The reticular thalamic nucleus: revealing a novel phenotype of neurons and describing changes in a rat model of Parkinson’s disease

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Abstract

The reticular thalamic nucleus (RTN) is a thin band of neurons that lies between the cortex and the rest of the thalamus. Most of the inputs to the nucleus are from collateral axons of thalamocortical and corticothalamic projections. The basal ganglia, basal forebrain and brainstem also have important connections to the RTN. The RTN has previously been characterized as an exclusively GABAergic nucleus that causes inhibition of thalamic relay neurons and modulates motor messages through thalamocortical projections. However, a small number of studies suggest that the RTN is not exclusively GABAergic, but did not define the phenotype of non-GABAergic neurons. The RTN is divided into sectors that represent all sensory modalities, and the limbic and motor systems. Each sector is closely connected to the functionally related thalamic regions. For example, the motor sector of the rostral RTN (rRTN) projects to the motor thalamus and receives inputs back from the motor thalamus, as well as from the motor cortex and the basal ganglia. This motor sector is active during movements and its activity changes with movements to coordinate the motor signals that are passed to the motor cortex. Despite its involvement in movement, the rRTN is not usually considered in movement related disorders such as Parkinson’s disease (PD). PD is a neurodegenerative disorder that is characterized by substantial loss of dopamine neurons in the substantia nigra pars compacta and the ventral tegmental area. Loss of these midbrain dopaminergic neurons underlies the hallmark motor symptoms in PD patients such as bradykinesia, akinesia, resting tremor and rigidity.

To advance the understanding of the RTN and its role in PD, this study aimed to characterize the phenotype of neurons within the rRTN of adult rats and investigate the effect of 6-hydroxydopamine (6-OHDA) induced lesions of midbrain dopaminergic neurons on the number and morphology of neurons in the rRTN. Chromogenic and fluorescent immunohistochemistry for the GABAergic marker GAD67 and the glutamatergic marker CaMKIIα confirmed that the majority of neurons were indeed GABAergic and a small novel population of neurons exist in the rRTN that are CaMKIIα positive. Furthermore, a lentiviral vector was injected into the rRTN that selectively labelled neurons expressing CaMKIIα with the fluorophore mCherry. Detection of mCherry in transduced neurons and GAD67 with fluorescent immunohistochemistry confirmed that there are two distinct populations of neurons in the rRTN. Chronic dopamine lesions were induced in the left hemisphere of the brain by the injection of the neurotoxin 6-OHDA. Stereological investigations into the
number and morphology of neurons in the rRTN revealed that 6-OHDA lesion induced a 20% increase in the number of GABAergic neurons and also produced a 20% decrease in the size of GABAergic neurons in the rRTN. These changes were not seen in glutamatergic neurons.

These data indicate that the rRTN is not exclusively GABAergic, as once thought, and although small, this glutamatergic population may play an important role in RTN function. Furthermore, dopamine depletion causes a number of changes in the rRTN, therefore, the rRTN may be a novel target in the treatment of PD.
Acknowledgements

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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>AMPA</td>
<td>amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AVVL</td>
<td>Ventrolateral portion of the anteroventral thalamic nucleus</td>
</tr>
<tr>
<td>BDA</td>
<td>Biotinylated dextran amine</td>
</tr>
<tr>
<td>BRNZ</td>
<td>Brain Research New Zealand</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CaMKIIα</td>
<td>Calcium/calmodulin-dependent protein kinase II alpha</td>
</tr>
<tr>
<td>ChR2</td>
<td>Channelrhodopsin-2</td>
</tr>
<tr>
<td>CI</td>
<td>Chloride ions</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin (B fragment)</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3’-diaminobenzidine chromogen stain</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DB</td>
<td>Diluting buffer</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAAC-1</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Authority</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamic acid decarboxylase 67 kDa molecular weight</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus externus</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus internus</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIAR</td>
<td>Heat induced antigen retrieval</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal injection</td>
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<tr>
<td>KDA</td>
<td>Ketamine domitor and atropine</td>
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<tr>
<td>L-DOPA</td>
<td>Levodopa</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>LTS</td>
<td>Low-threshold calcium spike</td>
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<td>mAChR</td>
<td>Muscarinic cholinergic receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neuron</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic cholinergic receptor</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline containing Triton</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Pf-CM</td>
<td>Parafascicular and centromedian thalamic nuclei</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus vulgaris</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRTN</td>
<td>Rostral portion of the reticular thalamic nucleus</td>
</tr>
<tr>
<td>RTN</td>
<td>Reticular thalamic nucleus</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulate</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TIF</td>
<td>Tagged image format</td>
</tr>
<tr>
<td>VA</td>
<td>Ventroanterior thalamic nucleus</td>
</tr>
<tr>
<td>VGLUT2</td>
<td>Vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>VL</td>
<td>Ventrolateral thalamic nucleus</td>
</tr>
<tr>
<td>VM</td>
<td>Ventromedial thalamic nucleus</td>
</tr>
<tr>
<td>VSVg</td>
<td>Vesicular stomatitis virus G glycoprotein</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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1.0 Introduction
1.1 The reticular thalamic nucleus

1.1.1 Overview

The reticular thalamic nucleus (RTN) is associated with regulating the flow of information from the thalamus to the cortex, and is involved in a number of processes such as movement, attention, and sleep (Pinault, 2004). The RTN is not often implicated in the pathophysiology of the movement-related Parkinson’s disease (PD), however, patients will experience problems with sleep and attention as well as the hallmark motor deficits associated to the disease (Jankovic, 2008). Furthermore the RTN has a functional relationship with the motor thalamus and the motor-related basal ganglia (Pinault, 2004; Lam and Sherman, 2015) and therefore the RTN becomes an interesting target in the study of the pathophysiology of PD. The literature below, about the RTN, will summarize its connections with nuclei throughout the brain, its basic anatomical and physiological features as well as its specific connections with motor related nuclei of the basal ganglia and the motor thalamus that are implicated in the pathophysiology of PD. This thesis will explore the phenotype of neurons in the rostral portion of the RTN and examine the changes that may occur to the number and morphology of these neurons in an animal model of PD.

The RTN is a thin sheet of neurons that lies between the thalamus and the cortex (Houser et al., 1980). The RTN surrounds the lateral portion of the anterior thalamus, covering both dorsal and ventral surfaces (Jones, 1975). It is located medial to the internal capsule and ventral to the zona incerta (Pinault, 2004). Almost all functional modalities are encoded by the RTN, which receives most of its inputs from the rest of the thalamus and the cortex. Information, from visual, auditory, somatosensory, gustatory, visceral sensations, limbic or motor systems, are topographically represented in the RTN in so called ‘sectors’ (Pinault, 2004). It is thought that these cortical and thalamic inputs to the RTN are collaterals from the corticothalamic and thalamocortical projections, respectively (Guillery and Harting, 2003), and give the RTN its characteristic reticulated shape (Figure 1.1). The RTN also receives input from areas of the brainstem, the basal forebrain and some of the basal ganglia nuclei (Ferrarelli and Tononi, 2011), which will be discussed in more detail below.

Francis Crick (1984) originally proposed the ‘searchlight’ hypothesis where the RTN searches through the thalamic inputs before they go to the cortex and ‘lights’ up the inputs that need to be attended to by inhibiting the inputs of lesser importance (Crick, 1984). Consistent with this idea, it is now thought that the RTN plays an important role in attention,
as well as the generation of sleep spindles – a characteristic pattern of oscillatory activity that is visible on an electroencephalogram (EEG) during sleep (Ferrarelli and Tononi, 2011). Currently, the RTN is primarily characterized as a nucleus that contains neurons that make the neurotransmitter γ-aminobutyric acid (GABA) thus is defined as a GABAergic nucleus (Houser et al., 1980), which accounts for its primary inhibitory function. It is important to stress, however, that early studies into the RTN stated that although the RTN is mostly made up of GABAergic neurons, it may not be the only neurotransmitter present (Houser et al., 1980). Despite this, many studies have been based on the premise that the RTN is exclusively GABAergic and the possibility of another phenotype of RTN neurons has been overlooked.

1.1.2 Anatomical description of the reticular thalamic nucleus

Early evidence suggested that the RTN contained at least 3 morphologically distinct populations of neurons that included both small (1) and large (2) fusiform shaped neurons with an elongated perikarya and (3) neurons with a round perikaryon that are multipolar (Spreafico et al., 1991). However, later studies found no evidence to distinguish between the small and large fusiform neurons (Lübke, 1993). The morphology of the neurons in the RTN remains controversial, as many descriptions are available, however, it seems that the description of RTN neuron morphology depends on the plane of sectioning used in a particular study (Pinault, 2004). It is likely that there are three types of RTN neurons: 1) large fusiform neurons that have 2 to 4 primary dendrites emerging from both poles of the

Figure 1.1. An example coronal image of glutamic acid decarboxylase (GAD) staining for GABAergic neurons in the RTN. This staining reveals the classical reticulated structure of the nucleus that is surrounding the lateral portion of the thalamus (Adapted from Houser et al., 1980). RT = reticular thalamic nucleus, ic = internal capsule, T = thalamus, P = putamen.
perikarya that branch into secondary dendrites a short distance from the cell body and tertiary
terminal dendrites at a long distance from the cell soma; 2) medium size fusiform neurons
with ellipsoid cell bodies, found mainly in the medial part of the RTN with primary dendrites
projecting from the somata that give rise to secondary and tertiary dendrites (Sprefafo et al.,
1991; Nagaeva and Akhmadeev, 2006) and 3) small multipolar cells with round perikarya,
which have up to 8 smooth primary dendrites that branch into two to three spiny secondary
dendrites and sometimes into tertiary dendrites (Sprefafo et al., 1991). This third morphology
has mainly been found in the rostral portion of the nucleus (Sprefafo et al., 1991).

Houser et al. (1980) identified that GABA was the primary neurotransmitter in the
RTN and stated that the majority of neurons in the nucleus were likely GABAergic.
Following this, many studies label the RTN as an exclusively GABAergic nucleus, however,
there is some evidence to the contrary and another minor population of neurons may exist in
the RTN. As mentioned above, 3 types of neurons are found to be in the RTN, 2 of which
match the morphological profile of GABAergic neurons, but the small round neurons are
different (Sprefafo et al., 1991). This third morphology of neurons has been found to
represent a small population in the RTN and is negative for staining against GABAergic
markers (Nagaeva et al., 2006; Çavdar et al., 2013), but to date, they have not been
characterised.

Ohara and Liberman (1985) observed that in adult rats the size of neurons in the RTN
changes through its rostro-caudal axis. Neurons in the caudal portion of the RTN had an area
of 115 - 125 µm² whereas rostral neurons had an area of 150 - 200 µm² (Ohara and
Lieberman, 1985). The morphological profile of each neuron was not determined, therefore,
these measurements would include small and large morphologies, which may account for the
range seen in the rostral portion (Ohara and Lieberman, 1985). Primary, secondary and
tertiary dendrites are found on neurons throughout the RTN (Sprefafo et al., 1991; Lübke,
1993). Primary dendrites are the longest, and often give rise to secondary dendrites that can
then branch into tertiary dendrites. Generally, primary dendrites are smooth, and it is the
secondary or tertiary dendrites that have dendritic spines, varicosities and hair-like projections
suggesting that this is where incoming information is targeted (Lübke, 1993). Synaptic
terminals in the RTN from its afferent inputs are described as small, large or GABA
containing (Nagaeva et al., 2006). Large and small-type terminals are from thalamocortical
and corticothalamic collateral axons and will be discussed with more detail blow.
GABA-containing terminals, presumably either from afferents to the RTN from the basal
ganglia, basal forebrain and brainstem or interconnections between RTN neurons, which are
discussed below, formed around 10% of synapses in the RTN (Liu and Jones, 1999; Pinault,
2004), however this description may need updating due to the diverse nature of inputs from these locations (see below).

RTN neurons express a variety of receptors to accommodate the heterogeneity of their inputs (Nagaeva et al., 2006). In this section the phenotype of these receptors will be described, the origin of the inputs to these receptors will be explained below and their actions on the RTN will be outlined in Section 1.1.3. Glutamatergic inputs to the RTN act via the fast acting ionotropic receptors: \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors, as well as the slower G-protein coupled, group I and group II, metabotropic glutamate receptors (mGluRs) (Cox and Sherman, 1999). GABAergic inputs to the RTN activate both ionotropic GABA\(_A\) and metabotropic GABA\(_B\) receptors, however, the main action is through the GABA\(_A\) receptor (Sun et al., 2012; Seo and Leitch, 2014, 2015). The substantia nigra pars compacta (SNc) has a dopaminergic projection to RTN neurons where dopamine D4 and D1 receptors are expressed in the RTN (Huang et al., 1992; Mrzljak et al., 1996; Florán et al., 2004). D4 receptor expression was later localized to the presynaptic terminals of afferent inputs from the basal ganglia (Gasca-Martinez et al., 2010; Erlij et al., 2012), however, D1 receptors have not been characterized further. RTN neurons also express the nicotinic (nAChRs) and muscarinic cholinergic receptors (mAChRs) (Beierlein, 2014), as well as receptors to noradrenaline \(\alpha\)1-receptors (McCormick and Wang, 1991). Serotonergic innervation from the brainstem acts via the G-protein coupled 5-HT\(_{1A}\) and 5-HT\(_{2A}\) receptors that are present on the soma and dendrites of RTN neurons (Rodríguez et al., 2011).

RTN neurons are thought to have a widely dispersed interneuron population, which has recently become controversial (Hou et al., 2016). Early evidence suggested that some neurons of the RTN had short axons that did not extend out of the nucleus and provided a pathway for communication between RTN sectors (Crick, 1984; Sun et al., 2012). These interneurons allegedly provide GABAergic inhibition to the interconnected network of neurons (Lam et al., 2006), however, there is limited evidence of this. Sun et al. (2012) stimulated slices of the RTN either electrically or with GABA\(_A\) agonists and found that synaptic GABA\(_A\) receptor activation led to burst responses in RTN neurons. In this kind of preparation electrical stimulation of the RTN could be activating any of the fibres that pass through RTN that give it the characteristic reticular appearance. Furthermore, GABA\(_B\) type receptors, which act in unison to shape the response to GABAergic stimulation in the RTN (Lam et al., 2006), were blocked with antagonists during the experiments performed by Sun et al. (2012) and could account for the responses seen by this group. Selective activation of GABAergic neurons in the RTN with optogenetics produced inhibitory response of RTN
neurons in mice under 2 weeks of age, but failed to elicit inhibitory responses in RTN neurons of older mice (Hou et al., 2016). Therefore, it seems that the interneuron population of neurons in the RTN is extremely complex and is strongly expressed at an early age. Although RTN interneurons may not exist in adults, they may communicate via electrical synapses with immediate neighbours (Blethyn et al., 2008). This local inhibition of RTN neurons may facilitate the processing of information that requires the most attention by inhibiting or silencing surrounding inputs that are less important (Blethyn et al., 2008; Ferrarelli and Tononi, 2011), tying RTN function back to the original ‘searchlight’ hypothesis proposed by Crick (1984).

Thalamic nuclei can be classified as either first-order or higher-order nuclei defined by the primary ‘driver’ input, that is the input that drives activity in the thalamic relay neurons (Sherman and Guillery, 1998). First order nuclei receive driver input from ascending sensory information (i.e. retinal ganglion input into the lateral geniculate nucleus) and then relay this information to the cortex (lateral geniculate nucleus to the visual cortex). Higher order thalamic nuclei receive driver input from layer V of the cortex and relay this information to other areas of the cortex. This is important to keep in mind, as the RTN sends axons to both first and higher order nuclei. However the RTN is not classified as a ‘driver’ nor does it receive any driver input. It is classified as a ‘modulator’, where it influences information processing by thalamic relay cells, but it does not directly trigger activity (Sherman and Guillery, 1998).

**Afferent connections to the RTN** The principal inputs to the RTN are from layer VI projections of the cortex and thalamic relay nuclei. However the brainstem, the basal forebrain, and the basal ganglia also have inputs to the RTN (Figure 1.2) (Guillery et al., 1998; Ferrarelli and Tononi, 2011).

The inputs from the thalamus and cortex are axon collaterals of excitatory glutamatergic thalamocortical and corticothalamic projections, respectively (Cornwall et al., 1990; Liu and Jones, 1999). Thalamic relay cell projections to the RTN typically terminate with asymmetric synaptic contact principally onto primary dendrites but also made contacts onto the distal second and third order dendrites as well as the somata (Liu and Jones, 1999; Deleuze and Huguenard, 2016). Thalamic relay axon terminals are derived from collateral axons of thalamic projection fibres as they make their way to the cortex and are large excitatory terminals that make up 30-40% of synapses found in the RTN (Liu and Jones, 1999). Cortical projections come from collaterals of neurons from layer VI that are on their way to the thalamic relay neurons. Cortical terminals are small excitatory terminals which, make asymmetric contact with neurons, mostly on secondary or tertiary dendrites and these
terminals form the majority of synapses in the rRTN (Liu and Jones, 1999). Synaptic contacts with proximal first-order dendrites and somata have also been observed, however these are less frequent than synapses with distal dendrites (Liu and Jones, 1999).

The basal forebrain and brainstem both have cholinergic projections to the RTN. The nucleus basalis of the basal forebrain and the pedunculopontine and laterodorsal tegmental nuclei in the brainstem send cholinergic projections to the RTN that contain numerous synaptic varicosities some of which, terminate with asymmetric synapses on secondary and tertiary dendrites of RTN neurons, however, many do not appear to be associated with RTN dendrites (Hallanger et al., 1987; Hallanger and Wainer, 1988). It has been suggested that cholinergic afferents to the RTN communicate via extrasynaptic and presynaptic receptors as well as conventional postsynaptic transmission indicating a possible role of the cholinergic system in the plasticity of RTN neuron responses (Beierlein, 2014).

Noradrenergic projections from the locus coerulus of the brainstem terminate with excitatory asymmetric synapses onto the primary, secondary and tertiary dendrites of RTN neurons (Asanuma, 1992). These projections have also been seen to make synaptic contact

Figure 1.2. A schematic diagram of the afferent and efferent connections of the RTN. Corticothalamic projections are represented by the light blue neuron and synapse onto secondary and tertiary dendrites of the RTN neurons. The dark blue neuron represents the thalamocortical projections that contact the somata and the dendrites of RTN neurons. The RTN projection to the thalamus is represented by the red neuron and is synapsing onto the primary dendrite of thalamic relay cells. (Adapted from Guillery et al., 1998).
with the somata of RTN neurons, and seem to come off axon collaterals that primarily project to the dorsal thalamus (Asanuma, 1992). However, noradrenergic projections have received little attention in studies of the RTN. Similarly, the serotonergic projections from the raphe nucleus of the brainstem have not been extensively studied. However, these serotonergic projections innervate the RTN with a moderate density and contain small varicosities that are regularly spaced (Rodriguez et al., 2011). The ventral tegmental area (VTA) has projections to the RTN (Kolmac and Mitrofanis, 1998), however the phenotype or action of these projections are yet to be defined.

From the basal ganglia, the external segment of the globus pallidus (GPe) and the substantia nigra pars reticulata (SNr) send GABAergic projections to the RTN (Paré et al., 1990; Kayahara et al., 1994; Kayahara and Nakano, 1998) and the SNc sends dopaminergic projections to the RTN (Anaya-Martínez et al., 2006). Pallidal projections arborize profusely around RTN dendrites, and synaptic varicosities make symmetric contacts with primary dendrites and the somata of RTN neurons (Hazrati and Parent, 1991; Paré and Steriade, 1993; Asanuma, 1994; Kayahara and Nakano, 1998). Similarly, SNr projections make symmetric contact onto the primary dendrites and somata of RTN neurons (Asanuma, 1994). GPe and SNr projections are mostly to the rostral portion of the RTN (Pare et al., 1990; Asanuma et al., 1994). Projections from the SNc are yet to be fully characterized. Due to the intimate involvement of the basal ganglia in the initiation and execution of movement, these inputs along with inputs from the motor thalamus and motor cortex will be considered in more detail in Section 1.1.7.

Efferent connections of the RTN Following Golgi impregnation, Scheibel and Scheibel (1966) confirmed that RTN axons project to thalamic nuclei. The RTN projects to most of the anterior, dorsal, intralaminar, posterior and ventral thalamic nuclei, however, no other targets outside thalamic nuclei have been discovered to date (Scheibel and Scheibel, 1966; Jones, 1975; Pinault, 2004). The axons of RTN neurons can emerge from the somata or primary dendrite and often project into the rest of the thalamus perpendicular to the thalamic-RTN boundary (Pinault and Deschenes, 1998).

RTN axonal projections to thalamic nuclei are organized in different ways that depend on the degree of overlap of the dendritic arbour of individual RTN neurons (see Figure 1.3 for a summary) (Pinault, 2004). Adjacent RTN neurons without an overlapping dendritic tree will project axons into two distinct sections of corresponding thalamic nuclei (Figure 1.3 A), however, adjacent RTN neurons with overlapping dendritic arbours will have overlapping axon terminals in corresponding thalamic nuclei (Figure 1.3 B) (Pinault et al., 1997). A small percentage of RTN neurons have an axon that splits into 2 branches that terminate in...
functionally related first-order and higher-order thalamic nuclei (Figure 1.3 C) (Crabtree, 1998; Pinault and Deschénes, 1998). Similarly a RTN neuron that has an overlapping dendritic tree with an adjacent neuron will each project one axon to a corresponding nucleus and have overlapping axon terminals, as above, plus another axon to a separate thalamic nuclei that is involved in a related function (Figure 1.3 D) (Pinault and Deschénes, 1998).

RTN projections terminate in an F type terminal with pleomorphic flattened synaptic vesicles and symmetrical synaptic contacts with postsynaptic neurons, indicative of an inhibitory functional connection (Ohara et al., 1980; Montero, 1983). These F terminals from the RTN have been immunohistochemically identified at light and electron microscope levels as GABA-positive terminals and electrophysiology confirms that RTN synapses have an

**Figure 1.3.** Schematic diagrams summarizing the organization of RTN axonal projections to the motor thalamus. (A) Two adjacent RTN neurons innervate two adjacent regions within motor thalamus (VA/VL). (B) Two adjacent RTN neurons with overlapping dendritic arbours project innervate VA/VL with overlapping axonal terminal fields. (C) A RTN neuron can have two distinct axonal fields within first-order and higher-order divisions of VA/VL (see Section 1.1.5). (D) A RTN neuron with an overlapping dendritic tree will have an overlapping axon terminal field in VA/VL, can also have another axon terminal field in the functionally related thalamic nucleus, such as the intralaminar thalamic Pf-CM complex (Pinault and Deschénes, 1998). Figure adapted from Pinault and Deschénes, 1998. RTN = Reticular thalamic nucleus, Pf-CM = Parafascicular-centromedian thalamic nuclei, VA/VL = Ventroanterior/ventrolateral thalamic nuclei.
inhibitory effect on thalamic relay neurons (Ohara et al., 1980; Liu et al., 1995). They terminate onto primary, secondary and tertiary dendrites, as well as the somata of relay neurons (Montero, 1983; Harting et al., 1991; Liu et al., 1995; Tsumori et al., 2002). GABAergic neurons of the RTN exert their action onto GABA_A and GABA_B receptors expressed on thalamic relay neurons (McCormick, 1992).

The RTN has an important role in modulating and integrating information from ascending (first-order) and descending (higher-order) information that will eventually be processed in the cortex (Pinault, 2004; Lam and Sherman, 2007). Efferent RTN connections to first-order thalamic nuclei are topographically organized and usually specific to a single thalamic nucleus (Lam and Sherman, 2007). In contrast, input to higher-order thalamic nuclei is a combination of topographic projections and more complex diffuse projections from throughout the RTN (Lam and Sherman, 2007). The information from first-order and higher-order areas must interact for a clear message of the desired task to be transmitted to the cortex, and this message is clearly modulated by the RTN (Pinault, 2004). The RTN’s axonal projections that terminate in first order and higher order provide a connection for these thalamic nuclei, and can form thalamo-reticulo-thalamic loops. By integrating RTN axonal projection patterns, these loops can shape the excitation and synchronization of multiple thalamic nuclei therein modulating the message relayed to the cortex (Guillery and Harting, 2003; Pinault, 2004; Lam and Sherman, 2007).

