

Using fast-scan cyclic voltammetry to investigate monoamine release in BAC transgenic rodent models of Parkinson's Disease

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Abstract:

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Nigrostriatal dopamine (DA) neurons are particularly susceptible to degeneration in PD, and the concomitant loss of DA signalling in the striatum is believed to underlie the motor symptoms of PD.

Transgenic rodent models expressing PD-related genes have previously revealed changes to DA synapse function as one of the earliest stages of PD progression, often preceding protein aggregation and cell death. However, such models often rely on foreign gene promoter elements that do not accurately replicate the genetic expression pattern and protein levels found in PD.

This thesis explores the electrically evoked release of two monoamines, DA and 5-HT (serotonin), in mice that express wild-type human α -synuclein at a range of levels, through a bacterial artificial chromosome vector (BAC), on a mouse synuclein-null background. Potential mechanisms underlying monoamine neurotransmission deficits were also investigated. Furthermore, this thesis explored the evoked release of DA in BAC transgenic rats that express the G2019S and R1441C mutant forms of the human Leucine-rich repeat kinase 2 (LRRK2) gene. All of the models covered express PD-related genes under regulation of endogenous promoter elements, which increases the physiological relevance of gene expression pattern and levels of gene expression in comparison to transgenes with foreign promoters. Fast-scan cyclic voltammetry, using carbon fibre microelectrodes, was used to sample electrically evoked DA and 5-HT in acute brain slices. High performance liquid chromatography was used to determine DA content.

Mice that express relatively high levels of human α -synuclein (SNCA-C mice), at approximately double the level of WT mice, have a ~30% deficit in evoked DA release from the dorsal striatum, but not the ventral striatum, when compared to control mice (*Snca* $-/-$). Investigations performed here showed that the DA release deficit depends on the level of human α -synuclein expression. It was also identified that SNCA-C mice do not have detectable changes to the responsiveness of DA D₂ autoreceptors that regulate DA release. However, DA release in SNCA-C mice was more responsive than *Snca* $-/-$ controls to the vesicle mobilising properties of cocaine, which suggests altered vesicle mobilisation as a possible mode of action for α -synuclein overexpression. No changes to 5-HT release, explored in substantia nigra, were observed in SNCA-C mice suggesting that the deficit in neurotransmission does not apply to all monoamines.

Rats that express the G2019S or R1441C mutant forms of LRRK2 revealed an age-dependent deficit of evoked DA release in the dorsal striatum, but not the ventral striatum. This deficit in DA release was not accompanied by changes to striatal DA content or the rate of DA uptake, which suggests a change in DA releasability.

Taken together, these data suggest that PD related forms of *SNCA* and LRRK2 cause changes to DA function by affecting DA releasability from axons. Findings presented here are in agreement with other literature to suggest changes in DA synapse function as one of the earliest stages of PD pathophysiology, and may contribute to a deeper understanding of the molecular mechanisms involved in PD.

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Abbreviations:

Abbreviation:	Meaning
[5-HT] _o	extracellular concentration of 5-hydroxytryptamine
[ACh] _o	extracellular concentration of acetylcholine
[Ca ²⁺] _o	extracellular concentration of calcium
[DA] _o	extracellular concentration of dopamine
4EBP1	eukaryotic initiation factor 4E-binding protein 1
5-HT	5-hydroxytryptamine
AADC	amino acid decarboxylase
AAV	adeno-associated virus
ACh	acetylcholine
AD	autosomal dominant
ANK	ankyrin repeat region
AR	autosomal recessive
BAC	bacterial artificial chromosome
CFM	carbon fibre microelectrode
CLi	caudal linear nucleus
COR	C-terminal of Ras
CPu	caudate putamen
DA	dopamine
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DR	dorsal raphe
EOPD	early onset Parkinson's disease
EPSC	excitatory post synaptic current
ER	endoplasmic reticulum
ERM	ezrin/radixin/moesin
FCV	fast-scan cyclic voltammetry
GABA	γ-Aminobutyric acid
GP	globus pallidus
GPe	external segment of globus pallidus
GPI	internal segment of globus pallidus
HVA	homovanillic acid
IPSC	inhibitory post synaptic current
IR	immunoreactive
L-DOPA	L-3,4-dihydroxyphenylalanine
LDTg	laterodorsal tegmental area
LOPD	late onset Parkinson's disease
LRR	leucine-rich repeat
MAO	monamine oxidase
MKK	Mitogen activated protein kinase kinase
MKKK	mitogen activated protein kinase kinase kinase

MLK	mixed-lineage kinases
MnR	median raphe
MPDP	1- methyl-4-phenyl-2,3-dihydropyridium
MPP+	1-methyl- 4-phenylpyridinium
MPTP	1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine
MSSN/MSN	medium sized spiny neuron
NAc	nucleus accumbens
NAcC	nucleus accumbens core
NAcS	nucleus accumbens shell
nTG	non transgenic
OPDC	Oxford Parkinson's disease centre
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PPN	pedunculopontine nucleus
PrP	prion promoter
RMg	raphe magnus
Rob	raphe oscurus
Roc	Ras of complex
Rpa	raphe pallidus
SCA2	spinocerebellar ataxia type 2
SERT	serotonin (5-HT) transporter
SN	substantia nigra
SNAP	soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	SNAP receptor
SNc	substantia nigra pars compacta
SNP	single nucleotide polymorphism
SNr	substantia nigra pars reticulata
STN	sub thalamic nucleus
SVZ	subventricular zone
TG	transgenic
TH	tyrosine hydroxylase
UPR	unfolded protein response
VMAT2	vesicular monoamine transporter 2
WPRE	woodchuck hepatitis post-transcriptional regulatory element
WT	wild type

CHAPTER 1: Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 1% of the population above age 60 (Samii et al., 2004). The financial cost of PD to the UK economy was estimated in 2010 to be around £2.3 billion per annum (Gustavsson et al., 2011), and with the average life expectancy estimated to be 80 to 85 years by 2015 (Hayflick, 2000), this cost is sure to increase. Although several treatments exist to alleviate the symptoms of PD, there is currently no cure and on going research is needed to understand the underlying pathology and progression of this disease.

This thesis focuses on how monoamine synapse function is affected in several transgenic models relating to PD. Monoamine content and evoked monoamine release was investigated in mice that express different levels of *SNCA*, and rats overexpressing different mutations of *Leucine Rich Repeat Kinase 2 (LRRK2)*. The rest of this introduction chapter will cover background information about the basal ganglia where monoamine release was investigated, PD, and the transgenic models used here to investigate early stages of PD.

1.1. Parkinson's disease: Symptoms

The main motor symptoms of PD are rigidity, bradykinesia, and postural instability. There is also a resting tremor present in about 70% of the cases, mostly in the upper limbs. As PD progresses, bradykinesia increases until people eventually develop akinesia. Not all PD symptoms relate to movement though, and other symptoms include absence of facial expression, sleep disturbances, and constipation. PD also has a 25 - 40% co-prevalence with dementia (Aarsland et al., 1996; Brown and Marsden, 1984; Hietanen and Teräväinen, 1988) and other psychiatric features such as depression (McDonald et al., 2003). PD symptoms get more severe as the disease progresses, and PD is often not diagnosed until later stages when symptoms have sufficiently progressed.

Several strategies exist for treating PD at different stages, but nothing is available thus far to stop disease progression. Understanding more about the earliest stages of PD pathology, and progression of the disease, remains vital for developing more effective treatment strategies. (Lees et al., 2009; Lesage and Brice, 2009; Postuma et al., 2012).

1.2. Parkinson's disease: Aetiology

Roughly 90% - 95% cases of PD are idiopathic, although various genetic and environmental factors that cause or contribute to PD have also been identified. Around 5% - 10% of PD cases are known to have genetic causes (Lees et al., 2009; Lesage and Brice, 2009).

1.2.1 Environmental factors

One of the first clues to the environmental causes of PD came about after four people developed chronic Parkinsonism from injecting a synthetic opiate, which had been wrongly manufactured to contain high levels of MPTP (Langston et al., 1983). This compound crosses the blood-brain barrier, and is metabolised by monoamine oxidase of glial cells to become MPP⁺, a toxin that is taken up into *dopamine* (DA) neurons by the dopamine transporter (DAT) (Gainetdinov et al., 1997). MPP⁺ kills the cells by interfering with mitochondrial function and by causing superoxide production (Fountaine et al., 2008; Nicklas et al., 1987).

Since the discovery of MPTP toxicity, other compounds, such as the pesticides rotenone and paraquat, have been linked to an increased risk of PD (Brown et al., 2006; Engel et al., 2001; Liou et al., 1997), along with other toxins, such as various heavy metals (Gorell et al., 1999a, 1999b) and solvents (McDonnell et al., 2003). Certain infections, such as viral encephalitis, have also been linked to PD (Takahashi and Yamada, 1999).

