled starter cells in (C) WT-VTA, (D) WT-SNC, (E) DAT-VTA, (F) DAT-SNC.

spread when compared to the wild-type mice. This is likely due to the large population of dopamine neurons in the VTA and SNc but not outside of these structures. Since infection is limited to dopamine neurons even if the injection spread beyond the target structures, non-dopamine neurons would still not express GFP. This means that the Cre-dependent viruses give a much more precise spread of infection even though the injection volumes were larger.

Next, to capture the 3D positioning of starter neurons we considered the anterior-posterior axis. We can see in Figure 3.5 A that in both DAT-Cre cases the range of slices containing starter neurons is small and all such slices are within the anterior-posterior boundaries of their target areas. The DAT-SNC range is slightly more anterior than the DAT-VTA, which could be because the SNc structure is present at those coordinates slightly closer to bregma. The centre of mass for both of these experiments are within 0.5mm of our injection coordinate, showing

our injections were precise and consistent between these experiments.

Comparing the DAT-Cre cases to the wild-type cases, we can see a clear difference between the anterior-posterior spreads. In the DAT-Cre cases the range of the data are much smaller than their wild-type counterparts which is likely to be because of the restriction of infection to dopamine neurons only.

In order to establish exactly what proportion of the starter neurons have spread to other areas such as the VTA, SN, and RN, the percentage of GFP expressing cells in each of these areas were calculated and displayed in Figure 3.5 B. Both DAT-Cre cases have a large majority of starter neurons located in the target region (83.2% for DAT-VTA, and 80.8% for DAT-SNc) which means that most of the mCherry signal seen in the cerebellum will be due to these areas. There is minimal signal seen in 'other' regions and 0% in the red nucleus for both DAT-Cre cases. This means that we don't need to consider red nucleus in being responsible for any transsynaptically labelled cells in the interposed cerebellar nuclei. However, due to the VTA and the SNc being in such close proximity and both containing a dense population of dopamine neurons there are starter neurons crossing this boundary in both directions. For DAT-VTA there are 15.9% of starter neurons in the SNc and for DAT-SNC there are 16.1% of starter neurons located in the VTA. This is a large enough proportion to be concerned that there will be signal in the cerebellum that is due to connection to the opposing target area.

Overall, differences in the starter cell distributions between the wild-type experiments and the DAT-Cre experiments are due to restriction of infection to dopamine neurons only. This cell-type specific infection gives a much more precise spread because the surrounding areas do not contain dopamine neurons. The wild-type experiments have a very small proportion of starter cells located in the red nucleus which could result in mCherry signal in the interposed cerebellar nucleus. Also, all experiment setups (especially the DAT-Cre experiments) have starter cell neurons either in the VTA when targeting the SNc or vice versa. Although these starter neurons may have an effect on the results, inflating the amount of signal in the cerebellar nuclei, due to the over estimation in starter neurons, and thus an over estimation in the spread of starter neurons, we believe the effect on results will be minimal.

3.2 Visualisation of neurons in cerebellum

Coronal sections of the cerebellum were imaged under green light (for mCherry expression) to identify transsynaptically labelled cells. Figure 3.6 A-D show the coronal sections obtained from the cerebellum of each mouse type. Cerebellar nuclei labels were added manually based on a standard mouse atlas [32] to better visualise the CN regions that have been labelled by the rabies virus. Strong monosynaptic labelling, with mCherry but not GFP, can be seen in all mice.

3.2.1 Is there a strong connection from the cerebellar nuclei to the VTA and SNc?

The first question this thesis aims to answer, before delving into the specifics of this connection, is whether a connection exists. Although this connection has been shown in previous work discussed in the introduction ([67] [68]) it is important for us to reaffirm these findings. Seeing evidence of this connection also confirms that the rabies tracing method is working as expected, and lays the foundation for analysis into the connection.

In all analysis of the rabies labelled neurons we calculate the convergence index for each slice of the cerebellum. The convergence index is a ratio of cerebellar rabies labelled neurons to starter neurons and should not be taken as an absolute value but as a measure of relative connectivity.

Strong evidence of contralateral connection from cerebellar nuclei to the VTA and SNc

Monosynaptic labelling, with mCherry but not GFP, can be seen in all mice and especially in the left hemisphere which is contralateral to our injection site. Figure 3.6 E shows the mean convergence indices for each experimental setup, with error bars representing the 95% confidence intervals and each point showing the convergence index for each brain slice. Due to the large variance in data, Figure 3.6 F gives a clearer image of the mean convergence indices. From this graph we can clearly see that the contralateral cerebellar nuclei gives a higher level of connectivity than the ipsilateral nuclei. In fact the difference between contralateral and ipsilateral convergence indices is very statistically significant for WT-SNC (0.00704 ± 0.00420 versus 0.000275 ± 0.000261, for contralateral and ipsilateral, respectively. Student T-test, $p < .05$) and statistically significant for WT-VTA (0.00262 ± 0.00264 versus 0.0000729 ± 0.000162, for contralateral and ipsilateral, respectively. Student T-test, $p < .10$), DAT-VTA (0.0163 ± 0.0197 versus 0, for contralateral and ipsilateral, respectively. Student T-test, $p < .10$), and DAT-SNC (0.0343 ± 0.0329 versus 0.00142 ± 0.00300, for contralateral and ipsilateral, respectively. Student T-test, $p < .10$).