1.1.3 General physiology of the reticular thalamic nucleus

Considering RTN neurons exclusively project to thalamic nuclei and have inputs from many areas in the brain (see above) the activity of RTN neurons is vitally important in optimizing the communication of messages between the thalamus and the cortex. Activity in RTN neurons has been extensively studied, however because the focus of this study is anatomical, the general electrophysiological features will be briefly explained and summarised below.

RTN neurons possess a range of different receptors that attest the range of inputs it receives. It also has a number of voltage-dependent ion channels that are vitally important for the membrane potential, firing pattern and synaptic transmission of RTN neurons. The nature of these receptors and ion channels endow each RTN neuron with the ability to fire in a specific pattern at a given moment independent of neighbouring neuronal activity; an ability uncommon in neuronal networks (Pinault, 2004).

RTN neurons are described as having 2 firing patterns that heavily depend on the attentional state of the animal. While the animal is awake, and actively attending to stimuli,
RTN neurons generally fire in a tonic state (Domich et al., 1986; Huguenard and Prince, 1992). During slow-wave sleep however, RTN neurons exhibit a bursty firing pattern, where neurons rapidly fire action potentials in ‘bursts’ for a short period of time that is subsequently followed by a period of silence (Wallenstein, 1994; Marlinski and Beloozerova, 2014).

Burstiness is a firing pattern that is seen throughout the nervous system. The close proximity of spikes in a burst allows a stronger signal to be sent to downstream nuclei resulting in reliable synaptic transmission with an increased release of neurotransmitter (Bosch-Bouju et al., 2013). The exact purpose of bursting is still unclear. However in the RTN, burstiness is thought to increase the inhibitory signal to thalamic relay neurons with precise timing as well as synchronise the neural circuit, which is important for the development and maintenance of slow-wave sleep (Pinault, 2004; Marlinski and Beloozerova, 2014).

Burstiness in the RTN is dependent on the low-threshold T-type calcium (Ca\(^{2+}\)) channels that are present on RTN neurons (Huguenard and Prince, 1992). T-type Ca\(^{2+}\) channels underlie the low-threshold calcium spike (LTS) burst that is exhibited by RTN neurons. LTS bursts are characterized by a high frequency (~200 Hz) burst of spikes that occurs following prolonged hyperpolarisation of the membrane potential (Bosch-Bouju et al., 2013). During prolonged hyperpolarisation of the membrane, T-type Ca\(^{2+}\) channels are de-inactivated, which allows the channel to become responsive to depolarizing events. If depolarization occurs following prolonged hyperpolarisation, Ca\(^{2+}\) ions are able to flow into the neuron through the now open T-type Ca\(^{2+}\) channel, further depolarizing the neurons and activating voltage-gated sodium channels and the neuron consequently fires an action potential. The T-type Ca\(^{2+}\) channel can keep the membrane potential of the cell depolarized for a relatively long duration, triggering multiple spikes in the LTS burst (Bosch-Bouju et al., 2013). After a period of time, the T-type Ca\(^{2+}\) channel inactivates and the membrane potential repolarizes.

RTN neurons that exhibit burstiness generally have hyperpolarized membrane potentials compared with those that fire tonically (Brunton and Charpak, 1997). As such, tonically active NMDA, GABA\(_A\) and mGluR2 receptors present within the RTN have different actions on RTN neurons (Crabtree et al., 2013). The combined effect of these receptors ultimately hyperpolarizes the membrane potential of RTN neurons, where tonically active presynaptic NMDA receptors facilitate GABA release from GABAergic terminals in the RTN, GABA\(_A\) receptors generate a persistent chloride ion (Cl\(^-\)) current that directly hyperpolarizes the membrane potential of RTN neurons, and presynaptic mGluR2 receptors reduced the glutamate release from cortical excitatory terminals (Crabtree et al., 2013).
Hyperpolarization of neurons increase their tendency to fire an LTS bursts following excitatory inputs whereas without prior hyperpolarization of the membrane potential, neurons will exhibit single spiking activity (Crabtree et al., 2013).

RTN activity is altered not only by tonically active receptors but also by the activity of its inputs. The major input to the RTN comes from the collaterals of corticothalamic and thalamocortical neurons that are glutamatergic in phenotype. Stimulation of these inputs leads to activation or inhibition of RTN neurons through the action of post-synaptic NMDA (Spreafico et al., 1988; Deleuze and Huguenard, 2016), AMPA (Deleuze and Huguenard, 2016), and mGluR receptors (Cox and Sherman, 1999; Wang et al., 2015). Stimulation of thalamocortical relay neurons and corticothalamic neurons in layer VI leads to a fast excitatory response in the RTN mediated by ionotropic activation of AMPA and NMDA receptors (Gentet and Ulrich, 2003, 2004), and these responses have a long decay time, thought to be due to the kinetics of the NMDA receptor (Gentet and Ulrich, 2003, 2004; Lacey et al., 2012). It appears that AMPA and NMDA receptors alter the response to thalamocortical or corticothalamic stimulation differently (Deleuze and Huguenard, 2016). In the presence of AMPA antagonists, no excitatory post synaptic potential could be elicited in the RTN following corticothalamic stimulation, whereas antagonism of NMDA receptors did not significantly alter the response of RTN neurons, suggesting that AMPA receptors are important for responses to cortical excitation in RTN neurons, while NMDA receptors have a small component to these responses (Gentet and Ulrich, 2004). Conversely, when AMPA and NMDA receptors kinetics were separated with a mathematical function, NMDA receptor kinetics were found to play a big role in the response of RTN neurons to both cortical and thalamic stimulation (Deleuze and Huguenard, 2016). Thalamocortical relay neurons elicit a stronger excitation of RTN neurons compared with corticothalamic and both AMPA and NMDA receptors have large components (Gentet and Ulrich, 2003; Deleuze and Huguenard, 2016).

As previously mentioned, cortically driven responses are directed mainly to the secondary and tertiary dendrites of RTN neurons (Liu and Jones, 1999), which may account for their longer lasting activations and this distal location promotes reticular cell burst firing via distally located T-type Ca\(^{2+}\) channels (Crandall et al., 2010; Deleuze and Huguenard, 2016). Whereas thalamic inputs are targeted to proximal dendrites, which evoke large, fast responses in RTN neurons (Lacey et al., 2012; Deleuze and Huguenard, 2016). It is thought that the NMDA receptor is important in activating T-type Ca\(^{2+}\) receptors and driving bursty activity in RTN neurons following thalamic stimulation (Gentet and Ulrich, 2003). Furthermore, the location of these synapses suggests that strong thalamic stimulation may
override cortically driven responses in neurons of the rRTN, switching firing from bursty back to single pulses (Deleuze and Huguenard, 2016).

The activation of mGluRs in the RTN produces both depolarization and hyperpolarization based on which group of mGluR is activated (Cox and Sherman, 1999). Group I mGluR activation couple to Gq protein leading to the downstream activation of intracellular calcium stores and depolarization of RTN neurons (Neyer et al., 2016), whereas group II mGluR activations leads to Gi protein coupled receptor mediated hyperpolarisation (Cox and Sherman, 1999). The function of mGluR activation often leads to longer lasting changes in neurons such as long-term potentiation (group I, LTP) or depression (group II, LTD). LTP and LTD modulate synaptic transmission, neurotransmitter release and synaptic plasticity (Neyer et al., 2016), however, the role of synaptic plasticity in the RTN is yet to be defined.

The nucleus basalis, the pedunculopontine and the lateral tegmental nuclei innervate RTN neurons with cholinergic projections. Cholinergic projections mediate their effects through both nAChRs and mAChRs that have opposing effects on the membrane potential of RTN neurons. Activation of cholinergic afferents leads to a fast depolarization mediated through nAChRs that is followed by a longer-lasting hyperpolarization mediated by mAChRs (Beierlein, 2014). This biphasic response required the activation of T-type Ca^{2+} channels indicating that cholinergic inputs modulate the activity of RTN neurons, and act to increase the length of time that LTS bursts may occur (Beierlein, 2014). Cholinergic inhibition of RTN neurons leads to disinhibition of thalamocortical neurons allowing them to better respond to sensory and motor information (Harris and Thiele, 2011; Beierlein, 2014).

Noradrenergic stimulation of RTN neurons from the locus coeruleus acts via \( \alpha_1 \)-adrenoceptors that are highly expressed on, and excite, RTN neurons (McCormick and Wang, 1991; McCormick, 1992). This excitation causes a slow depolarization of RTN neurons that inhibits the neuron’s ability to burst; however, smaller activation of noradrenergic inputs can enhance bursting activity (McCormick and Wang, 1991). Therefore the strength of noradrenergic input can modulate the firing pattern of RTN neurons. Serotonergic stimulation of RTN neurons from the raphe nucleus results in a, similarly, slow depolarization of RTN neurons that inhibits the burstiness of these neurons (McCormick and Wang, 1991; Rodríguez et al., 2011). This depolarization is indirectly mediated through a decrease in the outward ‘leaky’ potassium current activated by G-protein coupled receptors, which increases the membrane potential of RTN neurons promoting single spiking activity (McCormick and Wang, 1991; Rodríguez et al., 2011).
GABAergic input to the RTN comes from the basal ganglia and basal forebrain. Intrinsic cell-to-cell GABAergic input has recently been questioned in adult rats (see Hou et al., 2016). GABAergic inhibition of RTN neurons is mediated through post-synaptic GABA_A and GABA_B receptors (Gentet and Ulrich, 2003; Sun et al., 2012). While GABA_B receptors are blocked, activation of GABA_A receptors leads to a depolarization of RTN neurons that is in part due to the low expression of the potassium-chloride transporter, KCC2, that removes Cl^- from the cell (Sun et al., 2012). This low expression of KCC2 causes the intracellular levels of chloride to increase, and therefore neurons of the RTN generally have a high intracellular Cl^- concentration leading to a positive Cl^- equilibrium potential (Sun et al., 2012). Subsequent activation of GABA_A receptors leads to a Cl^- current down its concentration gradient and out of the cell, resulting in depolarisation (Sun et al., 2012). Furthermore, isolating the GABAergic synaptic input to RTN neurons can lead to bursts of action potentials, which is blocked with GABA_A antagonism (Sun et al., 2012). T-Type Ca^{2+} channel activation was found to be critical for this GABA_A mediated burstiness, where blockade of the low-threshold channel abolished GABA-mediated bursts of action potentials (Sun et al., 2012). It should be noted that the above results were garnered in rats of a relatively young age (P13-35). Aging has a number of effects on the RTN, as well as effects on GABA transmission throughout the brain. Rats that are P20 exhibit GABAergic responses following the optogenetic activation of RTN neurons, however fail to respond at P42 (Hou et al., 2016). Furthermore, the expression of the KCC2 transporter increases throughout neurons during maturation (Ben-Ari, 2002).

Although limited information is available on the action of GABA_B receptors on RTN neurons, it has been shown that activation of GABA_B receptors reduces the post-synaptic currents evoked by GABA_A receptors, and directly activates a potassium ion current leading to a hyperpolarisation of the resting membrane potential of RTN neurons (Ulrich and Huguenard, 1996). It is essential that the effects of GABA on mature adult neurons, especially through the activation of both GABA_A and GABA_B receptors, be investigated to get a better understanding of this nucleus.

Dopaminergic inputs from the SNc act via dopamine D4 receptors and modulate presynaptic release of GABA in the RTN. GABAergic neurons from the basal ganglia feature dopamine D4 receptors on their presynaptic terminals (Erlij et al., 2012), which when activated by SNc inputs lead to reduced GABA release (Florán et al., 2004; Govindaiah et al., 2010). GABAergic input from the basal ganglia and its modulation by dopamine from the SNc is likely to be important in the processing of motor information and will be discussed in more detail in Section 1.1.5.
RTN neurons exclusively project to thalamocortical relay neurons. Almost all projections of the RTN release GABA at their target thalamic nuclei and produce a strong inhibitory response (Huguenard, 1998). The GABAergic response in thalamic relay neurons is mediated by GABAA receptors that increase Cl− conductance and GABAB receptors that couple to G proteins to indirectly hyperpolarise the membrane of thalamic relay cells through K+ extrusion (Ulrich and Huguenard, 1996; Huguenard, 1998). High frequency bursts of GABA release from LTS bursting RTN neurons act to hyperpolarize the thalamic relay cell membrane quickly through GABAA receptors and for a prolonged period of time through GABAB receptors, thereby switching thalamic relay cell firing from tonic to bursty (Huguenard, 1998; Guillery and Sherman, 2002).

Most inputs to RTN neurons act to hyperpolarize the membrane and increase the likelihood of bursting activity. Although RTN neurons are thought to be bursty during slow-wave sleep, a small proportion of neurons exhibit bursty activity in the awake-state (Marlinski and Beloozerova, 2014), which is of particular importance to transmit signals to thalamic relay cells as the coding efficiency of bursts is much higher compared to single spikes (Marlinski and Beloozerova, 2014). It is, therefore, not surprising that most inputs to the RTN increase the likelihood of LTS bursts and also increase the likelihood of that message passing to thalamic relay cells. This functional control is reflected in thalamic relay cells, which switch their firing from tonic to bursting under the influence of RTN neurons (Huguenard, 1998). However, during slow-wave sleep, the bursting activity of RTN neurons occurs much more regularly in a higher proportion of neurons and becomes rhythmic (Pinault, 2004). This rhythmic bursting of RTN neurons synchronises thalamocortical relays and is important in maintaining slow-wave sleep (Pinault, 2004).

1.1.4 Sector organisation of the reticular thalamic nucleus

Most of the sensory modalities, the limbic and motor systems are represented in the RTN in distinct sectors. However, electrophysiological evidence suggests a small degree of overlap between the boundaries of adjacent sectors (Pinault, 2004). Visual, auditory, somatosensory, taste, and visceral sensations are all represented in the caudal RTN, whereas limbic and motor pathways are found in the rostral aspect. The specific thalamic nuclei and cortical areas that are associated with each specific RTN sector are described below. However, afferent projections from other locations in the brain such as the brainstem and basal ganglia will only be discussed as to how they relate to processing in the motor sector in Section 1.1.5.
The visual sector of the RTN is located in the dorsocaudal portion of the nucleus and receives projections from the visually related lateral geniculate nucleus of the thalamus (Crabtree and Killackey, 1989; Coleman and Mitrofanis, 1996). The visual sector of the RTN also receives inputs from the visual areas of the cortex; these are specifically mapped, and closely resemble the topography of the lateral geniculate (Crabtree and Killackey, 1989). The visual sector has reciprocal connections back to the lateral geniculate nucleus, and has efferent input to the higher-order pulvinar nucleus (Ohara et al., 1980; Conley and Diamond, 1990).

Represented ventrocaudally in the RTN are the auditory and somatosensory sectors. The auditory sector has reciprocal connections with primary auditory nucleus of the thalamus, the medial geniculate nucleus (Conley et al., 1991) and efferent input to the medial geniculate is topographic (Crabtree, 1998). The higher order auditory nucleus, the posterior nucleus, similarly has reciprocal connections with RTN (Conley et al., 1991). The primary auditory cortex in the temporal lobe has input into the auditory sector of the RTN from collateral axons that project to first and higher order auditory thalamic nuclei (Conley et al., 1991; Kimura et al., 2012).

The somatosensory sector has afferent input from the ventrobasal complex and the medial division of the posterior complex from the thalamus and from the primary somatosensory cortex (Lam and Sherman, 2010). Somatosensory inputs to RTN were somatotopic, where different parts of the body are represented by different parts of the somatosensory sector of the RTN (Shosaku et al., 1984). The somatosensory sector has reciprocal connections with the higher-order somatosensory thalamic nuclei in the posterior nucleus (Lam and Sherman, 2007). However the higher-order inputs to the RTN are not topographically defined like the first-order inputs, they are diffuse throughout the thickness of the RTN (Lam and Sherman, 2007).

Anterior to the auditory and visual sectors of the RTN lies the taste-related region. This sector has not been extensively studied, however, it receives input from and sends outputs to the thalamic taste-relay, the medial ventroposterior parvicellular nucleus (Hayama et al., 1994). The taste sector of the RTN also receives an input from the primary gustatory cortex on the insular lobe (Hayama et al., 1994). Presently, RTN connections to and from the higher-order nuclei of gustation have not been characterized.

Similarly information regarding the visceral sector of the RTN is lacking. The visceral sector lies next to the taste-related region and rostral to the visual sector. The RTN has reciprocal connections with the visceral thalamic relay, the lateral ventroposterior
parvicellular thalamic nucleus (Stehberg et al., 2001). The visceral sector also receives input from the visceral sensory area in the granular insular cortex (Stehberg et al., 2001).

At the very rostral pole of the RTN sits the limbic sector. Cortical innervation of the limbic sector comes from the cingulate and retrosplenial cortices (Lozsádi, 1994). The anteroventral and anteromedial thalamic nuclei have reciprocal connections with the rostral limbic sector of the RTN (Lozsádi, 1995), which is topographically ordered (Gonzalo-Ruiz and Lieberman, 1995).

The motor sector in the rostral portion of the RTN is of particular interest to this study and will be discussed in its own section below (Section 1.1.5).

1.1.5 Motor processing in the reticular thalamic nucleus

The motor region of the rostral RTN (rRTN) has been poorly defined to date. The available data and observations from the existing literature will be summarized below. The anatomy and physiology of the connections to the rRTN from the motor cortex, motor thalamus, the basal ganglia and the brainstem will be considered. These motor-related areas are implicated in the pathophysiology of PD, however, the rRTN has received limited attention with regards to the disease. Therefore these connections to the rRTN are of particular focus for this thesis.

The primary motor cortex and the ventral anterior (VA) and ventral lateral (VL) of the motor thalamus innervate the rRTN with glutamatergic projections (Cicirata et al., 1990; Lam and Sherman, 2015). These inputs are somatotopically organized similar to the somatosensory sector, where movements for different parts of the body are represented in distinct subsections of the motor sector (Cicirata et al., 1990).

In rats and mice, VA and VL are generally considered histologically indistinguishable and will henceforth be referred to as VA/VL. Although thought of as one nucleus, VA/VL can be divided into basal ganglia receiving and cerebellar receiving divisions (Kuramoto et al., 2009). Furthermore, the division innervated by the cerebellum is regarded as a first-order relay and the target of basal ganglia innervation constitutes a higher-order relay (Lam and Sherman, 2011). RTN neurons project to both of these first and higher-order relays, in a similar manner to their projections to first and higher order relays described in the somatosensory sector, where first order inputs is topographical and higher order inputs are more diffuse and thought to connect a range of neurons within VA/VL (Lam and Sherman, 2007, 2015).

Investigation of VA/VL neuron responses following rRTN activation is limited. Lam and Sherman reported that rRTN stimulation in slices evoked inhibitory post-synaptic
currents within VA/VL relay cells (Lam and Sherman, 2015). Recent data from our lab confirmed that rRTN neurons fire both tonically and exhibit a LTS bursting firing pattern (Little, 2014), therefore it is likely that the rRTN is acting in a similar mode to other parts of the RTN, and modulates thalamic relay cells through inhibition, and can change their firing pattern from tonic to bursty.

Data regarding the effect of motor cortex and VA/VL thalamic relay neurons on rRTN activity is completely lacking. Due to the similarity of connections between motor and other sectors of the RTN it could be assumed that stimulation of the motor cortex leads to the activation of rRTN neurons via AMPA and NMDA receptors and promotes bursty firing by activating distally located T-type Ca$^{2+}$ channels (Deleuze and Huguenard, 2016), similarly, VA/VL input to rRTN activates neurons via NMDA and AMPA receptors, but rather than promoting bursty activity, VA/VL activation would lead to powerful excitation of rRTN neurons (Gentet and Ulrich, 2003; Fuentealba and Steriade, 2005).

The rRTN receives inputs from the GPe and the SNr of the basal ganglia. Considering the involvement of these nuclei in motor control, it is expected that they modulate the projection of motor messages from the rRTN to the motor thalamus. The GPe sends a direct GABAergic projection to the rRTN, which arborizes profusely and primarily makes symmetric synaptic contact onto rRTN proximal dendrites and somata (Hazrati and Parent, 1991; Asanuma, 1994; Kayahara et al., 1994; Kayahara and Nakano, 1998). The SNr innervates the rRTN, and similar to the GPe, makes symmetrical GABAergic synaptic contacts with dendrites and somata of rRTN neurons (Asanuma, 1994; Tsumori et al., 2000, 2002). The SNr input is mainly to the ventral part of the rRTN and not topographic whereas the GPe input is topographic in nature and covers the whole portion of rRTN (Gandia et al., 1993). Limited studies have investigated the effect of stimulating GPe or SNr inputs to the rRTN. When the GPe is stimulated, the activity of tonically firing neurons in the rRTN is decreased without inducing burst firing of rRTN neurons (Pazo et al., 2013; Villalobos et al., 2016). In contrast, when the GPe is inhibited, firing rate of tonically active neurons in the rRTN increases (Villalobos et al., 2016), suggesting the GPe projection exerts a basal inhibition onto rRTN neurons that is potentially mediated by GABA$_{A}$ receptors on RTN neurons rather than GABA$_{B}$ receptors, which promote hyperpolarization and subsequent burst firing (Ulrich and Huguenard, 1996; Hallworth and Bevan, 2005). Similarly, SNr stimulation causes a decrease in the firing rate of tonically firing rRTN neurons but also inhibits the cortically evoked bursty activity of some rRTN neurons (Paré et al., 1990).

These GPe and SNr inputs also appear to be modulated by dopaminergic projections from the SNc. Presynaptic D4 receptors on GPe and SNr axon terminals, reduce the inhibition
that the GPe and SNr exert onto rRTN neurons, by inhibiting the release of GABA from these afferent projections (Gasca-Martínez et al., 2010; Erlij et al., 2012).

The rRTN also has projections to motor-related intralaminar nuclei, the parafascicular and the centromedian complex (Pf-CM) (Steriade et al., 1984; Cornwall and Phillipson, 1988). These inputs to the somata and dendrites of Pf-CM neurons form symmetrical synapses (Tsumori et al., 2002). The Pf-CM complex in turn, projects to the rostral portion of the rRTN and these projections are topographic. Connections to the rRTN were mostly located in the ventral portion, and coming from Pf rather than CM, whose connections were located more caudally (Sadikot et al., 1992). Although the exact nature of Pf-CM input to the rRTN is yet to be studied, the Pf-CM complex sends glutamatergic inputs to GABAergic neurons of the striatum, and it is possible that Pf-CM glutamatergic inputs also innervate rRTN (Sadikot and Rymar, 2009). The rRTN output neurons that project to Pf-CM receive inputs from the SNr, which are thought to provide an indirect pathway from the basal ganglia to intralaminar motor thalamic nuclei (Tsumori et al., 2000, 2002).

*In vivo* evidence on the activity of the rRTN during movement is limited. However, a couple of studies examined how locomotion in environments with different complexity affects the activity in the rRTN of cats (Marlinski et al., 2012; Marlinski and Beloozerova, 2014). During sleep, most neurons of the rRTN exhibit typical ‘full scale’ bursty activity, however, when the cat is freely walking, or walking on a horizontal ladder, the activity of neurons in the rRTN changes. As mentioned previously, the motor sector of the RTN is somatotopically organized and this is shown in their activity while walking. During the step cycle of a cat, the proximal limb (shoulder), represented in dorsal rRTN, and the distal limb (wrist-paw), represented in ventral rRTN, are modulated by the rhythm of the stride (Marlinski et al., 2012). The magnitude of this modulation and the tendency of RTN neurons to fire in bursts was higher for distal compared with proximal limb neurons. The difference in activity in RTN neurons transfers to an increased inhibition of thalamic neurons that are within the distal limb domain (Marlinski et al., 2012). This suggests that the pattern of rRTN neuron activity ensures that motor signals from the different parts of the limb arrive at the motor cortex with appropriate temporal precision (Marlinski et al., 2012), that is, during a step the distal limb representation in the rRTN is sending a strong bursty signal to the motor thalamus ensuring that the motor signal arrives at the cortex with precise timing to guarantee accurate placement of the paw.

