

**Changes in neuronal activity of the reticular thalamic nucleus in
anaesthetized parkinsonian rats.**

Shane Little

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Abstract

The reticular thalamic nucleus (RTN) is located between cerebral cortex, motor thalamus and the basal ganglia and thought to be implicated in the inhibitory-mediated regulation of motor control. Parkinson's disease is a neurodegenerative motor disorder caused by the significant degenerative loss of dopamine neurons from the substantia nigra pars compacta. This loss results in abnormal electrophysiological activity throughout the basal ganglia-thalamo-cortical network. Interestingly, the role of the RTN in modulating basal ganglia-thalamo-cortical network activity is poorly understood in control conditions and has not been explored at all in parkinsonism.

To address this, the present study characterized neuronal activity of rostral RTN (rRTN) neurons in urethane-anesthetized rats. Furthermore, this study investigated the effect that 6-hydroxydopamine (6-OHDA) induced chronic dopamine neurodegeneration of the substantia nigra pars compacta had on rRTN neural activity. Extracellular spike train data were recorded for at least five minutes using glass electrodes.

Consistent with existing literature, electrophysiological and immunohistochemical data indicate that the rRTN contains primarily putative GABAergic neurons (AP spike width ≤ 0.47 ms). These neurons had a mean firing rate of 4.9 ± 1.1 spikes/s. Approximately 60% of GABAergic neurons demonstrated low threshold calcium spike (LTS) bursts (~ 200 Hz), which are characteristic of thalamic activity. LTS bursting in GABAergic neurons of the rRTN was dominated by doublets. Dopaminergic lesion significantly decreased the firing rate ($p < 0.05$) and increased the incidence ($\sim 80\%$) of LTS bursting in GABAergic rRTN neurons ($X^2 = 38.37$, $p < 0.0001$) and non-doublet LTS bursts became dominant.

Interestingly, the electrophysiological and immunohistochemical data suggested the presence of non-GABAergic neurons in the rRTN (AP spike width ≥ 0.52 ms). Based on the available data these neurons were putatively defined as glutamatergic. These neurons had a mean firing

rate of 6.2 ± 1.9 spikes/s. Approximately half of these neurons demonstrated LTS bursts, which were dominated by non-doublet LTS bursts.

Parkinsonism appeared to affect the glutamatergic neuronal population in a similar manner to how it affected the GABAergic population. Parkinsonism significantly decreased ($p < 0.05$) the firing rate and increased the incidence of LTS bursting activity (~80%) in glutamatergic neurons, compared to control ($\chi^2 = 38.37$, $p < 0.0001$). Interestingly, the incidence of non-doublet LTS bursts was unaffected by parkinsonism, however the incidence of doublet LTS bursts was increased.

These data indicate that: 1) parkinsonism significantly affects the firing rate and LTS bursting characteristics of rRTN neurons and 2) there are possibly two distinct neuronal phenotypes present in the rRTN. Considering the reciprocal connections between RTN, basal ganglia nuclei and motor thalamus, it is possible that the RTN might be involved in producing some of the changes in the basal ganglia-thalamo-cortical network activity that underlie parkinsonian symptoms. Fully characterizing the neuronal characteristics of the rRTN, especially the novel population, will be important for understanding the role of the rRTN in information processing, particularly in future studies examining its role in movement control.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
ANOVA	analysis of variance
DAPI	4',6-diamidino-2-phenylindole
GABA	gamma-aminobutyric acid
GLUT	glutamate
GPe	globus pallidus externus
GPI	globus pallidus internus
ISI	interspike interval
ISI CV	mean interspike interval coefficient of variation
LFP	local field potential
LTS	low threshold calcium spike
LV	lentiviral
MFB	medial forebrain bundle
NGS	normal goat serum
PB	phosphate buffer
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing triton
PD	Parkinson's disease
PFA	paraformaldehyde
rRTN	rostral reticular thalamic nucleus
RS	rabbit serum
RTN	reticular thalamic nucleus
s.c.	subcutaneous
SEM	standard error of the mean
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
STN	subthalamic nucleus
TH	tyrosine hydroxylase
VTA	ventral trigeminal area

Introduction

Parkinson's Disease (PD)

Overview

Idiopathic Parkinson's disease (PD) is a debilitating age-related neurodegenerative disorder caused by the significant loss of dopaminergic neurons from the substantia nigra pars compacta (SNc). The cardinal symptoms of PD are characterized by motor disruption, and include; muscle rigidity, bradykinesia (slowed movement), akinesia (inability to initiate movement) and resting tremor (Parkinson, 1817; Bloem, 1992; Markus et al., 1992; Menza et al., 1993; Grill, 1999; Remy et al., 2005). Approximately three-quarters of parkinsonian patients are also afflicted by non-motor cognitive symptoms, such as; sleep disorders (Nausieda et al., 1982; Factor et al., 1990; Tandberg et al., 1998), mild cognitive impairment (Levy et al., 2002; Caviness et al., 2007; Aarsland et al., 2011), and dementia (Cummings, 1988; Aarsland et al., 2001; Aarsland et al., 2003).

The current prevalence of PD in New Zealand is approximately 8000 people; however, conservative estimates suggest that by 2040 the prevalence of PD will double due to our aging population (Tison et al., 1994; de Rijk et al., 1995; Abou-Sleiman et al., 2006; Statistics New Zealand, 2012, Statistics New Zealand, 2014). The increase in the population with parkinsonism will pose a significant burden on the New Zealand health system, as the estimated costs to care for each parkinsonian patient is approximately \$40,000 per year (Kowal et al., 2013). If new treatment options that improve patient independence, reduce hospitalizations and/or slow disease progression become available, then significant reductions in the costs associated with caring for parkinsonian patients could be achieved (Kowal et al., 2013; Johnson et al., 2013). However, before this can happen we need to better understand the pathophysiological changes that underlie the symptoms associated with parkinsonism.

Basal ganglia circuitry

The basal ganglia (Fig. 1) are a collection of seven nuclei that are implicated in motor program selection and encoding of motor related information. The seven basal ganglia nuclei are; SNc, ventral tegmental area (VTA), striatum, globus pallidus internus (GPi), globus pallidus externus (GPe), subthalamic nucleus (STN), and the substantia nigra pars reticulata (SNr) (Albin et al., 1989; Purves et al., 2001). The striatum and STN act as input nuclei for the basal ganglia, whereas, the GPi and SNr are the major output nuclei (Parr-Brownlie and Hyland 2005; Parr-Brownlie et al., 2009). As shown below in Figure 1, there are three neural pathways through the basal ganglia; the direct, the indirect and the hyper-direct. These pathways all have postulated glutamatergic input arising from cortex, however only the direct and indirect pathways from striatum are thought to be significantly influenced by dopaminergic modulation from SNc/VTA (Albin et al., 1989; DeLong, 1990; Ebrahimi et al., 1992; Smith and Villalba, 2008; Villalba and Smith 2013).

In PD, loss of dopaminergic modulation arising from the SNc results in altered neural activity throughout various regions of the brain. Loss of dopaminergic modulation to the basal ganglia nuclei has been extensively investigated, and studies report dopaminergic loss results in functional changes in the organization of the basal ganglia (Blandini et al., 2000) and is consistently associated with the neural pattern of the basal ganglia becoming irregular and bursty (Parr-Brownlie et al., 2007; Mallet et al., 2008; Parr-Brownlie et al., 2009; Avila et al., 2010; Bosch-Bouju et al., 2013). The changes in the basal ganglia nuclei upstream of the output nuclei results in increased neural activity and rhythmicity of the basal ganglia output nuclei (i.e. GPi and SNr; Bergman et al., 1998; Priori et al., 2002; Tai et al., 2003), which may ultimately lead to altered activity in the motor thalamus (DeLong, 1990; Magnin et al., 2000; Pessiglione et al., 2005; Parr-Brownlie et al., 2007; Bosch-Bouju et al., 2013). Disruption of normal motor thalamic activity is thought to have detrimental effects on

cortical excitability and the development of the motor program represented in motor cortex (Albin et al, 1989; Purves et al., 2001; Parr-Brownlie and Hyland, 2005; Parr-Brownlie et al., 2009; Pasquereau and Turner, 2011). It is these changes in neural activity of the basal ganglia-thalamocortical pathway which are believed to underlie the movement deficits of PD.

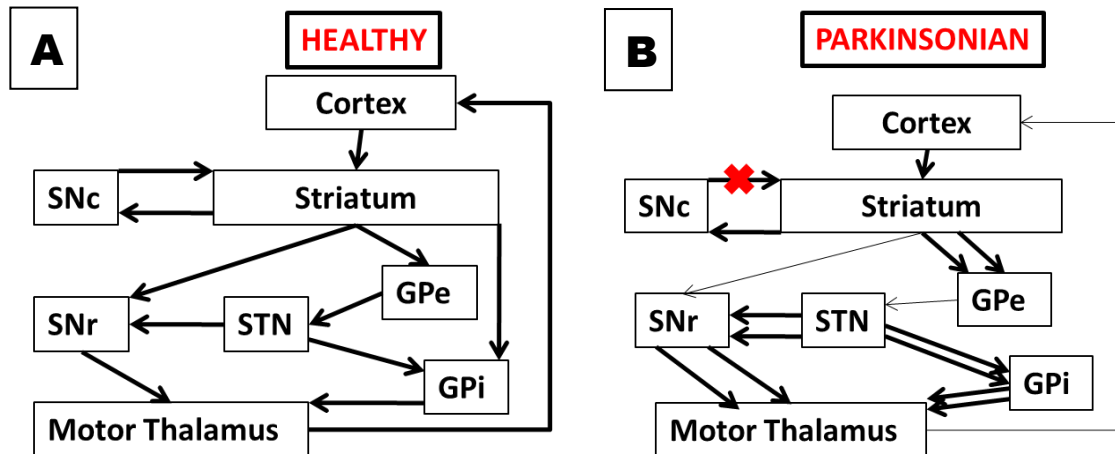


Figure 1: Schematic diagram of basal ganglia related structures and the pathways through the basal ganglia in healthy (A) and parkinsonian conditions (B). The red 'x' symbolizes the loss of efferent dopaminergic projections from the SNc. Double arrows indicate hypothesised increased neural activity whereas thin lines indicate hypothesised reduced neural activity (Adapted from Albin et al., 1989).

Chronic 6-OHDA induced model of parkinsonism

Much of the knowledge we have regarding the neurophysiological changes that occur in PD, is the result of the 6-hydroxydopamine (6-OHDA) chronic dopamine depletion animal model of PD. 6-OHDA is a hydroxylated analogue of dopamine that when injected into the brain accurately mimics many of the deficits in motor performance associated with PD (Lindner et al., 1999; Blum et al., 2001; Cenci et al., 2002). Normally, 6-OHDA is used to selectively lesion the SNc or striatum where it is preferentially taken up by dopaminergic and noradrenergic transporter proteins (Hokfelt and Ungerstedt 1973; Luthman et al., 1989; Berger et al., 1991). Within 12 hours of exposure to 6-OHDA, dopaminergic neurons begin to degenerate, due to the production of free radicals (Heikkila and Cohen, 1973; Cohen and Heikkila, 1974; Sachs and Jonsson, 1975). Two to three days after exposure, the levels of striatal dopamine are depleted (Faull and Lavery, 1969). While the 6-OHDA model accurately produces most of the motor deficits associated with PD, it causes rapid rather than

gradual dopaminergic neurodegeneration (Faull and Lavery, 1969; Betarbet et al., 2002). Furthermore, to prevent the animal from becoming reliant on experimenter intervention for feeding and grooming, 6-OHDA is administered unilaterally into the brain which leaves the contralateral hemisphere largely neurologically intact (Parr-Brownlie et al., 2009). Therefore, it is possible that the contralateral hemisphere could, to some extent, compensate for changes induced by the lesion. Unilateral lesions may not reflect the precise nature of PD for the majority of patients, although one hemisphere is often affected more than the other (Kempster et al., 1989; Lee et al., 1995).

The behavioral symptoms observed in PD have been attributed to the loss of dopaminergic modulatory inputs throughout the brain. Such loss is believed to disproportionately affect the neural activity of the basal ganglia nuclei. Although, the effect of PD on basal ganglia neuronal activity has been extensively studied; there is a distinct paucity of information pertaining to the implications of PD on the neurophysiology of the motor region of the reticular thalamic nucleus (RTN). It is possible that disruption of the inhibitory regulation, provided by the RTN, to the rest of the RTN-thalamo-cortical network might provide a pathophysiological mechanism responsible for some of the symptoms evident in PD (Buzsaki et al., 1990). Therefore, investigating the link between the RTN and PD is crucial for furthering our understanding of the pathophysiological changes that are associated with PD.

Reticular thalamic nucleus (RTN)

Overview

The RTN is a shell-shaped nucleus that is anatomically situated between cerebral cortex, basal ganglia and the motor thalamus. The RTN primarily envelops the anterior and lateral aspects of the thalamus, and to a lesser extent, the dorsal and ventral aspects (Guillery and Harting, 2003; Jones, 1975). The main role of the RTN has been described as the

regulation of thalamic activity and thus may influence the flow of thalamic information to the cortex (Yingling and Skinner, 1977). The RTN is conserved across all mammalian species, further supporting the importance of the RTN in normal brain functioning (Hines, 1929; de Biasi et al., 1986, Houser et al., 1980).

The RTN receives a vast array of inputs encoding nearly every functional modality. Three separate regions encoding functionally distinct information can be identified within the RTN; a sensory, a limbic and a motor encoding region (Pinault, 2004). The sensory region of the RTN is topographically mapped and occupies the caudal two-thirds of the RTN. This region has discrete sectors dedicated to visual, auditory, gustatory, somatosensory, and visceral information processing (Shosaku and Sumitomo, 1983; Shosaku et al., 1984; Villa, 1990; Hayama et al., 1994; Coleman and Mitrofanis, 1996; Stehberg et al., 2001; Pinault, 2004). An olfactory encoding sector has not been identified in the RTN but inputs pertinent to olfactory information processing are present in reciprocally connected nuclei (Cornwall et al., 1990; Groenewegen, 1988; Price and Slotnick, 1983; Pinault and Deschenes, 1998). To date the limbic region of the RTN has been poorly defined; however it appears to occupy the rostral pole of the RTN.

Unfortunately, the motor region of the RTN has also been poorly defined to date. The motor region is confined to the majority of the rostral aspect of the RTN (rRTN), with the exclusion of the rostral pole (Gonzalo-Ruiz and Lieberman, 1995a; Gonzalo-Ruiz and Lieberman, 1995b; Pinault et al., 1998). The rRTN receives significant glutamatergic innervation from layer VI of the cerebral cortex (Cornwall et al., 1990; Bourassa and Deschenes, 1995; Fitzpatrick et al., 1994; Aker et al., 2006) and possesses topographical representations of both motor cortex and motor thalamus (Gonzalo-Ruiz and Lieberman, 1995a). This suggests the rRTN may have a significant role in the processing of motor information. Afferent connections to the rRTN arising from the SNc, which also innervate the

striatum and/or the globus pallidus, have also been noted (Anaya-Martinez et al., 2006). This suggests a possible role for the rRTN in the generation of abnormal movement, particularly, that seen in PD (Anaya-Martinez et al., 2006). Given that the RTN lies between cerebral cortex, basal ganglia and motor thalamus, it is logical that there would be a region dedicated to the processing of motor information (Fig. 2). To the best of my knowledge, the exact nature of this role has not been examined although it likely has implications for the selection of relevant motor actions, given the reciprocal connections it has with motor encoding nuclei. If this region is indeed implicated in the processing of motor information, then it is probable that the rRTN will also be affected in PD. However, to the best of my knowledge, no study to date has specifically investigated the neural activity of the rRTN or investigated if PD induces pathophysiological changes. Therefore, the following is a brief review of what is known generally about the RTN and the possible implications of the structure in PD.

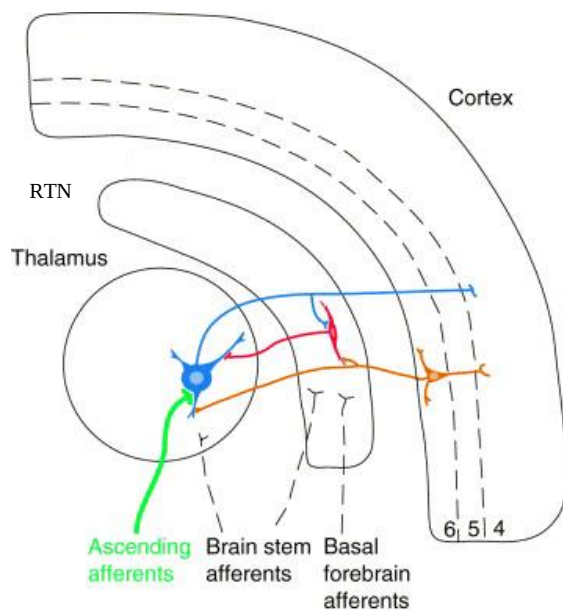


Figure 2: Schematic diagram of RTN connectivity (Adapted from Guillery et al., 1998).

Anatomical organization

GABAergic neurons are the predominant neuronal phenotype identified throughout the RTN (Houser et al., 1980; Steriade et al., 1984; Steriade et al., 1987; Steriade et al., 1993). Two morphologically distinct types of GABAergic neurons have been identified in the RTN.

The first type possesses elongated somata, extensive dendritic arborizations and axons with no branching collaterals (Spreafico et al., 1988). The second morphological type possesses large fusiform soma with horizontally branching dendritic arborizations and branching axon collaterals (Spreafico et al., 1988). In the rodent these large fusiform neurons have been identified as having intra-nuclear branching axon collaterals which allows these neurons to significantly affect the neuronal excitability of nearby RTN neurons (Spreafico et al., 1988). It is important to note that there is some evidence of non-GABAergic neurons being present within the RTN, but to date studies have failed to conclusively determine the phenotype of this non-GABAergic neuronal population (Houser et al., 1980), although some data indicate that this population might be glutamatergic or using a neurotransmitter that is a derivative of glutamate, such as glutathione (Shaw et al., 1996; Otterson and Storm-Mathisen, 1984).

Afferent connections

The rRTN receives significant glutamatergic innervation from layer VI of the cerebral cortex (Cornwall et al., 1990; Bourassa and Deschenes, 1995; Fitzpatrick et al., 1994; Aker et al., 2006). There are also extensive inputs arising from a variety of subcortical nuclei, including globus pallidus, pedunculopontine nucleus (PPN), paraventricular nucleus, VTA and substantia nigra (Cornwall et al., 1990; Bourassa and Deschenes, 1995; Bromberg et al., 1981; Feig and Harting, 1998; Fitzpatrick et al., 1994; Fonnum et al., 1981; Fosse et al., 1986; Kharazia and Weinberg, 1994). There is also evidence that the striatum is included in the circuitry of the rRTN, due to inputs relaying through the paraventricular nucleus (Cornwall et al., 1990). The cortical and thalamic inputs to the rRTN are topographically organized and appear to be primarily excitatory (Ahlsen and Lindstrom, 1982; Eaton and Salt, 1996; Golshani et al., 2001; McCormick and von Krosigk, 1992; Murphy and Sillito, 1996). The thalamus has two types of relays: 1) first-order relays which are peripheral or subcortical information going to primary cortical areas, and 2) higher-order relays which contain

information relaying through thalamus from one cortical area to another (Sherman and Guillery, 2006). However, in the RTN these relays are likely to be integrated as their topographically mapped inputs overlap with each other (Pinault, 2004). It has previously been speculated that the rRTN plays a significant role in visuomotor functioning (Cornwall et al., 1990).