Based on these comparisons we can conclude that there is strong evidence that connections from the cerebellar nuclei to the VTA and SNc do exist, consistent with previous findings. We can also conclude that there is strong evidence suggesting these connections are mainly contralateral with a weak ipsilateral connection also present. Therefore, going forward in this thesis when referring to the labelled cells in the cerebellar nuclei we refer to the contralateral connection only. All subsequent analysis ignores the ipsilateral labelled neurons and they are not included in any counts.

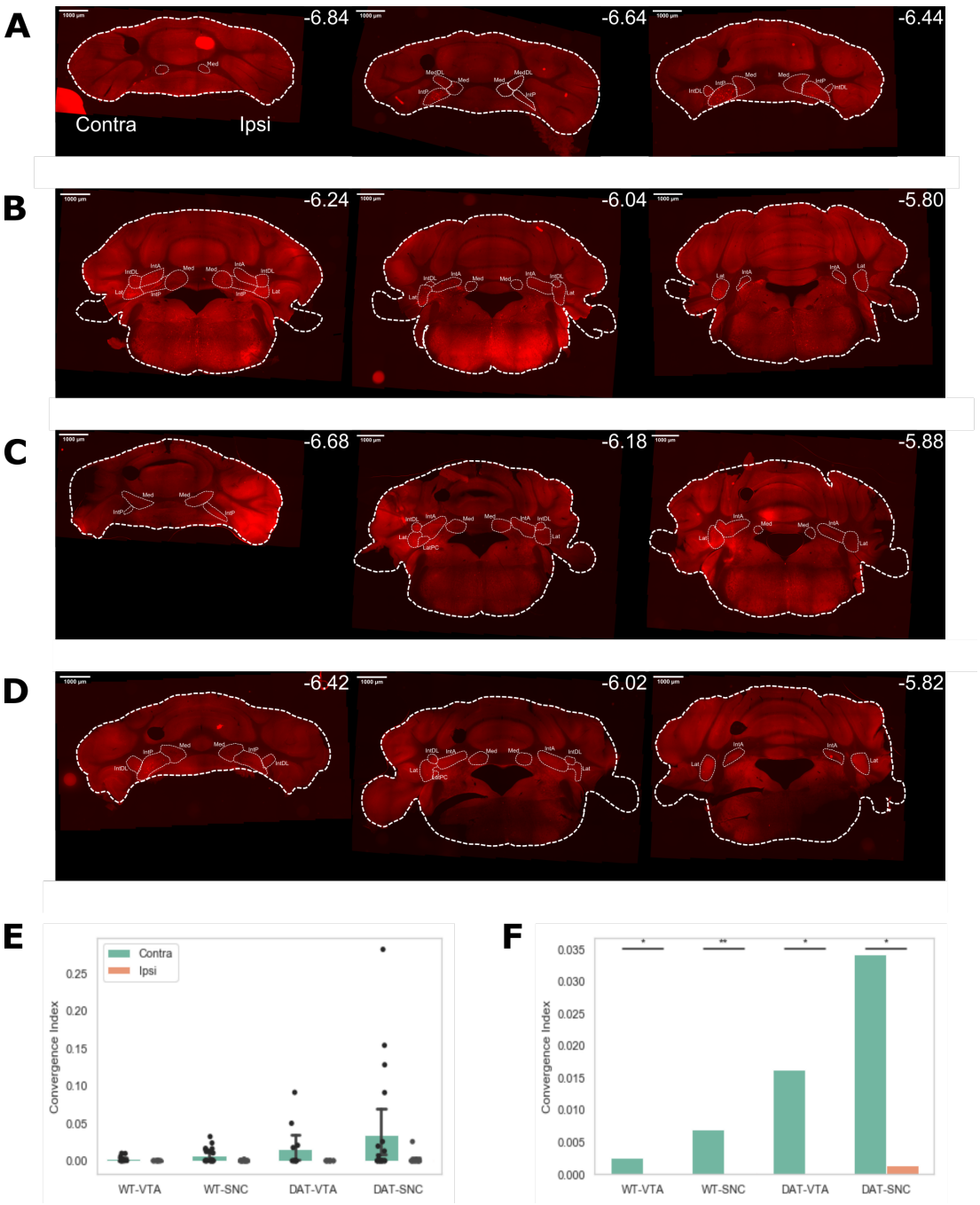

Figure 3.6: Comparing ipsilateral and contralateral rabies labelled neurons in the cerebellar nuclei.