Burstiness while walking freely or in a complex environment changes from a ‘full-scale’ burst that typically appears during sleep, which show an acceleration to maximal firing rate and then decelerate until the end of the bursty episode, to an atypical bursty pattern
that feature short initial bursts that start at their maximal firing rate followed by a characteristic deceleration phase (Marlinski and Beloozerova, 2014). The likelihood that bursty activity would occur did not differ between simple and complex environments, however the pattern that the bursty neurons exhibited changed. Walking on a horizontal ladder requires accurate placement of the paw with each step. During this complex task, the atypical short bursty pattern was more frequently observed in neurons of the rRTN compared with just walking freely (Marlinski and Beloozerova, 2014), suggesting that these changes in burst pattern allows brief, but temporally aligned, inhibition of the thalamic neurons, which is tuned to the pattern of the stride. Therefore, at the end of the stride, activity in the thalamocortical circuit is attenuated by intense episode of maximally firing burstiness in the rRTN, which ensures the command signal for the next step is relayed to the motor cortex without interference from other thalamocortical motor circuits (Marlinski and Beloozerova, 2014).

It seems likely that the motor cortex, motor thalamus and the motor sector of the RTN work in unison to ensure that the most appropriate motor signal is conveyed through the thalamocortical and corticothalamic circuits, so that smooth and accurate movements occur. While there is a current lack of in vivo evidence on the effect that other motor areas of the brain, such as the basal ganglia, have on the activity of rRTN and how that transfers to motor performance, it is likely that disruption of these signals will contribute to the pathophysiology of motor diseases such as PD.

1.2 Parkinson’s disease

1.2.1 Parkinson’s disease overview

PD is one of the most common neurodegenerative disorders in the world. It is a disease that mostly affects the elderly, and has no cure. Parkinson’s is characterised by four cardinal symptoms that are all motor related; bradykinesia or the slowness of movement, and akinesia or impairment of voluntary movement, tremor at rest most often occurring in distal limbs, rigidity or increased resistance in moving a limb, which may be accompanied by pain, and postural instability or abnormal posture (Bloem, 1992; Parkinson, 2002; Jankovic, 2008).

PD is initially caused by the loss of dopaminergic neurons in the SNc and VTA (Fahn et al., 1971; Uhl et al., 1985). Motor symptoms, however, are not experienced until approximately 70% of dopaminergic neurons in the SNc have been depleted (Fearnley and Lees, 1991). A number of patients with Parkinson’s also experience non-motor cognitive
symptoms such as sleep disorders, mild cognitive impairment, dementia, and depression (Factor et al., 1990; Tandberg et al., 1998; Aarsland et al., 2001, 2003; Levy et al., 2002; Remy et al., 2005; Caviness et al., 2007; Yarnall et al., 2013).

Currently, PD affects approximately 2% of the population aged 65 and older, leading to an estimated 13000 people with PD in New Zealand (Statistics New Zealand 2016; Aarsland et al., 2001, 2003). This number is expected to double in the next 25 years due to the increasing age of the general population (Kowal et al., 2013).

Oral administration of the dopamine precursor levodopa (L-DOPA) remains the gold standard treatment for PD and is effective in relieving most of the motor symptoms of the disease (Cotzias et al., 1967; Jankovic, 2008). However, the efficacy of L-DOPA varies between patients, the plasma levels of L-DOPA fluctuates so that patients exhibit so called “ON/OFF” effects, where motor symptoms return during the “OFF” period (Jankovic and Aguilar, 2008). Long-term treatment with L-DOPA narrows the therapeutic window of L-DOPA and causes dyskinesias and involuntary movements, which in themselves are disabiling (Jankovic and Aguilar, 2008; Poewe, 2009). Furthermore, L-DOPA is not effective against the non-motor symptoms of PD (Poewe, 2009).

Before the clinical application of levodopa, surgical ablation of the thalamus was the standard treatment for PD, however these were not effective in treating all the motor symptoms of PD and came with a number of complications in surgery and morbidity (Benabid et al., 1987; Poewe, 2009). Another surgical practice that is effective in treating the motor symptoms is deep brain stimulation (DBS), which can be used to treat patients who are refractory to L-DOPA treatment of those who are experiencing uncomfortable dyskinesias due to chronic drug treatment. (Benabid et al., 1987; Sprenger and Poewe, 2013). The subthalamic nucleus (STN) or the internal segment of the GP (GPi) are the most common targets for DBS, and electrical stimulation of these areas results in an improvement of motor symptoms (Poewe, 2009). However, DBS is an invasive therapy that can have a number of surgical complications, such as haemorrhage, and the electrical stimulation of the brain has effects on neuropsychological functions resulting in confusion, apathy and depression (Voon et al., 2006; Poewe, 2009; Sprenger and Poewe, 2013).

Emerging treatments are beginning to target non-dopaminergic pathways, such as glutamatergic NMDA receptor antagonists, and gene therapy, such as GAD gene therapy in the STN, is a promising treatment that has shown a reduction in L-DOPA related motor fluctuations in patients with no adverse side effects shown thus far (Kaplitt et al., 2007; LeWitt et al., 2011; Stayte and Vissel, 2014).
To further our understanding of the pathogenesis of PD, as well as discover novel therapeutic strategies, use of animal models offers a substrate to explore in vivo changes that may occur due to the disease. Although clinical data can be obtained from in vitro slices and post-mortem tissue of humans patients with PD, the in vivo changes that occur, prior to and during PD are crucial not only to optimise treatments for PD but also in the early detection and prevention of the disease.

Models of PD should meet several criteria that define Parkinson’s in patients. First and foremost dopaminergic neurons should be depleted to a degree that matches that of human patients (> 50%) and the cardinal symptoms that patients develop, such as the hallmark motor deficits, should be displayed in the animal model (Hirsch et al., 2003). Other desirable characteristics that could be modelled but are not essential are lesions to non-dopaminergic populations of neurons that are often evident in late-stage PD of humans, and the presence of alpha-synuclein containing Lewy bodies, which are present in patients with PD but are not specific to the disease and are found in other neurodegenerative diseases such as Alzheimer’s disease (Dauer and Przedborski, 2003).

Animal models of PD can be divided into a two broad categories; genetic models, that manipulate genes that have been related to PD and neurotoxic models, that may represent environmental risk factors which induce PD. However other models do exist, such as the intraparenchymal infusion of alpha-synuclein oligomers to induce Lewy bodies (Porras et al., 2012) and the use of pharmacological compounds to acutely antagonize dopaminergic transmission in the striatum such as haloperidol (Parr-Brownlie and Hyland, 2005; Seeger-Armbruster et al., 2015). The neurotoxic model is most commonly used, has been thoroughly studied, and is the specific focus of this section.

The two most frequently used neurotoxins to induce dopaminergic lesions are 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The pesticide rotenone is also frequently used to model PD. Each will be discussed below.

**6-OHDA** The neurotoxin 6-OHDA has been used to lesion dopaminergic neurons in the SNC for nearly 50 years (Ungerstedt, 1968). The VTA is also affected by 6-OHDA lesions but to a lesser degree than the SNC (Tseng et al., 2005). It is most frequently used in rats, however mice, non-human primates and other species can be made parkinsonian with 6-OHDA (Betarbet et al., 2002). The toxin does not cross the blood brain barrier, so is stereotaxically injected directly into the brain usually unilaterally, although bilateral injections do occur (Bové and Perier, 2012; Le et al., 2014). Due to its structural similarity to
catecholamines, 6-OHDA is taken up by catecholinergic neurons, such as dopaminergic and noradrenergic neurons, by a catecholamine transporter (Bové and Perier, 2012). Therefore to be selective for dopaminergic depletion, it is used in conjunction with a nor-adrenaline re-uptake inhibitor such as desipramine to protect noradrenergic neurons (Waddington, 1980). Once in the neuron, 6-OHDA accumulates in the cytoplasm and undergoes auto-oxidation yielding two toxic products, hydrogen peroxide and paraquione, which cause cell death (Le et al., 2014).

Following bilateral injections, rats are unable to look after themselves due to the severity of the lesion and must be intensively nursed. Therefore, unilateral injections are the most popular model (Blesa et al., 2012). 6-OHDA can be injected into the SNc itself, the striatum and the medial forebrain bundle (MFB), which is a bundle of nigral dopaminergic fibres ascending to the striatum (Le et al., 2014). When injected into the SNc and MFB dopaminergic neurons begin to degenerate within 24 hours (Jeon et al., 1995), however, retrograde degeneration takes 1-3 weeks when injected into the striatum (Przedborski et al., 1995). Following MFB injections, the dopaminergic content in the striatum is less than 20% after 3-4 days and the number of dopaminergic neurons in the SNc continues to decrease for up to 5 weeks (Bové and Perier, 2012). If a successful lesion has occurred, up to 90% of dopaminergic neurons will be lesioned in SNc (Bové and Perier, 2012).

Behaviour tests offer a quick in vivo check of unilateral lesion status with motor performance compared between paws. The non-lesioned hemisphere acts as a control, however, sham-lesioned rats should also be used as a more complete control for experiments (Hirsch et al., 2003). Rats will exhibit asymmetric rotations, as well as reduced motor activity, and preference for the unlesioned paw (Olsson et al., 1995; Vandeputte et al., 2010; Bové and Perier, 2012). Rotational behaviour can also be induced in unilateral lesioned rats by injecting a dopaminergic agonist, such as apomorphine, or drug that releases the remaining dopamine (e.g. amphetamine) that will cause rotations in the contralateral (away from the lesion) or ipsilateral (toward the lesion) directions, respectively (Ungerstedt and Arbuthnott, 1970). Following injections into the striatum, rats have also exhibited some non-motor symptoms of PD such as anxiety and depression, as well as gastrointestinal dysfunction (McDowell and Chesselet, 2012).

The behaviour of animals can be recovered with L-DOPA treatment, however, chronic long-term L-DOPA treatment leads to abnormal involuntary movements or dyskinesias, similar to human patients and indeed, this model has been used in the study of dyskinesias (Carey, 1991; Henry et al., 1998; Winkler et al., 2002; Smith et al., 2016). High frequency (≥130 Hz) DBS has also proved effect in the 6-OHDA model of PD, where symptoms of PD
were alleviated and neuronal transmission in the basal ganglia was recovered (Spieles-Engemann et al., 2010; So et al., 2012; Dorval and Grill, 2014).

Although 6-OHDA does not produce Lewy-body like neuronal inclusions and injections into the MFB acutely degenerate dopaminergic neurons, it is a highly reproducible model, and rats exhibit many of the symptoms that occur in patients with PD. It is widely used to study the pathophysiological changes that occur within the brain under parkinsonian-like conditions and it is with this in mind that the 6-OHDA model of PD was used in this study.

MPTP The neurotoxin MPTP was first discovered to induce symptoms, remarkably similar to Parkinsonism, in humans in the 1980s (Langston et al., 1983). Following this discovery, it was quickly adapted into animals as a model of PD. MPTP is most often used in non-human primates and mice but rats are relatively resistant to MPTP lesion (Chiueh et al., 1984). MPTP is usually administered systemically, will readily cross the blood-brain barrier and induce a bilateral lesion of dopaminergic neurons (Betarbet et al., 2002). MPTP can also be infused into the internal carotid on one side inducing a unilateral lesion that does not cause a complete destruction of the dopaminergic system and therefore animals are able to feed, drink, groom and maintain themselves (Blesa et al., 2012). Once MPTP is in the brain it enters astrocytes and is converted into its active metabolite 1-methyl-4-phenylpyridine (MPP+) by the enzyme monoamine oxidase B (Nakamura and Vincent, 1986). MPP+ is then released by the astrocytes and selectively taken up by dopaminergic neurons in the SNc due to its affinity with the dopamine transporter. MPP+ inhibits complex I of mitochondrial function subsequently leading to death of dopaminergic neurons through oxidative stress (Langston et al., 1983). Indeed, in patients with PD, systemic expression of complex I activity is reduced (Schapira et al., 1989), suggesting a similar mechanism between MPP+ and the pathogenesis of PD. Other neurotransmitter systems are sensitive to MPTP intoxication such as the noradrenergic and serotonergic systems, which are also affected in late-stage PD (Hirsch et al., 2003).

Non-human primate models of PD present with the hallmark symptoms of bradykinesia, rigidity, abnormal posture, and sometimes tremor (see Le et al., 2014) that closely mimic the human form of the disease. MPTP lesion is influenced by the age and gender of the primate model and can produce variable results. Motor symptoms improve with L-DOPA drug treatment, however dyskinesias may develop with prolonged treatment, similar with human patients (Blesa et al., 2012), and DBS stimulation of the STN or GPi relieve motor deficits associated with this model of PD (Benazzouz et al., 1993; Hahn et al., 2008; Tass et al., 2011). In some species of monkeys, non-motor symptoms of PD appear, such as
sleep disorders (Verhave et al., 2011), attention deficits and decision-making dysfunction (Le et al., 2014). However, MPTP administration in non-human primates is most often acute, and therefore does not mimic the slow progressive decline of PD over time. Chronic lower-doses of MPTP administration have been used but due to the time and cost of maintaining primate models it is used sparingly (Le et al., 2014). Furthermore, following chronic small-doses of MPTP motor symptoms reduce when treatment stops suggesting it may not progress to PD-like degeneration (Betarbet et al., 2002).

Mice models have been used more regularly than primate models, usually due to the lower financial cost, however, mouse strains differ in their susceptibility to the neurotoxin (Betarbet et al., 2002; Le et al., 2014). Motor symptoms in mice are not as characteristic as in the primates and in some cases mice become hyperactive following MPTP administration (Hirsch et al., 2003). These contradictory results may be due to the varying sensitivity of the mice to the toxin. Chronic exposure to MPTP in mice has led to the development of Lewy body-like inclusions and mice develop motor deficits as a result of dopamine depletion (Meredith et al., 2008).

MPTP does not work in rats, and although it represents a good model of PD in non-human primates and mice with acute and chronic exposure, there is a great financial and time cost to the primate models and it is variable with the strain of mouse used. Bilateral degeneration requires intensive post-lesion care but remains a standard toxin model of PD in mice and primates. MPTP was not used for this project because rats are the model species.

**Rotenone** This natural cytotoxic compound is extracted from tropical plants and is systematically administered most commonly in rats, however, monkeys have been used (Mastroberardino et al., 2009). It is highly lipophilic and readily crosses the blood brain barrier where it binds to and inhibits mitochondrial complex I much like MPP+ and at low doses has been shown to selectively degenerate dopaminergic neurons in the SNc (Betarbet et al., 2000; Dauer and Przedborski, 2003). Following administration of rotenone, animals develop abnormal posture, unsteady gait and bradykinesia, and can sometimes induce Lewy-body-like cytoplasmic inclusions that are alpha-synuclein positive. Rotenone can also be stereotaxically injected in the MFB and elicits dopaminergic lesions in the SNc that is associated with PD-like motor symptoms that are reversed with L-DOPA (Alam et al., 2004). However, rotenone lesions are a labour-intensive process, and is highly variable (Betarbet et al., 2002). Reproducibility of rotenone-induced lesions has been questioned, and has been found to lesion dopaminergic neurons and non-dopaminergic populations, suggesting a model of late-stage idiopathic PD (Höglinger et al., 2003). Not all strains of rats are sensitive to the toxin (Betarbet et al., 2000). Systemic administration of rotenone can also lead to a high
mortality rate of animals (Le et al., 2014), and similar to MPTP and 6-OHDA, bilateral injections leave the animal extremely sick and are difficult to maintain (Betarbet et al., 2002).

Rotenone appears to be a reasonably accurate model of Parkinson’s, especially late-stage PD, and with the appearance of dopaminergic, and non-dopaminergic lesions and neuronal inclusions rotenone induced PD remains a relevant model to study neuroprotective therapies. Although this may be useful to alleviate symptoms of patients with PD, it does not add to the early detection and prevention of PD. However, due to its difficult protocol and high variability, rotenone neurotoxicity was not used in this study.

1.2.3 Circuitry of the basal ganglia and Parkinson’s disease

Overview The basal ganglia are a group of nuclei that are intimately involved in the planning and initiation of movements. They include the striatum, the Gpi and GPe, the STN, the VTA and both the SNr and SNC (Beckstead et al., 1979; Albin et al., 1989). The basal ganglia are functionally connected with the motor cortex, the supplementary motor area and the motor portion of the thalamus including the VA/VL and the ventromedial (VM) nuclei, to make a cortico-basal ganglia-thalamocortical loop (Albin et al., 1989). The striatum receives inputs from most areas of the cortex (Albin et al., 1989) as well as a dopaminergic projection from the SNC and the VTA (Beckstead et al., 1979; Burns et al., 1983), and projects to the basal ganglia output nuclei, Gpi and SNr, via two pathways, the ‘direct’ and the ‘indirect’ pathway, which will be discussed below (DeLong, 1990). The Gpi and SNr send messages to the motor thalamus that in turn sends messages to the cortex, which are modulated by the RTN (Figure 1.4). Messages are then conveyed down the brainstem and spinal cord to innervate alpha motor neurons resulting in movement (Parent and Hazrati, 1995). The loss of dopaminergic neurons in the brain associated with PD has various pathological effects on the nuclei of the basal ganglia, and will be discussed in this section.

Anatomy As the name suggests, the ‘direct’ pathway is a direct projection from the striatum to the SNr/GPi. The indirect pathway involves a projection from the striatum to the STN, through a relay in the GPe, and a projection from the STN to the SNr/GPi. The SNr/GPi have output projections to the motor thalamus (VA/VL and VM) (Albin et al., 1989, DeLong, 1990). Dopaminergic projections from the SNC and the VTA modulate the incoming information from the cortex by differently affecting the output pathways of the striatum (Beckstead et al., 1979, Burns et al., 1983). Figure 1.4 shows the general connections of the basal ganglia that will be discussed in detail below where in each case, ‘normal’ anatomy is considered first followed by the changes that occur with dopaminergic depletion.
The output neurons of the striatum, called medium spiny neurons (MSNs) are inhibitory GABAergic neurons that can be classed into two sub-populations based on the expression of the neuropeptides, substance P and enkephalin (Gerfen et al., 1990). In addition, the striatum also contains at least three classes of interneurons; cholinergic interneurons, parvalbumin interneurons and somatostatin interneurons (Kawaguchi, 1997). The focus for this section will be on the output GABAergic MSNs. Cortical afferents to MSNs are glutamatergic and synaptic input is to the head and shaft of dendritic spines (Parent and Hazrati, 1995). MSNs of the direct pathway express substance P and dopaminergic modulation from the SNc and VTA is via D1-receptors whereas those of the indirect pathway projecting to the GPe express enkephalin and dopamine binds to D2-receptors (Gerfen et al., 1990; Surmeier et al., 2007). Dopaminergic depletion following PD results in a loss of dendritic spines on MSNs that decreases the surface area for cortical synaptic input and disrupts normal motor activity (McNeill et al., 1988; Stephens et al., 2005; Villalba et al., 2009; Villalba and Smith, 2010). PD also decreases D1-receptor expression and increases D2-receptors (Gerfen et al., 1990) resulting in abnormal modulation of striatal activity, again disrupting activity in the basal ganglia-thalamocortical pathway.

![Diagram](image_url)

**Figure 1.4. Connectivity of basal ganglia nuclei and the RTN.** Red arrows represent inhibitory projections, green arrows represent excitatory projections and blue arrows represent modulatory dopaminergic projections from the SNC and VTA. (Adapted from Albin et al., 1998).
Striatal input into the GPi/SNr via the direct pathway is GABAergic, and makes numerous synaptic contacts with dendrites and somata of GPi/SNr neurons (Bevan et al., 1994a, 1994b). Striatal innervation of the GPi/SNr is dense and directed to neurons that subsequently project to the motor thalamus (Bevan et al., 1994a). Similarly, striatal input through the indirect pathway is GABAergic. MSN output neurons densely innervate all parts of GPe neurons that project to the STN (Smith et al., 1998). The evidence regarding changes in striatal input to the GPi/SNr and GPe is divided. In rodent animal models of PD, only striatal input through the indirect pathway is affected (Ingham et al., 1997), whereas in primate models of PD, both direct and indirect pathways are affected (Ingham et al., 1998; Villalba et al., 2009). In either case, loss of spines is apparent in striatal inputs to the GPi/SNr or GPe (Villalba and Smith, 2010). The expression of the GABA synthesising enzyme GAD with the molecular weight of 67 kDa (GAD67) correlates to GABAergic activity of any particular neuron (Lau and Murthy, 2012). The expression of GAD67 in the striatum differs between subpopulations of striatal neurons where MSN output neurons generally have low expression of messenger ribonucleic acid (mRNA) that code the enzymes GAD67 and GABAergic interneurons have a high expression (Soghomonian et al., 1992; Laprade and Soghomonian, 1999). Following dopaminergic depletion, GAD67 mRNA expression in MSN output neurons is increases, whereas, in GABAergic interneurons it decreases (Soghomonian et al., 1992; Laprade and Soghomonian, 1999). These findings indicate that GABAergic synaptic transmission of striatal output neurons is altered and GABAergic transmission within the striatum itself is also changed (Soghomonian et al., 1992; Laprade and Soghomonian, 1999; Lau and Murthy, 2012). Furthermore, GABAergic transmission in the GPi/SNr is altered (Galvan and Wichmann, 2008), with an increased number of GABA receptors that are thought to account for the reduced GABAergic input from the striatum (Griffiths et al., 1990; Chadha et al., 2000; Katz et al., 2005). Whereas in the GPe, decreased numbers of GABAergic receptors indicate overactive GABAergic inputs from the striatum (Griffiths et al., 1990; Chadha et al., 2000; Katz et al., 2005).

Along the indirect pathway, GPe projections to the STN are topographically organized and input is made by symmetrical contacts onto proximal dendrites and somata (Smith et al., 1990). The GPe projection is GABAergic and makes numerous contacts with a STN neuron by ‘wrapping’ itself around its target neuron and synaptic varicosities provide multiple sites of synaptic transmission (Smith et al., 1990). Following depletion of dopamine, GABA release in the STN is decreased (Soares et al., 2004), presumably as a consequence of overactive striatal inputs into the GPe. In contrast to this, the amount of GAD67 mRNA per neuron and the number of neurons in the GPe that express GAD67 mRNA was increased.
following 6-OHDA lesions that would indicate increased GABAergic transmission (Kincaid et al., 1992; Soghomonian and Chesselet, 1992; Lau and Murthy, 2012). It is suggested that the activity pattern of GPe neurons (discussed below), which changes with dopaminergic lesions, is critical in the regulation of GAD67 mRNA expression (Soghomonian and Chesselet, 1992).

The STN projects to the output nuclei with asymmetric contacts with distal and proximal dendrites and somata (Bevan et al., 1994a, 1997). STN input is glutamatergic and convergent on GPi/SNr neurons that are projecting to the motor thalamus (Bevan et al., 1994a). In animal models of Parkinson’s, glutamatergic receptors are down regulated in the GPi/SNr presumably as a result of increased STN activity (Galvan and Wichmann, 2008).

The GPi/SNr output neurons are GABAergic, and densely innervate the motor thalamic nuclei, VA/VL and VM. Input from both GPi and SNr make synaptic contact onto the proximal dendrites and somata of thalamic neurons (Kha et al., 2000, 2001; Bodor et al., 2008). There is limited evidence regarding anatomical changes to this input, and most studies investigate the changes in activity of these basal ganglia output neurons, which will be summarised below. However, the levels of GAD67 expression in GPi and SNr projection neurons have been found to be increased in PD (Rodríguez Díaz et al., 2003; Conte-Perales et al., 2011), suggesting an increase in GABAergic signalling by these output nuclei.

**Physiology** Below, the physiological properties and firing patterns of the basal ganglia will be discussed with the normal activity considered first, followed by the changes that occur during PD. The pathophysiology of Parkinson’s is primarily thought to be due to abnormalities that arise in the basal ganglia. Generally, dopamine depletion in the SNc and VTA change the activity and firing pattern of neurons throughout the basal ganglia leading to an over-inhibition of the motor thalamus and results in the poverty of movement commonly seen in patients with PD (Galvan and Wichmann, 2008).