Of particular relevance to the present study, the output nuclei of the basal ganglia, namely GPi and SNr, project to the core and peripheral aspects of rRTN, respectively (Gandia et al., 1993). These afferent connections are relevant because they support the idea that the rRTN encodes motor information, as the basal ganglia has a significant role in the regulation and selection of appropriate motor programs (Graybiel et al., 1994; Takakusaki et al., 2004). Furthermore, a significant number (~50%) of SNc dopaminergic neurons have branching axonal terminals which directly innervate the RTN (Freeman et al., 2001; Anaya-Martinez et al., 2006). This dopaminergic input is important for normal functioning of the RTN as selective abolition results in increased messenger RNA encoding the enzyme glutamate decarboxylase, which is the rate limiting enzyme for GABA synthesis (Delfs et al., 1996).

Given that the rRTN has inputs arising from the basal ganglia and other nuclei that are implicated in encoding motor information it is highly probable that the RTN, at the very least, has a role in regulating the flow of motor information throughout the thalamocortical network. Furthermore, the presence of direct dopaminergic innervation of the RTN suggests that dopamine may have an important role in the regulation of RTN functionality, via; 1) direct innervation of RTN and/or 2) the inputs arising from the basal ganglia nuclei.

Efferent connections

The rRTN has extensive ipsilateral projections to subcortical nuclei implicated in the processing of motor information. These nuclei include the parafasicular, ventrolateral,

ventromedial, centro-lateral and centro-medial nucleus (Minderhoud, 1971; Sumitomo et al., 1976; Steriade et al., 1984; Ohara and Lieberman, 1985; Cornwall and Phillipson, 1988; Nanobashvili et al., 2012). RTN axon terminations synapse onto the dendrite shaft and neuronal cell bodies of thalamic neurons (Cucchiaro et al., 1991; Harting et al., 1991; Liu et al., 1995; Montero and Scott, 1981; Ohara et al., 1980; Peschanski et al., 1983). Interestingly, a small proportion of RTN neurons have been described as having two axons that project to the same thalamic target but originate from two distinctly different locations, somatic and dendritic (Pinault and Deschenes, 1998; Pinault et al., 1997). Some of the axons arising from the RTN dendrites were identified as having dendrite-like arborizing axons, the functional significance of which is unclear.

Identifying the efferent connections of the RTN is important for understanding the implications that the RTN has on the neural activity of other nuclei throughout the basal ganglia-thalamocortical network. For example, previous studies have identified significant changes in the spontaneous activity of PFN neurons and given that the RTN is upstream of this structure it raises the possibility that the rRTN may be partially responsible for the changes observed in the PFN (Parr-Brownlie et al., 2009).

Summary

At present the details of the connections of RTN, particularly the rostral region, are incomplete. There is however clear evidence that the rRTN has reciprocal connections with nuclei involved in the processing of motor information. These motor encoding nuclei have all, to some extent, been implicated in the dysfunction that underlies parkinsonism. This provides a tentative link that the RTN may be involved in the dysfunction observed in neurons of the basal ganglia-thalamocortical network.

Physiology of RTN neurons

The neural activity of RTN GABAergic neurons involved in the processing of sensory information has been extensively studied. These neurons possess several important voltage-dependent ion channels; a classical sodium and potassium, a calcium-dependent potassium channel, a non-inactivated sodium channel and a T-type calcium channel (Avazini et al., 1989; Bal and McCormick, 1993; Huguenard and Prince, 1992; Mulle et al., 1985; Spreafico et al., 1988). It has been identified that the rRTN is also rich in D1 and D4 dopaminergic receptors (Huang et al., 1992; Khan et al., 1998; Mrzljak et al., 1996; Floran et al., 2004; Govindaiah et al., 2010). The presence of these channels, particularly the T-type calcium channels, has significant functional implications on membrane potential, firing pattern and synaptic transmission (Pinault, 2004).

To the best of my knowledge only a few studies have specifically investigated the neural activity of rRTN neurons (Schlag and Waszak, 1970; Waszak, 1974; Domich et al., 1986; Marks and Roffwarg, 1993; Marlinski and Beloozerova, 2014). Due to the distinct paucity of information pertaining to the neural activity of rRTN neurons, the following is predominately a brief summary of the electrophysiological characteristics of neurons investigated in other regions of the RTN. It should be noted however, that evidence from anaesthetized and natural sleep recordings suggests that there are slight electrophysiological differences along the rostrocaudal axis of the RTN (Steriade et al., 1986; Marks and Roffwarg, 1993; Mukhametov et al., 1970). Therefore, the following summary is designed to give an impression of what the characteristics of the rRTN might be.

General burstiness pattern of neural activity

Burstiness is a general pattern of neural activity identified throughout the nervous system where there are periods of rapid action potential generation, subsequently followed by a period of silence. The close temporal and spatial proximity of the individual spikes within

these bursts allows for both temporal and spatial summation of the signal in the downstream target neuron effectively resulting in enhanced signaling and increasing the probability for action potential generation in the downstream neuron. As bursting is observed in many contexts the exact purpose of it is unclear, although three general functions of bursts have been proposed. The first is that bursts function as resonators. Resonating neurons exhibit sensitivity to specific frequencies of input. When exposed to these frequencies, resonant neurons typically respond by increasing their firing rate (Izhikevich, 2000). As a single neuron can innervate multiple downstream neurons, the notion of resonance allows for increased activation of specific target neurons within a network, as each downstream neuron can have different resonant frequencies (Izhikevich et al., 2003). The second function that bursty activity may serve is synchronization of neural activity in interconnected neurons (Butera et al., 1999; Izhikevich, 2000; Rulkov, 2001; Belykh et al., 2005). Neuronal synchronicity typically results in synaptic plasticity changes which can result in long-term potentiation of post-synaptic neurons consequently strengthening the relay of information throughout neuronal networks. A third function of burstiness is that it allows neurons greater flexibility to encode information (Izhikevich, 2000). Given that action potentials are all-or-nothing events, the pattern of neural activity is important for encoding information. This is because information is encoded by the individual action potentials and also the interspike intervals (ISI; Izhikevich, 2000). Regardless of the precise mechanism through which general bursting patterns of activity operate, it is clear that spontaneous bursting activity can significantly influence the properties of neuronal networks.

Two distinct firing patterns have been identified in the RTN that are dependent on the attentional state of the animal. During the awake state and paradoxical sleep a tonic firing mode dominates, whereas, during slow-wave sleep RTN neurons exhibit a predominately bursty firing pattern (Steriade et al., 1986; Marks and Roffwarg, 1993; Domich et al 1986;

Contreras et al., 1993; Kayama et al., 1986; Avanzini et al., 1989). The bursty firing pattern observed during slow-wave, and anaesthetized states, can be subdivided into an irregular slow-firing pattern with occasional bursts and high frequency bursts, accompanied by low threshold calcium spike (LTS) bursts (Steriade et al., 1986; Steriade et al., 1987; Pinault and Deschenes, 1992; Steriade et al., 1993; Contreras et al., 1993; Huguenard, 1996). The majority of RTN neurons typically exhibit one of the aforementioned firing patterns, however, a small proportion of neurons are able to switch between the firing patterns over time (Domich et al., 1986).

The change in firing pattern associated with the different attentional states can be attributed to increased hyperpolarization of the membrane potential of RTN neurons (Avanzini et al., 1989; Marks and Roffwarg, 1993). During the sleep state, RTN neurons are more hyperpolarized due to decreased cortical excitability, which is often described as driving bursty RTN neuronal activity (Lamour et al., 1982). The bursty firing pattern observed in RTN neurons recorded from anaesthetized animals is similar to the firing pattern observed during slow-wave sleep (Villa, 1990; Contreras et al., 1992; Lee et al., 2007; Alberi et al., 2013). Approximately 80% of neurons in the RTN exhibit bursty firing patterns with an average of 2-4 spikes per burst; this appears to be consistent across species (Villa, 1990; Alberi et al., 2013). Synchronous bursting activity is often observed in the RTN which produces powerful GABA-mediated inhibition of target neurons and can provoke spike wave discharges that resonate and cause oscillations throughout the neuronal network (Steriade et al., 1985; Steriade et al., 1987). It has been suggested that GABA-mediated inhibition is necessary for the generation of normal brain rhythmicity and induction of sleep (Bal and McCormick, 1993; Steriade et al., 1987; Steriade et al., 1993).

The few studies that have investigated the rRTN have predominately been performed in cats and generally, describe the rRTN as exhibiting bursty activity with a firing rate

between 7 and 20 spikes/s, during slow-wave sleep (Waszak, 1973; Domich et al., 1986; Marks and Roffwarg, 1993). It should be noted that, generally speaking, the RTN of the cat appears slightly more neurophysiologically active than the rat and so it is expected that lower firing rates (~7 spikes/s) will be observed in the present study, as opposed to the higher firing rates that dominate in cats during slow-wave sleep (Waszak, 1973; Domich et al., 1986; Marks and Roffwarg, 1993).

RTN low threshold calcium spike (LTS) burst activity

Of interest in the present study is the characterization of low threshold calcium spike (LTS) bursts. LTS bursts are a characteristic feature of thalamic nuclei and are frequently observed during slow wave activity (Hirsch et al., 1983; Llinas and Steriade, 2006). Previous studies have observed LTS bursts in RTN, but the frequency, size and spike adaptation of LTS bursts have not yet been fully characterized (Steriade et al., 1987; Steriade et al., 1993; Lacey et al., 2007). LTS bursts are of particular interest to the present study because they are likely to play an important role in the processing of information encoded by the RTN.

LTS bursts are characterized by high frequency bursts of spikes that occur after prolonged hyperpolarization of the membrane potential (Fig. 3). This characteristic feature of thalamic neurons relies on the distinctive properties of T-type calcium channels (Jahnsen and Llinas, 1984; McCormick and Huguenard 1992; Huguenard and McCormick, 1992). T-type calcium channels are depolarizing channels that are activated after prolonged hyperpolarization (~100 ms) of the membrane potential (-70 mV). This prolonged hyperpolarization is necessary to open the channels inactivation gate, thus making the neuron responsive to depolarizing events (Steriade et al., 1987; Steriade et al., 1993; Lacey et al., 2007; Parr-Bronlie et al., 2007, Bosch-Bouju et al., 2013). Once the membrane is depolarized, the T-type channel briefly activates, allowing influx of calcium ions which results in further depolarization of the membrane. When the neuronal membrane reaches threshold, voltage-

gated sodium channels are activated. As the T-type calcium channels prolong the depolarization of the membrane potential for a relatively long period (~50 ms), multiple action potentials are able to be generated. After ~50 ms the inactivation gate closes and the T-type calcium channel becomes inactivated, thus ending the LTS burst and permitting the membrane potential to repolarize (Fig.3; Jahnsen and Llinas, 1984; Huguenard and McCormick, 1992; McCormick and Huguenard, 1992; Bosch-Bouju et al., 2013).

Based on the available literature, LTS bursts observed in RTN neurons appear largely similar to the LTS bursts observed in other thalamic nuclei, (i.e. 100 ms of hyperpolarization prior to burst, first ISI <0.5 ms, subsequent ISI <0.7 ms) except the rate of spiking within RTN LTS bursts exhibits an initial acceleration followed by a subsequent deceleration in the rate of spiking (Domich et al., 1986; Steriade et al., 1986; Contreras et al., 1993). Evidence from acute RTN slice preparations demonstrated that the kinetics of T-type calcium channels are slower and greater depolarizing inputs are necessary to facilitate a similar level of recruitment, compared to other thalamic neurons (Mulle et al., 1986; Avanzini et al., 1989; Huguenard and Prince, 1992). This difference in kinetics is reported to be due to T-type calcium channels being widely distributed throughout the soma and dendrites of RTN neurons (Contreras et al., 1993; Huguenard and Prince, 1992; Destexhe et al., 1996; Crandall et al., 2010). This results in RTN neurons having unique voltage-independent calcium current inactivation kinetic properties, which consequently allow RTN neurons to generate longer duration LTS bursts, compared to other thalamic nuclei (Huguenard and Prince, 1992). Furthermore, it has been observed that after each LTS burst the firing mode in a subset of RTN neurons (~20%) switches from a bursty firing mode to a prolonged period of tonic spikes whereas the remaining neurons exhibit sustained approximately 1 Hz oscillations in activity (Domich et al., 1986; Steriade et al., 1986; Fuentealba et al., 2005). LTS bursts generated in RTN neurons have been described as incorporating more action potentials per

burst, compared to neurons in other thalamic nuclei (Sherman and Guillery, 2006). Consistent with other regions of the RTN, the rRTN exhibits LTS bursts, which have an acceleration-deceleration ISI pattern, and normally last between 2 and 6 ms (~3-8 spikes) (Schlag and Waszak, 1970; Domich et al., 1986; Marlinski and Beloozerova, 2014).

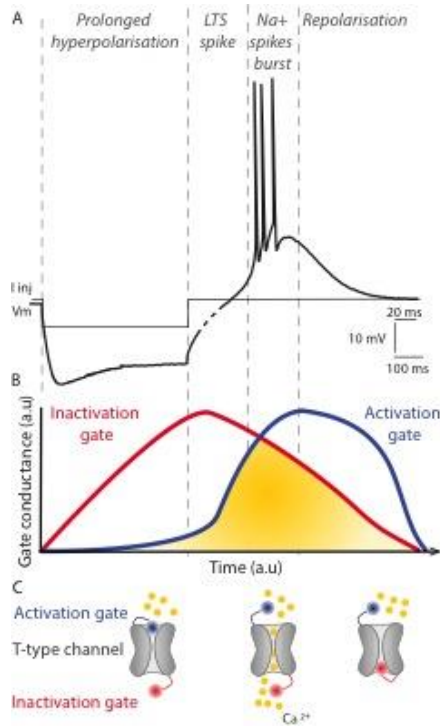


Figure 3: Schematic diagram of mechanism for LTS burst generation in thalamic neurons (Adapted from Bosch-Bouju et al., 2013).

A burst of spikes, particularly a LTS burst, will likely have different consequences on network activity than single spikes. This is because the probability of synaptic vesicular release by a single spike is generally low (Borst, 2010; Tarr et al., 2013). However, the probability of release is augmented by bursts of spikes which considerably increase the reliability of synaptic transmission (Lisman, 1997; Branco and Staras, 2009). This is true for LTS bursts and other types of bursts that are not triggered by prolonged hyperpolarization. However, compared to general bursts, LTS bursts exhibit relatively short ISIs which may allow LTS bursts to provide a more temporally precise signal than general bursts (McCormick and Huguenard, 1992). This temporally precise signal will likely further increase reliability of synaptic transmission and release of neurotransmitter (Branco and

Staras, 2009). Therefore, characterizing LTS bursts in the rRTN is important for understanding how this region of RTN is involved in the processing of motor information throughout the RTN-thalamo-cortical network.

Implications of parkinsonism on RTN neural activity

While there is a distinct paucity of studies investigating the neural activity of the rRTN under control conditions, there is an even greater paucity of information investigating the role rRTN has in parkinsonism. The following is 1) a summary of the studies investigating the link between parkinsonism and the rRTN, and 2) symptoms of parkinsonism that can be attributed to possible pathophysiology in the RTN, based on known functions of the RTN.

Previous studies by Picazo and colleagues (2009) demonstrated targeted 6-OHDA lesioning of rRTN results in the loss of up to one third of SNc dopaminergic neurons. Considering that the rRTN has afferent dopaminergic inputs arising from the SNc (Freeman et al., 2001; Anaya-Martinez et al., 2006) and is also rich in D1 and D4 dopaminergic receptors (Mrzljak et al., 1996; Khan et al., 1998; Govindaiah et al., 2010), loss of direct dopaminergic input is likely to significantly affect RTN neural activity. While PD is primarily characterized by deficits in motor function, a significant proportion of patients also suffer from sleep disorders (Nausieda et al., 1982; Factor et al., 1990; Tandberg et al., 1998). Sleep disorders are of concern as significant prolonged sleep disruption can severely impact on the patient's quality of life and could lead to psychosis or paranoia (Freeman et al., 2009; Freeman et al., 2010). Furthermore, the RTN has been implicated in providing the GABA-mediated inhibition that is necessary for the generation of spindle waves that induce sleep (Steriade et al., 1987; Bal and McCormick, 1993; Steriade et al., 1993). Further studies conducted by Weese and colleagues (1999) identified that unilateral ibotenic acid lesions to the rat RTN, significantly impaired the ability of the rat to react to the location of a visual

stimulus that was previously cued. This study while not directly assessing motor performance does clearly demonstrate the RTN has a role in regulation of behavioral control. It is therefore plausible that the loss of dopaminergic input to the RTN is a contributing factor for the generation of some of the symptoms that afflict parkinsonian patients (Nausieda et al., 1982; Steriade et al., 1986; Domich et al 1986; Factor et al., 1990; Tandberg et al., 1998).

Rationale and Aims of study

Based on the presented literature, it is highly probable that the rRTN contributes to the dysfunction observed within the basal ganglia-thalamo-cortical network during PD. This contribution to dysfunction is likely because of: 1) changes in dopaminergic SNc afferent neurons (Freeman et al., 2001; Anaya-Martinez et al., 2006), 2) changes in basal ganglia afferent nuclei (Cornwall et al., 1990; Gandia et al., 1993) and 3) reciprocal connections of the rRTN with nuclei encoding motor information (Steriade and Wyszinski, 1972; Steriade et al., 1984; Parr-Brownlie et al., 2009; Nanobashvili et al., 2012). To determine if the rRTN is affected by parkinsonism we studied rRTN neuronal activity in control rats, in order to understand the normal physiological activity of the rRTN, and also used the 6-OHDA induced chronic dopamine depletion rat model, to investigate the pathophysiological effects of parkinsonism on rRTN neuronal activity. Similar to changes observed in other structures affected by PD, it was hypothesized that unilateral 6-OHDA induced chronic dopamine depletion would cause significant changes in the neural activity of rRTN neurons, ipsilateral to the 6-OHDA lesion.

To examine this I recorded RTN neuronal activity, specifically investigating; 1) firing rate, 2) general bursting characteristics, 3) LTS burst characteristics and 4) the effects of parkinsonism. To fulfill these aims, the present study utilized the chronic 6-OHDA parkinsonian rat model. The 6-OHDA model is an established model for investigating parkinsonism in rats and allows for post-mortem verification of lesion extent. While PD

research is normally focused on the associated movement deficits in awake behaving animals we have opted for a urethane-anaesthetized model because the activity patterns in basal ganglia, cortex and motor thalamus have been well characterized, thus allowing comparison of RTN activity with other motor encoding structures. In addition, anesthesia limits any potential confounding impact of the sensory modalities, which is necessary because a significant number of inputs to RTN are sensory and the connections to rRTN are poorly defined, therefore recordings were conducted under urethane anesthesia.