(A-D) Representational coronal images of rabies (mCherry) labelling in the cerebellum of WT-VTA (A), WT-SNC (B), DAT-VTA (C) and DAT-SNC (D). (E) Convergence indices for the ipsilateral and contralateral neurons of the cerebellar nuclei. Individual data points (circles) indicate values for each brain slice (number of slices: WT-VTA, n=11; WT-SNC, n=22; DAT-VTA, n=11; DAT-SNC, n=21). Error bars represent the 95% confidence interval. (F) A zoom in on (E) to more clearly show the mean values. Statistically significant differences between pairs are indicated by the p value of the Student T-Test. $^{*}p < .10$, and $^{**}p < .05$.

3.2.2 Is there any difference between connections to the VTA versus the SNc?

The next stage is to compare the connectivity to the VTA versus the SNc in order to establish where the neurons in the cerebellum are sending information. Since these two areas have similar but different functions figuring out where the signal from the cerebellum is going could give an indication to what type of information is being sent.

Evidence of stronger connectivity to SNc than VTA for non-specific neurons

Figure 3.7 A and B show medium magnification images of the mCherry expressing cerebellar nuclei which are connected to the VTA (Figure 3.7 A) and the SNc (Figure 3.7 B) in wild-type mice. Strong synaptic labelling can be seen in the cerebellar nuclei of both VTA targeted and SNc targeted wild-type mice. Figure 3.7 E shows the mean convergence indices for each experimental setup, with the error bars indicating the 95% confidence interval and convergence indices for each brain slice plotted. Due to the large variance in data Figure 3.7 F gives a clearer image of the mean convergence indices.

In wild-type mice, targeting all neuronal sub-types, starter neurons originating in the SNc give a significantly higher convergence index than starter neurons in the VTA (0.00704±0.00420 versus 0.00262±0.00264, for SNc and VTA, respectively. Student T-test, $p < .10$). This evidence suggests that the cerebellum has a larger connection to the SNc when considering all neuronal sub-types. This means that we cannot be sure exactly which neurochemical sub-types are contributing to these connections, just that the SNc has an overall larger projection from the cerebellum than the VTA.

No evidence of differences in connectivity to SNc or VTA for dopamine neurons

Figure 3.7 C and D show medium magnification images of the mCherry expressing cerebellar nuclei which are connected to the VTA (Figure 3.7 C) and the SNc (Figure 3.7 D) in DAT-Cre mice. Strong synaptic labelling can be seen in the cerebellar nuclei connected to both of these brain areas.

Looking at 3.7 F we can see that the convergence index for DAT-SNC seems to be much higher than for DAT-VTA, in fact the mean is over 2x bigger. However, the difference is not statistically significant (0.0343±0.0329 versus 0.0163±0.0197, for SNc and VTA, respectively. Student T-test, $p = .329$). This means that there is no statistical evidence to suggest that there is a difference in connectivity between the cerebellum and VTA dopamine neurons versus the cerebellum and SNc dopamine neurons.

Assuming that there is no difference between the connectivity of the cerebellum to dopamine

neurons from the VTA or the SNc this means that the difference in connectivity in wild-type mice is due to the glutamatergic and GABAergic neurons present in these areas. In this case the cerebellum would be projecting more to non-dopamine neurons in the SNc compared to the VTA but projecting the same amount to dopamine neurons in both areas.

However, the statistical findings that suggest no significant difference between these two cases is surprising. We can see that the mean convergence index for DAT-VTA is over 2x larger than for DAT-SNC but due to the limited sample size and large variance of the data we lose some statistical power, leading to these surprising results. Therefore, we could assume that DAT-SNC does in reality have a significantly higher convergence index than DAT-VTA. In this case, it is impossible to tell without further investigation which neuronal sub-types cause the difference in the wild-type mice. It could just be due to the larger projection to dopamine neurons in the SNc, and non-dopamine neurons have similar connectivity in both areas, or both non-dopamine and dopamine projections could be less for the VTA, or there could be a weak or no projection to non-dopaminergic neurons at all and the signal we see in wild-types is also only from dopamine neurons. This last case (no projection to non-dopaminergic neurons) is unlikely since there are a lot more neurons labelled in the cerebellum in the wild-type mice. This could be because a higher number of dopamine neurons had been starter neurons in wild-types compared to DAT-Cre but is more likely because of the added contribution from non-dopamine cells. It is difficult to draw any solid conclusions because the data is limited and has a large variance.

3.2.3 Is there any difference between connections to non-specific versus dopamine neurons?

This leads to the next question we must answer, which is regarding the difference between projections to non-specific or dopamine cells. It is important to know what type of neurons are receiving information from the cerebellum in order to further investigate where the information is used and the pathways this connection is part of.

Due to differences in experimental methods between the wild-type and DAT-Cre groups it is not appropriate to make direct statistical comparisons across these groups. Therefore, we instead describe the trends we observe in the data, without statistical tests.