Physiological striatal activity can be difficult to characterize due to the heterogeneous population of MSNs in the striatum (Galvan and Wichmann, 2008). Focusing on striatal MSNs, activity of these output neurons is GABAergic and continuous at a low frequency, with rare episodes of burstiness (Dehorter et al., 2009). As stated earlier, burstiness is an important pattern for the communication between neuronal groups, and changes in the pattern of bursty activity can have an impact on downstream events (Bosch-Bouju et al., 2013). In dopamine depleted slices of MSNs, GABAergic currents shift from a low continuous drive, to giant spontaneous activity, occurring in single spikes as well as in bursts that changes the pattern of activity in the striatum from tonic to oscillatory (Walters et al., 2007; Dehorter et al., 2009). Currently there is little in vivo evidence of the changes to subpopulations of striatal
activity, however in 6-OHDA lesioned animals, direct pathway MSNs were found to have reduced activity than normal, and indirect pathway MSNs had increased discharges compared with normal (Mallet et al., 2006). Changes in activity in the striatum may affect the balance between the direct and indirect pathways and lead to downstream changes in the whole basal ganglia network.

The GPe, GPi, SNr and STN have similar tonic activity, and will be discussed together (Miller and DeLong, 1987; Filion and Tremblay, 1991; Walters et al., 2007; Galvan and Wichmann, 2008). Under normal physiological conditions, these nuclei display tonic discharges at a high frequency, however, some neurons of the GPe will exhibit pauses in activity (Miller and DeLong, 1987). In PD, the rate of firing changes in most of these nuclei (Miller and DeLong, 1987; Avila et al., 2010), which has consequences on the output from the basal ganglia to the thalamus. Following loss of dopamine, the firing rate in the GPi, and STN is increased (Miller and DeLong, 1987; Filion et al., 1988; Tremblay et al., 1989; Filion and Tremblay, 1991; Bergman et al., 1994; Rothblat and Schneider, 1995; Delaville et al., 2015), whereas in the GPe, the firing rate decreases (Miller and DeLong, 1987; Tremblay et al., 1989; Filion and Tremblay, 1991). The changes to the firing rate of SNr are controversial, some studies have found no changes in primate model of PD (Wichmann et al., 1999), however, increased firing in SNr has been observed 21-days post induction of PD in a rodent model of the disease (Avila et al., 2010). Under dopamine-deprived conditions, the GPi/SNr are released from their inhibition along the direct pathway, and are further activated by the disinhibited STN, causing over-inhibition of the motor thalamus. Consequently, relay of motor messages from the motor thalamus to the cortex becomes inhibited, leading to the classical motor symptoms of PD (Galvan and Wichmann, 2008).

Rate of firing is not the only change that occurs with PD. The GPe, GPi, SNr and STN demonstrate changes in their firing patterns, where bursty events and oscillations become more frequent. These nuclei all exhibit burst discharges normally (Miller and DeLong, 1987; Beurrier et al., 1999; Wichmann et al., 1999) which are a transient cluster of spikes and usually specifically timed to an event of physiological relevance, such as a motor task (Lobb, 2014; Marlinski and Beloozerova, 2014). In PD, the pattern of bursting activity of these nuclei become abnormal where the likelihood of bursty activity is increased and individual bursts of spikes are more persistent (Miller and DeLong, 1987; Filion and Tremblay, 1991; Parr-Brownlie et al., 2007). These bursts are either irregular or oscillatory in nature (Hammond et al., 2007). Oscillatory activity occurs in individual neurons as well as in neuronal populations and is thought to be important for the communication of messages within the brain (Galvan and Wichmann, 2008). Oscillatory activity in PD occurs in an
abnormal rhythm within the basal ganglia, and often pathologically synchronizes the nuclei together (Hammond et al., 2007; Quiroga-Varela et al., 2013). When synchronization does occur in a normal state, it is transient while the motor message is passed through the basal ganglia loop, however in PD, this synchronization becomes stable, therefore it persists and is thought to impair the motor message that is conveyed (Hammond et al., 2007).

The basal ganglia nuclei are extremely important in the proper generation and execution of movements. Normally they work in harmony with the thalamus and cortex to ensure normal development of the motor programme, and the dopaminergic system regulates this activity. When dopamine is depleted in the basal ganglia, changes in physiological properties including firing rate and firing pattern, disrupt the message that will be conveyed through this network, and impairs movement.

1.2.4 Parkinson’s disease and the reticular thalamic nucleus

The rRTN is perfectly situated to regulate the motor information going from the thalamus to the cortex. Considering that the RTN is involved in the generation of sleep spindles, and patients with PD frequently have problems with sleep (Factor et al., 1990), it is easy to see how the RTN could be implicated in the pathophysiology of this disease. Furthermore, excitotoxic lesions of the RTN with domoic acid is associated with attention deficits, and 6-OHDA lesions of the dopaminergic input to the RTN results in schizophrenia, and reduced motor activity, which patients with Parkinsonism may experience (Friedberg and Ross, 1993; Picazo et al., 2009).

The rRTN receives projections from the SNr and both the GPi and GPe that have been defined anatomically and physiologically in Sections 1.1 and 1.2.3 (see also Figure 1.4). The motor sector of the RTN also projects to VA/VL in the motor thalamus and should therefore be considered in basal ganglia-thalamocortical circuitry. The physiological characteristics of neurons in the rRTN are similar to the basal ganglia nuclei. Generally RTN neurons fire with relatively low tonic activity, compared with the high tonic activity in the GPe, GPi, SNr and STN, and some neurons of the rRTN demonstrate transient bursty activity similar to basal ganglia nuclei (Domich et al., 1986; Spreafico et al., 1988; Marlinski and Beloozerova, 2014).

The investigation into how PD affects the RTN is incomplete, however, the few changes that have been found will be described below. Following 6-OHDA lesions in the SNc, expression of GAD67 in rRTN is increased (Delfs et al., 1996), similar to increased expression of GAD67 in the striatum, GPe, GPi, and SNr (Soghomonian and Chesselet, 1992; Soghomonian et al., 1992; Delfs et al., 1995; Rodríguez Diaz et al., 2003; Conte-Perales et al., 2011), and indicates that GABA transmission in the RTN is altered (Delfs et al., 1996; Lau
and Murthy, 2012). Additionally, dopamine content within rRTN is decreased following depletion of dopaminergic neurons in the SNc (Anaya-Martinez et al., 2006), possibly causing an increase of transmitter release by GABAergic terminals from the GPe and SNr, which is normally modulated by presynaptic D4 receptors (Florán et al., 2004; Govindaiah et al., 2010; Erlij et al., 2012). A small decrease of dopaminergic neurons in the SNc and subsequent loss of dopaminergic innervation to the rRTN has also led to reduced motor activity and anxiety-related behaviours in the open-arm maze, however no affect was seen on rota-rod performance, possibly due to the small size of the dopaminergic lesion (Picazo et al., 2009).

Recent data reported in a thesis from this lab indicates that with 6-OHDA lesions of the SNc and VTA, the firing rate of rRTN neurons is decreased and the firing pattern of neurons in the rRTN becomes more frequently bursty (Little, 2014), again, similar to changes seen in basal ganglia neurons.

The RTN is a nucleus that is not normally considered important in basal ganglia function. However with its close anatomical and physiological link with a lot of the basal ganglia nuclei, it is very likely that the RTN plays a role in some of the symptoms of PD such as motor deficits, attention deficits or sleep disorders (Factor et al., 1990; Tandberg et al., 1998; Aarsland et al., 2001; Levy et al., 2002; Remy et al., 2005; Caviness et al., 2007; Yarnall et al., 2013).

1.3 Viral vector labelling of neurons

1.3.1 History of anatomical tracing methods

To understand fundamental aspects of disorders of the brain such as PD, it is essential to know how neurons connect and communicate with each other. Neuronal tracing allows us to look at the anatomy of connections, either the inputs to a particular area of interest or the target structures the area innervates. This mapping allows for detailed descriptions of neuronal projections that can reveal circuits within structures in the brain, such as the basal ganglia circuitry. Technology has massively advanced the ability to assess interconnections of neural systems and with the latest viral vector technology, we are able to selectively label a specific phenotype of a neuron, at a specific injection site within the brain and get substantial amplification of the signal that is easily detected. The history of anatomical tracing methods will briefly be summarised below.

Ramon y Cajal first pioneered anatomical descriptions of neuronal morphology and tract tracing with the Golgi impregnation method in the late 1800s and early 1900s (Lanciego
and Wouterlood, 2011). Although now considered coarse, Golgi staining paved the way to better and more specific methods of tract tracing. Utilising the uptake of macromolecules into neurons and their transportation within the neurons, tracers can be used to label the entire length of a cell from the soma to distal axon terminals (Kristensson et al., 1971). Transportation can either occur retrogradely, from the axon terminals towards the cell soma, or anterogradely, from the cell soma to the axon terminals. Neuroanatomical tracers can be stereotaxically injected into a desired area of the brain allowing a specific location to be targeted. Mechanical injection into the brain can cause damage of fibres of passage and uptake of tracers by these damaged neurons (Lanciego and Wouterlood, 2011). Whereas iontophoretic delivery limits this damage (Lanciego and Wouterlood, 2011). However, only small quantities of tracer can be delivered with this method, and not all tracers are compatible with iontophoretic delivery (Lanciego and Wouterlood, 2011).

The popular retrograde tracer horseradish peroxidase (HRP) has been used since the 1970s (Kristensson and Olsson, 1971). HRP is taken up in the axons and dendrites of neurons as well as at the cell body (LaVail and LaVail, 1972). The mechanism by which this happens makes HRP susceptible to being taken up by damaged axons and fibres of passage and, therefore, can label neurons away from the injection site (Lanciego and Wouterlood, 2011). HRP can be conjugated to the lectin, wheat germ agglutinin (WGA) and the bacterial non-toxic B fragment of cholera toxin (CTB) to produce a more sensitive tracer that has a limited capacity to be taken up by fibres of passage (Gonatas et al., 1979; Trojanowski et al., 1981; Wan et al., 1982). Compared with native HRP, CTB-HRP labels a greater number of neurons and more efficiently reveals the dendritic tree of labelled neurons (Wan et al., 1982). Both WGA-HRP and CTB-HRP are bi-directionally transported that can be a disadvantage when teasing out a difference between afferent and efferent fibres for example (Gonatas et al., 1979; Chen and Aston-Jones, 1995; Lanciego and Wouterlood, 2011). Another limitation is that the uptake of both conjugates relies on the availability of receptors on neuronal membranes, which is not always the case with viral vectors (Geerling et al., 2006). While the use of WGA-HRP is still popular, many studies do not account for the bi-directional nature of the tracer; it is generally regarded as a retrograde tracer (Lanciego and Wouterlood, 2011).

The lectin, *Phaseolus vulgaris* (PHA-L) is an anterograde tracer that is taken up exclusively at neuronal cell bodies and is transported towards axon terminals (Gerfen and Sawchenko, 1984). Uptake is receptor mediated so is subject to the same limitations as the HRP conjugates (Gerfen and Sawchenko, 1984). PHA-L detection results in detailed visualization of a neuron in its entirety. PHA-L is often combined with other tracers, such as the biotinylated dextran amines (BDA), which will be discussed individually below. The
combination of PHA-L and BDA allows the study of multiple circuits converging into one brain area (Lanciego and Wouterlood, 1994). Like HRP, PHA-L may be taken up by damaged fibres of passage when mechanically injected into the brain however uptake into fibres of passages occurs less frequently with delivery by iontophoresis (Lanciego and Wouterlood, 2011). PHA-L has also been seen to travel retrogradely in some systems suggesting that the nature of PHA-L transportation is not absolute and should be further investigated (Raju and Smith, 2006).

BDA was introduced as a quality anterograde tracer by Veenman et al. (1992) and initially used to trace neurons in the somatosensory cortex of the rat (Veenman et al., 1992). The mechanism by which BDA is taken up by neurons is still unknown, however, it seems to be taken up by both cell bodies and neuronal dendrites (Reiner et al., 2000). BDA remains stable in the rodent brain up to 4 weeks post injection making it a popular choice for longer-term neuroanatomical tracing studies (Veenman et al., 1992). BDA fills neurons by forming a homogeneous label inside neurons, in a way resembling classical Golgi impregnation (Lanciego and Wouterlood, 2011). This homogenous staining holds along the fibres to the terminals and allows clear visualization of neurons in their entirety and precisely maps fibre tracts. BDA may be transported retrogradely, the lower 3 kDa molecular weight BDA molecule is readily transported retrogradely and primarily used as a retrograde tracer, however, the more commonly used 10 kDa, infrequently travels retrogradely (Reiner et al., 2000). Although, labelling of the 10 kDa tracer in areas retrograde to the site is limited, this labelling could lead to false interpretation of anterograde labelling, and the same rings true for the 3 kDa molecule, which may label anterograde pathways (Reiner et al., 2000) Therefore, care should be taken in interpreting the results of pathway tracing using BDA. Fibres of passage infrequently take up BDA, again leading to unspecific labelling and false results. Despite this, BDA is an extremely versatile tracer that efficiently labels neurons in sections of a range of section thicknesses and can be easily paired with other tracers to label more than one population of neurons (Lanciego and Wouterlood, 2011). Its versatility makes BDA a popular choice for anterograde tracing studies.

Although these tracers can effectively label the entirety of neurons, in either retrograde or anterograde directions, they all come with their limitations; uptake by all cells in the area of injection, uptake in fibres of passage, unintentional bi-directional labelling or relying on the membrane components of a neuron for uptake. HRP, PHA-L and BDA are not as sensitive or specific as viral vectors and were therefore not used in this study.
1.3.2 Lenti and adeno-associated viral vectors

The properties of a virus that enable it to insert its genetic information into healthy host mean it can also be exploited as a neuronal tracer. Of course this requires removal of the genetic components that make the virus harmful, and taking advantage of the traits that allow a virus to enter neurons, begin replicating and act as a self-amplifying signal within a neuron (Ugolini, 2010). A virus’s ability to cross synapses can be especially important when studying the connective patterns of neural circuits (Lanciego and Wouterlood, 2011). Viral vectors act as a delivery medium for the expression of a chosen reporter gene, which is driven by a specific promoter gene (Sizemore et al., 2016). The use of viral vectors can still be dangerous due to nature of the virus within the host cell and the slim chance of infection, therefore, they must be handled carefully under strict biosafety protocols.

Since the early 1980s researchers harnessed aspects of the human immunodeficiency virus (HIV) and developed recombinant viral vectors that have become appropriate for use in neuroscience research (Parr-Brownlie et al., 2015). Lentiviruses enter a host cell through the intact nuclear envelope, where the viral RNA is reverse transcribed into deoxyribonucleic acid (DNA) before it is integrated into the host genome. Host cells can be dividing or non-dividing cells (Naldini, 1998), making lentiviral infection suitable for the typically non-dividing neural cells targeted in tracing studies. Recombinant lentiviral vectors contain long terminal repeats required for replication and integration at either end of a packaging signal and an exogenous promoter that can be used to express a cell-type specific promoter to target a specific population of neuron (Dull et al., 1998), such as the calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) promoter to specifically target glutamatergic neurons (Parr-Brownlie et al., 2015). Having this level of labelling specificity allows a particular neuronal phenotype to be distinguished from others in the brain (Figure 1.5). Viral vectors diffuse smaller distances than non-viral tracers, such as HRP, PHA-L and BDA, permitting the transduction and tracing in a smaller and more controlled area of the brain (Lerchner et al., 2014). Large populations of neurons can still be targeted by injecting larger volumes of virus, or with multiple injections (Parr-Brownlie et al., 2015).

Producing viral vectors with added glycoprotein envelopes from a different virus strain, a process called pseudotyping, affords the ability to modify the site of lentiviral vector uptake (Indraccolo et al., 1998). Specific targeting of uptake can begin to be exploited, depending on a study’s objectives and the physical damage resulting from the injection procedure can be avoided. For example, if the aim was to look at the projections of a specific population of cells, vesicular stomatitis virus glycoprotein (VSVg) pseudotyped vectors

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(Finkelshtein et al., 2013), can be injected into the vicinity of the cell bodies, and will anterogradely label the entirety of the neuron. In this scenario, the physical damage artefact caused by the injection at the cell bodies does not interfere with the results obtained at the projection sites (Parr-Brownlie et al., 2015). It is also possible to retrogradely label neurons with a lentiviral vector pseudotyped with the rabies glycoprotein (Schoderboeck et al., 2015).

Another option for viral vector tracing is the adeno-associated virus (AAV). AAVs are associated with high levels of expression and a larger site of diffusion around the injection site when compared to lentivirus (Packer et al., 2013). In some cases this can be more beneficial than the lentiviral construct, however not always, especially when delicate spatial specificity is required to trace a population of neurons in a small nucleus. The higher levels of AAV transduction may also lead to axonal malformations that could compromise tracing results (Miyashita et al., 2013), and AAVs are subject to inflammatory and immune responses to a greater extent than lentiviruses (Abordo-Adesida et al., 2005; Parr-Brownlie et al., 2015).

The evolution of viral vectors as anatomical tracers has been paramount to redefining the circuitry of the brain. The specificity that can be achieved at a sub-population level outmatches traditional tracing methods. Neural phenotypes can be targeted utilizing the expression of particular subcellular components, making it an ideal technique to investigate the specific phenotype of neurons in the rRTN.

![Diagram of viral vector tracing](image)

**Figure 1.5. An example of the specificity afforded by the injection of lentiviral vectors into an area of the brain.** In this case, the reporter fluorophores mCherry and green fluorescent protein (GFP) enable the dual labelling of glutamatergic neurons, promoted by CaMKIIα expression, and GABAergic neurons, promoted by GAD67 expression. (Adapted from Parr-Brownlie et al., 2015).
1.4 Rationale, aims and hypotheses

Presently, not a lot is known about how the RTN acts to modulate motor information between the motor thalamus and cortex. Converging inputs from the basal ganglia, motor cortex and motor thalamus to the rostral portion of the RTN suggest that it has a key role in the integration of messages relating to movement execution. Movement disorders, such as PD, have been extensively studied for decades, and although current treatments for PD can alleviate symptoms, the complete aetiology of the disease needs to be clarified. Due to the fact that the RTN receives inputs from basal ganglia nuclei, pathological changes occurring in the brain associated with PD may be associated with changes in the RTN.

Recently, electrophysiological data from our lab indicated that the RTN might contain at least 2 populations of neurons; a distinctive GABAergic population but also an undefined, non-GABAergic population. This second non-GABAergic population had a broad extracellular action potential width and some preliminary immunolabelling indicated that some RTN cells express markers such as the excitatory amino acid transporter EAAC-1, indicative of a glutamatergic phenotype. In light of this finding, I aimed to explore the phenotype of neurons in the RTN, often defined as being exclusively GABAergic, by utilizing dual immunohistochemistry to label multiple neuron-phenotype specific antigens. The small novel population of neurons were stained for CaMKIIα; a selective marker for glutamatergic neurons in the thalamus (Benson et al., 1991). Lentiviral vector technology was employed to selectively label this glutamatergic population of neurons to confirm the existence of these neurons in the rRTN and trace them to their potential synaptic targets. Due to the involvement that rRTN has with motor pathways, I examined the effect of dopaminergic lesions on the number and morphology of neurons in rRTN.

The project had the following hypotheses. As the literature suggest, the vast majority of neurons in rRTN would be GABAergic in phenotype, however, a small minor population of glutamatergic neurons would exist within the rRTN. Potential targets for this glutamatergic population may include other rRTN neurons, the motor thalamus, the globus pallidus or the striatum. Finally, in the 6-OHDA model of PD, no differences would exist in the number or morphology of neurons in the rRTN despite changes to the electrophysiology of these neurons as previously described.
2.0 Methods
The rRTN regulates activity in the thalamus and has a vast array of inputs from a number of regions of the brain, including many that are involved in movement. The literature often states that the rRTN is exclusively GABAergic, however, recent evidence suggests that a non-GABAergic population of neurons exists within the nucleus. The pathophysiology of PD has been linked to many structures that the rRTN has connections with, however, little is known about the effect PD has on the rRTN. The following section will highlight the protocols I used to explore the phenotype of RTN neurons immunohistochemically, examined the effect of the chronic 6-OHDA model of PD on the neuronal populations within the rRTN and utilized lentiviral vector technology to selectively label and trace glutamatergic neurons within the rRTN.

The following procedures were approved by University of Otago Animal Ethics Committee (AEC 10/13; AEC 03/16) and conform to their guidelines. Throughout the study every effort was made to reduce the number of animals used.

2.1 Animals

Twenty-six adult male Wistar rats (Hercus Taieri Resource Unit, University of Otago) were housed at 22°C in environmentally controlled conditions with a reversed 12 h light/dark cycle. Surgical procedures and behavioural testing were conducted during the animal’s dark cycle. Rats weighed between 290 g and 320 g at the time of the first surgery, and food and water were available ad libitum for the duration of the experiment. Rats were processed in two groups; one group underwent both 6-OHDA lesion, to induce a rat model of PD, and lentiviral injection surgeries and were used to determine neuronal phenotype and investigate neuronal tracing studies, and the second group underwent 6-OHDA lesions only and were used for stereology (see Figure 2.1). In both groups sham lesioned rats acted as a control group.

2.2 Rat model of Parkinson’s disease

To investigate the effects that the neurodegenerative PD has on rRTN neuronal populations, the chronic 6-OHDA rat model of PD was used. The reproducibility, high rate of success and vast experience this lab has with this model (Parr-Brownlie et al., 2007, 2009; Walters et al., 2007; Avila et al., 2010; Bosch-Bouju et al., 2014), it was deemed the most appropriate choice from the models listed in Section 1.2.2. Standard behavioural tests and
immunohistochemistry for tyrosine hydroxylase (TH) have been employed to assess the extent of the lesion and ensure the success of the model.

2.2.1 6-OHDA and sham lesions surgery

Unilateral lesions of the MFB were conducted in the left hemisphere of 28 rats. Roseanna Smither, a senior member of the lab, carried out 6-OHDA lesions and 5 of the 11 sham lesions in animals. I did the sham lesions in 6 rats. Rats were deeply anaesthetised with a subcutaneous (s.c.) injection of a cocktail of ketamine (75 mg/kg; Parnell Technologies, NZ), domitor (0.5 mg/kg; Pfizer Animal Health, NZ) and atropine (0.06 mg/kg; Phoenix Pharm, NZ) (KDA). The state of unconsciousness was closely monitored throughout the procedure and if the level of anaesthesia was too light a top up dose of 0.1 ml KDA (s.c.) was administered. Rats were administered desipramine hydrochloride with an intraperitoneal (i.p.) injection (15 mg/kg; Sigma-Aldrich, USA), 30 minutes prior to infusion of the neurotoxin 6-OHDA or vehicle to prevent uptake of 6-OHDA into noradrenergic neurons. Body temperature was maintained at 36-38 °C throughout the procedure and during the early stages of recovery by a heating pad. The area of incision was shaved and sterilized using three washes of betadine and ethanol. To provide local analgesia, lopaine (20 mg/mL, s.c.; Ethical Agents Ltd, NZ) was injected along the intended incision line and the antibiotic amphoprim (30 mg/kg s.c.; Virbac Animal Health, NZ) was administered to prophylactically prevent infection. Rats were mounted in a stereotaxic frame (Stoelting, USA), and eye ointment...
(Tricin; Jurox, Australia) was applied to prevent corneal dehydration. After ensuring rats were securely mounted, a midline sagittal incision exposed the cranial surface of the skull, the skin was retracted with small clips and subcutaneous tissue was carefully removed. The surface of the skull was cleaned with saline and dried to visualise bregma and lambda sutures. If bleeding persisted, the area was cauterised to ensure adequate visualisation of the sutures. A hole was drilled over the stereotaxic coordinates above the left medial forebrain bundle at +4.4 mm anteroposterior (AP) and -1.2 mm lateral (ML) from the lambda skull suture in the flat skull position. Bone chips were removed from the hole using fine forceps and the dura was reflected. The cannula was lowered 8.3 mm into the brain from the skull surface (DV). In lesioned animals, a solution of 6 \( \mu \text{g} \) of 6-OHDA (Sigma-Aldrich) in 3 \( \mu \text{l} \) of 0.9% saline containing 0.05% ascorbic acid was infused at a rate of 1 \( \mu \text{L}/\text{min} \) for three minutes. Control rats were sham lesioned with the same volume of vehicle solution that contained 0.9% saline and 0.05% ascorbic acid. After the infusion, the cannula remained in place for 5 min to prevent diffusion of 6-OHDA along the injection track. At the end of the procedure, the cannula was slowly withdrawn, the incision site was sutured and rats were administered carprofen (5 mg/kg s.c.; Norbrook, NZ) for long acting analgesic relief. To reverse the effects of the anaesthetic, rats were administered antisedan (2.5 mg/kg s.c.; Pfizer) and 5 ml of saline was injected to ensure rats did not become dehydrated overnight while the effects of the anaesthetic were wearing off.