Even though previous studies have suggested that the RTN solely contains GABAergic neurons, it was determined prudent, based on early electrophysiological evidence, to investigate the neuronal phenotype of the rRTN. To qualitatively examine this immunohistochemical and lentiviral transduction methods were utilized.

Methods

The following protocols were approved by the University of Otago Animal Ethics Committee and conform to their guidelines. Every effort was made throughout the study to minimize the number of animals used and their discomfort.

Animals

Thirty-five adult male Wistar rats (Hercus Tairei Research Unit, University of Otago) with free feeding weights between 180 g and 220 g the beginning of the experiment were used. Rats were maintained on a 12 hour dark/light cycle (lights off at 8:00 a.m.) at a constant temperature (22 °C) with ad libitum access to water and chow. For anaesthetized neural recordings rats were randomly assigned to control (n = 15), 6-OHDA lesion (n = 10) and sham (n = 7) groups. A further three rats were assigned to the viral vector transduction group. Rats were initially housed with their littermates. After surgery rats were housed in individual caging for one week before being housed again with their littermates, until they were euthanized.

Unilateral 6-OHDA lesion procedure and behavioral testing

Unilateral lesions of the medial forebrain bundle (MFB) were performed on rats weighing 190-260 g at the time of surgery. Ten rats underwent 6-OHDA lesion surgery. A further seven rats underwent sham surgery, where they received infusion of the saline-ascorbic acid vehicle solution. The 15 rats assigned to the control group did not undergo any form of surgery prior to anaesthetized recording.

For all lesion surgeries rats were deeply anesthetized using a cocktail of domitor (0.5 mg/kg; Pfizer Animal Health, NZ), ketamine (75 mg/kg; Parnell Technologies, NZ) and atropine (0.06 mg/kg; Phoenix Pharm, NZ) administered subcutaneously (s.c.). The surgical site was shaved and sterilized using three washes of betadine and ethanol. Lopaine (20

mg/mL, s.c; Ethical Agents Ltd, NZ) was injected along the intended incision line, to provide local analgesic protection. To protect noradrenergic neurons from the neurotoxic effects of 6-OHDA, by blocking uptake of the toxin into these neurons, rats were administered desipramine hydrochloride (15 mg/kg, i.p., Sigma-Aldrich, USA) 30 minutes prior to infusion of 6-OHDA or vehicle. Rats were mounted in a stereotaxic frame (Stoelting, USA), and ophthalmic ointment was applied to the eyes to prevent corneal dehydration. A midline sagittal incision exposed the cranial surface and the subcutaneous connective tissue was removed.

A hole was drilled through the cranium at the stereotaxic co-ordinates (4.4 mm anteroposterior, -1.2 mm lateral; relative to bregma) corresponding to the left MFB with the dorsal surface of the skull in a flat horizontal position and the underlying dura mater was reflected. A solution of 6 µg of 6-OHDA HCl (Sigma) in 3 µL of 0.9% saline solution, containing 0.1% ascorbic acid (Sigma), was infused via a cannula into the MFB. The 6-OHDA solution was infused at a rate of 1 µL/min for three minutes. To prevent diffusion of the 6-OHDA solution up the cannula track, the cannula remained in place for five minutes after infusion, and was then slowly removed. At the end of the surgical procedure the incision site was sutured and rats were administered amphotrim (30 mg/kg, s.c; Virbac Animal Health, NZ), for prophylactic antibiotic cover to prevent infection, and carprofen (5 mg/kg; s.c; Norbrook, NZ), for long acting analgesic relief. To reverse the effects of the anesthetic cocktail, rats were administered antisedan (2.5 mg/kg; s.c; Pfizer Animal Health, NZ). Body temperature was maintained at 36-38 °C throughout the surgical procedure by a heating pad. Sham surgeries followed an identical surgical procedure, except only the saline-ascorbic acid vehicle was infused into the medial forebrain bundle.

The rats were transferred to individual caging post-operatively for one week while their body weight, water intake, wound condition, behavior and general appearance was

monitored twice daily. The extent of the dopaminergic lesion was behaviorally assessed 5 to 7 days post-surgery using the step test method (Olsson et al., 1995). Briefly, the hindlimbs and the forelimb not being measured were restrained and the rat was moved slowly sideways along a one metre surface. The number of adjusting steps was counted in both the forehand and backhand directions and averaged across five trials for each rat. The forepaw contralateral to the 6-OHDA lesioned hemisphere has been identified as having significantly impaired motor performance, and is hereby referred to as the affected forepaw (Olsson et al., 1995). In contrast the forepaw ipsilateral to the lesioned hemisphere exhibits no significant impairment in motor performance, and is hereby referred to as the non-affected forepaw. Only lesioned rats that exhibited a strong lesion effect (steps affected forepaw/steps non-affected forepaw $\leq 10\%$) were selected for the present study. Previous studies have identified that severely impaired stepping behavior is associated with a 95% reduction in the number of dopaminergic neurons from the contralateral SNc (Olsson et al., 1995; Tseng et al., 2005; Parr-Brownlie et al., 2007; Avila et al., 2010). Between two and four weeks after the lesion procedure rats were prepared for anaesthetized neuronal recording.

Single-unit and local field potential (LFP) recording procedures

Extracellular single-unit and LFP recordings of the left RTN were performed between two and four weeks after unilateral infusion of 6-OHDA. Age-matched controls and age-matched sham rats underwent identical recording procedures to that of lesioned animals. Respiration rate and LFP activity were monitored throughout the recordings to ensure adequate cardiovascular performance, oxygenation and depth of anesthesia.

Recordings were performed under urethane anesthesia (1.4 g/kg, i.p., Sigma) and additional doses were given throughout the recording procedure if necessary to maintain a comparable depth of anesthesia for all rats, determined by a peak oscillatory frequency of approximately 1 Hz. This is necessary because different levels of anaesthesia are associated

with different dominant frequencies in LFP activity and thus can't be compared (Magill et al., 2000; Magill et al., 2004). The surgical site was shaved and lopaine (20 mg/mL) was injected along the intended incision line. Rats were mounted in a stereotaxic frame (Stoelting, USA) and placed on a heating pad (Harvard Apparatus, UK) to maintain body temperature. A midline sagittal incision was made and the underlying connective tissue was removed to expose the skull surface. A hole was drilled through the skull at the stereotaxic co-ordinates corresponding to the left RTN, relative to bregma in the flat skull position (AP -1.4 mm, lateral +3 mm; Paxinos and Watson, 2007) and the dura mater was reflected.

Borosilicate glass microelectrodes with impedances between 3 and 6 M Ω (measured at 135 Hz, tip diameter 1-2 μ m) were used to record extracellular action potentials and LFPs. The glass microelectrodes were produced by pulling borosilicate glass capillary tubes (Harvard Apparatus, UK) into the desired shape using a microelectrode puller and were filled with 2% Chicago Sky Blue dye (Sigma) in 2 M NaCl solution. A microelectrode was lowered into the brain until it was just above the RTN (dorsoventral 4.5-7.00 mm, relative to dura) and then advanced slowly ($\sim$ 200 μ m/min) through the RTN using a hydraulic microdrive (KOPF, USA) until a neuronal signal was detected. Signals were recorded at sampling rates of >20 kHz for spikes and 1 kHz for LFPs (Axoclamp 900A, Molecular Devices, USA; Model 3600, A-M Systems Inc., USA). Spikes were amplified (5000x), bandpass filtered (0.3-5000 Hz) and monitored using a Spike2 template matching window (version 7.10, Cambridge Electronic Design, UK) and an audiomonitor (Grass Technologies, USA). Similarly, LFPs were amplified (5000x), bandpass filtered (0.3-100 Hz) and recorded using Spike2 software. Discriminated signals were digitized, stored and analysed using the Spike2 data acquisition and analysis software. Recording sites were at least 20 μ m apart and recordings typically lasted for eight minutes.

Data analyses

For analyses, a 300 sec epoch of simultaneously recorded LFP and neuronal spike train activity that was free from artifacts was used from every neuron recorded within the boundaries of the RTN. At the beginning of the study it was decided that the ideal epoch would be 200-500 s as this would allow enough time for a stable recording to be established and any initial fluctuations in the firing of the neuron to stop. However, in some instances of artefact was present in this epoch so, alternative pre-determined 300 s epochs were used (100-400 s or 400-700 s).

Spike train regularity was assessed using the interspike interval coefficient of variation (ISI CV; Magill et al., 2000; Magill et al., 2001). ISI CV was calculated by dividing the standard deviation of ISI by the mean of the ISI for each 300 second epoch. Firing rates were calculated from the reciprocal of the mean ISI.

Spike train burstiness was investigated as a measure of firing pattern because changes in burstiness can have significant effects on the probability of neurotransmitter release (Izhikevich, 2000; Rulkov, 2001; Belykh et al., 2005; Butera et al., 1999). Two different types of burst analyses were used to analyze the spike train data. First, general spike train burstiness was assessed across all recorded neurons using the density discharge histogram method (Kaneoke and Vitek, 1996; Parr-Brownlie et al., 2007; Parr-Brownlie et al., 2009). This method is a statistical test to define bursts. It is based on the assumptions that a spike train is comprised of both burst and non-burst periods and that the burst periods are fewer than the non-burst periods. Briefly, the method identifies these burst periods by determining the mean ISI for the spike train and then constructing the discharge density histogram. For this analysis a burstiness index ≥ 0.5 was used, which would give the density discharge histogram a bin width of twice the mean ISI, thus is a conservative measure to detect significant bursts. The spike train epochs were classified as bursty if; 1) the discharge density

histogram distribution was significantly different to a Poisson distribution, 2) the histogram was positively skewed, 3) there was a minimum of three spikes per burst and 4) the baseline firing rate was greater than one spike per second (Magill et al., 2000, Magill et al., 2001; Parr-Brownlie et al., 2007). The measures of general spike train burstiness included; percentage of spikes in bursts, number of spikes per burst and number of bursts per 1000 spikes. To normalize the burst data across spike trains with differing average firing rates, the number of bursts per 1000 spikes per spike train was used instead of the number of bursts per spike train. This was calculated using the following formula;

$$\# \text{ bursts per 1000 spikes} = \frac{(\% \text{ spikes in bursts} \times 1000)}{\text{mean \# of spikes per burst}}.$$

The second burst analysis investigated the characteristics of LTS bursts. LTS bursts are a prominent feature of thalamic relay neurons in anaesthetized animals, and are initiated by sustained hyperpolarization of the neuronal membrane which de-inactivates T-type calcium channels (Bosch-Boujou et al., 2013). This allows the influx of calcium ions and generation of action potentials that have very short ISIs (Jahnsen and Llinas, 1984). In this regard LTS bursts can be distinguished from general bursting activity as they are based on physiological criteria as opposed to a statistical measure. The criteria used to define LTS bursts are based on initial patch-clamping experiments (Jahnsen and Llinas, 1984; Lu et al., 1992) but have since been reproduced using proxies in extracellular studies; 1) the first ISI was ≤ 5 ms, 2) subsequent ISIs were ≤ 7 ms and 3) the burst was preceded by a silent period >100 ms, which serves as a proxy for the period of hyperpolarization that is necessary to elicit LTS bursts (Steriade et al., 1985; Steriade et al., 1990; Lacey et al., 2007; Parr-Brownlie et al., 2009). The measures of LTS bursting activity were similar to those of general bursting and included; percentage of spikes in LTS bursts, number of LTS bursts per 1000 spikes, number of spikes per LTS burst and the proportion of each type of LTS burst (i.e. doublets, triplets, etc.). The number of LTS bursts per 1000 spikes was calculated using a

similar formula to that of general bursting activity. For all LTS bursting activity measures the data were analyzed in terms of mixed, doublet and non-doublet LTS bursts. This was performed to identify if different types of LTS burst are differentially affected by parkinsonism. The mixed group of LTS bursts contained both doublet and non-doublet LTS bursts.

To investigate any possible changes occurring in LFP power due to lesion, the LFP recordings from each neuron was smoothed to 20 Hz and high pass filtered at 0.3 Hz (Spike2). LFP power was calculated using 256 block Fast Fourier Transform, resulting in frequency resolution bins of 0.078 Hz (Spike2). Total LFP power was calculated for the entire 0.3-2.5 Hz range by averaging the sum of the power in each bin across each experimental group (Moran et al., 2006; Zaidel et al., 2009; Parr-Brownlie et al., 2009).

To categorize recorded RTN neurons as having either broad or narrow waveforms, the action potential width (ms) for each neuron between the first peak and trough of its mean action potential was measured, regardless of the order in which the peak and trough occurred (Wilson et al., 1994; Parr-Brownlie et al., 2007). In the present study all but six neurons exhibited a peak-trough pattern in the shape of their action potential; the other six neurons exhibited a trough-peak pattern. Previous studies have identified that neuronal phenotype can be distinguished based on action potential width (Tseng et al., 2006; Parr-Brownlie et al., 2007). Generally, studies consistently report glutamatergic and GABAergic neurons as exhibiting bimodal action potential width distributions, with GABAergic neurons having narrow waveforms and glutamatergic neurons having broad waveforms (Rao et al., 1999; Tseng et al., 2006; Parr-Brownlie et al., 2007). Figure 4 shows the mean action potential width from 189 neurons recorded in RTN. The weighted sum of two Gaussian curves, fitted using Prism, best fitted the action potential width distribution ($r^2 = 0.875$). Those neurons with action potential widths within two standard deviations of the mean of the narrow

Gaussian distribution were classified as narrow waveforms. Neurons with action potential widths within two standard deviations of the mean of the broad Gaussian distribution were classified as broad waveforms. Action potential widths that belonged to both individual Gaussian distributions formed an intermediate waveform group (unfilled bars). The narrow waveform group had action potential widths of ≤ 0.47 ms and were classified as putative GABAergic neurons (53.8% of neurons). The broad waveform group had action potential widths of ≥ 0.52 ms and were classified as putative glutamatergic neurons (33.5% of neurons). The intermediate group (12.6% of neurons) had action potential widths between 0.47-0.52 ms and remained undefined in terms of putative neuronal phenotype. This group of intermediate neurons was not used in any of the reported analyses. The mean ($\pm$ standard error of the mean; SEM) peak to trough widths for putative GABAergic and putative glutamatergic neurons were 0.37 ± 0.01 ms and 0.61 ± 0.02 ms, respectively. For the purposes of readability putative GABAergic neurons and putative glutamatergic neurons will henceforth be referred to as GABAergic and glutamatergic neurons respectively.

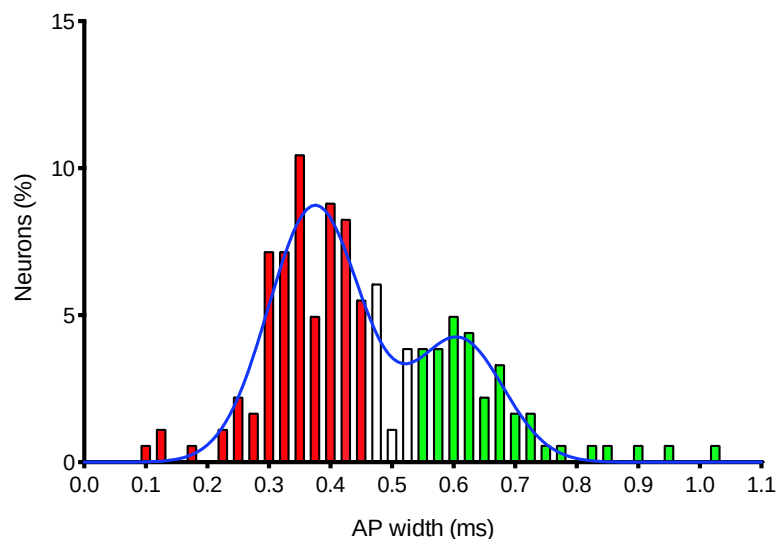


Figure 4: Determining putative neuronal phenotype from width of action potential waveform. Percentage of neurons with action potential widths (ms) between 0.09 ms and 1.05 ms. Three groups of neurons were identified based on their waveforms; narrow (red, 0.09-0.46 ms), intermediate (unfilled bars, 0.47-0.52 ms) and broad (green, 0.53-1.05 ms). Blue line demonstrates the fit of the weighted sum of two Gaussian distributions.

Statistical Analyses

The overall effects of lesion on firing rate, spike train regularity, the percentage of spikes in bursts or LTS bursts, number of spikes per burst or LTS burst, the number of bursts or LTS bursts per 1000 spikes and LFP power analyses were compared using two-way analysis of variance (ANOVA) with group (control, sham and lesion) and neuronal phenotype (GABA and GLUT) as factors. Tukey post-hoc comparisons were analyzed to determine significant effects. ISIs in LTS bursts were analyzed using a three-way ANOVA, with group (control, sham and lesion), size of LTS bursts (doublets to sextuplets) and position of the ISI in LTS bursts (first ISI vs second ISI, etc) as factors. The use of three-way ANOVA was necessary because when investigating LTS bursts in conjunction with the parkinsonian lesion, the burst type and the position of the interspike interval are independent of each other. The incidence of spike trains with LTS bursts in both GABAergic and glutamatergic neurons were compared using chi-squared statistical analysis (version 6, GraphPad Software, USA). Two-way ANOVAs and associated statistical analyses were performed using GraphPad Prism statistical software (version 6, GraphPad Software, USA). A three-way ANOVA to investigate the properties of ISIs was performed using Mathematica statistical software (version 9.01, Wolfram Research, USA). All data are presented as means $\pm$ SEM. Criterion for statistical significance was $p \leq 0.05$.

Histological verification of final recording sites

Following the completion of recordings, the last recording sites were marked by iontophoresis of Chicago Blue dye (-23 μ A for ≥ 25 mins, World Precision Instruments, USA). Animals were euthanized, brains extracted and post-fixed in 4% paraformaldehyde (PFA) for one week. Fixed tissue was transferred to 30% sucrose solution prior to being sectioned on a freezing microtome. Serial sections (40 μ m) were taken of the RTN and SNc from all rats. Sections were either mounted on glass microscope slides for staining with neutral-red

solution (FD Technologies, USA) or stored in cryoprotectant solution (0.04 M Tris-buffered saline, 30% ethylene glycerol, 30% glycerol) at -20°C until needed for immunohistochemical staining protocols.

To verify the last recording sites, a series of sections of the RTN from all recorded rats were stained with neutral red solution to help visualize the iontophoresed Chicago Blue dye with respect to the surrounding brain structures. Briefly, microscope slide mounted sections were submerged in neutral red solution for 10-12 minutes. Sections were rinsed in distilled water and air dried (20 mins). Sections were then dehydrated in 100% ethanol (3x2 mins), cleared in HistoClear (3x2 mins, Sigma-Aldrich) and coverslipped with Permount (Sigma). Sections were analysed using a light microscope to identify the last recording site and an image was digitally stored. The recording sites from earlier in each experiment were reconstructed for all neurons using the recording co-ordinates. Only neurons located within the confines of the RTN were included in analyses.