Weak evidence of differences in connectivity to non-specific or dopamine neurons in the VTA

Looking at Figure 3.7 F we can see that the convergence index for DAT-VTA is much higher than for WT-VTA, about 6x larger (0.0163 ± 0.0197 versus 0.00262 ± 0.00264, for DAT-Cre and wild-type VTA respectively). This suggests that the connection of cerebellar neurons to dopamine neurons in the VTA is more prevalent than the connection to other types of neurons such as

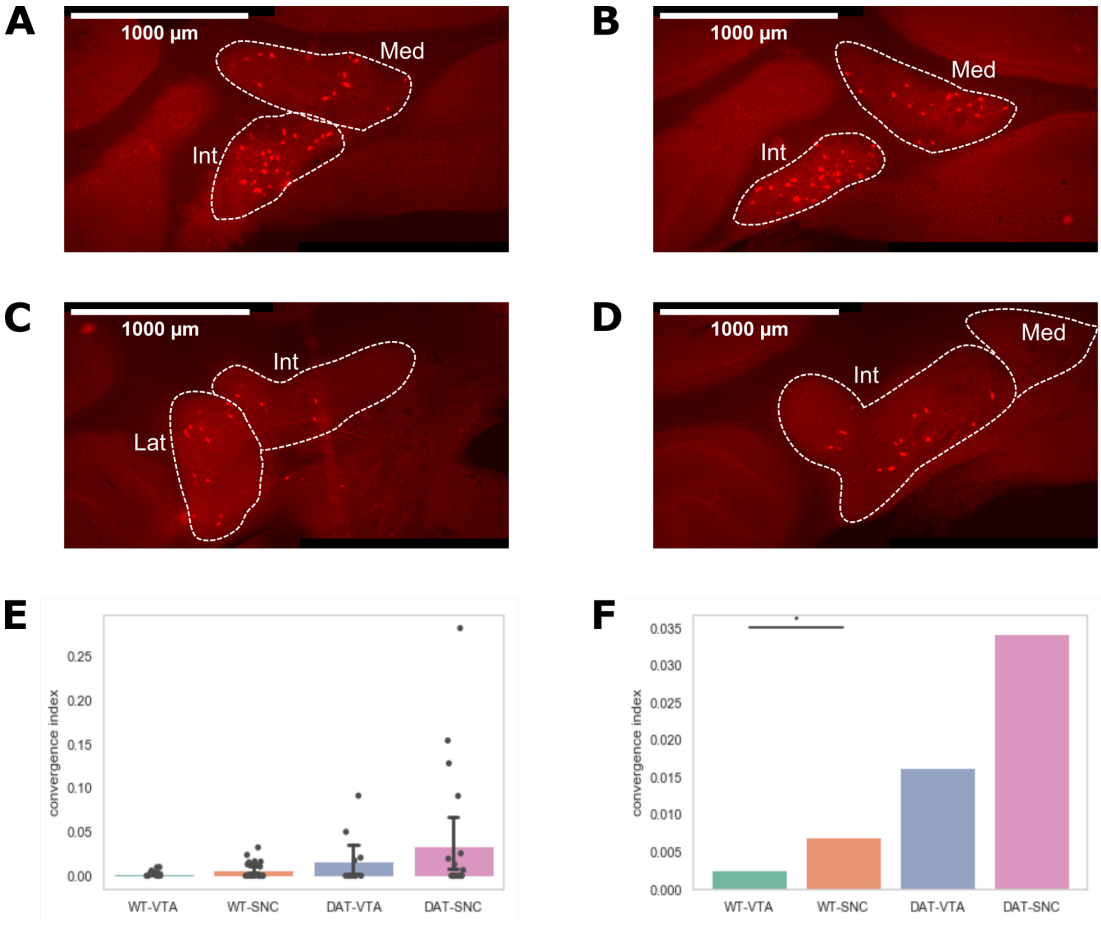

Figure 3.7: Comparing rabies labelled neurons in all cerebellar nuclei.
(A-D) Representational coronal images of rabies (mCherry) labelling in the cerebellar nuclei of WT-VTA (A), WT-SNC (B), DAT-VTA (C) and DAT-SNC (D). (E) Convergence indices for all cerebellar nuclei. Individual data points (circles) indicate values for each brain slice (number of slices: WT-VTA, n=11; WT-SNC, n=22; DAT-VTA, n=11; DAT-SNC, n=21). Error bars represent the 95% confidence interval. (F) A zoom in on (E) to more clearly show the mean values. Statistically significant differences between pairs are indicated by the p value of the Student T-Test. $^{*}p < .10$.

GABAergic or glutamatergic. In order to determine if this difference in means is statistically significant we would need to have a larger sample size and ensure consistent experimental conditions (e.g. the volume of virus injected) across both mouse genotypes. However, due to the large difference in means it is likely that with this extra data we would be able to confirm the trends that are visually apparent in the data. On the other hand, if this difference were not statistically significant this would suggest that non-dopamine cells have the same contribution to the connection that dopamine neurons have.