Rats were transferred to individual cages and post-operatively monitored twice daily for 1 week. For easy access, wet rat chow was put into the rat’s cage and extra treats were given to ensure a healthy weight was maintained. Animals that were lesioned with 6-OHDA often lost around 6% of their pre-surgery bodyweight and took a number of weeks to re-gain weight to pre-surgical levels due to the pathology of the model. If an animal lost more than 15% of their body weight post-operatively, the University of Otago veterinarian was advised to help choose the appropriate course of action, however, there was no instance where this occurred. In some cases, 24-48 h following surgery the suture had re-opened due to the rat scratching at it. In these instances a senior member of the lab assessed the animal, and the University of Otago veterinarian was informed of the situation. All cases resulted in the rat being re-sutured under the influence of the KDA anaesthetic following guidance and advice from senior members of the lab and close post-operative monitoring until the wound had fully healed.
To assess the extent of the dopaminergic lesion, rats were subjected to the behavioural motor tasks of step and cylinder testing at 5 to 7 days post-operatively (Olsson et al., 1995; Schallert et al., 2000). The forepaw contralateral to the hemisphere of 6-OHDA lesions was expected to have significantly impaired motor performance compared with the ipsilateral forepaw, whereas, no significant differences in step and cylinder test performance were expected for sham lesioned animals (Parr-Brownlie et al., 2007, 2009; Walters et al., 2007; Avila et al., 2010; Bosch-Bouju et al., 2014). From this point on, the contralateral forepaw will be referred to as the affected paw while the ipsilateral forepaw will be called the unaffected paw. Rats were considered to have had a successful lesion if the number of adjusting steps made with the affected forepaw was ≤10% of total steps and the forelimb was used for <10% of all touches made to the cylinder wall. If the behaviour of a lesion animal did not meet the criteria, the rat was considered unlesioned and was removed from the study. Previous studies imply that impaired motor performance on these tests is associated with a 95% reduction in dopaminergic neurons in the SNc of the lesioned hemisphere (Olsson et al., 1995; Fleming et al., 2005; Tseng et al., 2005; Avila et al., 2010).

**Step testing** Rats forelimb stepping performance is measured using the step testing (Olsson et al., 1995). The rats were trained for 3 days before lesion surgery, to acclimatise the animal and this researcher to the restraint and movement involved in this task (Figure 2.2 A). A senior member of the lab helped with the test on post-operative day 7 to ensure accurate analysis of the extent of PD lesion. The hind limbs and the forepaw not being assessed were restrained and the rat was moved slowly sideways along a one-metre bench surface in the lab. The number of adjusting steps for movements to the left and right were counted for the both unaffected and affected forepaw individually. With each trial the unaffected forepaw was tested first followed by the affected forepaw. The test was repeated 3 times in the same sequence for both forepaws and the result averaged. In general, 6-OHDA lesioned animals were expected to make a minimal number of adjusting steps with the affected forepaw compared to the unaffected forepaw and sham lesioned animals.

**Cylinder testing** Asymmetric forelimb use was examined using the cylinder test (Schallert et al., 2000). Rats were individually placed in a Plexiglas® cylinder (20 cm diameter x 30 cm height) for 5 minutes and scored for weight bearing limb touches with the wall of the cylinder (see Figure 2.2 B). The cylinder was high enough so that the animals could not touch the top edge when rearing occurred. Each session was video recorded from underneath the apparatus and scoring was assessed offline. The number of times the animal
placed their left forepaw, right forepaw or both forepaws together to the wall to support their weight was scored as a touch. Generally, 6-OHDA lesioned animals rarely use the affected forepaw for weight-bearing touches, instead placing the unaffected paw or both paws against the wall.

2.2.3 Tyrosine hydroxylase staining

To assess the extent of dopaminergic neuron loss in the SNc and the loss of dopaminergic terminals in the striatum, staining for TH was employed. TH is an enzyme involved in the synthesis of catecholamines. In particular, the enzyme catalyses the hydroxylation of L-tyrosine to the dopamine precursor L-DOPA (Molinoff and Axelrod, 1971). TH can, therefore, be used as a biomarker of catecholamine neurons throughout the brain and more specifically dopaminergic neurons in the SNc and dopaminergic terminals in the striatum. To label TH in the SNc and striatum, rats were perfused and 40 µm sections were obtained at the end of the experiments as described below in Section 2.3.1 and detection of TH was carried out with fluorescent immunohistochemistry. For a more detailed explanation of the immunohistochemistry procedure and the purpose of each step, see Section 2.3.2 and 2.3.4. Briefly, sections were first washed in a phosphate buffered saline (PBS) for 3 x 10 min, followed by 2 x 20 min permeabilization in PBS with triton added (PBS-T) and a 1 hour block in a 10% normal goat serum (NGS) solution diluted with PBS-T. Sections were then incubated in the primary antibody rabbit anti-TH (see Table 1 for dilution and supplier) diluted with a PBS-T solution containing 5% NGS at 4°C overnight. The following morning sections were washed for 3 x 10 min in PBS-T and incubated in the secondary antibody anti-rabbit dyelight 488 (see Table 1) for 4 hours at room temperature. Finally sections were washed in PBS for 2 x 10 min and 0.1 M phosphate buffer (PB) for 10 min before being

**Figure 2.2. Example images of the behavioural tests conducted during this study.** (A) An example of the restraint used to examine the performance of the unaffected paw of a lesioned animal during step testing. Image obtained from Olsson et al. (1995). (B) An animal conducting a weight bearing touch on the cylinder wall with the unaffected (left) paw of a lesioned rat.
mounted onto glass slides in 0.1 M PB. Tissue was then left to dry to slides for 40 min to ensure they adhered to the slide and then coverslipped with Vectashield mounting medium with the fluorescent nuclei stain 4’,6-diamidino-2-phenylindole (DAPI) incorporated (Vector, USA).

2.3 Characterising cell phenotype and tracing

To characterize the phenotype of neurons in the rRTN, the antigens CaMKIIα and GAD67 that are present in glutamatergic and GABAergic neurons, respectively (Benson et al., 1991; Kaufman et al., 1991) were immunohistochemically labelled. Immunohistochemistry utilizes antibodies that bind to antigens of interest in the rRTN and allows visualizing the location of these antigens with labels that can be detected with a microscope. Presently, the peroxidase based chromogenic immunohistochemistry and fluorescent immunohistochemistry were used to label RTN neurons. The use of chromogen staining enabled stereological investigation into the numbers of neurons in rRTN, and provided a cheap and long lasting method to visualize the antigens. In some rats, a lentiviral vector, which was driven by a CaMKIIα promoter and contained a mCherry fluorophore reporter, was injected into the rRTN to selectively label glutamatergic neurons at the injection site. If the injection was contained within the nucleus, the lentiviral vector enabled the tracing of rRTN neurons to their synaptic targets. For these rats, fluorescent immunohistochemistry provided confirmation of the location of CaMKIIα and GAD67 antigens at a much higher resolution as well as revealing the location of the mCherry fluorophore following lentiviral vector injections.

2.3.1 Perfusions and processing

Two weeks following 6-OHDA or sham lesion surgery or 6 weeks following lentiviral surgery, rats were deeply anaesthetised with sodium pentobarbitone (100 mg/kg i.p; Provent, NZ). Rats were transcardially perfused with 10% sucrose solution followed by 4% paraformaldehyde (PFA) solution. Following fixation, brains were carefully removed and placed into a 4% PFA solution at 4 °C overnight. Brains were then transferred to a solution of 30% sucrose to cryoprotect the tissue and stored at 4 °C for 2 days. Tissue was sliced into 40 µm thick coronal sections on a freezing microtome and stored for up to 14 days in a 24 well plate containing PBS at 4 °C until used for immunohistochemistry. For longer-term
storage, tissue was transferred to cryoprotectant containing 0.04 M Tris-buffered saline, 30% ethylene glycerol and 30% glycerol, sealed and stored at -20°C.

2.3.2 Chromogen based immunohistochemistry

To ensure that the best possible staining was achieved, a series of different immunohistochemical techniques that were learned through guidance, literature research and experience were trialled. A number of antibodies were also trialled to select the appropriate marker for GABAergic and glutamatergic neurons. For each antibody, a dilution series was completed to determine the ideal antibody concentration. Antibodies against a vesicular glutamate transporter (VGLUT-2; Synaptic Systems, Germany) were trialled however the resulting signal from the stain was inadequate, therefore staining with this antibody was not progressed any further. Antibodies for the markers used, GAD-67 and CaMKIIα that were raised in other species such as the chicken were initially trialled, however, these had more background staining than the mouse antibodies presented in the thesis. Techniques that I added to achieve the best antigen signal with the lowest background noise included peroxidase blocking, bovine serum albumin (BSA) combined with NGS blocking, and antigen retrieval, which are described and explained in the final protocol below. For full information on the antibodies and stains used including dilutions, host species and supplier, see Table 2.1.

**Single chromogen staining** For single chromogen immunohistochemistry sections were subjected to the following protocol. Firstly, tissue was washed for 2 x 5 min in PBS. Washing the tissue in PBS helps to produce the optimal environment for antibodies to bind to its antigens. At each washing step throughout the procedure, excess antibody, loosely bound non-specific antibodies and other debris are removed from the tissue to enable efficient and specific binding of the primary antibody. Due to the presence of endogenous peroxidases within some cells that may interfere with the HRP-conjugated antibody binding near the end of this protocol, which would cause non-specific background staining, tissue was pre-treated in a 0.3% hydrogen peroxide solution (H₂O₂) diluted with PBS for 10 min. Tissue was then washed in PBS for 2 x 5 min. To permeate the cell membrane and ensure access to antigens that exist within the cell, tissue was incubated in PBS-T, which contains the detergent Triton (Sigma-Aldrich, USA) at 2% for 2 x 20 min. Sections were then incubated in a blocking solution containing 10% NGS and 1% BSA diluted with PBS-T. NGS is employed as it is normal serum from the same species as the host of the secondary antibody (goat) and contains antibodies that will bind to non-specific reactive antigens that would increase background staining if they weren’t blocked. This allows the secondary antibody to exclusively bind only to the primary antibody. BSA is a protein buffer that will coat all proteins within the sample,
which means that it will compete with the primary antibody to bind to the specific antigen of interest (Wedege and Svenneby, 1986); therefore, it is used in small concentrations and in combination with NGS. After blocking, tissue is incubated at 4 °C overnight in primary antibody in a diluting buffer (DB) that contained 5% NGS and PBS-T (see Table 2.1 for antibody dilutions). The primary antibody was specific for the antigen that was being labelled. Specifically for GABAergic and glutamatergic neurons, mouse anti-GAD67 and mouse anti-CaMKIIα antibodies were used. The following day, sections were again permeabilized in PBS-T for 2 x 10 min and washed for 3 x 5 min in PBS before incubation in DB containing the secondary antibody (see Table 2.1) for 2 hours at room temperature. The secondary antibody targets and specifically binds to the antibody. The secondary antibody is also conjugated to enzyme horseradish peroxidase that catalyses the reaction between H2O2 and a chromogen subsequently producing the signal that allows the antigen of interest to be visualized (see Figure 2.3). Following the secondary antibody incubation, sections underwent 3 x 5 min washes in PBS followed by the application of the 3’3-diaminobenzidine (DAB) substrate kit (Vector) according to manufacturers instructions. The kit contained the DAB chromogen, a diluting buffer and hydrogen peroxide that were combined and then added to the sections for 2 – 3 min, resulting in a brown reaction product. Finally sections were washed

### Table 2.1 List of antibodies and peroxidase substrates

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Host</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single chromogen staining</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-GAD67</td>
<td>1/10000</td>
<td>Mouse</td>
<td>Merck Millipore, USA</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP conjugated Anti-mouse</td>
<td>1/800</td>
<td>Goat</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td><strong>Double chromogen staining</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CaMKIIα</td>
<td>1/500</td>
<td>Mouse</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Anti-GAD67</td>
<td>1/10000</td>
<td>Mouse</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP conjugated Anti-mouse</td>
<td>1/500</td>
<td>Goat</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Fluorescent staining</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CaMKIIα</td>
<td>1/300</td>
<td>Mouse</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Anti-GAD67</td>
<td>1/1000</td>
<td>Mouse</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Anti-RFP (5F8)</td>
<td>1/1000</td>
<td>Rat</td>
<td>Chromotek, Germany</td>
</tr>
<tr>
<td>Anti-TH</td>
<td>1/300</td>
<td>Rabbit</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse Alexafluor 488</td>
<td>1/500</td>
<td>Goat</td>
<td>Life-Technologies, USA</td>
</tr>
<tr>
<td>Anti-rabbit Dyelight 488</td>
<td>1/1000</td>
<td>Goat</td>
<td>Vector, USA</td>
</tr>
<tr>
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<td>Goat</td>
<td>Invitrogen</td>
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<tr>
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</tr>
<tr>
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<td>Vector</td>
</tr>
<tr>
<td>Vector SG substrate kit</td>
<td>Blue-grey</td>
<td>No</td>
<td>Vector</td>
</tr>
</tbody>
</table>
in PBS for 2 x 5 min and 0.1 M PB for 10 min before being mounted onto glass slides and left to dry for 48 hours.

**Heat-induced antigen retrieval** The use of aldehyde fixatives during the perfusion process (see *Section 2.3.2*) may result in cross-linking of protein amino acid residues by methylene bridges. These protein cross-links can block the antigen sites and reduce the sensitivity of antibody binding and, therefore, reduce the staining (D’Amico et al., 2009). Applying heat to these sections removes these protein cross-links and allows access antigen binding sites (D’Amico et al., 2009). When required, sections underwent heat-induced antigen retrieval (HIAR). The particular CaMKIIα antibody clone used in the present experiment is known to be susceptible to blockage by protein cross-links and sections underwent HIAR only when this antibody was used. The general protocol for HIAR was as follows. Sections were washed for 2 x 5 min in PBS and then placed in 10 mM sodium citrate buffer (pH 6.0). Sections in citrate buffer were then placed in a steamer (George Foreman, ‘Food Steamer’, model: GF3TSBLK) for 13 min, then set aside for 2 min and transferred to PBS to carry on with the immunohistochemical protocol.

**Dehydrate and clear** After tissue had been left to dry so that they were sufficiently adhered onto glass slides, they were put through a general dehydration and clearing protocol. Clearing the tissue allows better visualization of the staining and using the mounting medium before applying a coverslip increases the longevity of staining. However, the clearing agent and the mountant used were not miscible with water, therefore, dehydration is required to remove water from the tissue. The chromogens used in this study, DAB and Vector SG, were both alcohol insoluble, therefore ethanol (EtOH) was used in increasing dilutions to dehydrate tissue. To dehydrate tissue slides were put in distilled H₂O for 1 min, 50% EtOH for 1 min, 70% EtOH for 5 min, 2 x 95% EtOH for 2 min, and 100% EtOH for 1 min and to clear the tissue, slides were put in xylene for 8 min. Finally a coverslip was applied with the mounting medium, DPX (Sigma-Aldrich).

**Double chromogen staining** The primary antibodies used for double immunohistochemistry were both raised in the mouse species and staining was completed over 4 consecutive days with each primary antibody added and visualized sequentially. The order that the antibodies were visualised was important for achieving the best signal to noise ratio. It was recommended by the manufacturer that tissue undergo HIAR before the CaMKIIα antibody was applied. After a series of trials it was determined that visualization of the CaMKIIα antibody was best with the chromogen vector SG (Vector). The HIAR step was best placed between the rounds of sequential staining (Lan et al., 1995), so CaMKIIα staining was completed during the second sequence. The chromogen DAB is heat-resistant so was not
affected by the HIAR step and used first to visualise the antibody GAD67. DAB is a robust chromogenic dye and may shield other antigens of interest that are in close proximity (van der Loos, 2007), therefore, extra washing steps were added to ensure that excess chromogen was removed from the tissue to prevent this DAB sheltering. Furthermore, the HIAR step inactivates peroxidase enzymes, therefore, preventing further DAB colour development.

For double chromogen immunohistochemistry tissue underwent the following general protocol, and see Figure 2.3 for a simple schematic of the double immunohistochemistry procedure. Sections were first washed for 2 x 5 min in PBS followed by a 10 min peroxidase block in a 0.3% H2O2 diluted with PBS. Sections were then washed for 3 x 5 min in PBS, and permeabilized for 2 x 20 min in PBS-T, before going through a 1 hour block in a solution containing 10% NGS, 1% BSA and PBS-T at room temperature. Following blockade, sections were incubated in the first primary antibody, mouse anti-GAD67 diluted in DB (see Table 2.1 for antibody dilutions) overnight at 4 °C. The second day consisted of 2 x 10 min washes in PBS-T and 3 x 5 min washes in PBS before application of the secondary antibody, anti-mouse conjugated with HRP in DB for 4 hours at room temperature. After secondary antibody incubation, sections were washed for 3 x 5 min in PBS and application of the DAB substrate kit (Vector) according to manufacturers instructions, which produced a brown

![Figure 2.3](image)

**Figure 2.3.** A series of schematic diagrams that briefly describe double immunohistochemistry. The first primary antibody that is specific to an antigen binds (1), is tagged by the secondary antibody (2) and revealed with a chromogen (3). The second primary antibody is then added and specifically binds to a different antigen in another cell (4). This antibody is then revealed with a different coloured chromogen (5,6).
colour, before 4 x 5 min washes in PBS. The next day tissue underwent HIAR carried out as stated above, followed by 2 x 5 min washes in PBS, a 1 hour block in 10% NGS and incubation in the second primary antibody, mouse anti-CaMKIIα diluted with DB (Table 2.1), overnight at 4 °C. The following day sections were washed in PBS for 3 x 5 min and the secondary antibody, anti-mouse conjugated with HRP in DB was applied for 4 hours at room temperature. Sections were washed again in PBS for 3 x 5 min and put through a chromogen reaction with the Vector SG substrate kit (Vector), yielding a blue colour. Finally sections were washed in PBS for 2 x 5 min, 0.1 M PB for 1 x 10 min and mounted onto glass slides. The procedure outlined above to dehydrate and clear tissue was then carried out.

**Controls** The use of proper immunohistochemical controls was important for this study to ensure that staining was correctly interpreted and not falsely labelling other antigens, and to make sure that the staining was reproducible. To ensure that the primary antibodies were staining the antigens CaMKIIα and GAD67, tissue that had areas of the brain that have been previously shown to include these antigens in abundance were included in the staining procedures. The cortex and the hippocampus are known to express CaMKIIα (Colbran, 1992; Liu and Murray, 2012; Wang et al., 2013). Specifically in layers II, III, V and VI of the cortex and the pyramidal cell layer of region CA1 of the hippocampus (Colbran, 1992; Liu and Murray, 2012; Wang et al., 2013). Although the RTN itself is abundant in GAD67 staining, it could not be used as a control because it was the region of interest in this study; therefore the granule cell layer of the dentate gyrus was used. GAD67 and CaMKIIα are both present in the granule cell layer of the CA1 region of the hippocampus, however, do not colocalize in cells and form two distinct populations (Liu and Murray, 2012; Xiong et al., 2012; Wang et al., 2013). Therefore, sections were taken through the cortex and CA1 region of the hippocampus to use as positive controls for CaMKIIα and GAD67 antibody staining, respectively. These positive control sections underwent the same protocol for single chromogen immunohistochemistry. DAB is known to have a sheltering effect on the signal of the second primary antibody in dual-labelling studies (van der Loos, 2007), therefore, the CA1 region of the hippocampus also acted as a control to ensure that complete chromogen separation had occurred. To test the non-specific binding of the secondary antibodies, sections taken from the rRTN were put through the staining procedures, however, the primary antibody was omitted from the dilution buffer. For double chromogen immunohistochemistry, 3 negative controls for non-specific secondary antibody staining were tested. Both primary antibodies were omitted in the same section, and then each primary antibody was omitted while the other remained in the protocol. In each case, the protocol remained the same except primary antibody was replaced with DB.
### 2.3.3 Lentivirus surgeries

A lentiviral vector was injected into the rRTN of 15 animals by Roseanna Smither to selectively label glutamatergic neurons (Figure 2.4). The coordinates for the rRTN were based on previous reports in the literature (Mihaly et al., 1998; Delfs et al., 1996), and from the rat atlas (Paxinos and Watson, 2009). The lentiviral construct, pLenti.CaMKIIα.mCherry was packaged into a third-generation lentiviral vector by the Brain Research NZ (BRNZ) Viral Vector and Optogenetics platform. The vector was pseudotyped with VSVg glycoprotein (Linterman et al., 2011), to ensure that the viral vector was anterogradely transported throughout the whole neuron and that synaptic terminals at the targets of these neurons could be traced with the expression of the mCherry reporter fluorophore. Approval for the use of recombinant lentiviral vectors was obtained from the Environmental Protection Authority (EPA) New Zealand (GMD003091). The CaMKIIα promoter sequence ensures glutamatergic neurons selectively express the viral vector (Parr-Brownlie et al., 2015), and the mCherry reporter allowed the post-mortem analysis of the accuracy of viral vector transduction.

The lentiviral injection procedure closely resembled the unilateral lesion protocol outlined above with the following exceptions. Six rats received three injections of 100 nL (300 nL total volume) of lentiviral vector into rRTN. However, due to widespread transduction of the viral vector out of the rRTN, this was reduced to two injections of 100 nL (200 nL total volume) in the last 10 animals. Injections were made using a Hamilton microsyringe (World Precision Instruments Inc., USA) centred on the stereotaxic coordinates corresponding to the rRTN relative to bregma (-1.4 – 1.45 AP, +2.0 ML, -5.9 DV). The lentiviral construct was infused at a rate of 100 nL/min and the syringe remained in place for 10 min to prevent diffusion of the lentivirus along the injection track as the syringe was withdrawn. Animals were housed in isolation cages for at least 72 hours during the period they were considered infectious, before being re-housed together in conventional cages for a further 6 weeks.

![Figure 2.4. Schematic of the lentiviral construct and the target injection site in rRTN.](image)

*LTR pCaMKIIα mCherry LTR*

* = rRTN
2.3.4 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry works in much the same way as chromogen immunohistochemistry, with primary antibodies binding to the antigens of interest and secondary antibodies binding to the primary. The steps that ensure the best possible visualization of the signal are similar to that of chromogen immunohistochemistry and have been explained in detail in Section 2.3.2. Rather than using peroxidase enzymes and a substrate reaction to visualize the product, the antigens GAD67 and CaMKIIα were labelled with secondary antibodies that were conjugated to fluorophores (see Table 2.1). The fluorescent stain DAPI, which binds to DNA rich regions (Kapuscinski, 1995), was used to visualise the nuclei of neurons and was incorporated in the medium used to mount sections to the slides (Fluoroshield; Abcam, UK or Vectashield; Vector). These fluorophores emit a detectable signal when activated by a specific wavelength of light. The fluorescent signal in this study was, green, red or blue. Immunofluorescence enables the use of confocal microscopy rather than the light microscopy permissible with chromogenic immunohistochemistry. Confocal microscopy can generate an image of much higher optical resolution than that of light microscopy by eliminating out-of-focus light, which causes some background noise in images from light microscopy.