Age is by far the largest risk factor for PD (de Rijk et al., 1995), however DA cells are also lost during normal ageing, and there have been mixed opinions about the role that age plays in PD. Early investigation in humans found different patterns of cell loss in ageing in

PD brains (Fearnley and Lees, 1991; Hornykiewicz, 1989), and therefore suggested that the causes of cell loss are fundamentally different between ageing and PD. This idea, along with the way that different risk factors may contribute towards PD, gave rise to the 'multiple hit hypothesis'. This hypothesis states that multiple insults (from genetic or environmental factors), along with a lack of neuroprotective responses, all add up to cause selective neuronal death (Carvey et al., 2006; Sulzer, 2007). More recent investigations however, found robust similarities in various markers of cell pathology in ageing and PD (Chu and Kordower, 2007; Kanaan et al., 2007, 2008). A lot of variation has also been found within the patterns of cell loss for both normal ageing and PD brains (Brooks, 2010; Zaccai et al., 2008), which might explain the earlier differences observed by Hornykiewicz, Fearnley, and Lees. A more recent adaptation of the multiple hit hypothesis now suggests that the underlying mechanisms that cause cell loss in ageing and PD are varied and overlapping. Degeneration of SNc DA neurons is therefore believed to be a part of normal ageing, but can be accelerated stochastically by different insults (Collier et al., 2011).

1.2.2 Genetic factors

Families with inherited PD are relatively rare, and only about 5% to 10% of PD cases are monogenic (Lesage and Brice, 2009). Genome-wide association study (GWAS) analyses have identified at least 15 loci (Lill et al., 2012; Pihlstrøm et al., 2013), and other linkage analysis studies have identified several more genes associated with PD. Information on the most well-known genes associated with PD have been summarised in Table 1.1. Other genes also associated with PD through GWAS include CCDC62/HIP1R, DGKQ/GAK, MCCC1/LAMP3, STK39, SYT11/RAB25, GPNMB, STX1, ACMSD, and FGF20 (Lill et al., 2012; Pihlstrøm et al., 2013).

Early studies have shown up to 75% concordance of PD in monozygotic, and 28% concordance in dizygotic twins, suggesting that genetic mutations have a varied penetrance and that there is some interaction between genetic and environmental factors (Burn et al., 1992; Piccini and Brooks, 1999).

Genetic mutations linked to PD are often involved in molecular cascades that lead to cell death (Hindle, 2010), such as oxidative stress (Andersen, 2004), impaired mitochondrial function (Vincow et al., 2013), protein aggregation (Beyer, 2006), and impaired protein degradation. More about the specific roles of two genes in particular, SNCA and LRRK2, will be discussed in *sections 1.6 and 1.7*.

Table 1.1

Locus	Gene/Protein	Map Position	Form of PD	Protein Function	Associated Mutations	Reference
PARK1/PARK4	SNCA/ α -synuclein	4q21	EOPD, AD, sporadic PD	membrane dynamics	A30P, A53T, E46K, genomic multiplication	(Polymeropoulos et al. 1997)
PARK2	PARK2/Parkin	6q25.2-q27	Juvenile and EOPD AR and sporadic	Ubiquitin E3 ligase	>100 point mutations & exonic rearrangements	(Kitada et al. 1998)
PARK3	Unknown	2p13	LOPD AD	Unkown	Not identified	(Gasser et al. 1998)
PARK5	UCHL1	4p14	LOPD AD	Ubiquitin esterase	one mutation in single PD sibling pair, low penetrance	(Leroy et al. 1998)
PARK6	PINK1	1p36	ARPD	Ser/Thr protein kinase	>40 point mutations, rare large deletions	(Valente et al. 2004)

PARK7	PARK7/DJ-1	1p36	EOPD AR	Uncertain, putative antioxidant properties	>10 point mutations and large deletions	(Bonifati et al. 2003)
PARK8	LRRK2	12q12	LOPD AD, sporadic PD	Protein kinase	>40 missense variants, >pathogenic mutations including G2019S and R1441C	(Funayama et al. 2002; Zimprich et al. 2004)
PARK9	ATP13A2	1p36	Juvenile AR Kufor-Rakeb syndrome and EOPD	P-type ATPase	>5 point mutations	(Ramirez et al. 2006)
PARK10	AAOPD	1p32	GWAS association	Uncertain	11 SNPs	(Hicks et al. 2002; Farrer et al. 2006)
PARK11	GIGYF2	2q36-q37	LOPD AD, low penetrance	Uncertain	7 missense variants	(Sutherland et al. 2009)
PARK12	TSPAN6/ Tetraspanin 6	Xq22.1	Uncertain	Uncertain	Not identified	(Vahedi et al. 2010)
PARK13	Omi/HRTA2	2p13	Uncertain	Anti-apoptotic	2 missense variants	(Strauss et al. 2005)

PARK14	PLA2G6	22q13.1	Jevenile AR L-DOPA responsive dystonia parkinsonism	Ca ²⁺ independent phospholipase A2 β	2 missense mutations	(Paisan-Ruiz et al. 2009)
PARK15	FBX07	22q12-q13	EO AR parkinsonian pyromidal syndrome	F box containing protein (FPB)	3 point mutations	(Di Fonzo et al. 2009)
PARK16		1q32	Low Penetrance sporadic PD		unknown	(Li et al. 2011)
N/A	NR4A2/NURR1	2q22-23	sporadic PD	DA neuron differentiation	2 mutations in exon 1	(Le et al. 2003; Xu et al. 2002; Zheng, Heydari, and Simon 2003)
N/A	SNCAIP1/ Synphilin-1	5q23.1-.3	Unclear	Protein aggregation	Arg621Cys	(Chung et al. 2001; Marx et al. 2003)
	SCA2	12q24.1	Unclear, dominant for SCA2		Low range interrupted CAG expansion in SCA2	(Simon-Sanchez et al. 1; Lim et al. 2006)

GBA	1q21	Gaucher disease, α - synucleopathies	lysosomal hydrolase	Heterozygous mutations increase PD risk	(Tayebi et al. 2003; Goker- Alpan et al. 2010)
Mitochondrial DNA/ NADH complex 1			NADH complex 1	protective SNPs in mtDNA	(van der Walt et al. 2003; Pankratz et al. 2008)
BST1	4p15	GWAS association		no causative mutation found	(Satake et al. 2009; Saad et al. 2011; C. Wang et al. 2013)
MAPT	17q21	GWAS association, synucleopathies	microtubule dynamics	various SNPs	(Galpern and Lang 2006; K. S. Wang, Mullersman, and Liu 2010)

Table 1.1 main genes associated with PD. EO (early onset), LO (late onset), AD (autosomal dominant), AR (autosomal recessive), SCA2 (spinocerebellar ataxia type 2), SNCA (α -synuclein), UCHL1 (ubiquitin carboxy-terminal hydrolase L1), PINK1 (PTEN-induced kinase 1) LRRK2 (leucine rich repeat kinase 2), GIGYF2 (GRB 10-interacting GYF protein 2), PLA2G6 (group VI phospholipase A2), GBA (β glucocerebrosidase) (Table modified from (Lesage and Brice 2009; Pankratz et al. 2008))

1.3. Parkinson's disease: Pathology

PD is a progressive neurodegenerative condition. The Braak hypothesis suggests that PD pathology starts in the brain stem and advances in a topographically predictable manner to the midbrain, the basal ganglia, and the cortex (Braak et al., 2003, 2004). Some findings are however not in line with the pattern of pathology development as predicted by the Braak hypothesis, (Brooks, 2010; Zaccai et al., 2008). Certain pathological markers of PD, such as aggregated α -synuclein, can indeed spread between cells (Kordower et al., 2008), which supports the Braak staging. Some brain regions are however more vulnerable to degeneration than others, which means that PD pathology doesn't advance through all regions at the same rate (Saxena and Caroni, 2011). Furthermore, some variation in the progression of PD pathology can be found in more aggressive forms of PD, which often show early pathology beyond the brainstem (Eriksen et al., 2005; Langston et al., 1999).

In the basal ganglia, PD results in abnormal neurotransmitter release, axonal pathology, and cell loss. The death of DA neurons in the SNc and the concomitant loss of DA signalling in the dorsal striatum constitutes the main cause of motor disturbances in PD (Damier et al., 1999; Samii et al., 2004), although other cells are also affected. The surviving DA neurons often contain protein aggregations called Lewy bodies, which are about 15-30 μm in diameter and contain α -synuclein (Forno, 1996; Takahashi and Wakabayashi, 2001). PD pathology inside the cells involves many pathways, and a few emerging themes from the findings to date have been summarised in the following sections.

1.3.1 Protein aggregation

The presence of Lewy bodies in PD brains is one of the main signs that protein aggregation plays a key role in the disease. Lewy bodies are unable to degrade and they therefore displace other cellular components, which can be harmful to the cells (Bayer and Glimcher, 2005). It has however also been suggested that Lewy bodies provide protection against α -synuclein at earlier stages of aggregation (Tanaka et al., 2004; Tompkins and Hill, 1997), and Lewy body formation is not the only link between protein aggregation and cell death in PD. The ubiquitin proteasome system, and the autophagy lysosomal pathway, which are both critical in removal and degradation of proteins, are also affected during PD (Ebrahimi-Fakhari et al., 2012). Proteins in Lewy bodies are highly ubiquitinated (Kuzuhara et al., 1988), and these aggregations also contain numerous components of the ubiquitin proteasome system (Kwak et al., 1991; Lowe et al., 1990). Furthermore, accumulated autophagic vacuoles have been found in the SN of PD patients, suggesting that impaired autophagy is also involved in the degeneration pathway (Cuervo et al., 2004).