Weak evidence of differences in connectivity to non-specific or dopamine neurons in the VTA

Similarly to above, the mean convergence index of DAT-SNC is almost 5x larger than the convergence index of WT-SNC (0.0343±0.0329 versus 0.00704±0.00420, for DAT-Cre and wild-type SNc respectively). This suggests that connections from the cerebellum to the SNc converge on dopamine cells more than on non-dopamine cells such as GABAergic or glutamatergic. We would need more data with consistent experimental methods across both genotypes to provide statistical evidence that the convergence indices are significantly different and support these observations. Conversely, if the difference between the two convergence indices were found to be not statistically significant it would mean that there is no difference in connectivity between all neuronal sub-types versus dopamine cells only.

3.2.4 Is there any difference between connection from the lateral, interposed, and medial nuclei?

The rodent cerebellum contains four separate nuclei, the dentate (or lateral) nucleus, the posterior and anterior divisions of the nucleus interpositus, and the fastigial (or medial) nucleus. These nuclei are the sole output of the cerebellar cortex and are split not only by location but also by function. This means it is important to know exactly which nuclei send information to the mid-brain as this information could provide context as to what the function of the connection is.

Weak evidence of differences in connectivity between cerebellar nuclei

Strong synaptic labelling, with mCherry but not GFP, can be observed throughout each of the DCN. As you move through the brain in the anterior-posterior direction, the nuclei change shape and size. Figure 3.8 A-D gives a representation of these changes and how the extent of rabies labelling also changes in this direction.

It is interesting to see that for all experimental set-ups there is no mCherry expression in the medial nucleus from about -5.80mm to -6.40mm from bregma. This means that the posterior sections of the medial nucleus do project to the mid-brain but as you move towards the anterior portion these projections stop. The interposed nucleus seems to be the most consistent in containing a large amount of rabies labelled neurons throughout the entire cerebellum, especially in the wild-type mice. The DAT-Cre mice contain a lot fewer rabies labelled neurons compared to the wild-type but this is most likely due to there being less starter neurons in the DAT-Cre mice to begin with.

In order to compare the connections, rather than using the raw cell counts, we can calculate the convergence index for each cerebellar nuclei. The convergence indices of each brain slice are

plotted in Figure 3.8 E-H against the anterior-posterior coordinate from bregma to show the changes throughout the brain. This makes it clear that the neurons involved in the connection from the medial nucleus are located the most posterior and also have the weakest contribution to the connection in all four experimental setups. The lateral nucleus looks as though it has the most anterior connection which would make sense as the lateral nucleus is only present in the anterior parts of the cerebellum.

As well as how the connection changes throughout the brain we are also interested in comparing the overall connections between each nuclei and across the different experimental setups. Figure 3.8 I shows the mean convergence indices for each nuclei. Due to the large variance in the data, particularly for DAT-SNC, Figure 3.8 J shows a clearer image of the means without displaying the error bars or individual data.

A two-way analysis of variance (ANOVA) test was performed to identify any effects of target brain area (VTA or SNc) and nucleus on the convergence index. First, comparing the two wild-type experiment cases, this test showed that the nucleus had a statistically significant effect on the convergence index ($p = .019$). A post-hoc analysis using Tukey's test revealed that the mean value of convergence index between the interposed and medial nuclei in WT-SNC is the only comparison to give a statistically significant result (0.00426 ± 0.00288 versus 0.000596 ± 0.000712, for interposed and medial respectively. Tukey's post-hoc test, $p < .05$). This provides evidence that suggests the connection from the interposed nucleus to the SNc is much higher than the connection from the medial nucleus in wild-type mice. Secondly, we compared the two cases involving DAT-cre mice. The ANOVA test showed that neither different target areas ($p = .463$) nor different nuclei ($p = 0.282$) had a significant effect on the convergence index. This is a surprising result because looking at Figure 3.8 J we can see a large difference between the convergence index of different nuclei for both the DAT-Cre mice cases. In particular, we can see that the convergence index for the medial nucleus is much lower than the other two nuclei for both DAT-VTA and DAT-SNC. The large variance and small sample size in our data means that the statistical power is lowered and could be the reason for this result. The trend across all experimental setups is that the medial nucleus has the lowest convergence index. There is no statistical evidence that the medial convergence indices differ between experimental setups and if that is the case, this could indicate that the medial nucleus projects to both dopamine and non-dopamine neurons equally and to both the VTA and SNc. Therefore, any differences in connectivity between neuron types or areas would be due to the lateral and/or interposed nuclei.

Another trend across all the experimental setups is that the interposed nuclei gives the highest convergence index. the only exception to this is DAT-VTA, but this is likely to be due to missing data for this experimental case. You can also see this missing data in Figure 3.8 C. The real convergence index for the lateral nucleus is likely to be higher than what is shown and would fall into the same pattern as the other cases.

Overall, it is difficult to draw any solid conclusions due to the small sample size of experiments but it seems likely that the medial nucleus contributes to the connection the least, but projects to both dopamine and non-dopamine cells. The lateral and interposed nuclei send large projections to the VTA and SNc with the interposed nucleus making the largest contribution. Since we established that there were no starter cells located in the red nucleus for the DAT-Cre mice, we can be sure that this large projection from the interposed nucleus we can see is not due to that connection but is a connection to the VTA or SNc.