**Single fluorescent staining** To visualize neurons in rRTN that contain GAD67 or CaMKIIα, 40 µm sections were put through the following protocol for fluorescent immunohistochemistry. Solutions used in the present protocol were the same as those listed in Section 2.3.2 unless otherwise stated. All steps in this protocol were completed with the lights off and the blinds closed to ensure fluorescent signal was not bleached by external light. First, sections were washed in PBS for 3 x 10 min followed by permeabilization in PBS-T for 2 x 20 min. Sections were incubated in a 10% NGS blocking solution that was diluted with PBS-T for 1 hour, and then sections were transferred to wells containing primary antibody diluted in DB (see Table 2.1) overnight at 4 °C. The primary antibodies that were used were either mouse anti-GAD67 or mouse anti-ααα Following primary antibody incubation overnight, sections were washed for 2 x 20 min in PBS-T and incubated for 4 hours in secondary antibody diluted in DB (Table 2.1), at room temperature. The secondary antibodies that were used were either goat anti-mouse Alexa Fluor 488 or goat anti-mouse Alexa Fluor 594. Sections were then washed in PBS for 2 x 10 min and finally 0.1 M PB for 10 min before being mounted onto glass slides in 0.1 M PB. After allowing sections to adhere to the slide for 30-40 min a coverslip was applied with Fluoroshield mounting medium with DAPI incorporated (Abcam).
**Double fluorescent staining** To visualize the location of GAD67 positive neurons and virally transduced neurons expressing the mCherry fluorophore in the same section, tissue around the lentivirus injection site underwent double fluorescent immunohistochemistry. This protocol was also used to confirm GAD67 and CaMKIIα were not co-localizing in neurons and that two distinct populations of neurons existed in the rRTN. The protocol used follows the exact steps as those for single fluorescent staining listed above except for the following exceptions. Primary antibodies against GAD67 (mouse anti-GAD67) and the mCherry fluorophore (rat anti-RFP 5F8) were added simultaneously each diluted in DB (see Table 2.1). The secondary antibodies Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rat were diluted in DB and added simultaneously. At the end of the protocol, sections were mounted onto glass slides in 0.1 M PB, left to adhere for 40 min and a coverslip was applied with Vectashield mounting medium with DAPI incorporated (Vector).

### 2.4 Imaging tissue

All tissue was imaged with microscopes provided by the Otago Centre for Confocal Microscopy. Brightfield light microscopy and confocal fluorescent microscopy were both used during this study. Brightfield microscopy was employed to define the rRTN boundary, count neurons in the rRTN and image chromogen stained tissue, whereas confocal microscopy was used to investigate the cell phenotype of neurons within the rRTN as well as viral vector injections. Confocal microscopy offers a number of advantages over brightfield including the removal of out of focus objects that may obstruct the in focus fluorescent signal and the confocal microscope produces higher resolution, clearer images. Some disadvantages that were considered in the overall project design were that confocal microscope is expensive and the processing of high-resolution images with the available software is especially time-consuming.

#### 2.4.1 Defining the boundary of rostral reticular thalamic nucleus

As outlined in Section 1.1.4, the RTN is functionally split into sectors along its rostral to caudal axis, however, these sectors have no definitive boundary line. Therefore, we felt it prudent to define the rostral portion of the RTN, which would be involved in motor processing, before conducting neuronal cell counts. Previous literature studying rRTN suggest that the motor portion lies just before the rostral pole of the nucleus, and is adjacent to structures such as the ventrolateral part of the anteroventral thalamic nucleus (AVVL) at the
rostral part of rRTN and VA/VL thalamic nucleus at the more caudal part of rRTN (Cicirata et al., 1990; Delfs et al., 1996; Picazo et al., 2009; Villalobos et al., 2016). For the current study, we defined the motor portion of the RTN as -1.2 mm to -1.6 mm relative to bregma with regards to the Paxinos and Watson (2009) atlas (Figure 2.5).

![Figure 2.5. Sections of the rRTN revealed with DAB staining of the GAD67 antibody.](image)

Each image represents a 40 µm section and approximately 8 sections defined the rostral portion. Rt = rRTN. Scale bar = 100 µm.

2.4.2 Confocal microscopy

Sections of rRTN were examined on a microscope to determine the location of lentiviral transduction as well as to characterize the phenotype of neurons in the rRTN as described earlier. A confocal laser-scanning microscope (Nikon A1R MP, Japan), was used to assess lentiviral transduction and fluorescent immunohistochemistry using 4x (0.1 numerical aperture (NA)) Plan, and 40x (0.75 NA) Plan Fluor objective lenses. Lasers had excitation lines of 405 nm, 488 nm, and 561 nm to excite blue, green and red fluorophores respectively. Pictures were taken with Nikon Elements C (Version 4.13.01; Nikon, Japan), software running on a computer running Windows 7 (Microsoft, USA), that was connected to the microscope. ND2 files that held the imaging information from the NIS Elements C software were imported into FIJI, which is a distribution of the software ImageJ (Schindelin et al., 2012; Schneider et al., 2012), and saved as a tagged image file (TIF) for greater compatibility.
2.4.3 Brightfield light microscopy

Following chromogenic labelling of the rRTN, sections were viewed with a light microscope (Olympus BX61, Japan), using 4x (0.16 NA) UPlanSApo, 10x (0.4 NA) UPlanSApo and 40x (0.95 NA) UPlanSApo objective lenses. The microscope was attached to a computer running Mac OS X (Version 10.6.8; Apple, USA) with Volocity software (Version 5.2.0; Improvision, USA), installed to control a motor driven stage. Images were taken with a QImaging micropublisher (Diagnostic Instruments, Canada) camera that allowed precise image montaging of the rRTN. Montaged images were stitched together using AutoPano Pro software (Version 2.5.2; Kolor, France).

To estimate the volume of the rRTN and the number of GABAergic and glutamatergic neurons, Stereo Investigator software was used (Version 10.21.1; MBF bioscience, USA). The Windows 7 computer (Microsoft) was also connected to a light microscope (Olympus BX51) and the microscope stage was motor driven. The rRTN was delineated using a 4x (0.13 NA) UPlanFL N objective lens, while neurons were counted with a 40x (0.75 NA) UPlanFL N objective lens.

2.5 Stereology

All tissue that was used for stereological cell counts was processed as a group together to ensure that conditions for tissue processing were the same.

2.5.1 Cavalieri estimator

To get an unbiased estimate of volume of the rRTN the Cavalieri estimator probe was used. The Cavalieri estimator probe applied systematic random sampling to every 2nd section throughout the RTN to get the volume of the rRTN in which the GABAergic and glutamatergic neurons were counted. To apply the Cavalieri estimator, a point grid was overlaid onto the rRTN with each point spaced equally across and down at 50 µm. The investigator then counts how many points were selected within the rRTN and an unbiased estimate of the volume of the region was calculated (Figure 2.6).
Due to the complex shape of the RTN, the Optical Fractionator probe was employed to estimate the total number of GABAergic and glutamatergic neurons in the rRTN. The Optical Fractionator probe employs the Optical Disector with the fractionator method to count cells in a 3-dimensional space. The disector method allows the estimation of the number of objects within a region without making assumptions based on size, shape or orientation. The fractionator allows the sampling of objects in a number of thick sections from a known fraction of tissue that allows systematic random sampling of objects in the X, Y and Z-axis (Keuker et al., 2001).

By counting a subset of the total number of neurons in every 2nd section of the rRTN it is possible to estimate the total population within the rRTN that can then be assessed statistically. Firstly the boundary of the rRTN was delineated using the contour drawing tool based on the RTN figure in the Paxinos and Watson (2009) atlas. The RTN is easily distinguishable due to the high GABAergic immunoreactivity of the region. Once the rRTN had been outlined the Stereo Investigator software applied a random sampling grid, of 10,000 \( \mu \text{m}^2 \), across the region and a counting frame was placed onto the section. The counting frame penetrated the volume of the tissue and the user focussed through the section and counted cells throughout the volume of the region. This counting frame allowed unbiased counting of cells in three dimensions using a set of rules that remained consistent across all sections of the animals that were investigated. The mounted thickness was measured for each section as well as at every 10\(^{th}\) sampling point within the tissue.

Every 2\(^{nd}\) section of the rRTN was analysed using the Optical Fractionator probe. Once the rRTN had been delineated, random systematic sampling was used to apply sampling
points across the rRTN, up to 200 \( \mu m \) apart in the X and Y-axis directions so that a total of 30 sampling points were applied in each section. The Optical Disector penetrated 12 \( \mu m \) into the z-axis of each section excluding 4 \( \mu m \) at the top and bottom to account for tissue damage due to sectioning and processing. At each sampling point a 10,000 \( \mu m^2 \) counting frame was applied to the tissue within which GABAergic and glutamatergic neurons were counted. Due to some neurons crossing the counting frame, unbiased counting rules were applied to avoid the ‘edge-effect’ (Gunderson et al., 1987), which can cause the overestimation of neurons within the region. This required inclusion and exclusion borders of each of the edges of the counting frame (see Figure 2.7). Neurons intersecting the top and right borders of the counting frame were included in the count and those intersecting the bottom and left borders of the counting frame were excluded (Gunderson et al., 1987). This ensures that a neuron would only be counted once even when the counting frames were a minimal distance apart. To count each cell within the counting frame, different markers coded GABAergic and glutamatergic neurons. Each marker was placed at the top of a neuron if the neuron came into focus in the 12 \( \mu m \) distance of the z-axis and fell within the counting frame, or intersected the inclusion borders of the counting frame. They would not be marked if they were out of focus within the z-axis, or intersected the exclusion borders of the counting frame (Figure 2.7).

The total number of GABAergic and glutamatergic neurons was estimated in 4 sections per animal using the Stereo Investigator software. This software automatically calculates the total number using the following formula:

\[
N = \Sigma Q^- x t/h x 1/asf x 1/ssf
\]

Where ‘N’ = the estimated total population, ‘Q^-’ = neurons counted, \( t \) = section mounted thickness, \( h \) = counting frame height, \( asf \) = area sampling fraction, \( ssf \) = section sampling fraction. The area sampling fraction is defined as the distance between counting frames in the x and y direction squared, divided by the counting frame area and the section sampling fraction is the section increment (i.e. 1 of every 2 sections or 1/2).
2.6 Morphometric analysis

The area of GAD67 staining around soma was determined using the measure and label function of ImageJ (Schindelin et al., 2012; Schneider et al., 2012). Firstly, sections were selected from each animal that contained the same volume of rRTN, which had been measured previously. Each section was photographed with an Olympus BX51 microscope, using a 20x (0.5 NA) UPlanFl N objective lens and subsequently stitched together in Adobe Photoshop CC (Version 2014.0.0, Adobe Systems incorporated, USA). Although not directly stained for, the nucleus of each neuron was easily identified as the relatively unstained area within GAD67 immuno-positive neurons (Figure 2.8 A). Somal area measurements were made on 20 clear neurons randomly selected per rat resulting in 80 and 100 values for sham and 6-OHDA lesioned groups. As illustrated by Figure 2.8 B, all neurons that had sufficient immunoreactivity around their soma were outlined with the ellipse tool in ImageJ (Derecki et al., 2012; Schneider et al., 2012; Amorin and Calisi, 2015). A small proportion of somata could not be clearly outlined due to out-of-focus blur, background staining or clusters of neurons, therefore, these were not included in analyses. The area of the outlined neuron was then measured with ImageJ (Schneider et al., 2012), and exported to an excel spreadsheet. To ensure a neuron was not outlined twice, a small mark was added to each a neuron following measurement.
2.7 Data analysis

All data were analyzed using the software package Prism (Version 7.0a, GraphPad Inc, USA). A Shapiro-Wilk normality test was used to test whether the sampled data were normally distributed. If the normality test was passed (p ≥ 0.05), it confirmed that data were normally distributed and a parametric test was used to determine statistical significance. However, p ≤ 0.05 indicated that the data were not normally distributed and non-parametric tests were used to compare differences between groups. The following variables were compared between sham and lesion treatment groups using unpaired, two-tailed students t-tests: the number of affected forelimb adjusting steps made by a rat, number of touches made against a cylinder wall with the affected forelimb, the total number of GABAergic neurons in the rRTN, the total number glutamatergic neurons in the rRTN and the total measured volume of rRTN. A Mann-Whitney test was computed to compare differences in the mean area of the soma of neurons in the rRTN between sham and 6-OHDA lesioned groups. To assess the relationship between the number of GABAergic neurons and behavioural performance, the Pearson Product Moment Correlation coefficient was calculated in Prism. Data are presented as the mean ± the standard deviation (SD). The value of probability that defined a significant difference between groups was set at p ≤ 0.05. Throughout the results section, the p value presented was computed with an unpaired, two-tailed students t-test unless otherwise stated.

Figure 2.8. A representative figure showing GAD67 immunoreactivity alone and the same image with outlines of the soma of neurons that were measured. Scale bar = 100 µm (A) GAD67-positive neurons have a clear soma, where GAD67 staining (brown) is around the nucleus (clear). (B) The soma of neurons that were measured was outlined with a yellow ellipsoid. Examples that would not be counted due to out-of-focus blur, background staining or clustered neurons are illustrated by the red arrows.
3.0 Results
The present study aimed to define and characterise the phenotype of a novel population of neurons within the rRTN using general immunohistochemistry, stereology, morphometric analysis and lentiviral tracing technology. The effect of loss of midbrain dopamine neurons on neuronal numbers within the rRTN was also examined. To achieve this, 28 rats were subject to either 6-OHDA or sham lesions to the MFB, which was followed with lentiviral injections into the rRTN in 15 rats. It was deduced that a small novel population of neurons that were positive for CaMKIIα immunostaining existed within the rRTN and were presumed to be glutamatergic in phenotype. Finally, it is proposed that a dopaminergic lesion causes a significant increase in the estimated number, and decrease in the size of GABAergic neurons in the rRTN, however does not change the volume or population of glutamatergic neurons.

3.1 Model of Parkinson’s disease

3.1.1 Behavioural tests

**Behavioural assessment** The extent of 6-OHDA lesions was tested behaviourally using the step test to assess the stepping ability of the affected forepaw relative to the unaffected forepaw and the cylinder test that examines forepaw preference for weight bearing touches against a cylinder wall (Figure 3.1 A and B).

Normality tests confirmed that step testing data for sham and 6-OHDA lesioned animals was normally distributed (Shapiro-Wilk test; \( p = 0.31 \) and \( p = 0.71 \), respectively). In sham lesioned animals, the adjusting steps made by the affected forepaw and the unaffected forepaws were approximately equal (Figure 3.1 A) and no significant difference was seen between the two. In addition, sham lesioned rats used the affected forepaw to bear weight against the cylinder wall for approximately a quarter of weight bearing touches (Figure 3.1 B), which is the same for weight bearing touches with the unaffected forelimb (23 ± 8%; data not shown) and they used both limbs simultaneously for approximately 50% of weight bearing touches (58 ± 17%; data not shown).

In comparison, 6-OHDA lesion animals displayed a reduced ability to take an adjusting step with the affected forepaw during the step test. With a successful 6-OHDA lesion, the adjusting steps taken with the affected forepaw were severely reduced compared to the adjusting steps taken with the unaffected forepaw. This led to a significant decrease in the adjusting steps taken with the affected forepaw compared against the affected forepaw of sham lesioned animals (Figure 3.1 A, \( p < 0.0001 \)).
Normality tests confirmed that cylinder test data was sampled from a normal distribution for both sham and 6-OHDA lesioned animals (Shapiro-Wilk test; \(p = 0.32\) and \(p = 0.06\), respectively). Animals with 6-OHDA lesions showed a strong paw preference to the unaffected forepaw for weight bearing touches to the cylinder wall. Successfully lesioned animals barely used the affected forepaw to bear weight, preferring to use either the unaffected forepaw (40 ± 20%) or both paws simultaneously (59 ± 15%; data not shown). As shown in Figure 3.1 B, the 6-OHDA lesioned animals showed a definitive paw preference to avoid weight bearing with the affected forepaw compared against the same forepaw of sham lesioned animals (\(p < 0.0001\)).

3.1.2 Tyrosine hydroxylase staining

The effect of 6-OHDA lesions on dopaminergic neurons in rats was qualitatively assessed with TH immunohistochemical staining. Coronal sections, 40 µm thick, were obtained through the SNc and the striatum of sham and 6-OHDA lesioned animals and stained with an antibody against TH. Tissue that went through TH immunohistochemistry was from animals that had met the criteria for the behavioural assessments and indicated a successful lesion.

As shown in Figure 3.1 C, there is a distinct lack of TH immunostaining in the left SNc of 6-OHDA lesioned animals compared to the unaffected right SNc. This difference is not seen in sham lesioned animals indicating that dopamine neurons were lost in the SNc following 6-OHDA lesions. In the striatum, it is clear that the terminals of dopaminergic neurons have diminished in the left hemisphere of 6-OHDA lesioned animals compared with the right hemisphere, and sham lesioned animals exhibit no difference in staining.

3.1.3 Summary of 6-OHDA induced dopaminergic lesions

Overall, 5 rats were excluded from the experiment due to unsuccessful 6-OHDA lesions, as they did not meet the behavioural criteria stated in methods Section 2.2. Those rats that did meet the criteria showed a reduced motor performance with the affected forepaw in the step test and a paw preference asymmetry that favoured the unaffected forepaw or both forepaws for weight bearing touches in the cylinder test when compared with sham lesioned animals. These results were confirmed with qualitative analysis of dopaminergic immunohistochemistry in the SNc and the striatum. There was reduced staining of the dopaminergic neurons in the ipsilateral SNc and the dopaminergic terminals of ipsilateral striatum in 6-OHDA lesioned animals that also had reduced motor performance.
Figure 3.1. Behavioural and immunohistochemical assessment of 6-OHDA lesions in rats. (A) The adjusting steps made by the affected forelimb as a percentage of total adjusting steps in sham and lesioned rats. (B) Affected forelimb weight bearing touches against the cylinder wall expressed as a percentage of total touches in sham and lesioned rats. (C) Coronal sections of the SNc (left) and striatum (right) from example rats show cell immunoreactivity for tyrosine hydroxylase (TH) (green). TH immunostaining is similar between the injected (left) and uninjected (right) hemispheres in sham rats. In contrast, the lack of TH immunostaining in the left hemisphere of the SNc and striatum in rats who underwent 6-OHDA lesions illustrates that dopamine cells were lost in the ipsilateral hemisphere. Error bars represent SD. Scale bar represents 500 µm. Unpaired t-tests, *** = p < 0.001.
3.2 Characterizing neuronal phenotype and lentiviral vector labelling

3.2.1 GABAergic population in the rostral reticular thalamic nucleus

The rRTN has primarily been characterized as a GABAergic nucleus. To confirm that the rRTN studied in the present experiment did indeed contain GABAergic neurons, tissue underwent immunohistochemistry for the GAD67 antibody and was visualized with a green fluorescent secondary antibody (Figure 3.2 A) or the chromogen DAB (Figure 3.2 B). GABAergic neurons were stained throughout the rRTN in the characteristic reticulated arrangement. GAD67 staining occurred mostly around cell coma and revealed both medium and large sized fusiform shaped neurons (Figure 3.2 A and B).

Figure 3.2. GABAergic immunohistochemistry in a representative section of rRTN. (A) Neurons in the rRTN positively labelled for GAD67 (green) and nuclei stained with DAPI (blue) showed examples of medium fusiform (a) or large fusiform (b) neuron shapes. Image obtained using a 40x objective lens, scale bars = 100 µm. Inset imaged with a 10x objective lens shows the rRTN labelled with GAD67 neurons. (B) Neurons positive for GAD67 revealed with DAB in the rRTN. DAB staining was found around the perikarya and primary dendrites of neurons. Image obtained with a 10x objective lens, scale bar = 50 µm. (C) Schematic diagram representing the approximate location of rRTN in the presented images (Paxinos and Watson, 2009). Rt = rRTN, ic = internal capsule, AVVL = anteroventral thalamic nucleus, RtSt = Reticulostral nucleus, EGP = External globus pallidus.
3.2.2 Glutamatergic population in the rostral reticular thalamic nucleus

Previous electrophysiology research in this lab indicated that there was a novel subpopulation of neurons in rRTN, which had broad action potentials indicative of glutamatergic activity. To investigate this further, sections of rRTN were immunohistochemically stained using the antibody for CaMKIIα and detected with either a red fluorescent secondary antibody or the Vector SG (blue) chromogen. CaMKIIα staining in rRTN confirmed the presence of a small population of glutamatergic neurons. Glutamatergic neurons were sparse in the rRTN and had small round perikarya (Figure 3.3 A and B). Most often, neurons positively stained for CaMKIIα were found in the medial aspect of rRTN (Figure 3.3 A).

![Image of neurons in rRTN showing positive staining for CaMKIIα.](A) A neuron in the rRTN that positively stained for CaMKIIα (red, arrowhead) and nuclei stained with DAPI (blue) has small round soma and suggests a glutamatergic phenotype exists within rRTN. Image obtained with a 40x objective lens. Inset obtained with a 10x objective lens shows the rRTN is relatively absent of staining, however, the white arrow indicates the same arrowhead as in (A), located in the medial aspect of the rRTN. Scale bars = 100 µm. (B) Single chromogenic staining reveals a neuron positive for the CaMKIIα antibody (black arrowhead) in the rRTN. Image obtained with a 40x objective lens. Scale bar = 50 µm. (C) Schematic diagram representing the approximate location for rRTN of the presented images (Paxinos and Watson, 2009). Rt = rRTN, ic = internal capsule, AVVL = anteroventral thalamic nucleus, AM = anteromedial thalamic nucleus, RtSt = Reticulostrial nucleus, EGP = External globus pallidus.

Figure 3.3. Neurons in a representative section of rRTN showing positive staining for CaMKIIα.
3.2.3 Distinct population of neurons in the rostral reticular thalamic nucleus

**Double chromogen staining** To assess the extent of the glutamatergic population within rRTN, neurons were double-stained for GAD67 and CaMKIIα. A number of immunohistochemical trials were undertaken to ensure that the two chromogen-based stains were distinct and the best possible signal to noise ratio was achieved (Figure 3.4). The final result shows separation of the two chromogens, DAB and Vector SG, even at the close proximity of neurons (Figure 3.4 F) and the negative and positive controls that were processed alongside the regular staining procedures are represented in Figure 3.4 A-D. With the lack of background staining, negative controls suggest that the secondary antibody was binding to the primary antibody rather than non-specific binding sites and the chromogen was revealing the signal from the secondary antibody rather than endogenous peroxidase activity (Figure 3.4 A-C). Positive controls confirmed that the GAD67 and CaMKIIα antibodies were binding to the appropriate molecules in regions that are known to express these antigens (Figure 3.4 D and E).

![Figure 3.4](image-url)

**Figure 3.4. Example images illustrating the control tissue for double chromogen immunohistochemistry.** In all images scale bar = 100 μm. (A) The removal of both primary antibodies reveals no background staining and a successful negative control. (B, C) Omission of the GAD67 (B) or CaMKIIα (C) primary antibody from the regular immunohistochemistry procedure reveals no obvious DAB (B) or vector SG (C) background staining. (D) Positive staining in the hippocampus verified the GAD67 primary antibody staining. (E) Positive staining in the cortex verified the CaMKIIα primary antibody staining. (F) An example of the separation of the brown (DAB) and blue (Vector SG) colours of chromogenic staining even with close proximity of neurons in the CA1 layer of the hippocampus.
Following double staining for GAD67 and CaMKIIα, neurons in the rRTN were stereologically investigated using the Optical Fractionator probe in Stereo Investigator. Neurons that were positively stained for either marker were counted to get an estimate for the total population of neurons in rRTN. As shown in Figure 3.5 A, neurons positively marked for CaMKIIα, indicative of the novel glutamatergic population, made up a small percentage of the total population of neurons counted in rRTN. As stated in Section 2.5.2, every 2nd section of rRTN tissue was counted, therefore, the estimated total numbers stated below are ½ of all neurons in the rRTN. On average, sections contained 14 ± 2 neurons positively stained for CaMKIIα, whereas sections contained 561 ± 40 neurons that positively stained for GAD67.

Two examples of individual glutamatergic neurons that were counted are illustrated in Figure 3.5 B1 and B2. No colocalization of both GAD67 and CaMKIIα in individual rRTN neurons was observed. Neurons that were positive for CaMKIIα staining appeared to have discrete overlying positive staining for GAD67 on their soma, indicative of passing dendrites or axons near the soma.

Figure 3.5. Two distinct populations of neurons exist within the rRTN. (A) The population of GABAergic and glutamatergic neurons in the rRTN as a percentage of total number of stereology corrected neurons. (B1, B2) Representative images illustrating two individual glutamatergic neurons (black arrow) amongst GABAergic neurons in the rRTN. Image obtained with 40x objective lens. Scale bar = 50 μm.