The strong link between protein aggregation and PD poses a problem for animal models that attempt to replicate PD, but fail to present aggregates. The presence of Lewy bodies is a neuropathological criteria for PD diagnosis (Gelb et al., 1999; Lees et al., 2009), and the observation of Lewy bodies in presymptomatic cases suggest that Lewy bodies are involved in early stages of the disease (Braak et al., 2004; Gibb and Lees, 1988). However, PD-linked mutations, such as LRRK2, can lead to PD clinical diagnosis without the presentation of Lewy bodies (Gaig et al., 2007), and some models that express PD-linked genes only present protein α -synuclein aggregates during later stages (Eslamboli et al., 2007; Janezic et al., 2013). Some animal models have been able to provide clues of the early

stages of PD (Janezic et al., 2013; Nemani et al., 2010), but whether animal models of PD can be called as such without presenting α -synuclein aggregation remains an on going controversy.

1.3.2 Oxidative stress and mitochondrial dysfunction

Mitochondrial damage and oxidative stress are known to play a key role in ageing, and since age is the greatest risk factor for PD, we can certainly expect a link between PD and these two factors. Indeed, mitochondrial stress and oxidative damage are involved in PD pathogenesis in a rather complex manner.

The most direct role the mitochondria play in PD is through the inhibition of complex-I by MPTP or rotenone (Betarbet et al., 2000; Langston and Ballard, 1984), but the discoveries of mutations and polymorphisms in at least nine genes and mitochondrial DNA suggests that complex-I inhibition is not the only contribution to PD pathogenesis. Other mitochondrial damage causes cytochrome-C to be released, therefore triggering apoptosis. Parkin associates with the outer mitochondrial membrane to prevent cytochrome C release and caspase activation (Darios et al., 2003). Damaged mitochondria can also place a burden on the cell's autophagy mechanisms, and impaired mitophagy is another step towards PD pathology. PINK1 and Parkin act together to promote mitophagy and respiratory chain turnover (Vincow et al., 2013), and mutations in either of these genes have been known to lead to autosomal recessive PD. SNCA can also play a role in mitochondria dysfunction as overexpressing the A53T mutant form of α -synuclein in mice has been associated with mitochondrial DNA damage, mitochondrial degeneration and apoptosis (Martin et al., 2006).

Oxidative stress can reciprocally lead to mitochondrial damage, so these two factors often appear together. The oxidative state inside cells can impact protein structure, and investigations in cybrid cells have revealed that α -synuclein can be oxidised in conditions with mitochondrial dysfunction, but that antioxidants decrease α -synuclein aggregation (Esteves et al., 2009). Another potential mechanism where oxidative stress may contribute to PD pathology is DA itself, which can readily oxidise to produce quinones and free radicals. DA has been observed to affect α -synuclein aggregation (Watabe-Uchida et al., 2012), and VMAT2 can attenuate the toxic effects of DA and MPTP by sequestering them into vesicles (Guillot and Miller, 2009).

1.3.3 Immune responses, Braak stages, prion mechanisms, and region susceptibility

The underlying pathology in PD is complex and varied. Protein aggregation, oxidative imbalance and presynaptic dysfunction are all key features of PD pathology, but a few other mechanisms are also worth mentioning.

DA cell death in the SNc is associated with increased activation of microglial cells (McGeer et al., 1988; Teismann et al., 2003), which suggests that PD does not only affect DA neurons, but can involve changes to a range of different cells. It has also been observed that α -synuclein can transmit from one cell to another, not unlike prion proteins, (Grey et al., 2011; Hansen and Li, 2012), and transplanted embryonic SNc tissue can be 'infected' by α -synuclein from affected cells in a PD brain (Kordower et al., 2008). The spread of PD does however not follow a linear infection pattern (Brooks, 2010; Zaccai et al., 2008), which suggests that some cells are more vulnerable than others.

SNc DA neurons have several unique features that confer susceptibility to cell loss, including a high Ca^{2+} flux, exceptionally large axonal arbour, and a lack of protective factors. SNc DA neurons use Ca^{2+} for pacemaking activity (Saxena and Caroni, 2011). The intrinsic pacemaking activity of SNc DA neurons is driven by L-type Cav1.3 Ca^{2+} channels, which produce high Ca^{2+} flux loads (Chan et al., 2007), thereby contributing to oxidative stress (Chan et al., 2007; Guzman et al., 2010). Furthermore, SNc DA neurons lack the Ca^{2+} buffering protein calbindin, which is expressed in neighbouring VTA DA neurons (Damier et al., 1999). The lack of calbindin may further contribute to Ca^{2+} -related oxidative stress in SNc DA neurons. In addition to having a high flux of Ca^{2+} , SNc DA neurons also have unmyelinated axons and a uniquely high level of axonal branching, which create a substantial energy burden (Matsuda, 2009; Pissadaki and Bolam, 2013). Furthermore, insults to the cell, such as genetic mutations or toxins, may trigger compensatory homeostatic mechanisms, such as increasing the rate of protein degradation, thereby causing a further increase on the energy demand. It has therefore been suggested that the uniquely high energy burden of SNc DA neurons makes this population more susceptible, in comparison to other neurons, to be triggered into a pathological state by insults (Wellstead and Cloutier, 2011; Bolam and Pissadaki, 2012). In accordance with this idea, it has also been suggested that a range of insults to the nigrostriatal DA system can accelerate SNc degeneration in a cumulative manner (Sulzer, 2007; Collier et al., 2011).

The underlying aim of this thesis is to investigate presynaptic dysfunction as one of the earliest stages of PD pathology, so other mechanisms are beyond the scope of this introduction, and more about the susceptibility of SNc DA neurons will be discussed in Chapter 7.

1.3.4 Presynaptic dysfunction

Due to the progressive nature of PD, it can be expected that cell dysfunction, including abnormal neurotransmission would precede cell death. Several genes, including *SNCA* and *LRRK2*, have been associated with presynaptic dysfunction. The protein α -synuclein, coded for by *SNCA* is mainly found in presynaptic terminals (Lavedan et al., 1998; Yu et al., 2007), where it is involved in normal trafficking of presynaptic vesicles (Cabin et al., 2002; Murphy et al., 2000), and acts as a negative regulator of DA release (Abeliovich et al., 2000; Nemani et al., 2010). *LRRK2* can interact with synaptic vesicles in DA neurons (Biskup and Moore, 2006), and other presynaptic proteins (Piccoli et al., 2011), where it seems to play a role in DA neurotransmission (Li et al., 2010, 2009; Tong et al., 2009). More about the roles of α -synuclein and *LRRK2* in presynaptic dysfunction of DA neurons will be discussed in sections 1.6 and 1.7.

Other PD-related genes associated with presynaptic dysfunction are *DJ1* and *PINK1*. Although both the *DJ1* and *PINK1* proteins are mainly associated with mitochondria, *DJ1* can be found in the cytosol (Silvestri et al., 2005; Zhang et al., 2005). The *DJ1*, *PINK1* and Parkin complex can also interact with α -synuclein and loss of function mutations in these three genes cause autosomal recessive PD. Both *DJ1* and *PINK1* knockout mice show a clear deficit in DA neurotransmission (Gispert et al., 2009; Goldberg et al., 2003; Kitada et al., 2007), and $\alpha\beta\gamma$ -synuclein knockout mice show an increase in DA neurotransmission (Anwar et al., 2011). These observations suggest some antagonistic interactions between *DJ1*, *PINK*, and Parkin vs. α -synuclein, although the mechanism remains incompletely understood. Presynaptic dysfunction has been reported to precede other stages of PD pathophysiology (Lam et al., 2011; Lundblad et al., 2012). Exploring the mechanisms by

which α -synuclein and LRRK2 contribute to presynaptic dysfunction, such as deficits in DA release, could therefore contribute to a better understanding of early-stage PD pathophysiology.

1.4. Parkinson's disease in the basal ganglia and brain stem

1.4.1 Overview

The basal ganglia are a subcortical group of nuclei involved in cognitive, learning, and motor functions. The basal ganglia contain a complex network of connections and feedback loops that receive inputs from various regions, including cortex, thalamus, hypothalamus, and brainstem. The basal ganglia produce outputs to the cortex and subcortical regions including the superior colliculus (SC), pedunculopontine nucleus (PPN), lateral habenula (HBN), and reticular formation (RF) (Albin et al., 1989; DeLong, 1990; Smith et al., 1998). Dysfunction in the basal ganglia, caused by denervation of neuron fibres including DA neurons, contributes to the motor deficits of PD (Carlsson, 2002; Mallet et al., 2008; Wichmann and Dostrovsky, 2011). In addition to this, denervation in the brain stem, including the loss of Raphe *5-hydroxytryptamine* (5-HT) neurons, have also been found to play a role in both the motor and non-motor symptoms of PD (Braak et al., 2002; Halliday et al., 1990a; Henderson et al., 1992). A brief overview is given here of the structure and function of the basal ganglia and brain stem, followed by an introduction to some of the DA and 5-HT systems implicated in PD.