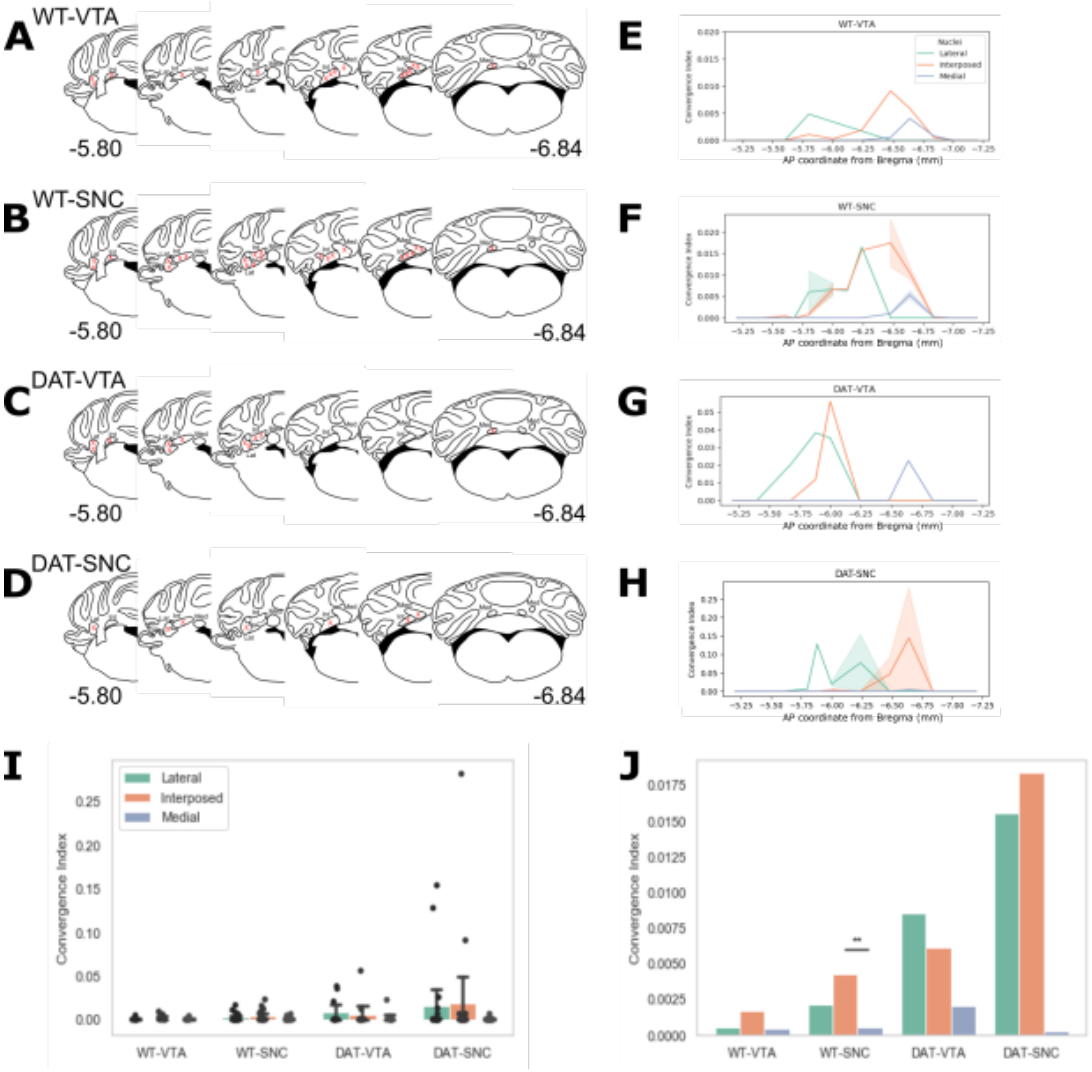

Figure 3.8: Comparing rabies labelled neurons across each cerebellar nucleus
(A-D) Anterior-posterior extent of mCherry rabies labelled neurons in the cerebellar nuclei. Extent of labelling represented using increasing number of symbols (1: <10 cells, 2: 10-20 cells, 3: >20 cells). (E-H) Change in mean convergence index across the anterior-posterior direction for each cerebellar nuclei. Shaded regions represent the standard error of the mean. (I) Convergence indices of each cerebellar nuclei. Individual points (circles) indicate values for each brain slice (number of slices: WT-VTA, n=11; WT-SNC, n=22; DAT-VTA, n=11; DAT-SNC, n=21). Error bars represent the 95% confidence interval. (J) A zoom in on (I) to more clearly show the mean values. Statistically significant differences between pairs are indicated by the p value of a post-hoc Tukey test. $^{**}p < .05$.

Chapter 4

Discussion

This chapter summarises and concludes the thesis, first by assessing the success of the experiments with regards to the objectives laid out in Chapter 1. Next, we evaluate the limitations of the project and their affect on results. Finally, we discuss the potential future direction of this research.