**Lentiviral transduction of rRTN neurons** To confirm the presence of a novel and distinct glutamatergic population of neurons, the lentiviral construct; pLenti-CaMKIIα-mCherry, was injected into rRTN. Rats were left for 6 post-surgical weeks to allow for sufficient time for viral vector transduction of rRTN neurons. Rats were injected in small groups and the location of virus transduction was reviewed before injecting the next group. The mCherry fluorophore allowed for quick detection to ascertain the injection success of the lentivirus into rRTN. No injection was fully contained within rRTN (for example
Figure 3.6 C), however the mCherry fluorophore can be seen in transduced neurons (Figure 3.6 A). The presence of the lentivirus confirms that the glutamatergic marker CaMKIIα is present in rRTN and as seen in Figure 3.6 B, and it does not co-localize with GAD67 further confirming that a population of glutamatergic neurons exists within rRTN. Transduced neurons were small with round perikarya, took a multipolar shape and were found in the middle and medial aspects of rRTN (Figure 3.6). These neurons were found in a line that seemed to follow the injection path, and were interspersed with GABAergic neurons.

The lentiviral vector was pseudotyped with VSVg to ensure the vector was taken up at the soma and the fluorophore was transported along the axon of transduced neurons to its synaptic terminals. However, due to the inability to confine lentiviral injections within the rRTN, it was deemed futile to trace transduced glutamatergic thalamic neurons to their synaptic targets because of the possibility that projection neurons from non-rRTN structures had transduced by the virus e.g. VA/VL and AVVL thalamic nuclei.

3.3 The effect of dopaminergic lesions on the rostral reticular thalamic nucleus

To assess the effect of 6-OHDA lesions on the number of neurons in rRTN, the Optical Fractionator probe in Stereo Investigator was used to count the neurons positively stained for the GABAergic marker GAD67 and glutamatergic marker CaMKIIα and compared in sham and 6-OHDA lesioned animals (Figure 3.7). The data obtained by stereological cell counts for GABAergic neuronal number were normally distributed in sham and 6-OHDA lesioned animal (Shapiro-Wilk test; p = 0.82 and p = 0.35, respectively). Glutamatergic cell count data were also normally distributed in sham and 6-OHDA lesioned animals (Shapiro-Wilk test; p = 0.25 and p = 0.23, respectively).

Before counting neurons in the rRTN, each section’s volume was measured using the Cavalieri estimator. To ensure the mounted thickness did not bias the tissue between groups, all processing was completed simultaneously. Due to the changing shape of the rRTN along its rostral to caudal axis, the volume of the rRTN in sham and 6-OHDA lesioned sections was variable (Figure 3.7 A). There was no difference found in the rRTN volume between sham and 6-OHDA lesioned animals suggesting that sections were taken from the similar aspects in both animal groups. Furthermore, although there was a small trend towards an increase in volume of the rRTN in the sections of 6-OHDA lesioned animals, this did not reach statistical significance (p = 0.46).
The results from the parkinsonian lesion experiment suggest that 6-OHDA altered the number of neurons positive for GAD67 (Figure 3.7 B). The mean number of GABAergic neurons counted in the rRTN of 6-OHDA lesioned animals was significantly increased compared to sham lesioned control rats ($p = 0.04$).

Large and medium sized fusiform GABAergic cells appeared throughout the rRTN in sham animals. Neuron soma area measurements were normally distributed for the sham lesioned group (Shapiro-Wilk test; $p = 0.0853$) and 6-OHDA lesioned data were not normally distributed (Shapiro-Wilk test; $p < 0.0001$). As illustrated by Figure 3.7 C, fusiform cells that were positive for GAD67 staining in the rRTN had a smaller mean soma area following

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**Figure 3.6. Lentiviral transduction of neurons in rRTN.** (A) The rRTN showing lentiviral transduced neurons containing CaMKIIα (red), GAD67 (green) and DAPI (blue). Image obtained with a 10x objective. Scale bar = 100 µm. (B) Image of rRTN taken with a 40x objective lens, indicating glutamatergic neurons (red) do not colocalize with GABAergic neurons (green). DAPI staining (blue) indicates cell nuclei. Scale bar = 100 µm. (C) Schematic diagram representing the approximate location (red) of lentiviral transduced neurons in the thalamus. Rt = rRTN, ic = internal capsule, AVVL = anteroventral thalamic nucleus, AM = anteromedial thalamic nucleus, RtSt = reticulostral nucleus.
6-OHDA lesions compared to neurons of sham lesioned animals (Two-tailed, Mann-Whitney test; p < 0.0001).

Following 6-OHDA lesions, the number of neurons positively stained for GAD67 in the rRTN had increased by approximately 20%, while the mean soma area of these GABAergic neurons had decreased by 20%, suggesting that 6-OHDA lesions leads to an increased number of smaller GABAergic neurons in the rRTN.

The relationship between a strong behavioural deficit, indicative of a greater dopaminergic lesion, and the change of GABAergic transmission in the rRTN, was assessed. However, no correlation between the step testing scores and the number of GABAergic neurons in the rRTN was observed (Pearson’s r correlation; r = -0.29, n = 5, p = 0.63). Therefore, the increase in rRTN GABAergic neurons appeared to be independent of dopamine neuron loss.

To investigate whether 6-OHDA lesions had a similar effect on the novel glutamatergic population of neurons in the rRTN, neurons positive for the marker CaMKIIα were counted in sham and 6-OHDA lesioned animals (Figure 3.7 D). Interestingly, despite an increased number of GABAergic neurons, no differences were observed in the mean number of glutamatergic neurons in sham and 6-OHDA lesioned rats suggesting that these neurons were relatively unaffected by 6-OHDA lesions (p = 0.18). Consequently, I did not assess the size of glutamatergic neurons nor did I test if the glutamatergic neuron count correlated with step test performance in lesioned rats.
Figure 3.7. Comparison of stereology corrected neuron counts and soma area measurements in sham and 6-OHDA lesioned animals. (A) The mean volume (µm$^3$) of the rRTN was measured with the Cavalieri estimator in sham (n = 4) and 6-OHDA lesioned rats (n = 5). (B) The mean total number of GABAergic neurons counted in the rRTN in sham and 6-OHDA lesioned rats. Stereology corrected numbers. * = p ≤ 0.05. (C) The mean measured soma area (µm$^2$) of GABAergic neurons in the rRTN in sham and 6-OHDA lesioned animals. *** = p ≤ 0.0001. (D) Mean total number of glutamatergic neurons in sham and 6-OHDA lesioned animals.
4.0 Discussion
The RTN is thought of as an exclusively GABAergic nucleus that provides inhibitory input to the rest of the thalamus and has a large impact on the messages that are travelling to and from the cortex. The rRTN is involved with the processing of motor information with numerous afferent inputs from motor related areas of the basal ganglia, SNc, motor thalamic nuclei and motor cortex, and has efferent projections to the motor thalamus. However there is a distinct scarcity of information regarding the role that the pathology of the rRTN has in movement related disorders such as PD.

The present study investigated the phenotype of neurons in the rRTN hypothesizing that the majority of neurons in the rRTN would be GABAergic and a small novel population that has a glutamatergic phenotype exists based on a previous electrophysiology study in our lab. Following characterization of these neurons, it was hypothesized that chronic 6-OHDA induced dopamine depletion in the SNc and VTA would not have an effect on the number or morphology of neurons in the rRTN.

To characterize the phenotype of neurons in the rRTN, GAD67 and CaMKIIα antibodies were used as markers for GABAergic and glutamatergic neurons, respectively, and were revealed with fluorescent and chromogen immunohistochemistry. Both types of neurons were found in the rRTN revealing a novel glutamatergic population of neurons. Furthermore, a lentiviral vector was injected into the rRTN driven by a CaMKIIα promoter to selectively label these glutamatergic neurons. Lentiviral transduction occurred for 6 weeks, after which, dual-labelling immunohistochemistry for GAD67 and the mCherry fluorophore of transduced neurons confirmed that the glutamatergic population existed within the rRTN and that this population was distinct from GABAergic rRTN neurons.

To investigate the effect of dopamine loss on the morphology and number of neurons in the rRTN, the dopaminergic neurons in the midbrain were lesioned with the neurotoxin, 6-OHDA. Dopaminergic lesions of the SNc and VTA were confirmed behaviourally and with qualitative immunohistochemistry. Degeneration of the dopaminergic fibres surprisingly led to an increase in the number of neurons positive for GAD67 staining. Furthermore, we quantified the soma area of GABAergic rRTN neurons and found that it was 20% smaller in 6-OHDA lesioned rats than in sham lesioned animals. Dopaminergic lesions had no effect on the volume of the rRTN or the small population of glutamatergic neurons in the rRTN.

The existence of a novel population of neurons raises numerous questions about the current understanding of the rRTN. However, despite being an aim for this study, technical difficulties meant that the targets of these neurons remain unknown. Furthermore, 6-OHDA induced changes in the rRTN may add important information to the ever growing evidence regarding the pathophysiology of PD. The rRTN may stand as a promising new therapeutic
target for intervention strategies such as electrical or optogenetic deep brain stimulation. Targeting this novel population of neurons might be a key factor in easing the symptoms of PD and improving the quality of life of those who are exposed to the disease.

4.1 Distinct novel population of neurons in the rostral reticular thalamic nucleus

The majority of neurons in the rRTN are GABAergic (Houser et al., 1980). Indeed staining for the GABAergic marker, GAD67 was seen throughout the nucleus in the classical reticulated arrangement caused by the thalamocortical and corticothalamic fibres that penetrate through the RTN. These GABAergic neurons were either large or medium-sized with a fusiform shape to the soma, which are similar to the reported shapes for other RTN sectors (Yen et al., 1985; Sprefico et al., 1988).

Early studies into the RTN stated that the majority of cells in the RTN were GABAergic but suggested that a minor population was not (Houser et al., 1980). Sprefico et al., (1991) identified 3 morphological types of cells in the RTN - small and large fusiform types, and small round neurons. The present study positively identified the first two morphological types that were stained for GAD67 and the third type as immunoreactive to CaMKIIα. This third shape of neuron has also been reported previously in an electron microscopic study on a model of absence epilepsy, however the phenotype of these cells was not recorded (Nagaeva et al., 2006). Similarly, Cavdar et al., (2013) have recently reported a small population of neurons in the RTN that did not show staining for GABAergic markers. These neurons were smaller than GABA-positive cells, however the phenotype of GABA-negative cells was not investigated further (Çavdar et al., 2013).

Here we report that a novel population of neurons exists in the rRTN that are positive for CaMKIIα indicating they are glutamatergic (Colbran, 1992; Hanson and Schulman, 1992). This novel population presumably accounts for some the previous records of a heterogeneous population of cells in the RTN (Houser et al., 1980; Gonzalo-Ruiz et al., 1996; Nagaeva et al., 2006; Çavdar et al., 2013). Previous work in our laboratory recording activity of rRTN neurons found a population with broad extracellular action potentials indicative of glutamatergic neurons (Little, 2014). CaMKIIα-positive neurons were sparse, accounting for less than 5% of neurons counted in the rRTN. Although this constitutes a small proportion of neurons in the rRTN, it does not mean this population is insignificant. For example, striatal cholinergic interneurons make up 1 – 3% of the total population of neurons in the striatum (Houser et al., 1985; Goldberg and Reynolds, 2011), however, these cholinergic interneurons
have a large role in modulating the firing rate of the MSN output neurons causing diverse and complex responses in MSNs mediated through post- and pre-synaptic mACh and nACh receptors (Gonzales and Smith, 2015). Cholinergic interneurons regulate attentional and reward learning behaviours of animals (Goldberg and Reynolds, 2011; Gonzales and Smith, 2015), as well as, synaptic plasticity within the striatum (Gonzales and Smith, 2015). This raises the possibility that the sparse population of glutamatergic neurons in the present experiment may have an important functional role in the rRTN or wider thalamus, despite being a small proportion of the total population of neurons within the rRTN.

CaMKIIα-positive neurons had round perikarya and generally appeared smaller than GAD67-positive neurons within the rRTN. Glutamatergic neurons are known to exhibit this shape, which is sometimes referred to as ovoid, and is present in other thalamic nuclei containing glutamatergic neurons such as the VA/VL (Sawyer et al., 1989), and the Pf-CM complex (Tseng and Royce, 1986; Deschénes et al., 1996).

The lentiviral vector that was injected into the rRTN allowed selective labelling of neurons driven by a promoter for the CaMKIIα protein as well as immunohistochemical detection of the mCherry fluorophore that was part of the genetic makeup of the viral vector. Selective labelling of neurons with the CaMKIIα proteins indubitably confirmed the presence of a distinct population of glutamatergic neurons within the rRTN. Injection of lentiviral vectors may lead to a limited immune or inflammatory response that may alter the morphology of neurons (Annoni et al., 2007; Parr-Brownlie et al., 2015), but transduced neurons in this study exhibited a similar shape to those immunostained with the CaMKIIα antibody suggesting that limited or no immune or inflammatory response following injections. However, the transduced neurons seemed to follow the path of injection, perhaps a sign that some damage to the tissue occurred following the mechanical injection of the virus or the path of least resistance of diffusion.

At present it is not clear what function these glutamatergic neurons in the rRTN have. Nevertheless, glutamatergic neurons with a fusiform shape in the VA/VL were identified as thalamocortical projection neurons (presumably with collaterals to the rRTN). However these neurons also had “bushy” appearance with a number of dendrites extending from the soma that is commonly described in most thalamic nuclei (Sawyer et al., 1989), which the rRTN glutamatergic neurons did not show. Therefore, rRTN glutamatergic neurons may exhibit some unique characteristics compared to glutamatergic neurons in other thalamic nuclei.

It has previously been thought that interneurons of the rRTN play an important role in the communication between the different sectors of the rRTN (Crick, 1984). However, questions regarding the presence of a GABAergic population of interneurons in the RTN have
been raised, with new evidence suggesting that GABAergic interneurons are not present in the rRTN in adult rats but are found in rats under 2 weeks of age (Hou et al., 2016). While further evidence would be required, this novel glutamatergic population of neurons could indeed act in an interneuron capacity, linking the different sectors of the RTN together to ensure proper communication.

Unfortunately the attempt to trace these neurons with this viral vector was unsuccessful due to the virus diffusing into other structures and subsequently transducing neurons in that structure. Although targets could not be specifically identified, a number of structures could be speculated to be the targets of these glutamatergic neurons based on the action it would have in the cortico-BG-RTN-thalamocortical circuits.

With regards to movement, the activity of the rRTN is thought to ensure temporally precise inhibition of the thalamus allowing clear passage of the appropriate motor signals to the motor cortex (Marlinski et al., 2012). GABAergic neurons of the rRTN are thought to achieve this with changes in their bursting activity pattern. Recent evidence obtained in our laboratory found that putative glutamatergic neurons in the rRTN also fire in a bursty pattern (Little, 2014). It could therefore be assumed that this temporality is achieved through activity in both glutamatergic and GABAergic neuron populations. The most logical synaptic target for the glutamatergic neurons in the rRTN would be to the motor thalamus, as the only GABAergic output from the RTN that has been defined to date is to other thalamic nuclei (Pinault, 2004). Excitatory input from the rRTN to the motor thalamus could act in two ways. Firstly, to enhance the signal that is going up to the cortex through excitatory activation of thalamic relay cells, meanwhile other non-important signals are inhibited by the RTN. This again achieves a precise temporality to the appropriate motor signals for the intended target. Secondly, this glutamatergic population may be acting as a functional reserve, so that if a high priority event occurs that requires immediate attention or alteration of the projected movement these neurons could reverse the effects of the inhibitory GABAergic input to the motor thalamic neurons.

As suggested earlier, RTN neurons may act as interneurons that allow for communication between sectors of the RTN. Glutamate can cause excitation or inhibition of RTN neurons depending on the receptor that it activates. Glutamatergic activation of RTN neurons causes fast excitatory post synaptic potentials mediated through AMPA and NMDA receptors (Lacey et al., 2012; Deleuze and Huguenard, 2016). These responses are sufficient to activate T-type Ca\(^{2+}\) channels that can lead to a change from tonic to bursty activity in RTN neurons if they were previously hyperpolarised (Deleuze and Huguenard, 2016). Therefore, this glutamatergic population acting as interneurons would promote the burstiness of their
interconnected neurons, which would act to enhance the inhibitory output of particular neurons, in a particular sector and ensure that the each sector and their functionally connected thalamic relay neurons are responding to external stimuli appropriately (Pinault, 2004). Additionally, glutamatergic action through group I and group II mGluRs leads to LTP and LTD, respectively, induced synaptic plasticity (Wang et al., 2015; Neyer et al., 2016). If this glutamatergic population of neurons were to act as interneurons, this mGluR mediated mechanism of synaptic plasticity provides a form of communication where the strengthening and weakening of particular synapses by LTP and LTD, respectively, may be driven by local modulation of these interneurons.

The CM nucleus is part of the thalamo-striatal-basal ganglia loop and is important in the changing of attention and reward-related motor tasks (Henderson et al., 2005; Minamimoto et al., 2005; Smith et al., 2014; Galvan et al., 2015). CM thalamic efferents to the striatum synapse onto GABAergic MSNs of the striatum, and increase the firing rate of MSN output neurons (Smith et al., 2014). Single pulses from CM neurons do not elicit responses in MSNs, which require burst firing to be activated (Nanda et al., 2009). It is believed that this system helps to alter activity in the basal ganglia in response to attention-related stimuli (Smith et al., 2014). This raises the striatum as another potential target for this glutamatergic population of neurons in the rRTN. The RTN is commonly described to act as a gateway for information coming from the thalamus and is able to focus on stimuli that require our immediate attention while simultaneously inhibiting irrelevant information (Pratt and Morris, 2015). The RTN is known to send GABAergic projections to the CM (Steriade et al., 1984). Speculatively, if the glutamatergic population in the rRTN were to project to the striatum and act to ‘highlight’ stimuli that require attention, a direct loop would be formed that allows simultaneous inhibition in the CM, therefore reducing the CM-mediated excitation of the striatum, as well as excitation of the striatum by the rRTN itself.

4.2 Parkinsonism affects the rostral reticular thalamic nucleus

The effects of 6-OHDA induced Parkinsonism on the number and morphology of GAD67 and CaMKIIα stained neurons in the rRTN was investigated and compared against a sham lesioned control group. Following lesion of the SNc and VTA via 6-OHDA injections into the MFB, the number of cells positive for GAD67 staining increased compared to sham injections into the same area. This increase in rRTN GABAergic neuronal number did not correlate to the degree of dopaminergic lesion that was indicated by the behavioural deficit
displayed by lesioned animals. Previous reports have shown that behavioural performance can indicate the extent of dopaminergic neuron loss in the SNC and VTA (Tseng et al., 2005). This suggests that the increase in GAD67-positive neurons may occur early in the progression of dopaminergic lesions and does not require a substantial lesion to midbrain dopamine neurons. The immunohistochemistry completed in this thesis was targeted for GABAergic or glutamatergic neurons in the rRTN. While counting, it was noted that this staining seemed to account for most of the neurons in the structure, however, this would have been made clearer with a general neuronal stain such as Nissl staining. It is presented in this thesis that the rRTN contains approximately 1200 cells. To the best of my knowledge, only one other group has reported a total neuronal count in the RTN (Sabers et al., 1996). Sabers et al., (1996) counted up to 30,000 total cells in the entire RTN structure, which covers up to 3 mm in the rostro-caudal axis. In contrast, the rRTN that was counted in this thesis was only 0.4 mm in the same axis, which would account for the difference in neuronal number.

Although research into the effects of a dopaminergic lesion on the rRTN is scarce, it has previously been found that the mRNA encoding the enzyme GAD67, which is involved in the synthesis of GABA, is increased following 6-OHDA induced lesions in rats (Delfs et al., 1996). This increase in GAD67 mRNA was independent of the dose of 6-OHDA given, suggesting that the changes to expression of GAD67 mRNA are independent to the size of dopaminergic lesion (Delfs et al., 1996). The CM/Pf complex is similarly affected by dopaminergic depletion of the midbrain. In 6-OHDA and MPTP lesioned animals, and human patients with PD, loss of neurons in this intralaminar thalamic complex is not associated with the degree of dopaminergic lesion (Henderson et al., 2000a, 2000b; Kusnoor et al., 2012; Villalba et al., 2014). One interpretation is that the rRTN may have a role in the non-motor symptoms of PD, which occur earlier than the motor symptoms that become apparent when up to 70% of dopaminergic neurons are lesioned (Fearnley and Lees, 1991). For example, it is possible that the disruption of normal activity in the rRTN as seen presently and in previous work in our lab (Little, 2014), of the RTN spreads to the other sectors of the RTN through electrical gap synapses (Blethyn et al., 2008), or the proposed glutamatergic RTN interneurons (see above). Through this route, the pathological changes to the RTN could affect other sectors of the RTN, such as the limbic sector, which may contribute to deficits in motivation as well as the depressive symptoms seen in some patients with PD (Remy et al., 2005; Park and Stacy, 2009). Furthermore, global synchronization of bursty activity in the RTN that occurs during the development of sleep spindles (Fuentesalba and Steriade, 2005), may be disrupted by the spread of pathology to all sectors of the RTN. The role that the RTN may have in the sleep disturbances experienced with PD is further discussed below.
The increase in GABAergic neurons did not coincide with an increase in the volume of the rRTN. However, the soma of identified fusiform GABAergic neurons were smaller compared with the sham lesioned group. Typically, when somal area is measured a marker for the nucleus is used (Derecki et al., 2012; Amorin and Calisi, 2015). GAD67 immunoreactivity clearly labelled the soma and around the nucleus of GABAergic neurons in this study, despite not using a nucleus marker. Furthermore, sham lesioned animals had neuronal soma area measurements similar to what has previously been reported in the rRTN of adult rats of around 150 – 200 µm² (Ohara and Lieberman, 1985), indicating that use of the nuclear marker is not essential for this analysis.

Together these data suggest that although dopamine loss is required to change the number and the morphology of GAD67 expressing GABAergic neurons and it does not require a large lesion to induce these effects. The number of neurons positive for CaMKIIα was not affected by 6-OHDA lesions, nor was the morphological appearance.

The findings of Delfs et al. (1996) raises the issue of whether or not an actual increase in neuronal number was found or whether the increase of GAD67 mRNA confounded the neuronal cell counts. The levels of GAD67 mRNA are directly correlated with the levels of the GAD67 enzyme (Gierdalski et al., 2001; Lau and Murthy, 2012), which was targeted with immunohistochemistry in the present experiment. However, Delfs et al. (1996) employed in situ hybridization methods to measure mRNA expression of GAD67, and the number of neurons in the rRTN was not reported. All care was taken to ensure that stereological cell counts of the rRTN were unbiased and potential confounding elements were reduced with the use of the Optical Fractionator method alongside strict use of unbiased counting rules. Although it is possible that due to the ambiguity between different portions of the RTN, small parts of the caudal neighbours of rRTN could have been included in the neuron counts, adding to the total. Kincaid and colleagues report that 6-OHDA caused increased expression of mRNA of GAD67 per neuron in the GPe as well as an increased percentage of neurons in the GPe that express immunoreactivity to GAD staining (Kincaid et al., 1992). Similarly, mRNA expression of GAD67 and the number of neurons expressing GAD67 were increased in the barrel cortex of mice following sensory learning (Gierdalski et al., 2001) indicating the GABAergic neuron numbers are altered under physiological and pathological conditions.

In the RTN, however, the majority neurons are already immunoreactive to GAD67 staining and if a small percentage of GAD67 negative neurons started to express the enzyme, the difference is unlikely to account for the increases seen presently. Interestingly, during normal ageing of a rat, the RTN undergoes changes in its neuronal density. From young adulthood at 3 months of age to the later age of 24 months the number of neurons per mm² of
RTN tissue progressively declines. As a rat enters old age at 24 months to 30 months, the number of neurons per mm$^2$ in the RTN increases (Ramos et al., 1995; Çavdar et al., 2013). The rats in the present study were around 4 months old at the end of their experimental life and therefore into young adulthood and should be in a period of decline in the number of neurons per mm$^2$ in the RTN. One interpretation is that 6-OHDA lesions of dopaminergic neurons in the SNc cause premature ageing in rats, thereby disrupting the normal ageing process and accounting for the increased number of GABAergic neurons in the rRTN presented in this thesis. Indeed, ageing is a major risk factor for the development of PD with an increased prevalence of PD in human patients over 60 years of age (Aarsland et al., 2001).