1.4.2 Anatomy of the basal ganglia

The basal ganglia mainly consist of the striatum, internal and external segments of the globus pallidus, subthalamic nucleus (STN), and the substantia nigra (SN) (Parent and Hazrati, 1995a, 1995b). There are however a few differences between species, for example, the external segment of the globus pallidus (GPe) in humans is often referred to as simply the globus pallidus (GP) in rodents, and the internal segment of the globus pallidus (GPi) in

humans is the entopeduncular nucleus in rodents. The dorsal part of the striatum contains the caudate nucleus and putamen, which are separated by the internal capsule, but in rodents this part is jointly known as the caudate putamen (CPu). The ventral part of the striatum contains the nucleus accumbens (NAc), which can be further subdivided into the NAc core (NAcC) and NAc shell (NAcS), and also portions of the olfactory tubercle. The SN can be divided into SN pars compacta (SNc) with the adjacent ventral tegmental area (VTA), and the SN pars reticulata (SNr).

The majority of the inputs to the basal ganglia are glutamatergic cortical and thalamic inputs to medium spiny neurons (MSNs) in the striatum (Kemp and Powell, 1971). Other inputs to the striatum include the PPN, midbrain, STN, GPe, raphe, amygdala, and hippocampus. About 90 to 95% of striatal neurons are γ -aminobutyric acid (GABA)-ergic MSNs (Oorschot, 1996), and the remaining cells are large cholinergic interneurons, and at least three different populations of GABA interneurons (parvalbumin-positive, nitric oxide synthase, and calretinin-expressing). MSNs can project to cells within the striatum, but also project to the other nuclei of the basal ganglia. One sub-population of MSNs preferentially project to the SNr and GPi, the output nuclei of the basal ganglia, thus forming the direct pathway, which disinhibits the thalamus and facilitates motor functions. These MSNs selectively express dynorphin, substance P, and mainly D₁-type DA receptors, therefore receiving excitatory input from DA neurotransmission in the striatum. The other sub-population of striatal MSNs project to the GPe, which signals either straight to the output nuclei, or through the STN. Outputs from this set of MSNs therefore comprise the indirect pathway, causing inhibition to motor functions. These MSNs express enkephalin and D₂-type DA receptors, therefore receiving inhibitory input from DA neurotransmission in the

striatum (Bolam et al., 2000; Chevalier et al., 1985; Parent and Hazrati, 1995b; Smith et al., 1998). The output nuclei of the basal ganglia project back to the cortex via the thalamus, and also to other sub-cortical structures such as the SC, PPN, LH, and RF (Figure 1.1).

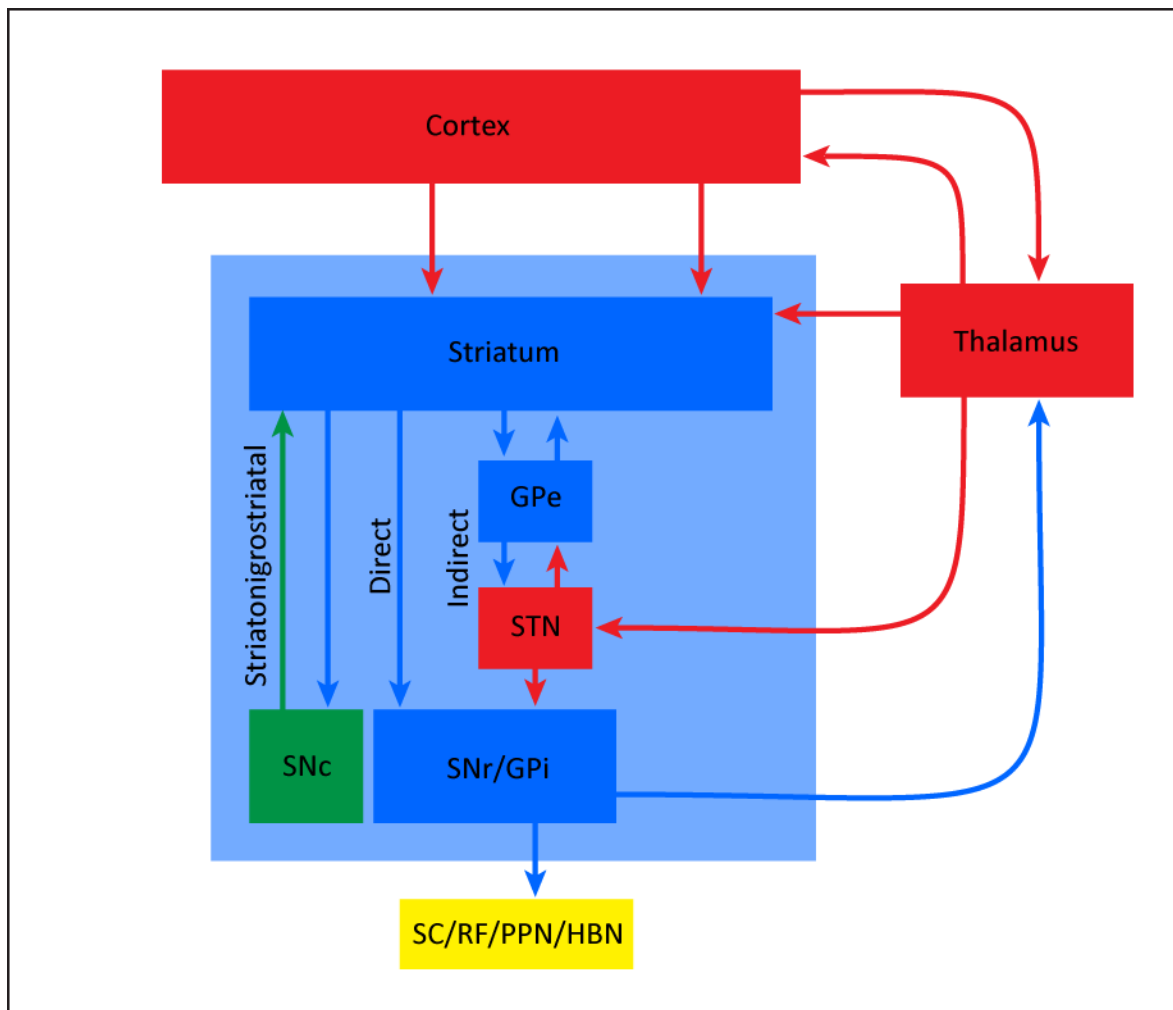


Figure 1.1: Simplified diagram of the basal ganglia and the main connections. The basal ganglia consist of the striatum, internal and external segments of the globus pallidus (GPi and GPe), sub thalamic nucleus (STN), substantia nigra pars reticulata (SNr), and substantia nigra pars compacta (SNc). Red arrows are excitatory glutamatergic projections, blue arrows are inhibitory GABAergic projections, and green arrows are dopaminergic projections. Cortex and thalamus project to basal ganglia nuclei including the striatum and STN. Some striatal MSNs send inhibitory GABAergic projections to the output nuclei SNr and GPi, forming the indirect pathway. Other striatal MSNs send inhibitory projections to the GPe, thereby disinhibiting the output nuclei and forming the indirect pathway. The SNr and GPi can inhibit the thalamus, superior colliculus (SC), reticular formation (RF), pedunculopontine nucleus (PPN), and lateral habenula (HBN). Striatal MSNs also send GABAergic projections to the SNc, and receive dopaminergic input from the SNc and VTA.

1.4.3 Dopamine in the basal ganglia

Midbrain DA neurons send large projections to the striatum (Matsuda, 2009), but also send smaller projections to other nuclei including the GP and STN (Cragg et al., 2004; Fuchs and Hauber, 2004). Projections of midbrain DA neurons travel through the medial forebrain bundle to the striatum, and form *en passant* varicosities, with some having no post synaptic specialisation, and others making synaptic connections onto dendritic spines, shafts, and cell bodies (Descarries et al., 1996; Groves et al., 1994). DA signalling acts by volume transmission, so DA release from one bouton can reach more than one cell or dendrite (Cragg and Rice, 2004; Cragg et al., 2001; Rice et al., 2011).