Qualitative assessment of neuronal phenotype

To assess neuronal phenotype, selected sections of rRTN slices were labelled with GAD67, EACC1 and 4',6-diamidino-2-phenylindole (DAPI). GAD67 is a neuronal marker specific to GABAergic neurons. EACC1 is a neuronal marker for the vesicular glutamate transporter protein. DAPI is a fluorescent stain that binds to regions of DNA and thus allows visualization of nuclei. The details of the labelling procedure are as follows. Slices were selected and stored overnight in phosphate buffered saline (PBS) to remove the cryoprotectant solution. To remove any residual cryoprotectant solution the tissue was washed in PBS (3x10 mins). Slices were permeabilised with PBS containing 0.2% Triton (PBS-T; 2x20 mins) and blockaded for one hour in 5% rabbit serum (RS) containing PBS-T. Slices were incubated overnight in 2% RS PBS-T that contained both mouse anti-GAD67 (1:1000; Abacus ALS) and goat anti-EACC1 (1:1000; Abacus ALS) antibodies. Tissue was

washed in PBS-T (2x20 min) and incubated for four hours in 2% RS PBS-T containing donkey anti-mouse Alexa 488 (1:1500; Life Technologies) and Cy3 (1:800; Abacus ALS). After incubation sections were washed in PBS (2x10 min) and phosphate buffer (PB; 10 min). Slices were mounted on glass slides and allowed to air dry for 30 minutes. Slices were mounted in vectashield hardset with DAPI (Invitro Technologies) and coverslipped. Slides were stored at 4°C until required for viewing. The immunolabelled tissue sections were visualized using a Zeiss LSM 710 Upright Confocal Microscope to determine the presence and co-localisation of DAPI, GAD67, and EACC1. The confocal images were analysed using ZEN (2009) microimaging software (Carl Zeiss, USA).

Qualitative assessment of dopaminergic lesion

To qualitatively assess the extent of 6-OHDA induced dopaminergic lesion another set of rat brain slices (4 slices per rat, 10 rats) containing the SNc were selected and stored for tyrosine hydroxylase (TH) and DAPI immunolabelling. The tissue storage and immunolabelling protocol were similar to that stated above, with the following key differences. Slices were blocked in 10% normal goat serum (NGS) for one hour at room temperature. Slices were incubated overnight in 5% NGS PBS-T solution containing the rabbit anti-TH antibody (1:300). After incubation, tissue was washed in PBS-T (3x10 min) and incubated for four hours at room temperature with 5% NGS PBS-T containing the Alexa 488 antibody (1:1000). Slices were washed in PBS (3x10 min) and PB (1x10 min). Slices were mounted and covered with vectashield hardset with DAPI and coverslipped. Slides were stored at 4°C until required for viewing. Visualization of the immunolabelled sections was the same as that mentioned previously for determining neuronal phenotype.

Lentiviral (LV) mediated anatomical study

To assist in determining the neuronal phenotype present in the rRTN; a group of three rats, that were not used for electrophysiology, were used for an anatomical study using the lentiviral construct LV-CaMKII α -mCherry. This lentiviral construct specifically targeted, and allowed subsequent transduction of, glutamatergic neurons, due to the CaMKII α promoter (Naldini et al., 1996; Watson et al., 2002). The use of the VSVg glycoprotein on the viral envelope allowed for only those neurons with somata and/or dendrites in the rRTN to be transduced (Naldini et al., 1996; Watson et al., 2002). Expression of the lentiviral construct was regulated by the CaMKII α promoter which would result in expression of the construct in CaMKII α positive, and likely glutamatergic neurons (Kennedy et al., 1983; Liu and Jones, 1996; Gradinaru et al., 2009). The mCherry reporter protein enabled post-mortem identification and verification of transduced neurons. The phenotype of transduced neurons was confirmed by co-expression of mCherry with glutamatergic markers and the lack of co-expression for markers of GAD67.

Lentiviral vector production

Approval for the use of recombinant LV vectors was obtained from the Environmental Protection Authority (EPA) New Zealand (GMD03091). The LV-CaMKII α -mCherry construct was produced, amplified and packaged by the Neural Development and Disease Laboratory at the University of Otago. Briefly, lentiviral packaging followed a modified protocol of Linterman and colleagues (2011) in which HEK293 cells were transfected with packaging and transgene plasmids, using the lipofectamine 2000 reagent transfection system (Invitrogen, USA). The resulting mixture was incubated overnight at 37°C in an atmosphere containing 5% CO₂. Virus was harvested by centrifuging media at 3000 rpm for 15 minutes. Virus was purified by supernatant filter sterilization and

centrifuging at 25000 rpm for 90 minutes. Supernatant was removed and the pellet was re-suspended in lactose (40 mg/mL), aliquotted (5 μ l/cryotube) and stored at -80°C.

Lentiviral surgical procedure

The three rats used for the anatomical study were bilaterally infused with the LV-CaMKII α -mCherry construct. Three injections of 300 nL per hemisphere (900 nL total volume) centered around the stereotaxic co-ordinates corresponding to the rRTN (AP -1.4 $\pm$ 0.3 mm, ML $\pm$ 2.0, DV -6.0) were made using a microsyringe (World Precision Instruments Inc., USA). All surgical procedures followed the survival surgery procedure outlined above for 6-OHDA lesion surgery with the following exceptions. Viral construct was infused at a rate of 100 nL/min for three minutes using a Hamilton syringe and pump. The syringe remained in place for a further five minutes after infusion to prevent unwanted diffusion of the viral construct along the cannula track as the syringe was withdrawn. Animals were transferred to isolation cages for one week before being re-housed together in conventional cages for the remainder of the study.

Qualitative assessment of lentiviral transduced neurons

Eight weeks after transduction with LV-CaMKII α -mCherry, rats were euthanized by administration of an overdose of the anesthetic sodium pentobarbitone (100 mg/kg; i.p; Provent, NZ), mixed with 0.9% saline. The rats were transcardially perfused with 100 mL of 10% sucrose solution followed by 400 mL of 4% PFA solution. Once the tissue was fixed, the rat was decapitated, the brain extracted and stored in 4% PFA overnight at 4°C. After 24 hours, brains were transferred into 30% sucrose solution and stored at 4°C until sectioned on a freezing microtome. Serial coronal sections (40 μ m) were collected of the RTN and surrounding structures. Sections were transferred to cryoprotectant solution and stored at -20°C until required for immunohistochemistry.

To prepare for the immunohistochemical staining procedure selected sections were transferred to PBS overnight, to remove cryoprotectant solution. Sections were washed in PBS (3x10 min), permeabilized in PBS-T (2x20 min) and blocked for one hour in 5% RS PBS solution. After blockade, sections were incubated overnight with the mouse anti-GAD67 antibody (1:1000) in 2% RS PBS at 4°C. After the primary incubation, sections were washed in PBS-T (2x20 min) and incubated with PBS-T-RS 2% for four hours containing donkey anti-mouse Alexa 488 antibody (1:1500). After the secondary incubation, sections were washed in PBS (2x10 min) and PB (1x10 min). Sections were mounted on glass microscope slides with PB and allowed to air dry (~30 min). Finally, sections were mounted in vectashield hardset with DAPI and coverslipped. RTN tissue sections obtained from the virally transduced rats were visualized using a Zeiss LSM 710 Upright Confocal Microscope to determine the expression pattern of GAD67 and the LV-CaMKII α -mCherry, as well as co-localization with DAPI. The confocal images were collected and analysed using ZEN (2009) microimaging software (Carl Zeiss, USA).

Results

The present study aimed to characterize the neural activity of the rRTN and elucidate whether the rRTN is affected by parkinsonism. To achieve this data were obtained from 189 neurons (99 control, 59 lesion, 31 sham) recorded from 32 anaesthetized rats (15 control, 10 lesion, 7 sham) was analysed. As expected there were no significant difference between control and sham groups for most measures investigated and therefore average data in the text are given across the control and sham groups, but statistics are shown for the full analyses. Data were also obtained from three rats injected with a viral vector to verify the presence of a population of non-GABAergic neurons in the rRTN.

Assessment of 6-OHDA induced chronic dopamine depletion

The extent of 6-OHDA lesion was tested behaviorally using the step-test to ascertain if the lesion had been successful (Fig. 5). The step-test measures the ability of the lesion affected forepaw to step relative to the non-affected forepaw. There was no difference in the ability of control or sham animals to step, because on average they had a step-test score of 1.00 ± 0.01 . This demonstrates that both paws are equally adept at stepping. In comparison, lesioned animals exhibited a 92% reduction in the ability of the lesion affected paw to step relative to the non-affected paw, as they had an average step test score of 0.08 ± 0.01 . One-way ANOVA with group (control, sham and lesion) as a factor revealed a significant effect of lesion on the mean stepping score ($F_{(2, 20)} = 1554$, $p < 0.0001$). Post-hoc analysis revealed a significant decrease in the mean stepping score of lesioned rats compared to both control ($p < 0.0001$) and sham ($p < 0.0001$). This is consistent with previous literature demonstrating a unilateral 6-OHDA lesion directed at the MFB results in a significant reduction in the stepping ability of the affected paw (Olsson et al., 1995; Parr-Brownlie et al., 2007). It has previously been demonstrated that a >90% 6-OHDA lesion in the parkinsonian rat model is

equivalent to the 80-85% neurodegeneration of the SNc observed in advanced PD patients (Fearnley and Lees, 1991; Nandhagopal et al., 2011; Djaldetti et al., 2011).

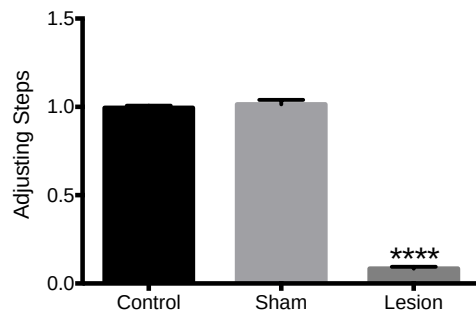


Figure 5: Behavioral assessment of 6-OHDA induced chronic dopamine depletion of the SNc. Parkinsonian rats demonstrated a significant reduction in adjusting steps by the lesion affected forepaw relative to the non-affected one, compared to both control and sham. Significant level: **** = $p < 0.0001$.

The extent of 6-OHDA lesion of dopamine neurons was verified via TH immunohistochemical staining. A series of 40 μm thick coronal slices of the SNc was obtained from each lesioned rat post-mortem. Figure 6 represents a typical image of the SNc obtained from the confocal microscope, which shows many TH stained neurons were located in the SNc of the non-lesioned hemisphere whereas staining was absent in the lesioned hemisphere of the same rat. Both the behavioral and TH immunohistochemical staining indicate significant depletion of dopamine in chronic 6-OHDA lesioned rats in this study.

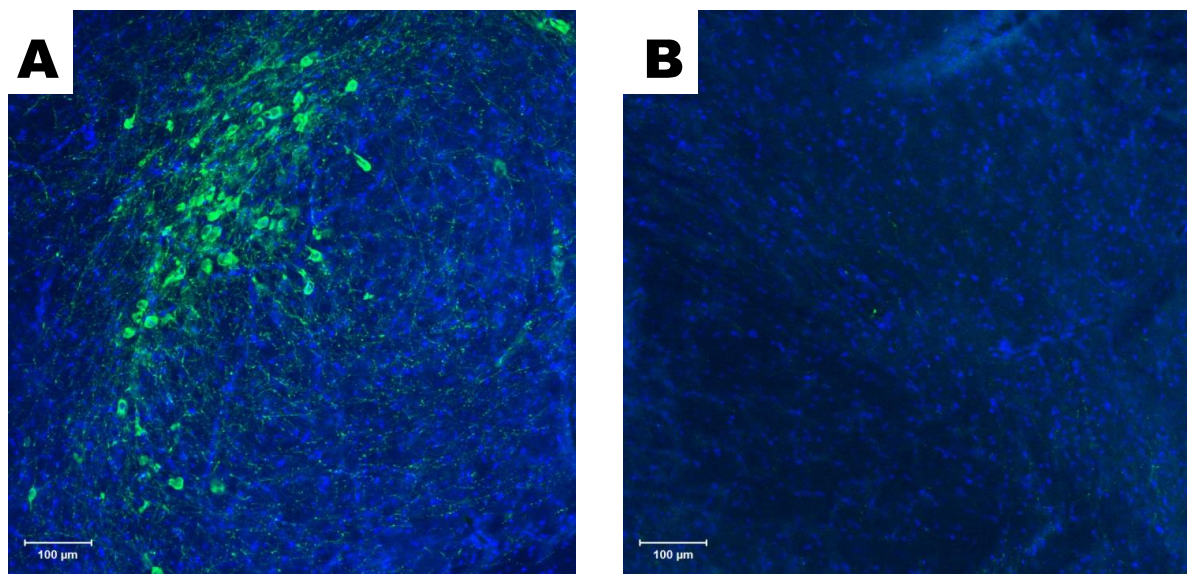


Figure 6: A) TH staining (green) in the SNc of the non-lesioned hemisphere of parkinsonian rats shows many TH stained neurons. B) No TH staining occurred in a comparable region in the lesioned hemisphere of the same parkinsonian rat. DAPI (blue) indicates the presence of nuclei. Scale bars equal 100 μm .

Histological assessment of final recording sites and neuronal phenotype

Final recording sites in the RTN were marked by iontophoresis of Chicago blue dye and the locations were verified by collecting a series of 40 μm thick coronal slices from each rat post-mortem. Slices were stained with neutral-red solution to aid identification of the RTN and surrounding structures. All recording sites were reconstructed for each rat based on the position of the final recording site and are summarized in Figure 7. All recording sites were between -1.3 mm and -1.5 mm relative to bregma, and were predominately clustered around -1.44 mm. A total of 160 neurons across the control, sham and lesion groups were phenotypically identified as either GABAergic (59 control, 12 sham, 34 lesion) or glutamatergic (20 control, 15 sham, 20 lesion). Therefore, 34.7% of recorded neurons are thought to be glutamatergic.

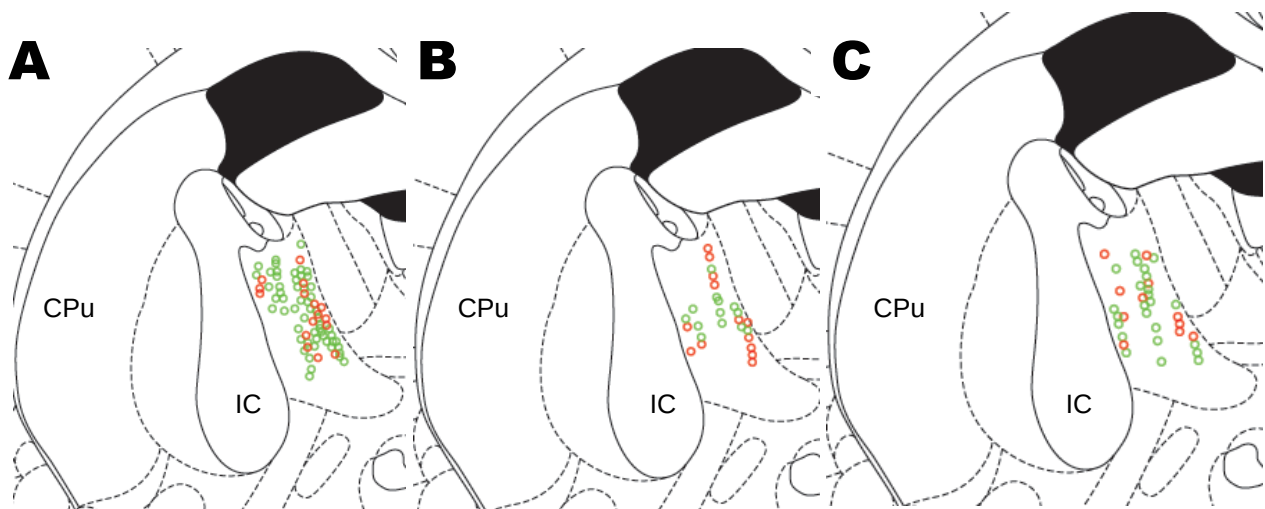


Figure 7: Summary of RTN recording sites from control (A), sham (B) and lesioned (C) rats. Green circles indicate the position of putative GABAergic neurons ($n= 59, 12$ and 34 for control, sham and lesioned groups, respectively). Red circles indicate the position of putative glutamatergic neurons ($n= 20, 15$ and 20 for control, sham and lesioned groups, respectively). All recordings were between -1.3 mm and -1.5 mm relative to bregma. Recording sites have been placed on schematic coronal plates of -1.44 mm relative to bregma (Paxinos and Watson, 2007). CPu = Caudate putamen, IC = Internal capsule.

To ascertain the phenotype of neuronal populations present in the RTN, 40 μm thick coronal slices were immunohistochemically stained with DAPI, EACC1 and GAD67 to identify nuclei, glutamatergic neurons and GABAergic neurons, respectively (Fig. 8).

Confocal microscopy confirmed that the soma of the majority of neurons in the RTN were positive for GAD67 and EACC1, consistent with the idea that the nucleus primarily contains GABAergic neurons. Considering that EACC1 stains the vesicular glutamate transporter it is expected that it would be present in GABAergic neurons because GABA is a derivative of glutamate. However, the soma of a number of RTN neurons were EACC1 positive and GAD67 negative, suggesting the presence of a population of putative glutamatergic neurons (Fig. 8).