4.1 Assessment with regards to objectives

1. Accurately label a population of starter cells in the VTA and SNc using the modified rabies tracing technique.

   We have successfully performed accurate and precise injections into the target brain areas of the VTA and SNc. However, the distribution of starter cells is larger than we would have liked, labelling a large proportion of cells outside of the target area, especially in wild-type mice. Furthermore, there were some problems with the rabies mCherry expression in starter neurons which led to an over estimation of the number of them.

2. Perform fluorescence microscopy in perfused brain slices to identify cells in the cerebellum which have been labelled by the virus and are thus pre-synaptically connected to the starter cells.

   We have successfully identified cells in the cerebellar nuclei which express mCherry but not GFP, indicating that these have been labelled via the transsynaptic movement of the rabies virus. We have identified that the connection is largely a contralateral connection.

3. Identify which regions of the DCN provide projections to the VTA and SNc.

   We have successfully shown that projections to the VTA and SNc originate in all cerebellar nuclei. We have shown that the medial nucleus has the smallest contribution to this connection and that the connectivity changes along the anterior-posterior axis of the medial nucleus. We have also shown that it is likely that the interposed nucleus has the

largest contribution to the connection to the VTA and SNc.

4. Identify which neuronal sub-types are receiving the projections in the VTA and SNc.

   We have successfully shown that there is a large projection from the cerebellar nuclei to dopamine neurons in both the VTA and SNc since there is evidence of a connection in DAT-Cre neurons. We have also shown that it is most likely that there is a connection to non-dopamine neurons such as GABAergic and glutamatergic neurons due to the difference in connectivity between the VTA and SNc in wild-type mice. Since we have shown these two areas are likely to have similar connectivity in DAT-Cre mice, the difference would be because of differences in connection to non-dopamine neurons.

5. Calculate, analyse, and compare convergence indices to determine the relative connectivity.

   We have successfully computed and compared the convergence indices of the connections. However, it is difficult to draw any solid conclusions due to the minimal data we are comparing.

Hypothesis

The lateral and interposed nuclei provide projections to both the SNc and VTA. This projection converges to all neuronal sub-types, GABAergic, glutamatergic and dopaminergic but there will be a larger convergence to dopamine neurons.

Our experimental results give evidence for connections to the SNc and VTA originating in all cerebellar nuclei, that is the lateral, interposed and medial nuclei. This is in line with our hypothesis. However, there is not enough evidence to concretely conclude that there are projections to all neuronal sub-types although it is highly likely to be shown with more data and there is evidence of a large projection to dopamine neurons in the VTA and SNc.

4.2 Critical Evaluation

Overall, the results achieved by this project are in line with what we expected and also with previous work ([67] [68]). However, there are three main caveats which may have affected results and need to be addressed in future iterations of this work. The main problems were as follows:

1. There was not enough data obtained to draw many meaningful conclusions based on comparisons.
2. The overestimation of starter cells, based on GFP expression in cells of the VTA and SNc.
3. The convergence index is already an underestimation of connectivity and it's usefulness is affected by the above two points.

We will now go into detail about each of these short-comings, and the extent to which they affected the results of experiments.

4.2.1 Minimal data

Due to the nature of the project, it is difficult to gather large volumes of data so unfortunately the data presented in this thesis is based off 6 mice; 1 WT-VTA, 2 WT-SNC, 1 DAT-VTA and 2 DAT-SNC. A small sample size means that our estimates and analysis is more uncertain and therefore it is difficult to draw concrete conclusions.

Ideally, we would have a larger sample size and would be able to compute the overall convergence index of each mouse rather than using the convergence index of each brain slice. This would give us a more meaningful convergence index as it would represent the overall connectivity between the two areas which could then be broken down further to look at individual nuclei. This wouldn't work with our data since you cannot meaningfully compare only two data points. To get around this caveat we chose to calculate the convergence index for each brain slice. This leads to a huge amount of variance in the data since the connectivity changes along the anterior-posterior axis. This can be seen largely in the medial nuclei (3.8 A-D) which contains no mCherry expressing cells in some slices, but up to 20 cells in others.

4.2.2 Overestimation of starter cells

Chapter 3.1 gives a description of how we visualised the starter cells in the VTA and SNc. We defined our starter cells as all GFP expressing cells in these areas. This results in a large over estimation of starter cells as not all of the GFP expressing cells would have also been infected by rabies virus via injection, meaning that they would not have contributed to the transsynaptic labelling. An overestimation of starter cells leads to an underestimation of the convergence index as the denominator of the ratio is bigger than expected. This would not be a huge problem if it affected all experimental setups equally, but this is not the case.

The wild-type mice are shown to have a much larger spread of GFP expressing neurons compared to DAT-Cre mice. Assuming that the rabies virus injection would spread to a similar diameter to the AAV viruses, it is likely that the cells on the edge of the range would not be infected by the rabies virus. The level of overestimation for the DAT-Cre mice is likely to be less than that of the wild-type mice because the viruses are limited to the dopamine neurons. The injection volumes in DAT-Cre mice are much higher (200nl vs 75nl) yet the spread of GFP expressing neurons in much lower because of the specificity. This high volume means that the secondary injection of rabies virus is likely to cover all neurons which were infected the first time, leading to a higher proportion being infected with rabies compared to the wild-type mice. This means the convergence indices for wild-type mice might be artificially low.