Mesostriatal dopaminergic projections are arranged topographically so that neurons from the ventral tier of the SNc mainly project to the dorsolateral striatum, and DA neurons in the dorsal tier SNc and VTA mainly project to the central and ventromedial striatum. The topography is however rather complex with overlapping projections so that there is a ventrolateral to dorsomedial gradient in the midbrain projecting towards a dorsolateral to ventromedial gradient in the striatum respectively (Fallon and Moore, 1978; Joel and Weiner, 2000; Lynd-Balta and Haber, 1994; Prensa and Parent, 2001). There is another organisation superimposed on top of the topographical arrangement projecting preferentially to either patch or matrix compartments in the striatum (Gerfen et al., 1987, 1985; Graybiel et al., 1981; Martin et al., 1993). DA neurons in the ventral tier of the SNc project preferentially to the patches defined by areas of dense μ -opiate receptor binding and areas of low acetylcholinesterase labelling (Graybiel et al., 1981; Herkenham and Pert, 1981) whereas the dorsal tier of the SNc preferentially projects to matrix compartments,

which are mainly composed of neurons that contain 28 kD calbindin and a rich plexus of somatostatin immunoreactive fibres (Gerfen, 1985; Gerfen et al., 1985).

Striatal MSNs can feed back to midbrain DA neurons either by direct projections, or through striato-pallido-mesencephalic, or striato-pallido-thalamo-cortical circuits (Ikemoto, 2007). MSN projections from the striatum to the midbrain are also topographically arranged so that ventromedial striatum projects to both tiers of the SNc and the VTA, but projections from the central and dorsolateral striatum are gradually more focused towards the ventral tier SNc, and ventral columns in primates (Haber, 2000; Joel and Weiner, 2000). The reciprocal projections between the midbrain and striatum therefore form an ascending spiral whereby the ventral striatum, associated with limbic functions, can influence DA input to the associative central striatum, which in turn influences DA input to the motor dorsolateral striatum (Haber, 2000). In addition to receiving inputs from the striatum, SNc and VTA DA neurons also receive inputs from other areas including the GP, PPN, STN, SNr, laterodorsal tegmental area (LDTg), dorsal raphe, hypothalamus and cortex (Bolam and Smith, 1990; Clarke et al., 1987; Van der Kooy and Hattori, 1980; Watabe-Uchida et al., 2012).

DA can be released from DA axons in the striatum, or at the somatodendritic level (Cragg et al., 1997a, 1997b; Stamford et al., 1988). The amount of DA released in the striatum can be influenced locally by various factors at the synaptic terminals, including availability of frequency of action potentials, DA uptake by DAT, synaptic vesicle organization, D2 autoreceptors, and Ca^{2+} concentration, GABA, glutamate, cannabinoids, hydrogen peroxide (H_2O_2), acetylcholine (ACh), opioids, and nitric oxide (NO). Part of the aim of this thesis will

be to investigate the mechanisms by which PD-related genes affect DA release. Some of the mechanisms mentioned here will therefore be discussed in greater detail in the results chapters, particularly Chapter 4.

Midbrain DA neurons are tonically active *in vivo* at frequencies of 2-10 Hz, but also have phasic bursts at 15 -30 Hz that may cause more DA to be released, and these are believed to be involved in the neural activity encoding of the salience of experiences, as well as the value of rewarding experiences and the anticipated value of rewards (Matsumoto and Hikosaka, 2009; Mirenowicz and Schultz, 1994). Regional differences can be seen in the frequency dependence of electrically evoked DA release, with the CPu showing little frequency dependence and the NAc being more dependent of stimulation frequency (Cragg, 2003; Exley et al., 2007; Trout and Kruk, 1992). The activity of DA neurons can be influenced at the somatodendritic level by several signalling molecules including glutamate, GABA, H₂O₂, ACh, 5-HT, and substance P (Bolam and Smith, 1990; Chen et al., 2002; Gérard et al., 1996, 1997; Morikawa and Paladini, 2011; Weiner et al., 1990).

DA is stored in presynaptic vesicles that are not distributed in clearly defined pools. However, presynaptic vesicles in DA neurons can be observed in a range of distributions, with some vesicles primed for exocytosis at the plasma membrane, and others tethered to the cytoskeleton, and unavailable for release (Thomson, 2000). There is also a functional distinction between synaptic pools of vesicles, with some available for immediate release, and others not immediately available, but that can be detected once the immediately available pool has been depleted. For example, it has been observed that α -methyl-p-tyrosine can deplete the readily releasable pool of DA (Venton et al., 2006), thereby

quenching electrically evoked DA release. However, the application of cocaine can reinstate DA release by mobilising a reserve pool of vesicles. Interestingly, the action of cocaine depends on availability of synapsins (Venton et al., 2006), which are a family of proteins involved with the mobilisation of synaptic vesicles (Hosaka and Südhof, 1998; Feng et al., 2002).

Extracellular DA is taken up by the DA transporter (DAT) and recycled back into vesicles through vesicular monoamine transporter 2 (VMAT 2). DAT is only expressed by DA neurons (Hersch et al., 1997; Nirenberg et al., 1996), although DA can be taken up by the 5-HT transporter (Larsen et al., 2011). The decrease in extracellular concentration of DA ($[DA]_o$) after exocytosis is however not dominated by DAT mediated uptake, but rather by diffusion away from the cell (Cragg and Rice, 2004; Cragg et al., 2001). The population of presynaptic DAT is greater in the CPu than the NAc (Cragg et al., 2000; Stamford et al., 1988), leading to differences in the rate of DA uptake between these regions.

DA release is auto regulated by D_2 receptors with a time lag of about 250ms-300ms (Benoit-Marand et al., 2001), and their effect can last for up to 5 seconds (Lee et al., 2002b; Phillips et al., 2002). The location of striatal D_2 autoreceptors is primarily extrasynaptic, and they decrease DA release by acting at the Kv1 family of voltage gated K^+ channels (Fulton et al., 2011; Martel et al., 2011). However, D_2 autoreceptors can also have different long term effects in the cell body to chronically increase DA pacemaker through cyclic AMP and protein kinase A signalling (Hahn et al., 2006). In acute striatal slices, D_2 agonists such as quinpirole cause a concentration dependent suppression of electrically evoked DA release (Kennedy et al., 1992; Stamford et al., 1991). On the other hand, D_2 antagonists

have no effect on single stimulation DA release, but relieve D₂ mediated suppression of DA release from pulse-train stimulations (Cragg and Greenfield, 1997; Kennedy et al., 1992; Limberger et al., 1991)

1.4.4 Parkinson's disease in the dopamine pathways

Although PD pathology in the brain can be widespread, the death of DA neurons in the SNc and a deficit of DA release in the striatum, remains one of the key features (Lees et al., 2009; Meissner et al., 2011). It is the preferential death of SNc DA neurons, and the concomitant loss of DA signalling in the dorsal striatum, which is responsible for the main symptoms of PD (Albin et al., 1989; DeLong, 1990; Langston et al., 1999). Understanding what happens to DA neurons in the nigrostriatal pathway during PD can therefore play a key role in understanding more about the disease and working towards treatments. The loss of DA can be preceded by dysfunction in DA signalling where DA release, uptake, and presynaptic regulatory mechanisms all seem to play a role (Lam et al., 2011; Lundblad et al., 2012; Nuber et al., 2013). Part of the aim of this thesis is to investigate the effect of certain PD-related genes on DA release.

1.4.5 The brain stem and raphe nuclei

The brain stem is the lower extension of the brain, which connects to the spinal cord and cerebellum. It's made up of four sections namely medulla oblongata, pons, midbrain, and diencephalon (Ángeles Fernández-Gil et al., 2010). Flanking the midline of the brain stem, from the medulla oblongata to the midbrain, lie the raphe nuclei (Meessen and Olszewski, 1949; Olszewski and Baxter, 1954). 5-HT neurons make up the largest population of cells in the raphe nuclei, preferentially located near the midline, but other neurons can also be

found in this area, including neurons positive for monoamines and neuropeptides (Dahlstroem and Fuxe, 1964; Hornung, 2003).

The raphe nuclei consist of a rostral group which contains about 85% of the 5-HT neurons in the brain, and a caudal group which contains a maximum of 15% of the population of 5-HT neurons in the brain (Hornung, 2003). The rostral group contains three main nuclei known as the caudal linear nucleus (CLi), dorsal raphe nucleus (DR), and median raphe nucleus (MnR). The CLi is located in the VTA, dorsal and caudal to the interpeduncular nucleus, where 5-HT neurons can be found alongside others that contain tyrosine hydroxylase (TH) and substance P (Halliday et al., 1990a, 1990b). The DR overlaps with the CLi at the oculomotor nucleus, and extends caudally to the middle of the pons. The DR contains one of the largest populations of 5-HT neurons in the brain stem, although a significant amount have also been found positive for substance P (Baker et al., 1991).

Afferent connections to the rostral group of raphe nuclei include glutamatergic projections from the ventral tegmental area, lateral habenula, several hypothalamic nuclei, interpeduncular nucleus, the laterodorsal tegmentum nuclei, and the cingulate cortex (Behzadi et al., 1990; Peyron et al., 1998), but also GABAergic and other projections (Wang et al., 1992). Efferent projections from the rostral group ascend towards the forebrain in two parallel pathways known as the dorsal- and ventral tracts (Azmitia and Gannon, 1983; Azmitia and Segal, 1978). Both these pathways meet in the caudal diencephalon, where they mix and split again into two new projections. One of these projections passes through the internal capsule to the lateral cerebral cortex, and the other travels within the medial forebrain bundle to innervate several areas including the hypothalamus, basal forebrain,

amygdala, and medial cerebral cortex (Azmitia and Segal, 1978; Imai et al., 1986; Kievit and Kuypers, 1975; Steinbusch et al., 1980; Waterhouse et al., 1986).