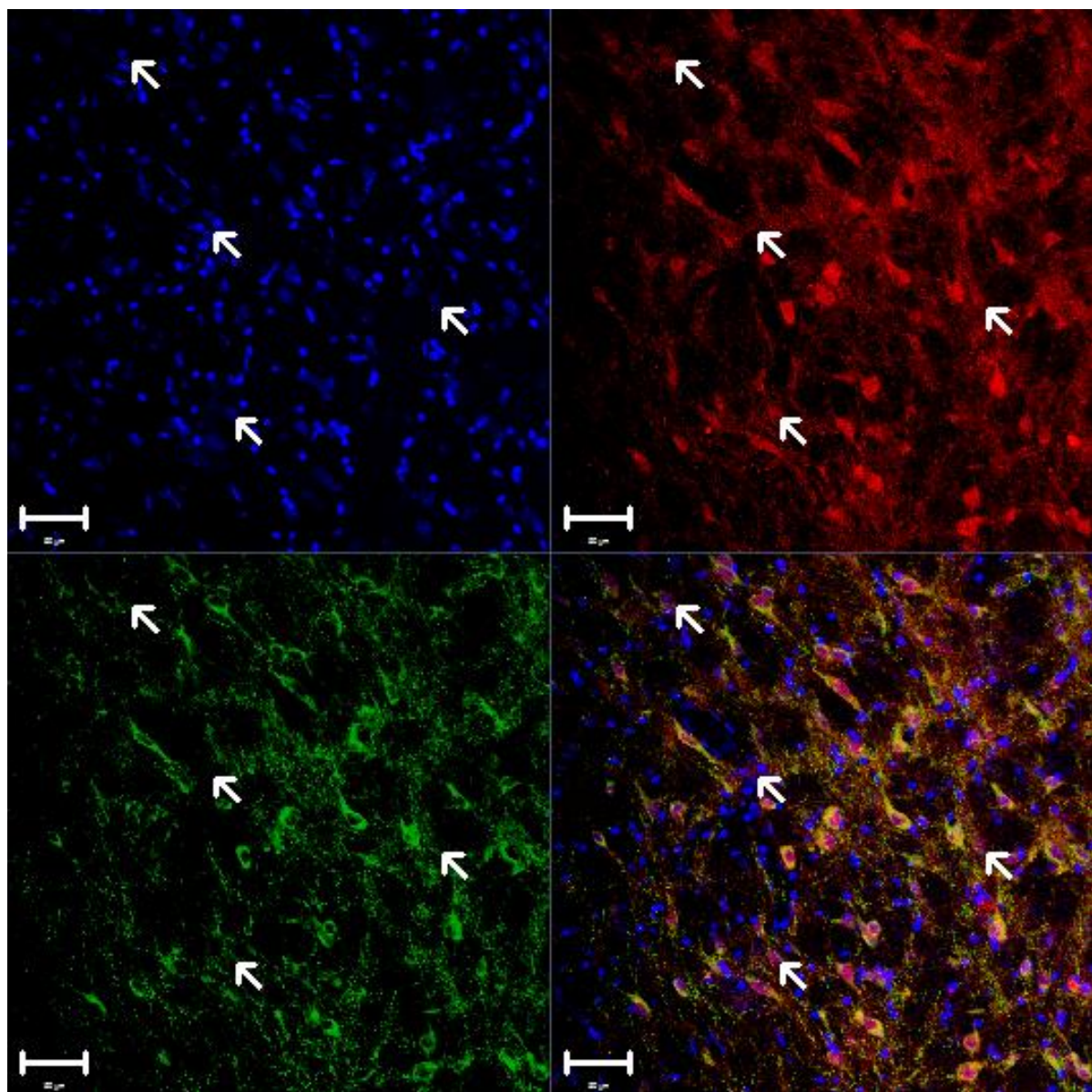


Figure 8: Example immunohistochemical staining of neurons in the RTN. DAPI (blue) indicates the presence of nuclei. GAD67 (green) and EACC1 (red) indicate the presence of GABA and glutamate respectively. Yellow staining (bottom right) corresponds to neurons that co-express glutamate and GABA. Arrows indicate neurons that are likely glutamatergic as they co-express DAPI and EACC1, but are GAD67 negative. Images are taken from 40 μm thick coronal slices. Scale bars equal 50 μm .

To further explore the possibility that there is a population of glutamatergic neurons present in the RTN, the LV-CaMKII α -mCherry construct was injected into the same approximate area of the RTN as the recording sites. Coronal slices (40 μ m) were collected from rats that were transduced with the viral construct and stained with DAPI. Punctate staining of innate mCherry was observed in a small number of neurons that were transduced within the RTN. This staining confirmed the presence of a population of putative glutamatergic neurons in the RTN (Fig. 9).

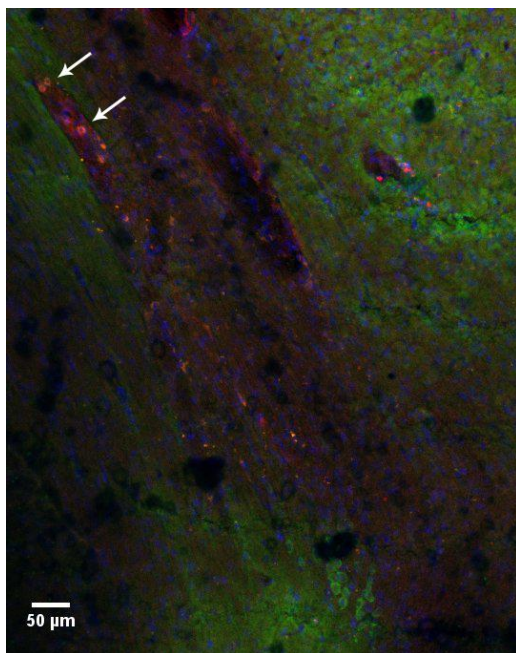


Figure 9: Example of immunohistochemical staining of lentiviral transduced glutamatergic neurons in the RTN. Innate mCherry (red), CaMKII α (green) and DAPI (blue) indicate the presence of lentiviral transduced neurons, glutamatergic neurons and nuclei, respectively. Arrows indicate neurons that are likely glutamatergic as they exhibit co-expression of mCherry, CaMKII α and DAPI. Images are taken from 40 μ m thick coronal slices. Scale bars equal 50 μ m.

Electrophysiology of RTN neurons

Neuronal activity was recorded in anaesthetized rats to determine the effect that parkinsonism had on the firing rate and pattern of rRTN neurons. Electrophysiological recordings were obtained from control, sham and lesioned anaesthetized rats. Only electrophysiological recordings with LFPs that were dominated by $\sim$ 1 Hz activity, had neurons with 300 second epochs free from artifact and were identified to be within the boundaries of the RTN were used for analyses. The power spectra and total LFP power

analysis across all recorded neurons is demonstrated in Figure 10. One-way ANOVA with group (control, sham and lesion) as a factor identified no significant effect ($F_{(2, 153)} = 2.199$, $p = 0.1144$) of group on the total LFP power.

Due to the immunohistochemical (Fig. 8 and 9) and electrophysiological (Fig. 4) evidence which suggests that there were both GABAergic and glutamatergic neuronal populations present within the rRTN, the effect of parkinsonism on these two putatively defined populations was investigated. While the neuronal populations remain only putatively determined, in the interests of readability the two populations are hereby referred to as GABAergic and glutamatergic.

For the following analyses, neurons that formed an intermediate population, defined as having an action potential width lying within two standard deviations of the mean of the two defined populations, were excluded from analyses (Fig. 4). This intermediate population included 23 neurons obtained from 10 control, 4 sham and 6 lesioned rats. Data from the GABAergic (101 neurons; AP width <0.47 ms) and glutamatergic (63 neurons; AP width >0.52 ms) neuronal populations were assessed using a two-way ANOVA to analyze the effects of experimental group (control, sham and lesion) and neuronal phenotype (GABA and GLUT) on neural activity. Example spike trains and LFP recordings from both GABAergic and glutamatergic neuronal populations are shown in Figure 11.

The measures of neuronal activity investigated include firing rate, spike train regularity, general bursting activity and LTS bursts. Spike train regularity was assessed using ISI CV. General bursting activity was assessed using the density discharge histogram method (Kaneoke and Vitek, 1996). From this analysis we obtained the percentage of spikes in bursts, number of bursts per 1000 action potentials and number of spikes per burst. LTS bursting activity included the percentage of spikes in LTS bursts, number of LTS bursts per 1000

spikes, number of spikes per LTS burst and the percentage of LTS burst type. The spike frequency adaptation of LTS bursts was also explored in control and parkinsonian rats.

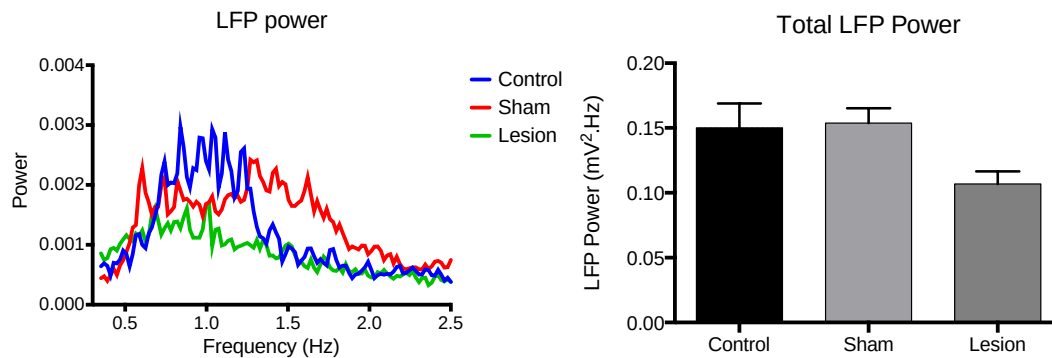


Figure 10: LFP power spectra in control, sham and lesioned animals (left). Total power (right) did not significantly differ between control, sham or lesioned animals. The peak LFP frequency was approximately 1 Hz for all groups.

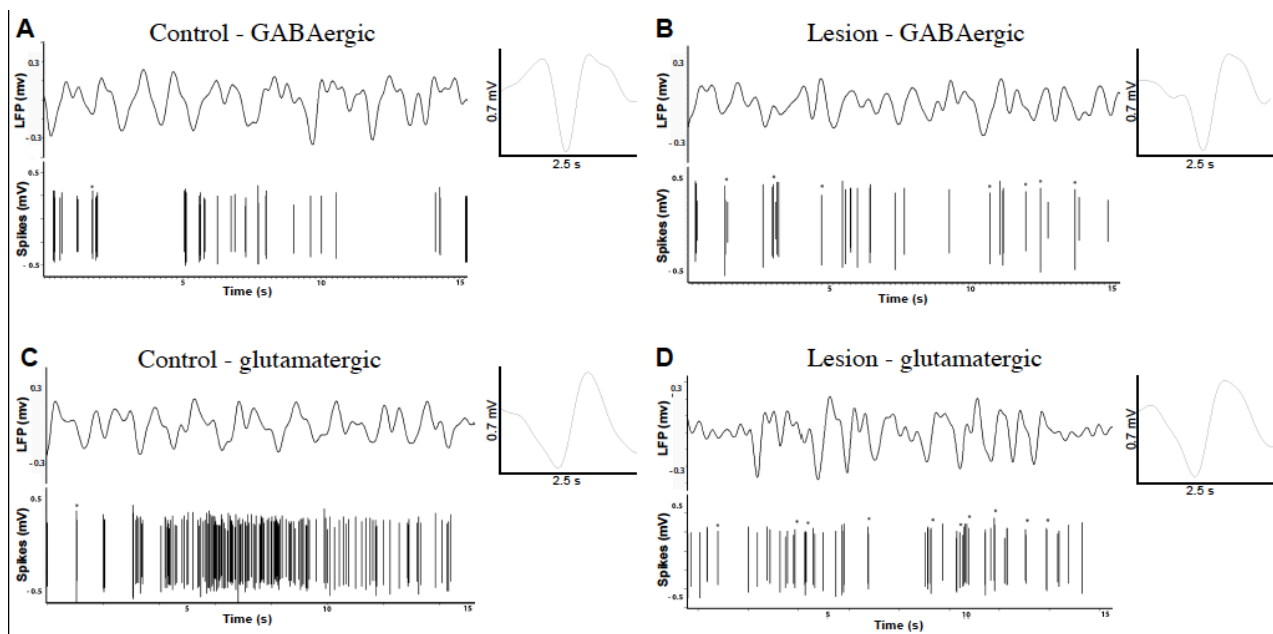


Figure 11: Fifteen seconds of simultaneously recorded RTN spike trains and LFPs from the GABAergic (top) and glutamatergic (bottom) neuronal phenotypes. Insets (right) demonstrate the AP width. The firing rate of GABAergic control (A) was 3.97 Hz with an AP width of 0.4049 ms, whereas for lesion (B) it was 2.61 Hz with an AP width of 0.4498 ms. The firing rate for the glutamatergic control neuron (C) was 6.19 Hz with an AP width of 0.5372 ms, whereas for lesion (D) it was only 2.14 Hz with an AP width of 0.5464 ms. The dominant LFP frequency for all four neurons was ~1 Hz.

Effect of 6-OHDA lesion and neuronal phenotype on firing rate

Figure 12 illustrates the effect of 6-OHDA induced chronic parkinsonism on mean firing rate of GABAergic and glutamatergic RTN neurons. GABAergic neurons had a mean firing rate of 4.9 ± 1.1 spikes/s across the control and sham groups, whereas glutamatergic

neurons had a mean firing rate of 6.2 ± 1.9 spikes per second. The lesioned animals demonstrated lower firing rates in both GABAergic and glutamatergic neuronal populations. A two-way ANOVA with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.92$, $p = 0.4019$) or effect of neuron phenotype ($F_{(1, 154)} = 0.92$, $p = 0.3383$) on the mean firing rate. However, there was a significant effect of group ($F_{(2, 154)} = 4.45$, $p = 0.0132$) and post-hoc analyses revealed that the mean firing rates were significantly reduced by 6-OHDA induced parkinsonism, compared to control ($p < 0.05$) and sham ($p < 0.05$) groups. There were no significant differences between the control and sham groups ($p > 0.05$). The observation that there was no significant difference between control and sham recordings was consistent for *all* variables analysed in the present study.

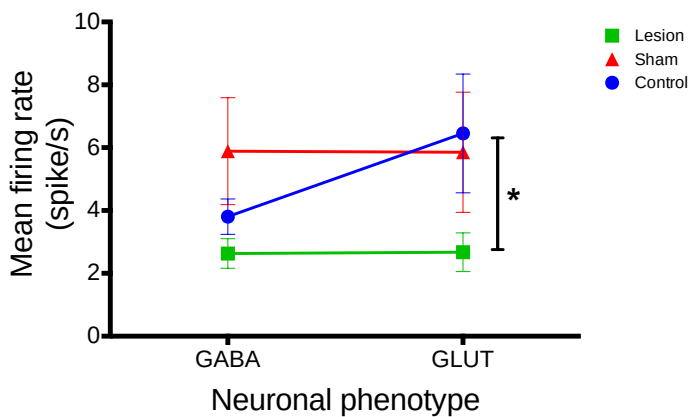


Figure 12: Mean ($\pm$ SEM) firing rate (spike/s) of RTN neurons in control (blue), sham (red) and 6-OHDA lesion (green) groups. Firing rate was significantly lower in the neurons recorded in lesioned rats ($n = 79$) compared to control ($n = 54$) and sham ($n = 27$) groups. Significant level: * = $p < 0.05$.

Effect of 6-OHDA lesion and neuronal phenotype on firing pattern

Figure 13 illustrates the effect of 6-OHDA induced chronic parkinsonism on the spike train regularity, measured by ISI CV, of recorded RTN neurons. GABAergic neurons from control and sham had an average ISI CV of 1.4 ± 0.1 , whereas the average for glutamatergic neurons was 1.6 ± 0.2 . A two-way ANOVA with group (control, sham, lesion) and neuronal phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 1.60$, $p =$

0.2043), effect of neuronal phenotype ($F_{(1, 154)} = 0.68$, $p = 0.4097$) or group ($F_{(2, 154)} = 1.65$, $p = 0.1946$) on spike train regularity. Therefore, 6-OHDA chronic dopamine induced lesion did not affect spike train regularity for either the GABAergic or glutamatergic neuronal populations.

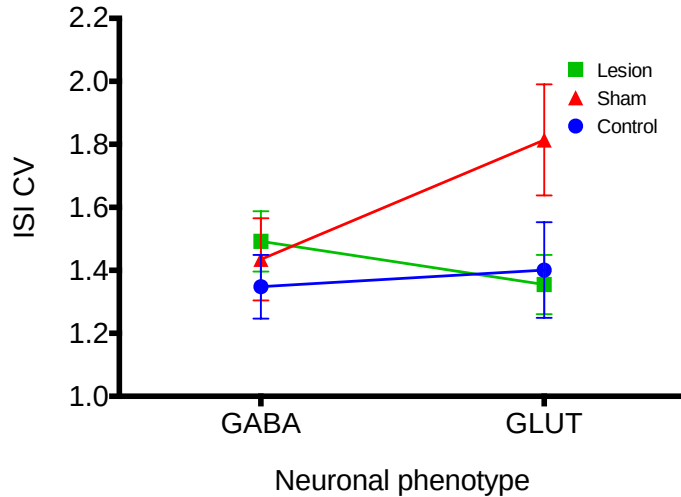


Figure 13: Mean ISI CV ($\pm$ SEM) of GABAergic and glutamatergic RTN neurons in control (blue), sham (red) and lesion (green) groups. ISI CV was not significantly affected by parkinsonism or neuronal population.

Effects of PD and neuronal phenotype on general bursting activity

The second measure used to assess firing pattern was an analysis of general burstiness using the Kaneoke and Vitek (1996) method. This method is a statistical test to determine if any ISIs within the 300 second epoch were significantly shorter than the average ISI across the entire epoch. Three variables of general burstiness were investigated; 1) number of bursts per 1000 spikes, 2) percentage of spikes within bursts and 3) the mean number of spikes per burst. Similar to ISI CV, there were no significant effects of parkinsonism on general burstiness.

Figure 14A illustrates the effect of 6-OHDA induced chronic parkinsonism on the mean number of bursts per 1000 spikes in GABAergic and glutamatergic RTN neurons. On average, RTN neuronal spike trains from control and sham groups had 96.85 ± 20.2 and 115.95 ± 23.7 bursts per 1000 spikes, for the GABAergic and glutamatergic populations respectively. A similar number of bursts per 1000 spikes were observed in 6-OHDA lesioned

rats. A two-way ANOVA with group (control, sham, lesion) and neuronal phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 1.10$, $p = 0.3361$) on the number of bursts per 1000 spikes. Likewise, there was no significant effect of neuronal phenotype ($F_{(1, 154)} = 0.72$, $p = 0.3963$) or group ($F_{(1, 154)} = 0.30$, $p = 0.7415$).

Similarly, Figure 14B demonstrates the effect that 6-OHDA induced chronic parkinsonism had on the mean percentage of spikes contained within bursts for GABAergic and glutamatergic RTN neurons. On average control and sham GABAergic neurons had 32.92 ± 7.1 percent of spikes contained within bursts and glutamatergic neurons had 38.91 ± 8.2 percent of spikes contained within bursts. Similar values were observed in 6-OHDA induced chronic dopamine depleted parkinsonian recordings for both GABAergic and glutamatergic neurons (Fig. 13B). A two-way ANOVA with group (control, sham, lesion) and neuronal phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.51$, $p = 0.5999$) on the percentage of spikes in bursts. Likewise, there were no significant effects of neuronal phenotype ($F_{(1, 154)} = 0.67$, $p = 0.4152$) or group ($F_{(1, 154)} = 0.23$, $p = 0.7955$).

Figure 14C illustrates the effect of chronic parkinsonism on the mean number of spikes per burst in GABAergic and glutamatergic RTN neurons. The control and sham GABAergic neurons had an average of 3.7 ± 0.5 spikes/burst whereas glutamatergic neurons had an average of 3.5 ± 0.4 spikes/burst. Comparable data were obtained for 6-OHDA lesioned rats. A two-way ANOVA with group (control, sham, lesion) and neuronal phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.65$, $p = 0.5231$) on the mean number of spikes per burst in recorded rRTN neurons. Likewise, there were no significant effects of neuron type ($F_{(1, 154)} = 0.10$, $p = 0.7558$) or group on this measure ($F_{(1, 154)} = 0.42$, $p = 0.6617$).