The wild-type mice convergence indices are also lowered due to the large amount of starter neurons located in other areas outside of the VTA or SNc. These starter neurons are unlikely to contribute to transsynaptic labelling in the cerebellum but are still counted and included in the convergence index, increasing the denominator of this ratio.

4.2.3 Convergence index

The convergence index itself in an underestimate of the connectivity. This is because it does not account for the fact that not all starter cells will receive direct inputs from the cerebellar nuclei, which increases the overestimation of the starter cells and further increases the denominator of the ratio. Similarly, the efficiency of transsynaptic labelling using rabies virus is not 100%, meaning that not every pre-synaptic neuron will be transsynaptically labelled. This leads to an underestimation of cerebellar nuclei cells involved in this connection, and an underestimation of the numerator of the convergence index. This underestimation is why the convergence index should not be taken as an absolute value, but rather interpreted as a relative measure. It is also important to note that this thesis does not aim to make any conclusions about the strength of functional connectivity and that is not what the convergence index gives a measure of as the ratio between post and presynaptic cells is not necessarily related to functional strength.

4.3 Future Work

Future work in this area is needed to further investigate and conclude which neuron sub-types are involved in this connection and to start to understand the functional role of these connections. This thesis provides a strong foundation to build on, including confirming that the rabies virus technique works in this context.

Firstly, more data regarding different neuron sub-types is required. This monosynaptic rabies tracing technique could be performed using different transgenic mouse strains expressing Cre recombinase in different cell types such as GABAergic or glutamatergic. This will allow us to further specify which cells are being targeted. We could also use a transgenic line to target all neuron types excluding dopamine which would provide a more direct comparison of the connectivity to dopamine versus non-dopamine cells.

The injection volumes for wild-type mice could be reduced to get a more precise starter cell population, or a different tracing technique in the anterograde direction could be used to elucidate exactly which structures are receiving projections from the cerebellar nuclei. Additionally, to reveal which neuronal sub-types are receiving projections, immunostaining techniques using molecular markers such as tyrosine hydroxylase (TH) and $GABA_A$ receptors could be used to label cells post tissue fixation. In this way you would be able to see the post-synaptic partners labelled due to anterograde tracing in the VTA or SNc and establish the neurochemical sub-type

of these cells due to the overlap of labelling with the relevant molecular marker. Immunostaining methods could also be used in conjunction with the rabies retrograde tracing method to mark dopamine neurons and more accurately establish the boundaries of the VTA and SNc. This means we would be able to more accurately establish the proportion of starter neurons contained within the target structures and check that the DAT-Cre mice have successfully restricted starter cell labelling.

The next stage would be to investigate the function associated with this connection in relation to reward behaviours. To do this, a rabies virus expressing channelrhodopsin-2 (ChR2) would be injected into the VTA or SNc and an optical fibre implanted over the cerebellum. In this way we can use light to excite cerebellar neurons and test the consequences of activating this connection. Similarly, we could use an inhibitory opsin to test the consequences of suppressing the activity of cerebellar nuclei neurons. Using a Cre-dependant virus in parallel with different transgenic mice means we can also specify the neurochemical sub-type that we are targeting in the VTA or SNc. This means that we could identify functional differences between connections terminating on dopamine neurons versus GABAergic or glutamatergic neurons. The choice of behavioural task during these optogenetic manipulations will be important for determining functional connectivity, for example determining if these connections are active during reward omission, prior to reward, during unexpected reward etc. A popular hypothesis is that this connection to the VTA is providing information about timings [48] so it will be necessary to have a task which involves predicting rewards at different time intervals. Also interesting would be to compare tasks which involve motor input at specific timings and tasks which do not and see if there is a difference in cerebellar activity or if cerebellar activity/inhibition affects the outcomes of these tasks.

Since dopamine neurons from the VTA project to numerous other structures, including the nucleus accumbens and prefrontal cortex and these neurons have been shown to be divided into functionally and spatially organised subgroups it would also be beneficial to determine which subgroup the cerebellum projects to so we can continue to follow the circuit [85]. Since it has been established that the mesolimbic pathway is involved with addiction [22], if the cerebellum projects to dopamine neurons involved in this circuitry it could reveal potential functionality of this cerebellar connection. To do this would involve juxtacellular tagging of neurons in order to determine the location of cells after electrophysiological experiments.

Investigating how the cerebellum is involved in the reward circuitry of the brain, and how the connections from the cerebellum influence behaviour is an important step to understanding how the cerebellum modulates higher order processes. Since the reward system plays an important role in many different behaviours including aversion [20], addiction [19], and motivation [16] this circuitry has an impact on many aspects of every day life. Understanding what information the cerebellum provides to the VTA and SNc and how this influences behaviour could have important implications for cognitive and social impairments associated with these areas, such as schizophrenia, ADHD, and autism spectrum disorder.

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