1.4.6 5-HT in the brain stem and basal ganglia

5-HT is widely distributed in the CNS and involved in a range of functions like sleep, arousal, feeding, motor functions, mood and emotion (Fox et al., 2009). 5-HT cell bodies located in the MnR and DR project widely through the brainstem, basal ganglia, cortex, and cerebellum. In mice, 5-HT projections to the substantia nigra make direct synaptic contacts with various dendrites, including those from DA neurons (Corvaja et al., 1993; Nedergaard et al., 1988). 5-HT receptors on DA neurons, such as 5-HT_{1A}, 5-HT_{2A}, and 5-HT₆ receptors (Doherty and Pickel, 2001; Gérard et al., 1996, 1997; Nocjar et al., 2002), are however expressed in relatively low amounts. With various other 5-HT receptor ligands influencing DA release (Alex and Pehek, 2007), it seems that 5-HT also regulates DA release indirectly by acting on SN GABA-ergic and other neurons. For example, 5-HT_{1B} agonists decrease GABA in the VTA (Parsons et al., 1999), inhibits K⁺ induced [³H] GABA release from VTA slices (Yan and Yan, 2001), and concurrently reduces the amplitude of GABA-B IPSPs in VTA DA neurons (Cameron and Williams, 1995; Johnson et al., 1992).

Electrically evoked 5-HT release in the SNr depends on stimulation frequency and pulse number, with the highest amounts released at frequencies of 30Hz– 50Hz (Jennings et al., 2010; John et al., 2006). 5-HT transporters (SERT) can affect the frequency sensitivity of SNr 5-HT release, and also reduce the extracellular 5-HT concentration [5-HT]_o by taking it back up into the cells (Jennings et al., 2010). 5-HT release in the SNr is Ca²⁺ dependent, is under local presynaptic regulation of the 5-HT_{1B} autoreceptor (Threlfell et al., 2010a), and

can also be suppressed by histamine, through H3 and constitutively active H2 histamine receptors on 5-HT axons, in a manner that is independent of glutamate or GABA (Threlfell et al., 2004, 2008).

1.4.7 Parkinson's disease in the 5-HT pathways

The loss of 5-HT neurons have not been implicated in PD to the same extent as the loss of nigrostriatal DA neurons, but it is worth noting that 5-HT systems can also be severely affected in PD, and studying the early stages of PD development in other cells such as 5-HT can help elucidate the underlying pathological mechanisms of PD. Both humans and rodents express α -synuclein in raphe 5-HT neurons (Giasson et al., 2001; Maroteaux et al., 1988). Post-mortem analyses of PD patients have shown a reduction in 5-HT levels, 5-HT related enzymes, and 5-HT markers throughout the raphe, basal ganglia and cortex (Halliday et al., 1990a; Kish et al., 2008; Scatton et al., 1983). The earliest stages of PD seem to affect the lower raphe nuclei more, which project to the spinal cord, whereas the dorsal raphe nuclei, which project to the basal ganglia and cortex, are not affected until later stages of disease progression (Braak et al., 2002, 2003; Del Tredici et al., 2002). PD has been associated with a reduction in cortical, striatal, and hippocampal 5-HT, and a reduction in striatal and midbrain 5-HT transporters, which suggests loss of 5-HT boutons (Kish et al., 2008; Roselli et al., 2010). Part of the aim of this thesis is to investigate the effect of certain PD-related genes on 5-HT release.

1.5. Treatments for Parkinson's disease

Various treatment options are currently available for PD, but to date there is nothing that can stop or reverse the neurodegeneration process. The following section summarises some of the most relevant and latest developments in treating PD.

1.5.1 Pharmacological treatments

By far the most commonly used pharmacological treatment for PD is *L*-3,4-*dihydroxyphenylalanine* (L-DOPA), a compound produced in the body from L-tyrosine by TH, which is the rate-limiting enzyme in DA production. L-DOPA increases the levels of DA production in surviving DA neurons by being converted to DA through a reaction catalysed by DOPA decarboxylase, and therefore overcoming the rate-limiting step in DA synthesis. L-DOPA is often given with amino acid decarboxylase (AADC) inhibitors, that don't cross the blood brain barrier, to prevent excessive DA production in the peripheral nervous system (Rascol et al., 2002). L-DOPA does however cause dyskinesias in about 50% of patients within 5 years of treatment (Hely et al., 1994; Nutt and Nygaard, 2001), and 20% - 50% of patients experience fluctuations in motor performance after 5-6 years (Hely et al., 1994; Koller et al., 1999).

An alternative to L-DOPA treatment involves taking DA agonists, such as apomorphine. These drugs tend to have a lower incidence of dyskinesias (Holloway et al., 2004), but can cause psychological disturbances and cardiovascular problems (Singh et al., 2007; Wong et al., 2003). DA agonists are not quite as effective as L-DOPA, which normally has to be given after 2-5 years of DA agonist treatment (Silver and Ruggieri, 1998). Other dopaminergic drugs include monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT)

inhibitors. These are often used in conjunction with L-DOPA and prevent the degradation of DA *in vivo* (Singh et al., 2007). Like other dopaminergic drugs, MAO and COMT inhibitors have serious side effects including dyskinesias and cardiovascular problems.

Another class of drugs called anticholinergics, which act as antagonists at ACh receptors in the brain. ACh and DA work in conjunction to modulate MSN activity in the striatum (Cragg, 2006), and anticholinergics therefore counteract the imbalance caused by reduced DA signalling. Muscarinic antagonists can be used to treat the tremor in PD (Katzenschlager et al., 2003), but side effects, including drowsiness, confusion and agitation, limits drug dosing (Singh et al., 2007).

Other pharmacological treatments for PD also include antiapoptotic drugs, promitochondrial drugs, glutamate agonists, growth factors, vitamins and anti inflammatory agents (LeWitt and Taylor, 2008), although the full extent of pharmacological treatments available for PD is beyond these cope of this introduction.

1.5.2 Surgical and other treatments

Surgical treatments have mainly focussed on lesioning certain neural pathways to disinhibit movement. Early surgical ventroposterolateral pallidotomy treatments showed marked improvements in some patients (Laitinen et al., 1992) and more recent non-destructive lesions in the GPi and STN by deep brain stimulation have seen improvements in patients that can last several years (Rodriguez-Oroz et al., 2005). The effects of deep brain stimulation can be varied though, and worsen some symptoms whilst improving others (Fasano et al., 2012). Deep brain stimulation can however be a very effective

treatment for PD if patients are carefully selected to avoid complications (Bronstein et al., 2011).

Cell replacement therapy can be used to replace DA innervation in the striatum. Some transplantations of foetal SNc tissue into the striatum of PD patients have been attempted but overall, it has not been very successful (Freed et al., 1992; Freeman et al., 1995; Lindvall et al., 1990; Olanow et al., 2003), and the best results of such transplants don't seem significantly better than deep brain stimulation or pharmacological treatments (Obeso et al., 2010; Olanow et al., 2009). The vast distance between the SNc and striatum is too great for axons from SNc neurons to innervate the striatum once the brain has completed development. Therefore, DA neurons have to be implanted directly into the striatum, which means that they can't fulfil the same role as the SNc neurons that were lost. Transplantation of foetal SNc tissue also has ethical challenges and the tissue is prone to be rejected by the immune system, which makes this therapy an ideal candidate for induced pluripotent (iPS) stem cells, which are reprogrammed cells taken from the patient (Kiskinis and Eggan, 2010; Soldner et al., 2009). The role for iPS cells to treat PD is however still under development with some studies having success in showing observing stem cells forming innervations and relieving motor symptoms in rodent and monkey models of PD (Björklund et al., 2002; Cai et al., 2009; Redmond et al., 2007). One of the major obstacles in this area remains specific programming of the cells and avoiding tumour formation.

Another interesting avenue for treating PD is gene therapy. This strategy attempts to restore DA synthesis through inducing the expression of enzymes such as AADC (Eberling et al., 2008), or neurotinin (Marks et al., 2008). Gene therapy treatments have shown

success in MPTP primate models (Hadaczek et al., 2010) and several clinical trials have been undertaken worldwide (Ginn et al., 2013).

Although there are many different approaches to treating PD, nothing has been able to stop or reverse the progression of cell death. By far the most effective strategy to treating PD is early diagnosis and treatment, which means that understanding the early stages of PD pathology, including synaptic dysfunction is of great interest. The rest of the introduction will cover SNCA and LRRK2: structure and physiological function, role in PD, and a summary of transgenic models to date, along with briefly comparing our model and explaining why it's necessary.