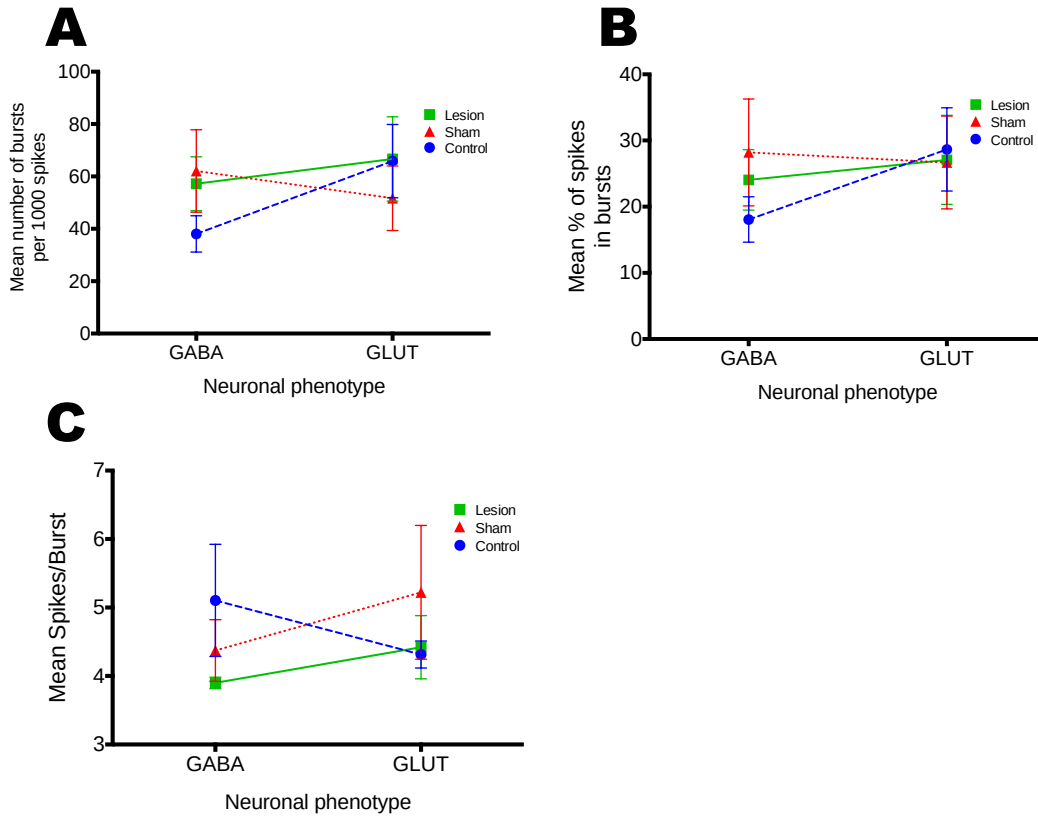


Figure 14: General bursting activity in GABAergic and glutamatergic RTN neurons control (blue), sham (red) and 6-OHDA lesion (green) groups. Chronic 6-OHDA lesion did not produce any significant change in the mean number of bursts per 1000 spikes (A), percentage of spikes in bursts (B), or the mean number of spikes per burst (C) compared to control and sham groups.

Neuronal phenotype effects of 6-OHDA lesion on LTS activity

The third measure to assess firing pattern was an analysis of the LTS characteristics of rRTN neurons. LTS bursts are a prominent feature of thalamic relay neurons and have been identified, but not fully characterized, in the RTN (Contreras et al., 1992; Contreras et al., 1993; Bazhenov et al., 1999; Coulon et al., 2009). Furthermore, to the best of my knowledge the characteristics of LTS bursts have not been explored at all in parkinsonism. Figure 15 shows representative examples of LTS bursts (i.e. doublet, triplet, etc.) recorded from RTN neurons. The analysis for LTS bursts can be distinguished from general bursting activity as it is based on physiological criteria rather than a statistical measure. Three measures of LTS burst characteristics were investigated; 1) number of LTS bursts per 1000 spikes, 2) percentage of spikes within LTS bursts and 3) mean number of spikes per LTS

burst. For the purposes of analyses all LTS bursts, regardless of the size of the burst, were initially analyzed with other LTS bursts from their respective neuronal phenotype. LTS bursts were then categorized as either doublets (two spikes per LTS burst) or non-doublets (more than two spikes per LTS burst). These doublets and non-doublets were also analyzed individually to determine if parkinsonism produced any differential effects on LTS burst type.

Figure 16 demonstrates that for GABAergic neurons recorded from control rats, of the 69% spike trains that exhibited LTS bursts in the rRTN, the majority (61%) were dominated by doublets. In contrast, 76% of GABAergic spike trains recorded from lesioned animals exhibited LTS bursts and of these spike trains the majority (72%) was dominated by non-doublet LTS bursts. For glutamatergic neurons 50% of control rats demonstrated LTS bursts. These LTS bursts were dominated by non-doublet LTS bursts (80%) whereas 80% of lesioned neurons exhibited LTS bursts and were instead dominated by doublet LTS bursts (56%). It was identified that 6-OHDA lesion significantly altered the proportion of spike trains demonstrating LTS bursts in both GABAergic ($X^2 = 35.37$, $p < 0.0001$) and glutamatergic ($X^2 = 38.37$, $p < 0.0001$) rRTN neurons. In both populations clear significant differences were present in the incidence of LTS bursts between the control and lesioned neurons. While sham was similar to the control group in glutamatergic neurons interestingly the sham group was more similar to the lesioned group in GABAergic neurons. It is likely that this discrepancy with the sham group is due to the small sample size of the group. However, differences in the incidence of LTS bursts across the experimental groups between the two phenotypes were evident and due to these differences it was determined to conduct a mixed analysis first, followed by subsequent doublet and non-doublet LTS burst analyses.

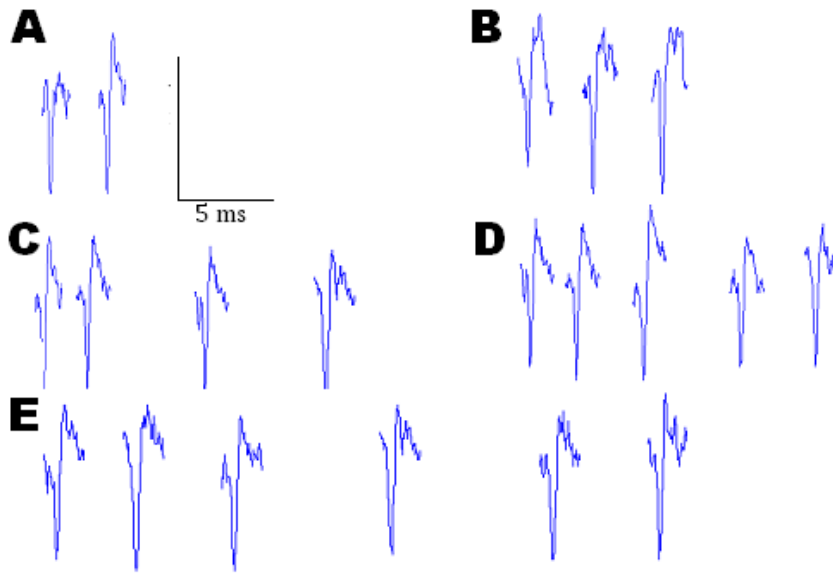


Figure 15: Representative examples of LTS A) doublets, B) triplets, C) quadruplets, D) quintuplets and E) sextuplets obtained from rRTN neurons. Representative scale (black) was the same for all example traces of LTS burst.

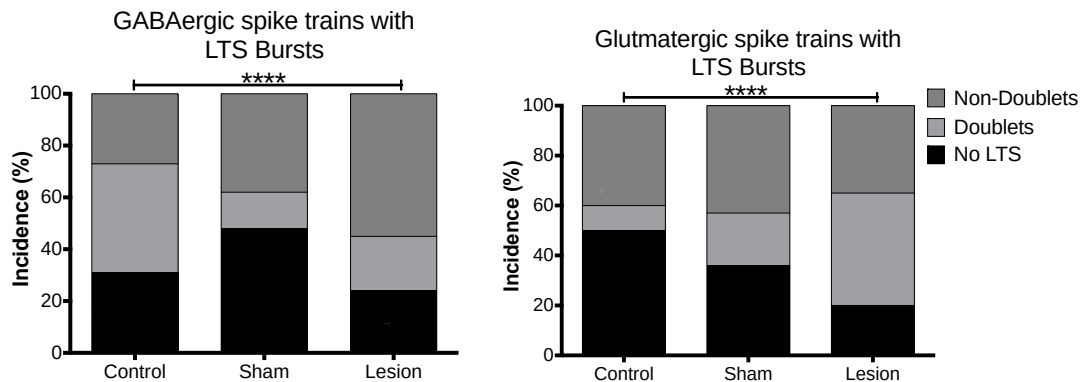


Figure 16: Incidence of spike trains exhibiting no LTS bursts (black), doublets (light grey) and non-doublet LTS bursts (dark grey) in GABAergic (left) and glutamatergic (right) rRTN neurons. Parkinsonism significantly altered the incidence of spike trains exhibiting LTS bursts, compared to control and sham groups. Significant level: **** $p < 0.0001$.

To determine the rate of LTS bursts in rRTN spike trains, the mean number of mixed (combined doublet and non-doublet LTS bursts) LTS bursts per 1000 spikes in GABAergic and glutamatergic RTN neurons and the effect of 6-OHDA induced chronic parkinsonism on this measure are shown in Figure 17. On average, control and sham GABAergic neurons, including those that had no LTS bursts, demonstrated a mean of 9.9 ± 3.0 mixed LTS bursts per 1000 spikes. Glutamatergic control and sham neurons demonstrated 13.1 ± 6.3 mixed

LTS bursts per 1000 spikes. Figure 17 shows that the number of LTS bursts per 1000 spikes was consistently higher in 6-OHDA lesioned rats. A two-way ANOVA with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.02$, $p = 0.9813$) on the number of mixed LTS bursts per 1000 spikes. Likewise, there was no significant effect of neuron phenotype ($F_{(1, 154)} = 0.10$, $p = 0.7499$). However, there was a significant effect of group ($F_{(1, 154)} = 11.18$, $p < 0.0001$). Post-hoc analysis revealed that the number of mixed LTS bursts was significantly increased in lesioned neurons relative to both control ($p < 0.01$) and sham ($p < 0.01$) treated animals, regardless of neuronal phenotype.

LTS bursts can exhibit variability in the number of spikes present in bursts (i.e. doublets, triplets, etc.). Here we analyzed doublets and non-doublet LTS bursts separately to determine if there were any differential effects. The increase in LTS bursts due to parkinsonism outlined above, were consistent for both doublet and non-doublet LTS bursts. Two-way ANOVA with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors performed on doublet only LTS bursts revealed no significant interaction ($F_{(2, 154)} = 0.09$, $p = 0.9131$) or effect of neuron phenotype ($F_{(1, 154)} = 0.01$, $p = 0.9373$) on the mean number of doublet only LTS bursts per 1000 spikes. A significant effect of group ($F_{(2, 154)} = 16.44$, $p < 0.0001$) was identified on the mean number of doublet LTS bursts per 1000 spikes. Post-hoc analysis revealed lesion significantly increased the mean number of doublet only LTS bursts per 1000 spikes in both the GABAergic and glutamatergic neuronal populations, relative to their respective control ($p < 0.0001$, $p < 0.05$) and sham ($p < 0.01$, $p < 0.05$) groups.

The equivalent two-way ANOVA, performed on non-doublet LTS bursts, with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors also revealed no significant interaction ($F_{(2, 154)} = 0.64$, $p = 0.5297$) or effect of neuron phenotype ($F_{(1, 154)} =$

1.96, $p = 0.1632$). However, a significant group effect ($F_{(2, 154)} = 28.90$, $p < 0.0001$) was observed. Consistent with the doublet analysis, post-hoc analysis revealed the number of non-doublet LTS bursts in both the GABAergic and glutamatergic neuronal populations was increased by parkinsonism, relative to their respective control ($p < 0.0001$, $p < 0.0001$) and sham ($p < 0.01$, $p < 0.0001$) groups. Therefore it is evident that 6-OHDA lesion profoundly increased the number of LTS bursts per 1000 spikes. Such a dramatic change in the number of LTS bursts with more action potentials might profoundly affect downstream structures.

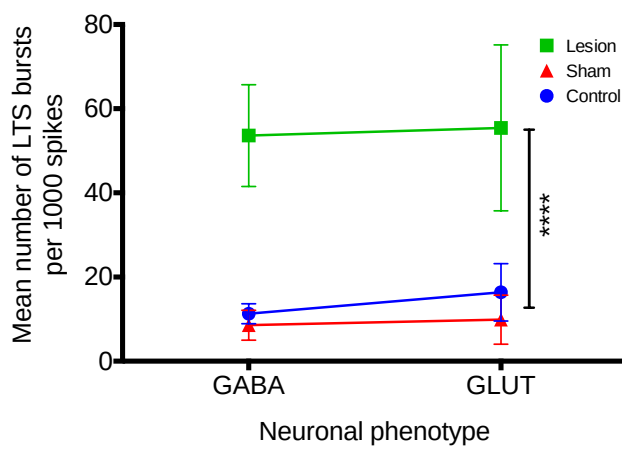


Figure 17: LTS bursting activity in GABAergic and GLUTamatergic RTN neurons control (blue), sham (red) and 6-OHDA lesion (green) groups. Chronic 6-OHDA lesion significantly altered the mean number of mixed LTS bursts per 1000 spikes, compared to control and sham groups. Significant levels: **** $p < 0.0001$.

The second LTS characteristic investigated was the mean percentage of spikes in LTS bursts for both GABAergic and glutamatergic neurons and the subsequent effect of 6-OHDA lesion (Fig. 18). The average percentage of spikes in mixed LTS bursts (i.e. containing both doublet and non-doublet LTS bursts) recorded from the control and sham groups for GABAergic neurons was 2.1 ± 0.6 percent, whereas, for the glutamatergic neuronal population the mean was 3.1 ± 1.4 percent. Lesioned rats exhibited a significantly higher percentage of spikes in mixed LTS bursts in both the GABAergic and glutamatergic neuronal populations. A two-way ANOVA with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.07$, $p = 0.9339$) or effect of neuron phenotype ($F_{(1, 154)} = 0.37$, $p = 0.5435$) on the percentage of spikes in mixed

LTS bursts. There was however, a significant main effect of group ($F_{(2, 154)} = 12.62$, $p < 0.0001$) on the percentage of spikes in mixed LTS bursts. Post-hoc analysis revealed that the 6-OHDA lesion significantly increased the percentage of spikes in mixed LTS bursts in both the GABAergic and glutamatergic neurons compared to their respective control ($p < 0.001$, $p < 0.05$) and sham ($p < 0.05$, $p < 0.05$) groups.

A two-way ANOVA performed on doublet LTS bursts with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors demonstrated no significant interaction ($F_{(2, 154)} = 0.07$, $p = 0.9357$) or effect of neuron phenotype $F_{(1, 154)} = 0.01$, $p = 0.9097$) on the mean percentage of spikes in doublet LTS bursts. However, a significant effect of group ($F_{(2, 154)} = 17.84$, $p < 0.0001$) was observed. Post-hoc analysis identified lesion significantly increased the mean percentage of spikes in doublet LTS bursts in both the GABAergic and glutamatergic neuronal populations, relative to their respective control ($p < 0.0001$, $p < 0.05$) and sham ($p < 0.01$, $p < 0.05$) groups. Likewise, a two-way ANOVA of non-doublet LTS bursts with group (control, sham, lesion) and neuron phenotype (GABA, GLUT) as factors revealed no significant interaction ($F_{(2, 154)} = 0.13$, $p = 0.8790$) or effect of neuronal phenotype ($F_{(1, 154)} = 0.67$, $p = 0.4138$). A significant group effect ($F_{(2, 154)} = 6.03$, $p = 0.0030$) was observed on the mean percentage of spikes in non-doublet LTS bursts. Post-hoc analysis revealed no identifiable significant effect. This is likely because there are only a small number of LTS bursts in this subgroup. Therefore, it is evident that 6-OHDA induced chronic dopamine depletion results in a significant increase in the percentage of spikes in LTS bursts.

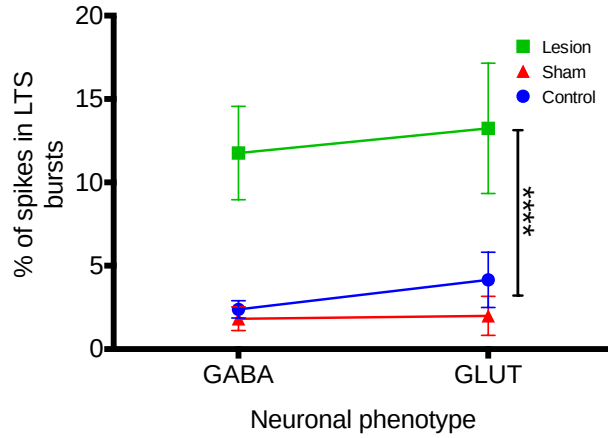


Figure 18: LTS bursting activity in GABAergic and glutamatergic RTN neurons control (blue), sham (red) and 6-OHDA lesion (green) groups. Chronic 6-OHDA significantly altered the percentage of spikes in mixed LTS bursts compared to control and sham. Significant level: **** $p < 0.0001$.

The third variable of LTS bursts was the mean number of spikes per mixed LTS burst recorded from GABAergic and glutamatergic RTN neurons in control and parkinsonian rats are shown in Figure 19. The control and sham groups of GABAergic neurons had an average of 2.2 ± 0.1 spikes per mixed LTS burst, whereas glutamatergic neurons had an average of 2.7 ± 0.3 . Low numbers of spikes per LTS burst were expected for GABAergic neurons as their LTS bursts were dominated by doublets (Fig. 16). In contrast GABAergic and glutamatergic neurons recorded from lesioned animals had an average of 2.1 ± 0.1 and 2.2 ± 0.1 bursts per mixed LTS burst, respectively. A two-way ANOVA with group (control, sham, lesion) and neuronal phenotype (GABA, GLUT) as factors revealed there was a significant interaction ($F_{(2, 114)} = 3.34$, $p = 0.0389$) on the mean number of spikes per mixed LTS burst. Likewise, there were significant neuronal phenotype ($F_{(1, 114)} = 10.68$, $p = 0.0014$) and group ($F_{(2, 114)} = 3.92$, $p = 0.0226$) effects. Post-hoc analysis revealed control glutamatergic neurons have more spikes per burst than control GABAergic neurons ($p < 0.05$). Post-hoc analysis also revealed that 6-OHDA lesion caused a significant decrease in the mean number of spikes per mixed LTS burst in glutamatergic neurons compared to control rats ($p < 0.05$).