In addition to this idea, during normal ageing, neurons of the basal forebrain in humans and the cortex of rats go through a period where their size increases, which is followed by a subsequent 10% reduction in size in older age (Diamond et al., 1977; de Lacalle et al., 1991). Therefore, this premature ageing may account for the decrease in the size of GABAergic neurons seen presently. We minimised this factor by ensuring that sham lesioned control animals were of the same age as 6-OHDA lesioned animals and all tissue was processed together under the same conditions. There is currently a lack of research on the effects of 6-OHDA lesions on ageing in rats. However, in a highly used model of ageing in the worm, C. Elegans, genetic techniques were used to increase the lifespan of PD-mutant worms and it was found that PD-like symptoms were alleviated and α-synuclein aggregates were prevented (Cooper et al., 2015). This suggests that a dopaminergic deficit may alter neurons of the brain and make them more susceptible to the processes of ageing. In the rRTN, this may manifest as an increase in the number of GABAergic neurons and a decrease in the size of these neurons.

It becomes interesting to speculate on which inputs to the rRTN are responsible for the changes in the levels of GAD67, and indeed it is likely a combination of all inputs – most of which are also altered following 6-OHDA intoxication. The SNc sends dopaminergic input to the rRTN (Florán et al., 2004; Gasca-Martinez et al., 2010), which would likely be lost with dopaminergic lesions leading to direct changes in the rRTN. Dopaminergic input from the SNc acts to reduce GABA inhibition of rRTN neurons (Gasca-Martinez et al., 2010), so loss of dopamine will increase the inhibition of rRTN neurons by GABAergic inputs from the overactive SNr, among others. This may lead to hyperpolarization of the rRTN membrane potential resulting in an increased bursty pattern of activity, which is accompanied by an increase in GABA synthesis enzymes i.e. GAD67, GABA synthesis and GABA transmitter release (Delfs et al., 1995; Bosch-Bouju et al., 2013). Pharmacological antagonism of the dopaminergic D4 receptors, which are present in the rRTN (Florán et al., 2004), leads to a reduction of motor activity (Zhang et al., 2002). In the present study D4 receptors would not
be activated due to a lack of dopamine rather than by antagonism, however, a similar reduction in motor activity was seen, suggesting that the changes to the rRTN that contribute to movement deficits are a consequence of reduced dopaminergic activity from the SNc input to the rRTN, at least in some capacity. However, to confirm this assumption, the rRTN would need to be isolated in the pathology of the disease.

As previously described D4 receptors in the rRTN are located on presynaptic terminals of GABAergic inputs from the GPe, GPi and SNr (Gasca-Martinez et al., 2010) suggesting that dopamine loss may not directly affect rRTN neurons but instead modulates GABAergic signalling in the nucleus. Furthermore, increases in GAD67 mRNA expression following 6-OHDA lesions have been reported for the striatum, GPi, GPe and SNr (Kincaid et al., 1992; Soghomonian and Chesselet, 1992; Soghomonian et al., 1992; Delfs et al., 1995; Rodríguez Díaz et al., 2003). Of these structures, the primary dopaminergic projections are to the striatum, although the SNc also has some projections to the GPe (Freeman et al., 2001; Anaya-Martinez et al., 2006). However, none of these reports have quantified neuronal number. It seems that dopamine loss in the SNc leads to an increase of GAD67 expression, which leads to increased GABA neurotransmission in most nuclei of the basal ganglia and the motor thalamus (Galvan and Wichmann, 2008). For example in the GPe, where an increase in the mRNA of GAD67 has lead to an increase in the likelihood of burst firing (Pan and Walters, 1988; Filion et al., 1991; Delfs et al., 1995).

The size of GABAergic neurons was reduced in the rRTN in this thesis. A reduction in the size of neurons occurs with old age as previously mentioned (Diamond et al., 1977; de Lacalle et al., 1991), and in pathological conditions, such as, Rett syndrome which is an autism spectrum disorder, where in a genetically modified mouse model the somal area of hippocampal cells are decreased compared with normal wild-type mice (Derecki et al., 2012). It is not presently known what causes this reduction, however, in PD, the increased bursty activity in the GPe, as well as the overactive SNr would lead to an increase in the release of GABA from GPe and SNr terminals in the rRTN. The GPe and SNr inputs are mainly to primary dendrites and somata of rRTN neurons (Paré et al., 1990; Asanuma, 1994). Therefore it could be speculated that in PD the changes to these nuclei leads to a compensatory decrease in rRTN neuronal soma size in an attempt to normalize the inhibition from GPe and SNr neurons by decreasing the available area for GABA to have its actions. Although this yet to be substantiated, the opposite of this idea is true in the hippocampus, where decreases in input activity to hippocampal cells in culture resulted in a compensatory increase in the size of neurons to increases synaptic reliability and strength (Murthy et al., 2001).
Recent evidence recorded in this laboratory found an increase in the likelihood of any individual rRTN neuron to exhibit LTS bursting activity following 6-OHDA induced dopamine lesions (Little, 2014). Therefore, increases in the number of GABAergic neurons in the RTN, as found in this thesis, combined with increases in LTS bursting activity within each neuron would cause a large increase in the inhibitory signal that modulates activity of the thalamic relay neurons. The consequent disruption of the temporality that is required for normal movements (Marlinski and Beloozerova, 2014), may underlie some of the motor symptoms seen in patients with PD. Additionally, a decrease in the area of the somata reduces the area available to receive messages of inputs that synapse onto the soma of RTN neurons such as the thalamocortical and corticothalamic collateral terminals and inputs from the basal ganglia (Paré et al., 1990; Asanuma, 1994; Liu and Jones, 1999). The “noisy signal” pattern of activity is a mechanism that has been suggested to underlie dysfunction in basal ganglia networks, where pathological activity in the nuclei of the basal ganglia disrupts the normal pattern of activity required for movements (Brown and Eusebio, 2008), therefore, the changes to the rRTN found in our lab suggests that the rRTN may add to this noise and contribute to the disruption of movement in PD.

Despite the limited range of evidence regarding changes to the RTN in Parkinsonian conditions, other neurological conditions may help understand how changes to the RTN may affect the wider networks of the brain. The RTN is implicated in the generation of the oscillatory activity through the thalamocortical-thalamic loop, that is thought to be important in the normal functioning of the brain but can become pathological (Pinault, 2004). Spike and wave discharge (SWD) patterns are a characteristic feature of absence epilepsy and develop when oscillatory activity in this network is abnormally expressed (Steriade, 2005). GABAergic neurotransmission from the RTN is thought to play an important role in the generation and maintenance of this SWD pattern (Steriade, 2005). In animal models of absence epilepsy, the mRNA expression of GAD67 has been reported to be increased (Lin et al., 1999), a change which is similarly seen in PD. Furthermore, it was suggested that this increase in GAD67 expression and subsequent increase of GABAergic synaptic transmission by RTN neurons projecting to thalamic relay neurons reduced the ability of the relay neurons to participate in synchronized burst firing (Lin et al., 1999). Normal synchronized activity of thalamic relay neurons in the motor thalamus is essential for the development of movement and is transiently allowing the temporally precise flow of motor message to the cortex (Hammond et al., 2007). In PD, the increased GABAergic synaptic transmission from the rRTN as well as from nuclei of the basal ganglia would override this transient
synchronization, impeding the development of a motor pattern and could account for some of the bradykinetic and akinetic features of the disease.

The current study did not find any differences in the number of neurons positively stained for CaMKIIα, however, it must be noted that the ratio of GABAergic and glutamatergic neurons counted in the rRTN was fairly constant across sham and 6-OHDA lesion groups. Previously this lab has found that the activity of presumed glutamatergic neurons decreased and the incidence of LTS bursts was increased in the PD model (Little, 2014). These changes to the firing pattern of glutamatergic neurons are similar to those described in the STN (Delaville et al., 2015). Although an increase in glutamatergic neurons was not teased out with statistical testing, the constant ratio of glutamatergic neurons to GABAergic neurons suggests that both types of neurons within the rRTN may have increased. It is hard to know what this would do to the wider system, without knowing where the glutamatergic outputs are going, however, it could be stipulated that this increase in neuronal number as well as a change to the firing pattern would upset the messages being conveyed through the rRTN. However, to fully understand the effects of PD on this small population of neurons in the RTN, additional investigation is required.

4.3 Exploring the rostral reticular thalamic nucleus as a novel targets in the treatment of Parkinson’s disease

Research into the changes in the rRTN in PD has been limited. However, it appears from this study that more GABAergic neurons are present in the rRTN, as well as increased expression of GAD67 (Delfs et al., 1996). The consequence of this increased GABAergic tone may increase the inhibition of thalamic relay neurons and change the temporal pattern of activity that is required for a number of processes throughout the brain. During skilled movements in non-lesioned rats, the RTN fires in bursts of action potentials that provide thalamocortical neurons with inhibition in a pattern that is precisely timed with the movement (Marlinski and Beloozerova, 2014). Additionally, it was found that neurons of the rat motor thalamus fire in a precise pattern of activity that is specifically timed to executing a skilled reaching movement, where there are periods of inhibition before and after the movement and a peak of excitation as the movement is occurring (Bosch-Bouju et al., 2014). This modulation is abolished in 6-OHDA lesions (Bosch-Bouju et al., 2014), which could theoretically be due to altering the precise temporal nature of the inhibitory input from the RTN. Therefore the rRTN offers a potential novel target for therapies that alleviate the
symptoms of PD. Furthermore, this nucleus may offer a target for the treatment of not only the motor symptoms, but also the non-motor features of the disease due to the involvement of the RTN in processes such as attention and sleep.

The increases in the numbers of GAD67 positive neurons, seen presently, and the expression of GAD67 mRNA (Delfs et al., 1996), and subsequent changes in GABAergic synaptic transmission occurs not only in the rRTN, but in the striatum and SNr, where this change has received a lot more attention. It is thought that by modifying the GAD gene in PD it is possible to return GABA transmission back to normal and indeed a number of studies in a rat model of PD have reported that behavioural symptoms are reduced following GAD gene therapy (Horvath et al., 2011; Wettergren et al., 2014). These studies utilize interference RNA (RNAi), which are incorporated into the GAD67 mRNA and disrupt production the enzyme (Wettergren et al., 2014). Injecting lentiviral vectors, expressing RNAi to knockdown GAD67 isoforms, into the striatum or SNr restored normal GAD67 levels and alleviated the motor symptoms of PD in rats (Horvath et al., 2011; Wettergren et al., 2014). The present results indicate that GAD67-expressing neurons are increased in the rRTN, therefore, GAD gene therapy targeted to the rRTN may improve the symptoms of PD similar to the effects seen in the striatum and SNr. By reducing the levels of GAD67 in the rRTN, through RNAi induced knockdown, GABAergic inhibition of the thalamic relay neurons by the rRTN will be reduced and the appropriate motor signals that are impaired in PD might be restored. GAD gene therapy is a promising upcoming treatment with trials of viral-vector induced expression of GAD67 in human patients already underway (Kaplitt et al., 2007), although targeted in the STN. However, further rRTN studies are required before conducting trials that modify GABAergic rRTN transmission.

Non-motor symptoms occur in up to 90% of patients that live with PD, and most non-motor symptoms are not responsive to the gold standard treatments offered such as L-DOPA. Additionally, non-motor symptoms can occur earlier in the progression of the disease than the hallmark motor symptoms and often go undiagnosed (McDowell and Chesselet, 2012). It is critical that the non-motor symptoms of PD are better understood to ensure early detection of PD and to improve the quality of life of those already living with the disease.

Sleep disturbances are a common non-motor symptom of PD and affect around 80% of patients (Factor et al., 1990; McDowell and Chesselet, 2012). As mentioned in Section 1.1, the RTN has a role in the generation of sleep spindles – a hallmark oscillatory pattern that occurs during light non-rapid eye movement sleep (Steriade et al., 1993; McDowell and Chesselet, 2012). Therefore, the RTN represents an interesting nucleus to target treatment to
alleviate sleep disturbances in Parkinsonian patients. Burstiness of the rRTN is a key activity pattern that synchronizes the rRTN and thalamocortical relay neurons leading to the slow oscillatory rhythm important for non-rapid eye movement sleep (Fuentealba and Steriade, 2005). As previously mentioned, the pathology of absence epilepsy is characterized by SWDs that oscillate at an increased frequency compared with sleep oscillations (Fuentealba and Steriade, 2005). During SWDs, the neurons of the RTN synchronise in firing with bursty pattern more regularly, and this burst is much longer than before seizures (Steriade and Contreras, 1995). PD increases the number of GABAergic neurons in the rRTN as reported presently, which likely leads to increased bursty activity of the neurons in this nucleus (Little, 2014). Therefore, disturbing the sleep oscillations with increases in the number and the likelihood of burst firing in rRTN neurons (Little, 2014), which is induced by dopamine depletion, may underlie the sleep disturbances and poor sleep patterns of PD patients. In Parkinsonian patients, low-frequency stimulation of the rRTN did not alter sleeping patterns but did alleviate resting tremor, however, these patients did not have a previous history of sleep disturbances (Arnulf et al., 2000). At low-chronic doses of 6-OHDA, the so called ‘clock’ gene PER2 that regulates the circadian rhythm of rats is altered in the striatum (Gravotta et al., 2011). This disruption of normal rhythmic function may provide a model that could investigate sleep disturbances in PD (Gravotta et al., 2011). However, to the knowledge of this researcher there have not been investigations looking at RTN activity with regards to sleep disturbances in animals with 6-OHDA lesions.

Advancing our understanding of the RTN in the pathophysiology of motor and non-motor symptoms of PD is vitally important to improve treatments for patients, as well as early identification of Parkinsonian symptoms. Changes to the normal anatomy and physiology of neurons that occur in PD are complex and affect a number of areas of the brain. The RTN receives considerable inputs from many of these areas, and projects to thalamic relay neurons, but it has often been ignored in the treatment of PD. Restoring normal activity in the thalamus could normalize activity throughout the brain (Seeger-Armbruster et al., 2015), and the RTN represents an important target for future treatment endeavours.

4.4 Technical considerations

4.4.1 Definition of the rRTN boundary

The RTN is functionally segregated into many sectors of different modalities. Represented at the rostral end of the nucleus are the limbic, and motor sectors (Jones, 1975;
Pinault, 2004). The sector pertaining to visceral information lies just caudal to the motor related rRTN and the limbic sector is the rostral neighbour of the motor sector (Jones, 1975; Cicirata et al., 1990; Stehberg et al., 2001). Each sector is reasonably singular in its functional afferent and efferent pathways with little or no overlap (Pinault, 2004), however there is no strict anatomical delineation that separates individual sectors. Therefore, in the present study, the definition of the rRTN was designed to be consistent with previous literature that had specifically looked at the motor sector of the RTN (Cicirata et al., 1990; Delfs et al., 1996; Picazo et al., 2009; Villalobos et al., 2016). Despite this, it is possible that portions of the limbic and visceral sectors may have been included in the reported cell counts. The motor portion of the RTN receives inputs from motor thalamus, the GPe and the SNr, which are found only in the rostral portion of the RTN (Delfs et al., 1996), the SNr may also project to limbic sectors of the RTN (Gandia et al., 1993; Tsumori et al., 2000). The activity of these nuclei is altered in 6-OHDA lesions and the rRTN seems to be similarly altered. The increased number of neurons in the rRTN seen in the present study may have been underreported if the visceral sector of RTN was included in neuronal counts because it has no known projections from nuclei mentioned above. Nevertheless, the results of the present study are consistent with previous GAD67 mRNA results investigating the affect of a 6-OHDA lesion on the rRTN (Delfs et al., 1996).

4.4.2 Chromogenic versus fluorescent immunostaining

Fluorescent immunohistochemistry is a very good method to distinguish two different antigens of interest at a high-resolution due to the different wavelengths of light that are employed. Each wavelength can be viewed individually, and then merged together to form a coherent image. However, the labelling of antibodies with fluorescent antibodies is much less sensitive than chromogenic labelling and requires high concentrations of primary antibodies and therefore is more expensive. Moreover, high-power fluorescent microscopy that produces these high-resolution images is time-consuming and expensive to use on an hourly basis. Due to the constraints of an MSc project, fluorescent immunohistochemistry was used sparingly in this project and chromogenic labelling was primarily used. Furthermore, access to the Stereoinvestigator software was only available in conjunction with chromogen-based light microscopy for this study. The detection of antigens that are in close proximity is a lot more difficult using the chromogenic system because of the higher sensitivity of the reaction products and the limited supply of different colours. DAB is a chromogen that has been used for decades and is readily used by this laboratory group. Vector SG was chosen as a second label colour due to its compatibility with dual peroxidase stain labelling according to the
manufacturer. Vector SG is a less sensitive chromogen than DAB, and staining with this chromogen in dual-labelling experiments showed a faint reaction product. This combination of reaction products has not been previously reported for dual label neuronal antigens but we were able to achieve colour separation. DAB may also cause sheltering of the secondary reaction product due to its high sensitivity leading to a high yield of reaction product as well as the tendency for the brown DAB reaction product to aggregate producing a more intense stain (van der Loos, 2007). The inclusion of antigen-retrieval before application of the second primary antibody and vector SG staining and the extremely dilute concentrations of the first primary antibody prevented sheltering effects of DAB (Tornehave et al., 2000; van der Loos, 2007).

An alternative to DAB that could have been used in these experiments is 3-amino-9-ethylcarbazole (AEC), which produces a red reaction product and provides a good contrast against the blue vector SG. However, AEC requires mounting with an aqueous mounting medium such as glycerol gel and fades over time (van der Loos, 2009), whereas DAB and vector SG can be mounted and viewed permanently.

4.4.3 Lentiviral vector injections

To irrefutably confirm the presence of a distinct glutamatergic population of neurons in the rRTN, a lentiviral vector was injected into the brain at the stereotaxic coordinates that represented the rRTN. Lentiviral vector transduction was driven by a CaMKIIα promoter and could easily be identified with the mCherry reporter. The viral vector was also pseudotyped with VSVg that ensured transportation of the vector throughout the whole neuron enabling anterograde tracing of the neuron. While expression of transduced neurons in the rRTN was observed, the injections of the viral vector were not contained within the rRTN and therefore any terminals that were subsequently identified could not be accurately reported to be from the rRTN. Initially, the coordinates were modified according to the injection path of the previous group of rats, beginning with coordinates from previous studies in the lab. Then, a smaller volume of virus was injected, which showed some promise. However, transduction of the virus was still extended beyond the rRTN boundaries. With each lentiviral injection requiring 6 weeks to transduce neurons, on top of 2 weeks between lesion surgeries and lentiviral surgeries, the process taken to selectively hit the rRTN was extremely time consuming. Due to time constraints inherent to a Masters project, we were unable to complete this aim of the project.
4.5 Future studies

This study has revealed a sparse novel population of neurons in the rRTN that have a glutamatergic phenotype. A large number of previous studies have generally believed the RTN to be made up of exclusively GABAergic neurons and therefore this glutamatergic population may play an important role in the function of the RTN. However, we were not able to identify the synaptic targets of the RTN due to the injection path of the viral vector entering into other structures. To further understand the function of this novel population it is vital to identify the projection patterns of these neurons. Accurately identifying the rRTN before injections of the viral vector would be essential and may address the issues of misplaced transduction. The changing shape of this structure along its rostro-caudal axis makes the accurate injection in any individual rat difficult, to ensure that viral vector transduction is limited to the RTN, a small (100 nL) injection volume should be used and a large number of rats would be required to eliminate the possibility of transduction of neurons in other structures. Stereotaxic electrophysiological mapping before lentiviral vector injections would allow precise placement of the viral vector into the rRTN. The RTN has a characteristic electrophysiological profile that can be identified while recording activity in live anaesthetised animals, and electrophysiological mapping has been used successfully to identify the motor and premotor cortex as well as inject lentiviral vectors directly into these areas (Galvan et al., 2016). This technique requires a recording microelectrode to be attached to the injection tubing for viral delivery. This exact application of the lentiviral vector used in the present study would allow post-mortem tracing of the glutamatergic projections and possibly reveal a non-thalamic target for the RTN.

To extend this idea further, combining electrophysiological mapping with optogenetics would enable the *in vivo* investigation of the glutamatergic neurons in the rRTN. Firstly the precise transduction of RTN neurons must occur by using a lentiviral vector, which has the capability to add a light-activated cation-selective membrane channel, channelrhodopsin-2 (ChR2) specifically into the glutamatergic neuron (Nagel et al., 2003; Seeger-Armbruster et al., 2015). Then by stimulating the transduced neurons with light and recording in downstream nuclei it is possible to distinguish the functional synaptic targets of these neurons (Hira et al., 2013). To avoid the possibility of movement-related artefacts in potential target locations, animals could be anaesthetised. Similarly, thalamic relay nuclei projections have been specifically mapped to their cortical targets using stimulation of ChR2 expressing thalamocortical neurons (Cruikshank et al., 2010).
Following the identification of the target nuclei of the glutamatergic population it would be interesting to study the effects that optogenetic stimulation has on the behaviour of awake animals, which may help clarify the function of these glutamatergic neurons. Lentiviral vectors that induce the expression of ChR2 in the motor thalamus have previously been shown to change the behaviour of Parkinsonian rats (Seeger-Armbruster et al., 2015). Therefore, it would be interesting to see the effects of a similar scenario applied to the rRTN in animal models of PD. Although no change to the number of glutamatergic neurons was found, previous research conducted by members of this laboratory found that presumed glutamatergic neurons exhibited decreased firing rate and increased bursty firing pattern following 6-OHDA lesions (Little, 2014). Attempting to restore these neurons back to normal activity using optogenetic stimulation may offer a novel treatment paradigm for PD.

Presently, the number of GABAergic neurons in the rRTN was increased following dopaminergic lesions with 6-OHDA and previous reports indicate an increase in the expression of GAD67 mRNA as well as increased bursty activity in the rRTN (Delfs et al., 1996; Little, 2014). Recently, a channelrhodopsin, which is normally cation-selective and causes depolarisation (Nagel et al., 2003), has been engineered that allows the conductance of chloride ions that causes light-activated inhibition of neurons (Berndt et al., 2016). Alternative light-activated inhibitory channels are available for use, such as halorhodopsin, that acts as a chloride pump (Tonnesen et al., 2009) but this chloride-conducting channelrhodopsin exerts inhibition more faithfully than halorhodopsins under the control of optogenetic stimulation (Berndt et al., 2016). The ability to selectively inhibit rRTN GABAergic transmission may have important therapeutic effects in the treatment of PD and indeed knockdown of GAD67 in other structures, such as the SNr, has shown to improve Parkinsonian symptoms (Horvath et al., 2011; Wettergren et al., 2014). Having the ability to provide patterned stimulation, such as theta-burst stimulation (Larson et al., 1986), to these neurons may help reduce the consequences of pathological changes in the precisely timed GABAergic transmission of the rRTN onto thalamic relay neurons (Marlinski and Beloozerova, 2014). Theta-burst patterns of stimulation delivered with transcranial magnetic stimulation to the motor cortex has been shown to be effective in the treatment of some neurological conditions, such as PD and stroke (Huang et al., 2005), and optogenetic theta-burst stimulation of excitatory neurons in the motor thalamus reduced behavioural deficits in Parkinsonian rats (Seeger-Armbruster et al., 2015). Therefore, optogenetic inhibition delivered to the rRTN may act to silence GABAergic transmission and return function back to normal. Furthermore, theta-burst stimulation of the inhibitory channelrhodopsin could
effectively return the precise temporal pattern of firing exhibited by rRTN neurons under normal physiologic conditions (Marlinski and Beloozerova, 2014).

4.6 Conclusions

In conclusion, the current study used immunohistochemistry-staining techniques and lentiviral vector technology to specifically characterize the phenotype of neurons in the rRTN. Although the majority of neurons in the rRTN were GABAergic, consistent with early reports on this nucleus (Houser et al., 1980), a small distinct population of neurons was identified in the rRTN and were characterized as glutamatergic cells, a novel finding of this thesis. Additionally, stereological methods were utilized to assess the effects of the 6-OHDA lesion model of PD on the neuronal population in the rRTN. Dopaminergic depletion caused a surprising increase in the number of neurons positively stained for GAD67 in the rRTN, which were smaller than GABAergic neurons in sham lesioned animals. No changes to the glutamatergic population of neurons in the rRTN were observed. It is further suggested that optogenetic inhibition of overactive GABAergic neurons in the rRTN could be a forthcoming method in the treatment of PD to improve, not only motor but also non-motor, deficits in patients with PD.
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