1.6. Structure, expression, and function of α -synuclein

The synuclein family of proteins was first isolated from Pacific electric ray using antiserum against purified cholinergic terminals (Maroteaux et al., 1988). α -Synuclein, which is coded for by the *SNCA* gene, was initially linked to neurodegenerative disease as a non-amyloid component in amyloid plaques of Alzheimer's disease patients (Jakes et al., 1994; Uéda et al., 1993). It was however later revealed that α -synuclein does not always associate with amyloid plaques, but rather that α -synuclein aggregations, including Lewy bodies, can sometimes be found in brains affected with Alzheimer's disease (Culvenor et al., 1999; Wirths and Bayer, 2003).

SNCA is found on chromosome 4q21.3-q22, where it spans 117kb DNA, and has 6 exons, although the first exon is untranslated. α -Synuclein contains 140 amino acids (Touchman et al., 2001; Xia et al., 2001), and is a member of the highly conserved synuclein family along with β - and γ -synuclein (Lavedan, 1998; Spillantini et al., 1995). The mouse *SNCA* gene is found on chromosome 6, and there is a relatively high level of similarity between human and mouse α -synuclein, with the sequences being 95.3% identical (Eliezer et al., 2001; Lavedan, 1998; Touchman et al., 2001). The α -synuclein protein can be divided in three domains according to its primary structure, namely the N-terminal-, central-, and C-terminal domains. The N-terminal domain, consisting of amino acids 1 - 61, contains 11 imperfect amino acid repeats with the core sequence: KTKEGV, which is very similar to class A2 apolipoproteins and allows it to bind to membranes (Davidson et al., 1998; Westphal and Chandra, 2013). The central domain, consisting of amino acids 62 to 95 was originally the part believed to be the non-amyloid component in amyloid plaques (Culvenor

et al., 1999; Uéda et al., 1993). This region is hydrophobic, and involves lipid binding, and helical structure formation (Spillantini et al., 1998). The C-terminal domain, consisting of amino acids 96 - 140, contains many aspartate and glutamate residues, which therefore makes it acidic. The C-terminal does not form a helical structure like the rest of the protein and has no association with vesicles (Eliezer et al., 2001; Ostrerova et al., 1999).

The conformation of α -synuclein depends largely on the environment. α -Synuclein has been observed as an unfolded monomer in solution (Eliezer et al., 2001; Jakes et al., 1994). α -Synuclein monomers have also been observed in vivo when interacting with phospholipids (Davidson et al., 1998; Westphal and Chandra, 2013). Fibrillary beta sheets of α -synuclein have been observed in aggregates, such as Lewy bodies, where it can interact with other proteins like 14-3-3 proteins and parkin (Kawamoto et al., 2002; Schlossmacher et al., 2002; Spillantini et al., 1998). The secondary structure of membrane bound α -synuclein can also depend on membrane curvature. Low curvature induces a straight helix, and very curved membranes cause α -synuclein to adopt a broken helix (Westphal and Chandra, 2013).

The expression of α -synuclein is predominantly restricted to neurons in the central nervous system, and can be found in areas including the cortex, hippocampus, olfactory bulb, striatum, substantia nigra, thalamus, amygdala, and cerebellum of the adult brain (Galvin et al., 2001; George et al., 1995; Iwai et al., 1995; Jakes et al., 1994; Maroteaux and Scheller, 1991; Masliah et al., 1996; Mori et al., 2002; Shibayama-Imazu et al., 1993). The intracellular localisation of α -synuclein changes throughout the development period, initially confined to the cell bodies of neural precursors, and later redistributed to

presynaptic nerve terminals where it can either be found in the cytosol or associated with synaptic vesicles (Bartels et al., 2011; Maroteaux and Scheller, 1991; Westphal and Chandra, 2013).

The earliest observations of a causal link between α -synuclein gene mutations and PD were the A30P, and A53T point mutations identified in familial cases of PD (Krüger et al., 1998; Polymeropoulos et al., 1997). Shortly thereafter, α -synuclein was discovered to be the primary component of Lewy bodies (Spillantini et al., 1998), and the E46K mutation was also later reported (Zarranz et al., 2004). More recently, the H50Q mutation was also discovered in a PD patient with a family history of Parkinsonism and dementia (Appel-Cresswell et al., 2013). The autosomal dominant inheritance of these forms of PD suggests that the A30P, A53T, H50Q, and E46K point mutations, which are all in the central domain, cause a gain of function that leads to PD. More recently, GWAS studies have linked α -synuclein to PD, and also identified a few low penetrance α -synuclein single nucleotide polymorphisms (Edwards et al., 2010; Pankratz et al., 2008; Satake et al., 2009; Simon-Sanchez et al., 2009). Interestingly, duplications and triplications of the unmutated *SNCA* gene locus has been shown to cause familial PD, suggesting that an overabundance on wild type (WT) α -synuclein can also cause PD (Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibáñez et al., 2004; Singleton et al., 2003). Furthermore, people with *SNCA* triplications have shown higher levels of cortical α -synuclein, earlier disease onset, and more severe symptoms than people with *SNCA* duplications, suggesting a dose dependence for the pathophysiology of α -synuclein (Eriksen et al., 2005; Farrer et al., 2004). The idea that unmutated α -synuclein can contribute towards PD is further supported by the

observations that α -synuclein KO mice have greater resistance to MPTP toxicity (Dauer et al., 2002; Robertson et al., 2004; Schlüter et al., 2003).

As mentioned previously, α -synuclein is involved in several pathogenic processes in PD, and the following sections will further discuss the roles of α -synuclein in health and disease.

1.6.1 α -synuclein function at the synapse

Various investigations have revealed that α -synuclein can be localised at synaptic boutons, and associated with presynaptic vesicles (Clayton and George, 1999; Kahle et al., 2000; Lee et al., 2008; Zhang et al., 2008). α -Synuclein can reversibly bind phospholipid membranes, which have been shown to stabilise the alpha helical confirmation of the N-terminal- and central domains, whilst the c-terminal domain remains unfolded (Li et al., 2001; Perrin et al., 2000). Furthermore, the membrane curvature, and therefore the size of the vesicles that α -synuclein interacts with has recently been shown to influence the secondary structure of α -synuclein. Large vesicles and membranes with a low curvature promote a long single helix structure, whilst the steep curvature of small vesicles promotes a structure with two antiparallel helices (Chandra et al., 2003; Ferreón et al., 2009; Jao et al., 2004; Ulmer et al., 2005). By default, α -synuclein preferentially binds to highly curved membranes, such as those of synaptic vesicles (Davidson et al., 1998; Middleton and Rhoades, 2010). It has therefore been proposed that monomeric α -synuclein can act as a membrane curvature sensor in low concentrations, and influence the shape of vesicle membranes at higher, but still physiological, concentrations (Mizuno et al., 2012; Varkey et al., 2010; Westphal and Chandra, 2013). It is worth noting however, that α -synuclein can also be found in solution

as a tetramer that is resistant to membrane binding and aggregation (Bartels et al., 2011; Westphal and Chandra, 2013).

α -Synuclein can interact with intracellular vesicles through membrane-bound GTP-Rab3a, and has been shown to play a role in the localisation and trafficking of presynaptic vesicles (Chen et al., 2013; Murphy et al., 2000). An investigation in cultured hippocampal neurons found that suppressing the expression of α -synuclein caused a reduction in the distal pool of synaptic vesicles, but did not report any change to vesicles in the active zone (Murphy et al., 2000). Another investigation in α -synuclein KO mice reported no changes to DA release or reuptake in acute striatal slice preparations, but found increased DA release from the second pulse of a paired pulse stimulation train (Abeliovich et al., 2000), suggesting changes in the replenishing rate of the readily releasable pool. Removing α -synuclein therefore allows for a quicker recovery of releasability, suggesting that α -synuclein plays a role in stabilising the reserve pool of synaptic vesicles.

In addition to α -synuclein playing a role in vesicle trafficking, it can also be involved in exocytosis and endocytosis during neurotransmission. A recent investigation by Burre et al. revealed that α -synuclein interacts with synaptobrevin 2, and promotes SNARE complex assembly in vivo and in vitro (Burré et al., 2012). The N- and C- terminal domains are especially crucial for this function, whereas alterations to the central domain were more tolerable. α -Synuclein has also been shown to compensate for dysfunctional SNARE assembly, and rescue synapse degradation in mice lacking CSP α , a protein involved in endo- and exocytosis in the presynaptic terminal (Chandra et al., 2005; Zhang et al., 2012). Furthermore, suppressed α -synuclein expression in rat hippocampal cultures is

accompanied by a decrease in the immunofluorescence intensity of synaptophysin, which interacts with the SNARE protein synaptobrevin (Becher et al., 1999; Lotharius and Brundin, 2002; Murphy et al., 2000). This observation provides some support for the idea that α -synuclein has a role in chaperoning SNARE complex function. In addition to interacting with exocytotic proteins, α -synuclein can bind to phospholipid PIP₂, which is a key regulator of endocytosis (Di Paolo and De Camilli, 2006; Stöckl et al., 2008), and loss of $\alpha\beta\gamma$ -synucleins in mice is accompanied by an increase in levels of a key endocytic protein called protein endophilin 1A (Westphal and Chandra, 2013). Moreover, α -synuclein has also been associated with clathrin-coated vesicles, suggesting in some involvement in endocytosis (Blondeau et al., 2004). If the function of α -synuclein on membranes is taken in conjunction with its involvement in neurotransmission, then it could be suggested that α -synuclein acts as a chaperone between the membrane and the exocytotic machinery to regulate reserve presynaptic vesicle dynamics (Burré et al., 2012; Hui et al., 2009; McMahon et al., 2010).