In contrast, two-way ANOVA performed on non-doublet LTS bursts revealed no significant interaction ($F_{(2, 57)} = 1.70$, $p = 0.1919$) or effect of neuron phenotype ($F_{(1, 57)} = 0.50$,

$p = 0.4836$) on the mean number of spikes per LTS burst. However, there was a significant effect of group ($F_{(2, 57)} = 7.45$, $p = 0.0013$). Post-hoc analysis revealed that the lesion significantly decreased the mean number of spikes per non-doublet LTS burst in glutamatergic neurons compared to control ($p < 0.05$). It is therefore evident that parkinsonism significantly affects the mean number of spikes per LTS burst in glutamatergic rRTN neuronal phenotypes. Furthermore, it is evident that parkinsonism significantly affects the characteristics of LTS bursts in both GABAergic and glutamatergic neurons. Doublet only LTS bursts were not investigated for this variable because they always contain two spikes, therefore it is not possible for there to be any difference in the number of spikes per burst.

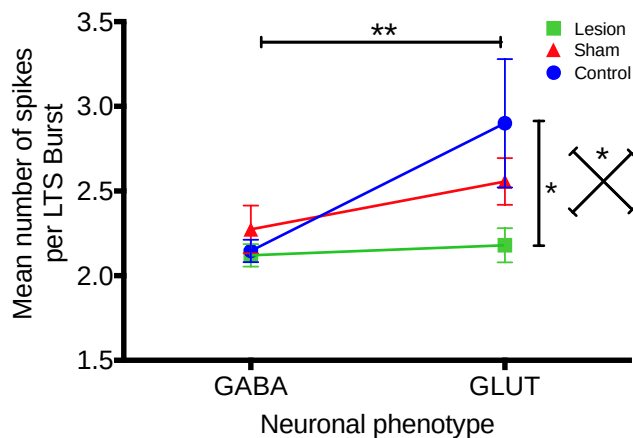


Figure 19: LTS bursting activity in GABAergic and glutamatergic RTN neurons control (blue), sham (red) and 6-OHDA lesion (green) groups. Chronic 6-OHDA lesion significantly decreased the mean number of spikes per mixed LTS burst in glutamatergic neurons, compared to both control and sham groups. Significant levels: * $p < 0.05$, ** $p < 0.01$.

Neuronal phenotype effects of 6-OHDA lesion on ISIs in LTS bursts

The ISI properties of GABAergic and glutamatergic RTN neurons and the effect of 6-OHDA induced chronic parkinsonism on the mean ISI is shown in Figure 20. A three-way ANOVA with group (control, sham, lesion), ISI position (ISI 1, ISI 2, ISI 3, ISI 4, ISI 5) and number of ISIs (1, 2, 3, 4, 5) as factors revealed there was no significant three-factor

interaction ($F_{(12, 10915)} = 1.08$, $p = 0.376$). There was a significant two-factor interaction between ISI position and type of LTS burst ($F_{(6, 10915)} = 7.546$, $p < 0.0001$). No significant two-factor interactions were identified between group and ISI position ($F_{(8, 10915)} = 1.55$, $p = 0.1342$) or group and type of LTS burst ($F_{(8, 10915)} = 0.65$, $p = 0.7336$). There were significant effects of all three main factors; group ($F_{(2, 10915)} = 24.834$, $p < 0.0001$), ISI position ($F_{(4, 10915)} = 24.79$, $p < 0.0001$) and type of LTS burst ($F_{(4, 10915)} = 14.18$, $p < 0.0001$). Post-hoc analysis revealed that the duration of the ISI significantly differed based on position of the ISI within LTS bursts. Within LTS bursts there was a trend for ISI duration to increase as the position of ISI increased, with significant differences between ISI 1 and ISI 3 ($p < 0.0001$), ISI 1 and ISI 4 ($p < 0.0001$) and ISI 1 and ISI 5 ($p < 0.0001$) (Fig. 20G). It was expected that ISI 1 would be significantly different to other ISIs as they are based on different physiological criteria (i.e. ISI 1 < 5 ms whereas ISI 2,3,4,5 < 7 ms), however they were included in the analyses to allow comparison with previous studies. There were also significant differences observed between ISI 2 and 3 ($p < 0.01$), ISI 2 and ISI 4 ($p < 0.001$), ISI 2 and ISI 5 ($p < 0.0001$), ISI 3 and ISI 5 ($p < 0.0001$) and ISI 4 and ISI 5 ($p < 0.01$). These differences are notable because they are based on the same criteria (i.e. ISI < 7 ms) and demonstrate a robust adaptation in spike frequency. Post-hoc analysis also demonstrated that the average duration of ISIs were significantly longer in quadruplets and quintuplets compared to doublets ($p < 0.0001$, $p < 0.0001$; Fig. 20H), and also in quadruplets and quintuplets compared to triplets ($p < 0.001$, $p < 0.01$), there were no other significant differences in the duration of the ISI with regard to the number of LTS bursts. For completeness post-hoc analysis revealed that ISIs were significantly shorter in lesioned rats compared to control ($p < 0.0001$) and sham ($p < 0.0001$) rats (Fig. 20I). It is therefore evident that parkinsonism significantly affects the ISI properties of LTS bursts in both GABAergic and glutamatergic neurons in the RTN.

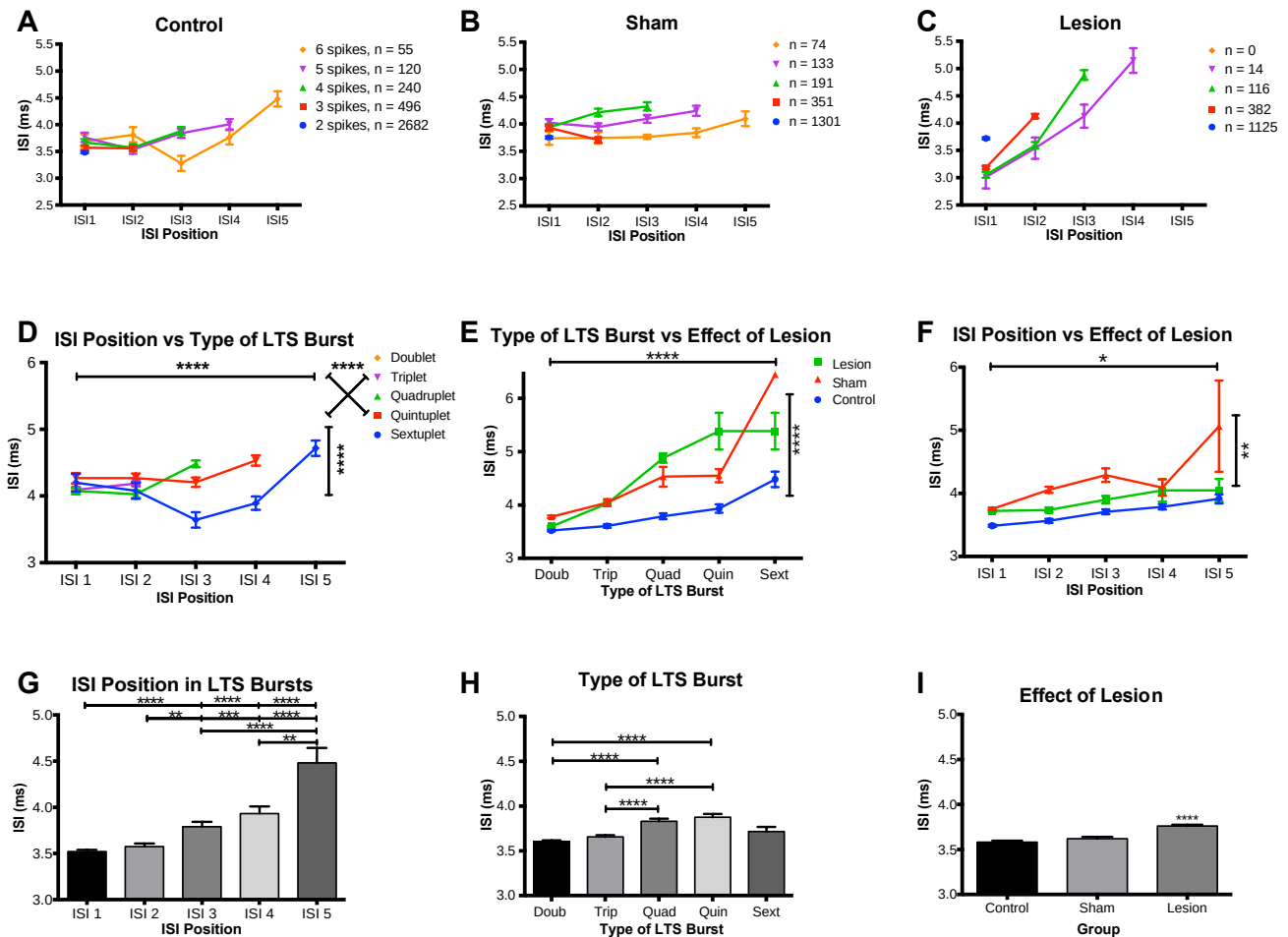


Figure 20: Properties of interspike intervals (ISIs) in LTS bursts. (A-C) Line graphs of mean ISIs in LTS bursts in control (A), sham (B) and lesioned (C) rats. The number of LTS bursts containing doublets (2 spikes), triplets (3 spikes), quadruplets (4 spikes), quintuplets (5 spikes) and sextuplets (6 spikes) are shown within each graph. (D-F) Two way interaction plots demonstrating; the effect of lesion on the position of the ISI in LTS bursts (D), the effect lesion has on the number of ISIs in LTS bursts (E), and the effect spike position and number of ISIs in LTS bursts (F). (G-I) Effect of the position of the ISI in LTS bursts (G), number of ISIs in LTS bursts (H), and lesion (I) on mean ISI. A 3-factor ANOVA revealed that all single factors (position of ISI, number of ISIs and lesion) were statistically significant ($p < 0.0001$). Likewise a significant two-factor interactions were identified between ISI position and type of LTS burst. There were no further significant two-factor interactions or significant interactions between all three factors. Significant levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Summary

In summary, the electrophysiological data clearly demonstrate parkinsonism significantly affects the firing rate and LTS burst characteristics of neurons within the rRTN. The immunohistochemical and electrophysiological experiments performed in the present study identified the presence of a GABAergic and a glutamatergic neuronal population in the rRTN. Parkinsonism significantly affected both RTN neuronal phenotypes, although there were

some differential effects. Likewise, parkinsonism was identified to significantly affect spike frequency adaptation and the ISI properties of LTS bursts.

Discussion

The RTN has long been described as a nucleus solely containing GABAergic neurons, and research has implicated the RTN in the regulation of thalamic activity (Yingling and Skinner, 1977). However, there is currently a distinct paucity of research investigating the role of the RTN in the pathophysiology underlying the symptoms associated with Parkinson's disease.

The present study investigated the hypothesis that chronic 6-OHDA induced dopamine depletion of the SNc would significantly alter the firing rate, pattern and LTS burst characteristics of recorded rRTN neurons. To investigate this, rRTN neuronal activity was recorded in urethane anaesthetized control and 6-OHDA dopamine depleted parkinsonian rats. Spike trains were recorded from parkinsonian animals two-four weeks after unilateral lesioning of the MFB. The results of the present experiment identified parkinsonism significantly decreased the firing rate of neurons in rRTN. In addition, parkinsonism significantly affected the LTS characteristics of rRTN neurons. However, there were no significant changes in the general bursting characteristics after 6-OHDA lesion. Furthermore, the present study identified a novel population of non-GABAergic neurons present in the rRTN, and immunohistochemical staining and viral transduction indicated that these were glutamatergic neurons. The presence of a glutamatergic population in the rRTN raises important questions regarding how these neurons might alter information processing in rRTN and the influence rRTN has on downstream nuclei.

RTN neuronal activity in control and sham rats

Electrophysiological data obtained from neuronal spike train recordings of control and sham rats did not significantly differ in their firing rates, ISI CV, general burstiness or LTS burst characteristics. Urethane induced-anesthesia is often described as generating slow-wave activity patterns, which are often characterized by spindle rhythms similar to that

observed during slow-wave sleep (Steriade et al., 1986; Steriade et al., 1987; Fuentealba et al., 2005). Spindle rhythms in other regions of the RTN have been strongly associated with firing rates of 8-12 spikes per second (Destexhe and Sejnowski, 2001). However, in the present study, the firing rate observed in the rRTN of neurologically intact control rats was 4-6 spikes per second. This lower firing rate in the rRTN was not surprising, because; 1) a substantial input to this region arises from motor cortex and anesthesia has previously been shown to suppress the excitability of (Steriade et al., 1993), and 2) the neuronal activity of other motor encoding regions of the brain are normally suppressed during anesthesia (Sukhotinsky et al., 2005). It is however surprising that there was no significant difference in the firing rate between the two putatively defined neuronal phenotypes. In cortex glutamatergic neurons often exhibit slower firing rates than GABAergic neurons (Fellous et al., 2001). The reason why this is not evident in the present study of the rRTN could be due to where these neurons project or the make-up of the afferent connections. We do not know if this novel population of rRTN glutamatergic neurons is projecting to other structures or acting as interneurons but if they possess a similar array of inputs as the GABAergic phenotype then this may account for the lack of a significant difference in firing rate between the two phenotypes. Also a similarity in some of the basic firing properties of these two neuronal populations could help explain why this small glutamatergic population has gone unnoticed before.

Studies have previously reported that RTN neurons exhibit predominately one of two discharge patterns, bursty and tonic. The bursty discharge pattern of neuronal activity has been described as exhibiting burst discharges of 3-7 spikes per burst (Mulle et al., 1985; Domich et al., 1986; Steriade et al., 1986; Spreafico et al., 1988; Destexhe and Sejnowski, 2001). The remainder of RTN neurons exhibit slow tonic neuronal activity with irregular bursts of action potentials (Contreras et al., 1992; Brunton and Charpak 1997; Pinault, 2004; Lee et al., 2007). The rRTN neurons investigated in the present study were predominately

bursty, with burst discharges similar to those previously described. Whilst under deep anesthesia, some RTN neurons are capable of alternating between the bursting and tonic firing patterns (Contreras et al., 1992). While not quantitatively examined in the present study, observations of neuronal spike train recordings demonstrated a small proportion of rRTN neurons did change from one firing pattern to the other. The bursty nature of these neurons is reflected in the ISI CV data.

LTS bursts are a characteristic phenomenon of thalamic nuclei and the RTN is no exception. LTS bursts likely play an integral role in the processing and encoding of neuronal firing and thus were of particular interest in the present study. Previous studies have identified LTS bursts throughout the entire RTN, although no study has yet fully characterized LTS bursts in the rRTN. In the present study the majority (~60%) of rRTN neurons exhibited LTS burst activity, and the proportion of spikes in LTS bursts per spike train was similar to that reported for other thalamic nuclei (Coulter et al., 1989; Huguenard, 1996; Destexhe et al., 1998; Parr-Brownlie et al., 2009). The majority (~75%) of these LTS bursts contained two spikes (doublets). LTS bursts that contained between 3 and 4 spikes were rarer (~20%) than doublets and bursts with 5 or 6 spikes were extremely rare (~5%). This is in contrast to previous studies, which have identified that other regions of the RTN are dominated by 3-5 spikes per LTS burst (Coulon et al., 2009; Destexhe and Sejnowski, 2001).

The characteristics of LTS bursts in the RTN appear largely similar to LTS bursts described in other thalamic structures, with a few key differences (Destexhe and Sejnowski, 2001; Llinas and Steriade, 2006). One unique characteristic of RTN LTS bursts is the acceleration-deceleration pattern in the ISIs in LTS bursts (Steriade et al., 1993; Steriade et al., 1986; Domich et al., 1986; Contreras et al., 1993). However, the present study found no evidence of an acceleration-deceleration pattern within LTS bursts in the rRTN. This difference is likely because, to date, the research has primarily focused on the caudal RTN. It

is therefore unsurprising that no evidence of an acceleration-deceleration pattern was identified because the caudal RTN is implicated in sensory information processing whereas the rRTN is implicated in processing motor information. The present study identified a significant increase in the duration each ISI with respect to the ISI immediately preceding it, with the exception of the second ISI. The increase in ISI duration relative to the first ISI was expected as they are based on different criteria (i.e. ISI 1 < 5 ms, ISI 2,3,4,5 < 7 ms). The increase in duration relative to the other ISIs is notable as the second, third, fourth and fifth ISIs are all based on the same criteria (i.e. ISI < 7 ms), and demonstrates a robust adaptation in spike frequency. This LTS burst pattern is reminiscent of that described for other thalamic nuclei (Magill et al., 2000; Magill et al., 2001; Parr-Brownlie et al., 2009;) but is in stark contrast to other regions of the RTN which are reported to demonstrate an acceleration-deceleration LTS burst pattern (Steriade et al., 1993; Steriade et al 1986; Domich et al., 1986; Contreras et al 1993).

Furthermore, control and sham electrophysiological data demonstrated that there are possibly two populations of neurons present in the rRTN. Previous studies have suggested that while GABAergic neurons are the dominant population present throughout the RTN, there is also a small population of neurons that are non-GABAergic (Houser et al., 1980). However, to date there is a paucity of information investigating the phenotype of a non-GABAergic population present in the RTN.

Identification of putative glutamatergic population in RTN

The electrophysiological and immunohistochemical data demonstrated that there is a small population of neurons within the rRTN that appears to be glutamatergic as they have broad action potentials and express proteins associated with glutamate biosynthesis, namely EACC1, while also absent for GABAergic neuronal markers, such as GAD67. The presence of a novel glutamatergic neuronal population within the rRTN raises questions regarding how

this population might influence neuronal activity throughout the wider RTN-basal ganglia-thalamo-cortical neuronal network. The current study did not identify whether this population are projection neurons or interneurons, so further study is necessary to elucidate this. However, while the connections of this neuronal population were not investigated in the present study, a number of speculative effects on downstream nuclei can be proposed, if certain assumptions are made. The speculative projections of these neuronal populations are summarized below (Fig. 21).

In theory there are six possible arrangements for the connections of these two populations, and are dependent on the arrangement of both the afferent and efferent connections. Two possibilities exist for afferent projections; either they arise from similar nuclei or they arise from different nuclei. If they arise from similar nuclei then they will encode similar information, whereas if they arise from different nuclei they might encode different information. In terms of efferent projections three possibilities exist, the projections can be divergent, convergent or the glutamatergic population could be interneurons. If the projections are divergent then the RTN will likely stimulate one target neuron while simultaneously inhibiting another. If the projections are convergent then the information encoded by the neuronal populations will become integrated to differentially modulate the neuronal activity of target neurons. Finally, if this small novel glutamatergic population acts as interneurons for the rRTN, then this population will modulate the neuronal activity of the GABAergic population.

While the presence of a small glutamatergic population in the rodent rRTN is novel, there is previous evidence of a small population of non-GABAergic neurons being present in the RTN (Houser et al., 1980). However, further research investigating the afferent and efferent connections of this novel neuronal population, in contrast to the GABAergic

population, is necessary for identifying which of the proposed neural circuitry maps is correct (Fig. 21).

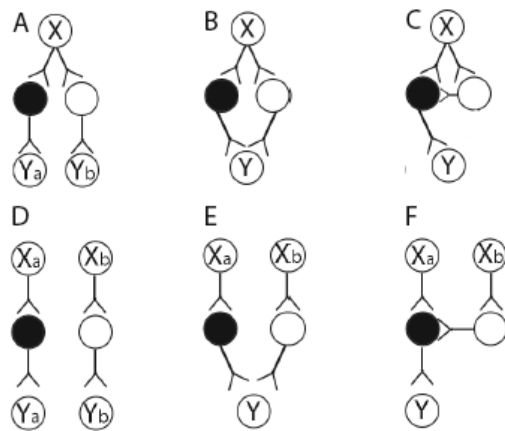


Figure 21: Schematic diagram of hypothesised GABAergic (filled circles) and glutamatergic (unfilled circles) RTN neuronal circuitry. X represents afferent nuclei and Y represents efferent nuclei. A and D demonstrate divergent afferent projections, B and E demonstrate convergent projections, while C and F demonstrate glutamatergic neurons as interneuronal populations.

Parkinsonism alters rRTN neuronal activity

To explore how parkinsonism alters neuronal activity in the rRTN, spike train data were obtained from urethane anaesthetized 6-OHDA induced parkinsonian rats, and were compared to age-matched control and sham rats. The present study identified that parkinsonism reduced the firing rate of rRTN neurons and significantly increased LTS bursting activity. However, parkinsonism did not alter the general bursting activity of rRTN neurons. Specifically, chronic dopaminergic depletion of the SNc significantly increased the number of LTS bursts, the number of spikes per LTS burst and the percentage of spikes in LTS bursts.

While we do not possess the data to know definitively what has happened, we speculate that the changes in LTS bursting characteristics could be explained by the idea that the membrane potential of rRTN neurons is hyperpolarized in parkinsonism. Increased RTN hyperpolarization would increase the probability of activating the T-type Ca^{2+} channel, as prolonged hyperpolarization is necessary for the inactivation gate to open (Bosch-Bouju et al.,

2013). This would increase the responsiveness of RTN neurons to depolarizing events which would result in generation of LTS bursts. The subsequent effect of neurotransmitter release onto the post-synaptic cell will also likely be dramatically altered, as there would be increased and sustained neurotransmitter release. Increases in LTS bursting activity could produce powerful effects on downstream neuronal targets, by increasing the probability of neurotransmitter release and synaptic reliability (Lisman, 1997; Branco and Staras, 2009).

The changes in RTN neuronal activity, induced by parkinsonism, can be attributed to a number of possible mechanisms. One mechanism is associated with the loss of direct dopaminergic input (Pare et al., 1990; Khan et al., 1998; Tsumori et al., 2002; Anaya-Martinez, 2006; Picazo et al., 2009; Govindah et al., 2010; Cebrian and Presna, 2010; Gulcebi et al., 2012). The RTN is rich in both the excitatory D₁ and inhibitory D₄ dopamine receptors (Huang et al., 1992; Mrzljak et al., 1996, Floran et al., 2004). D₁ receptors produce excitation by increasing adenylyl cyclase activity, whereas D₄ receptors suppress adenylyl cyclase activity leading to inhibition (Rang et al., 2007). The physiological effect of D₁ receptor activation, independent of D₂-like receptor activation (i.e. D₄ receptors), has not been clearly established in the RTN (Huang et al., 1992). However if we assume it is similar to receptor activation in other regions of the brain, then under normal physiological conditions exposure to dopamine would likely result in inhibitory modulation of RTN neuronal activity. This is because the two receptors appear to have a similar abundance throughout the RTN, but the D₄ receptor has a greater affinity for dopamine (Mrzljak et al., 1996; Rang et al., 2007). This difference implies that exposure to dopamine will result in a greater activation of the D₄ receptor than the D₁ receptor, resulting in dopamine producing a net inhibitory effect. Therefore, the loss of direct dopaminergic input in parkinsonism might remove the modulatory effect of dopamine, consequently, altering the firing pattern of the rRTN.

Loss of dopaminergic innervation in parkinsonism significantly alters the firing rate and pattern throughout the basal ganglia nuclei (Parr-Brownlie et al., 2007; Mallet et al., 2008; Parr-Brownlie et al., 2009; Avila et al., 2010; Rubin et al., 2012) and motor thalamus (Magnin et al., 2000; Pessiglione et al., 2005). These changes in firing rate and pattern provide a second mechanism by which the changes in LTS burst characteristics can be explained. Considering the rRTN receives extensive afferent connections from the basal ganglia, any disruption of normal neuronal activity in these structures will likely have flow-on effects on the pattern of rRTN neurons. As these connections are reciprocal it is also highly probable that the changes in neuronal activity will become self-perpetuating throughout the RTN-basal ganglia-thalamic network.

Parkinsonism has previously been associated with the suppression of motor cortex excitability (Albin et al., 1989; Ridding et al., 1995; Purves et al., 2001; Parr-Brownlie and Hyland 2005; Parr-Brownlie et al., 2009; Pasquereau and Turner, 2011). Suppression of motor cortex excitability provides a further mechanism through which the observed changes in LTS characteristics can be explained. Decreased tonic excitatory input from the cortex may pre-dispose the RTN to the hyperpolarizing effects of other nuclei, consequently, if sufficient excitatory inputs, from any afferent structure, were to innervate the RTN during this period of maintained hyperpolarization, it may lead to a rebound in the membrane potential and the generation of LTS bursts.

Each of these mechanisms can, to some degree, explain the increase in the number of LTS bursts, the proportion of spikes in LTS bursts and the number of spikes per LTS burst. It is highly probable that the parkinsonian induced changes in the characteristics of rRTN LTS bursts are due to several, or all, of these mechanisms working in combination.

As the predominant neuronal phenotype within the RTN is GABAergic, increased LTS bursting activity may produce several distinct effects on downstream nuclei, that are

dependent on the amount of neurotransmitter released and the type of post-synaptic receptor present. The two predominant types of GABA receptor are the ionotropic GABA_A and the metabotropic GABA_B receptors (Rang et al., 2007). GABA_A receptors are directly linked to chloride ion channels, so in the presence of GABA will typically result in neuron hyperpolarization, due to the influx of chloride ions (Rang et al., 2007). GABA_A receptors produce two subtly different affects depending on the location of the receptor. Postsynaptic GABA_A receptors mediate the fast postsynaptic hyperpolarization of the neuronal membrane by increasing the permeability of the associated chloride ion channel (Rang et al., 2007). In contrast, peri-synaptic GABA_A receptors act as neuromodulators; producing slow inhibitory effects on the postsynaptic neuron (Rang et al., 2007). Macdonald and Olsen (1994) have suggested that GABA_A receptors have two recognition sites for GABA and that increased synaptic release of GABA results in increased duration of chloride channel opening. Considering the results from the present experiment demonstrate changes in pattern, specifically increased LTS bursting activity, it is hypothesized that the increased synchronicity observed throughout the basal ganglia network (Mallet et al., 2008; Avila et al., 2010) might be, at least partially, due to increased GABA being released from the RTN onto the basal ganglia-thalamocortical network.

The second class of GABA receptor is the metabotropic GABA_B receptor. When activated these receptors inhibit the conductance of pre-synaptic calcium and the conductance of post-synaptic potassium, via inhibition of adenylyl cyclase (Rang et al., 2007). Thus, GABA_B receptors provide a mechanism through which neurotransmitter release can be decreased (Kaupmann et al., 1997). This can further explain the changes in pattern that are observed in efferent motor encoding nuclei, such as the motor thalamus and basal ganglia, as excess release of GABA would stimulate both post-synaptic GABA_B receptors and pre-

synaptic auto-receptors to decrease the release of GABA, thus increasing the susceptibility of the post-synaptic neuron to depolarizing events and the generation of action potentials.

General bursting activity

While parkinsonism produced significant changes in the characteristics of LTS bursts, it is interesting that general bursting activity was unchanged in the rRTN. It is not entirely surprising that changes might be observed in the characteristics of LTS bursts but not in general bursting activity, as the measure used in the present study for general bursting activity is a statistical measure of bursting activity (Kaneoke and Vitek, 1996). The method used identified periods where the ISI of at least three spikes is significantly shorter compared to the ISI across the entire epoch. In contrast, the method employed to analyze LTS bursting activity is based on the physiological properties of the T-type calcium channels. The changes observed in LTS bursting characteristics were dominated by changes in doublets and these changes would not have been detected by the general bursting analysis as a minimum of three spikes are required in each burst. Furthermore, it is plausible that while significant changes were observed in LTS bursts with three or more spikes, this does not mean that significant changes will occur in general bursting activity.

Differential effects of PD on neural populations

Electrophysiological recordings provided preliminary data that raised the possibility of two neuronal populations in the rRTN. This was subsequently confirmed using immunohistochemistry and injection of viral vectors targeted to transduce glutamatergic neurons. Therefore, electrophysiological data were further examined for effects of neuronal phenotype, in both control and parkinsonian conditions, by separating neuronal spike train recordings obtained from urethane anaesthetized 6-OHDA lesioned rats into populations based on action potential width.

The results from the present study demonstrated that parkinsonism significantly affected the firing rates and firing pattern of both neuronal phenotypes equally. Chronic dopamine depletion was also identified to increase the number of LTS bursts and the proportion of spikes in LTS bursts in both neuronal phenotypes. This suggests that parkinsonism significantly increased the relative proportion of doublet LTS bursts, compared to triplets or quadruplet LTS bursts, and that rRTN neurons in parkinsonian rats are more likely to exhibit LTS bursts. In contrast, parkinsonism did not significantly affect the general burstiness or spike train regularity of either neuronal population. These changes are consistent with the notion of increased RTN hyperpolarization, but can also be explained by reduced cortical and thalamic excitatory input to the RTN. Notably, the glutamatergic neuronal phenotype exhibited significantly larger number of spikes per LTS burst, than the GABAergic neuronal phenotype. This differential effect lends further support to the notion that there is more than one rRTN neuronal phenotype.

Implications of PD on understanding pathophysiology

The findings of the present study have two important implications for understanding parkinsonism. First, the present study clearly identified that the neural activity of the RTN is affected by parkinsonism. Therefore, it is plausible that some of the symptoms associated with Parkinson's disease can be attributed to RTN pathophysiology. One likely non-motor symptom that could be associated with disruption of RTN neuronal activity is the sleep disturbance a significant proportion of Parkinson's patients suffer from (Nausieda et al., 1982; Factor et al., 1990; Tandberg et al., 1998). This is because the RTN has previously been suggested to provide the inhibitory regulation necessary for generating normal brain rhythmicity and sleep (Bal and McCormick, 1993; Steriade et al., 1987; Steriade et al., 1993), thus any changes to the neural activity of RTN would likely contribute to abnormal brain rhythmicity and disruption of sleep. It is also plausible that the changes observed in RTN

neural activity are critical in the production of abnormal neural activity in other nuclei already implicated in Parkinson's disease due to disrupted GABA-mediated inhibition of these structures, such as the motor thalamus (Magnin et al., 2000; Pessiglione et al., 2005). To definitively test this hypothesis, experiments would need to be conducted in freely moving parkinsonian rats, which display movement deficits.

Evaluation of Experimental Techniques

Urethane induced anaesthesia

Urethane induced anesthesia was necessary to create a neurologically stable brain state in the present study to; 1) reduce any potential confounding effects arising from the sensory modalities, and 2) prevent changes in rRTN neuronal activity as a result of movement. The urethane anaesthetized model provided a stable neurological state in the rodent brain to investigate rRTN neuronal activity, and the subsequent effects of parkinsonian induced pathology. It should be noted that neurons can exhibit different sub-threshold membrane potentials for the generation of action potentials (Wilson, 2008). The first state is a hyperpolarized (down) state, whereas the second state is a more depolarized (up) state. Neurons exhibit this two-state behaviour as part of their intrinsic neuronal properties or because they are part of a network that imposes it on them (Wilson, 2008). The down state observed in the cortex during sleep, anaesthesia or as a result of direct cortical or thalamic stimulation appears to be due to disfacilitation of the cortex, or hyperpolarization as a response of a reduction in synaptic excitation to the cortex (Contreras et al., 1996; Timofeev et al., 2000; Shu et al., 2003). Considering cortical pyramidal cells receive the majority of their excitatory inputs from thalamocortical neurons or other pyramidal cells, it becomes evident that the down state must arise from some influence that reduces these inputs (Wilson, 2008). In contrast, the up state in cortical neurons is predominately explained by virtue of the

features of the thalamocortical network (Wilson, 2008); although some potential cellular properties have also been proposed such as nonlinearity in the somato-dendritic membrane (Waters and Hemchen, 2006), and NMDA-dependent plateau potentials (Milojkovic et al., 2005). The up state however appears as a transiently stable state that gradually loses stability and returns the cortical network to the down state (Wilson, 2008).

Urethane anaesthesia has previously been reported to induce strong fluctuations in cortical excitability ultimately leading to this two-state behaviour, which can have a significant impact on downstream neurons, such as the rRTN (Steriade et al., 1993; Steriade et al., 2001; Destexhe et al., 2007). By restricting selection of neurons to only those that exhibited an approximate 1 Hz LFP frequency, it was ensured that the fluctuations urethane anaesthesia is capable of inducing in cortical excitability were kept consistent across experimental groups and phenotype, and hence minimized the impact that these fluctuations could have on the rRTN. In a few cases additional doses of urethane were necessary to maintain the appropriate depth of anaesthesia, this was determined by an increased dominant LFP frequency and/or the presence of a hindlimb withdrawal reflex. If the dominant frequency increased above 1.2 Hz then the experiments did not progress, as different LFP frequencies are associated with different attentional states and therefore different levels of neuronal activity (Destexhe et al., 1999; Destexhe and Sejnowski, 2001). The urethane induced model is an important first step in investigating the possible relationship between the RTN and any changes in neuronal activity due to parkinsonism. Further experiments in the awake behaving animal are now necessary to elucidate how the rRTN contributes to the motor and cognitive symptoms associated with Parkinson's disease.

Lentiviral vector mediated gene transfer

As demonstrated by the immunohistochemical data, a small population of RTN neurons did not possess markers associated with the GABAergic phenotype. This population

was positive for the vesicular glutamate transporter protein, EACC1, which is associated with GABA/glutamate biosynthesis. However, the EACC1 neuronal marker was also co-localized with a marker associated with GABAergic neurons, GAD67. This is not surprising given that glutamate is used in the biosynthesis of GABA. However, therein lies one of the problems with solely relying on immunohistochemical techniques for the identification of neuronal phenotype. If the neuronal phenotype of interest shares a biochemical pathway with another neuronal phenotype in the structure of interest, there will likely, but not necessarily, be significant co-localization.

To overcome this problem the present study utilized the lentiviral vector mediated gene transfer technique. This technique specifically allowed the expression of mCherry in glutamatergic neurons. This was achieved by controlling the expression of mCherry under the CaMKII α promoter sequence which is a protein associated with glutamatergic, compared to GABAergic, populations (Kennedy et al., 1983; Liu and Jones, 1996; Gradinaru et al., 2009). This technique reinforced the findings that the RTN possesses a population of non-GABAergic neurons, and that these neurons are probably glutamatergic.

The use of the lentiviral mediated gene transfer technique did present some technical difficulties. The major difficulty arose from the fact that a very small population of neurons was being targeted. Only small quantities of viral vector were injected, to prevent uptake of the vector into non-target nuclei, therefore, the ability to successfully transduce neurons was limited. Furthermore, accurately targeting these neurons was difficult, as there was no discernable pattern to the position of glutamatergic neurons throughout the rRTN. These additional surgeries, combined with the other immunohistochemical and electrophysiological data, confirm the presence of a non-GABAergic neuronal population in the RTN, which is likely glutamatergic.

Future studies

A natural progression for the present study would be to examine how chronic dopamine depletion induced by 6-OHDA neurotoxic lesion affected the rRTN in an awake behaving rat model of Parkinson's disease. The present study demonstrated that the rRTN is affected by parkinsonism whilst under anesthesia, however, the proposed study would extend these findings by examining; 1) whether the rRTN has a role in the production/selection of functional motor tasks and 2) the specific changes in rRTN firing rate and pattern induced by parkinsonism in the awake behaving animal model.

The present study identified a novel glutamatergic population in the RTN, which requires further investigation, to understand the effect that changes in the neuronal activity of this population might have on the local neuronal networks. The first question that should be investigated is whether this novel population of glutamatergic neurons project out of the RTN or function as interneurons. Investigating this would provide valuable insight into the local neuronal circuitry of the RTN and the effects that this structure might have on downstream nuclei. One experimental technique that could answer these questions would be optogenetics. This technique would be extremely useful for studying the glutamatergic population as this population is dispersed throughout the RTN and accounts for only a small proportion of the neurons. If optogenetics was used in concert with electrophysiological recordings of the target structures, it would allow identification of how changes in rRTN firing rate and pattern directly affect target nuclei.

While the 6-OHDA induced dopamine depletion model has been extremely useful over the years in furthering our understanding of how parkinsonism affects the brain, one of the major problems with the model is that it does not mimic the progressive nature of the disease. To address this, a possible alternative would be the design of a progressive neurodegenerative model, which would allow the investigation of how Parkinson's disease

affects neural activity and behavior as the disease progresses over time. The concept for such a progressive model extends from early research which identified that α -synuclein, a protein implicated in Parkinson's disease, has prion-like properties (Braak et al., 2003). Such a progressive model would pose many technical difficulties but also has the potential to drastically advance the development of targeted treatment strategies which may allow us to halt or reduce disease progression.

Conclusion

Due to the aging global population, overstretched health-care systems and a dearth of effective medications, there is significant interest in the investigation of neurodegenerative disorders, such as Parkinson's disease. However, there are still fundamental questions pertaining to the neural circuitry implicated in the disease. The present study identified that the rRTN is affected by parkinsonism. Furthermore, considering the rRTN has reciprocal connections with basal ganglia nuclei and the motor thalamus, it becomes evident that the rRTN might be involved in the production of some aspects of parkinsonian symptoms. This raises the interesting possibility of targeting the rRTN with existing and novel treatment strategies to alleviate the symptoms associated with parkinsonism.

The present experiment argues for further research investigating the implications of parkinsonian pathophysiology on rRTN neural activity. Given that the present study also identified a novel neuronal population in the rRTN, further characterizing of this population in the neurologically intact brain may yield valuable insight into the neuronal circuitry associated with the rRTN. The investigation of this novel population under various pathological conditions may also further our understanding of the neurological processes underlying disease symptoms.

In the wider context, the present study suggests that while SNc dopaminergic neurodegeneration is the root cause of Parkinson's disease, the rRTN could be important for

fully understanding the pathophysiology in the basal ganglia-thalamo-cortical circuitry. The findings from the present study may provide the necessary link for the development of novel therapeutic strategies for alleviating both motor and non-motor cognitive dysfunction.

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