Recent studies on transgenic cell, rodent, and primate models have revealed that overexpressing WT α -synuclein affects several different markers of synapse dysfunction (See Table 1.2 for summary). Overexpressing α -synuclein caused reduced DA release in PC 12 chromatin cells (Larsen et al., 2006), and reduced striatal DA release in rodents (Garcia-Reitböck et al., 2010; Gaugler et al., 2012; Lundblad et al., 2012). Striatal DA release also progressively reduced with age in some of the rodent models, suggesting a progression of presynaptic dysfunction over time (Garcia-Reitböck et al., 2010; Lam et al., 2011; Lundblad et al., 2012). It is however not just DA release that is affected, but overexpressing WT α -synuclein reduced spontaneous EPSPs in striatal MSNs, suggesting dysfunction in

glutamate release from corticostriatal synapses (Lam et al., 2011; Wu et al., 2010), and also reduced glutamate release from acute hippocampal slices (Nemani et al., 2010).

Overexpressing α -synuclein has also been associated with reduced DA content (Lam et al., 2011; Nuber et al., 2013; Tofaris et al., 2006), reduced rate of DA uptake (Lundblad et al., 2012; Unger et al., 2006), and reduced availability of DAT, VMAT2 and TH (Decressac et al., 2012; Lam et al., 2011; Lundblad et al., 2012; Unger et al., 2006).

The changes in DA release observed in PD models has been of particular interest as it often presents before other signs of PD pathophysiology, and has been suggested to be involved in the early stages of PD (Garcia-Reitböck et al., 2010; Gaugler et al., 2012; Lundblad et al., 2012). However, current transgenic models of α -synuclein overexpression often express α -synuclein at high quantities, which may not be physiologically relevant, and through promoter elements that do not recapitulate an endogenous expression pattern (Lam et al., 2011; Lundblad et al., 2012; Nemani et al., 2010). Further investigation into the earliest stages of PD pathogenesis is therefore needed to understand how DA release and synapse function is affected in transgenic models that represent overexpression of α -synuclein at levels and anatomical distributions comparable to human PD, which will comprise part of this thesis.

Table 1.2 briefly summarises recent literature on rodent and cell models that overexpress human α -synuclein.

Reference	Species & promoter	Protein expression level	Cell death	Aggregation/ pathology	Synapse dysfunction
(Zhou et al. 2000)	Rat primary culture and cell lines: AAV overexpression of A53T, human WT, or rat WT α -synuclein	4 to 5 fold above rat	A53T caused cell death, Human WT alone caused no cell death but increased cell death after 6-OHDA		
(Kirik et al. 2002)	Rat: AAV overexpression of A53T and WT α -synuclein	not specified		Cellular and axonal pathology, α -synuclein positive cytoplasmic inclusions, swollen dystrophic neurites similar to PD patients. Only in DA neurons and mainly in SN	
(Lo Bianco et al. 2002)	Rat: lentiviral overexpression of rat WT, human WT, A30P, or A53T α -synuclein	level not quantified, but equal for each genotype	30% and 10% loss of SN TH-IR cells from human WT and rat WT respectively	α -synuclein positive inclusions and neuritic pathology	
(Lee et al. 2002; Unger et al. 2006)	Mouse: mouse PrP overexpression of human WT, A53T, A30P mutant α -synuclein	4 - 15 fold of nTG mouse	A35T (but not WT or A30P) had adult onset neurodegeneration, and early death	Pathological inclusions of α -synuclein and ubiquitin, abnormal locomotor activity in A53T mutants, reversed by application of a D ₁ receptor antagonist.	Decreased DAT expression and reduced striatal DA uptake

(Rockenstein et al. 2002)	Mouse: murine Thy-1 and PDGF promoter expression of human α -synuclein	5- 10 fold of human brain		α -synuclein accumulation in synapses, neurons and glial cells throughout the brain	
(Thiruchelvam et al. 2004)	Mouse: expression under rat TH promoter of human WT and A30P+A53T double mutant α -synuclein on mouse WT background	1.3 to 1.5 fold of nTG mouse	Significant loss of SNc TH positive cells over 19 months in TG but not nTG mice; double mutant, but not human WT	Reduced locomotor activity and abnormal responses to apomorphine in TG mice	Pesticide induced greater loss of DA, DOPAC, and HVA in double mutant mice vs nTG
(Yamada et al. 2004)	Rat: AAV overexpression of human WT α -synuclein	level not specified, but similar for α -synuclein and EGFP	At 13 weeks post injection: 50% decrease of TH positive cells in SN (vs contralateral) of α -synuclein group (only 5% loss in EGFP control)	No changes to apomorphine induced rotations (vs EGFP cohort); some S129-phosphorylated α -synuclein aggregates and caspase-9 activation	No significant differences in DA/DOPAC/HVA content
(Larsen et al. 2006)	PC12 and chromaffin cells: pCDNA vector overexpression of A30P and human WT α -synuclein	12 fold for A30P and 19 fold for WT of nTG		Accumulation of docked vesicles	All α -synuclein inhibited catecholamine release (no change to Ca^{2+} dependence) and lower number of exocytotic events per stimulus
(Tofaris et al. 2006; Garcia-Reitböck et al. 2010)	Mouse: truncated α -synuclein under rat TH promoter on <i>snca</i> $-/-$ background	not specified	No significant increase in cell death	Pathological inclusions in SNc, reduced locomotion	Reduced striatal DA and HVA content, striatal synaptic accumulation of α -synuclein, redistribution of synaptic SNARE proteins, age dependent reduction in DA release

(Eslamboli et al. 2007)	Marmoset monkey: AAV overexpression of human WT, and A53T α -synuclein	not specified	Degeneration of striatal DA fibres and DA cell bodies in midbrain, greater cell death in A53T mutant	Motor abnormalities 15 weeks post injection, protein aggregation in striatum and SN.	
(Nemani et al. 2010)	Mouse: PrP overexpression of human α -synuclein, Transfected primary hippocampal and midbrain (rat) cultures	3 fold of nTG mouse		No observed pathology or α -synuclein deposits	In acute hippocampal slices: lower baseline neurotransmission, reduced vesicle density at the active zone, and vesicles had reduced clustering after endocytosis in TG vs nTG. Hippocampal culture: impaired synaptic vesicle exocytosis, reduced readily releasable and synaptic vesicle recycling pools. Midbrain culture: impaired synaptic vesicle exocytosis in TG vs. nTG
(D. A. Scott et al. 2010; Rockenstein et al. 2005; D. Scott and Roy 2012)	Primary mouse neuron cultures: PDGF promoter overexpression of human α -synuclein.	mRNA 2 – 3 fold human brain	No overt loss of cells or synapses	Proteinase K resistant aggregates in a subset of boutons, lysosomal aggregates and pathology	Reduction in glutamate-induced post-synaptic currents, enlarged synaptic vesicles, loss of presynaptic proteins including synapsin1 and synaptobrevin 2, smaller reserve pool of synaptic vesicles and reduced trafficking between boutons
(Wu et al. 2010)	Mouse: Thy1 promoter overexpression of human WT α -synuclein	5- 10 fold of human brain	No observable cell loss	Progressive sensorimotor anomalies, non-motor abnormalities.	Higher EPSC stimulation threshold and decreased spontaneous EPSC in striatal MSSNs for TG vs. nTG mice

(Cabeza-Arvelaiz et al. 2011; Fleming et al. 2004)	Mouse: Thy1 promoter overexpression of human WT α -synuclein	5- 10 fold of human brain		Progressive reduction in spontaneous Activity and impaired fine motor function	Changes in transcription levels relating to synaptic plasticity, signalling, Transcription, apoptosis, and neurogenesis
(Lam et al. 2011)	Mouse: Thy1 promoter overexpression of human WT α -synuclein	5- 10 fold of human brain			Young mice: increased tonic striatal [DA] _o (but unchanged DA clearance and amphetamine evoked [DA] _o), increased open field activity, reduced MSSN EPSCs, opposite effects of quinpirole and sulpiride on MSSN EPSCs in TG vs. nTG. Old (14months): reduced striatal DA and TH content, L-dopa sensorimotor deficit in TG mice.
(Gaugler et al. 2012)	Rat: AAV + WPRE enhancer overexpression of human WT and A30P α -synuclein	2 to 3 fold increase above control hemisphere		Motor impairments in human WT (but not A30P) rats vs. control	Reduced striatal DA release, decreased DA vesicle density
(Decressac et al. 2012)	Rat: AAV + WPRE enhancer overexpression of human α -synuclein	2 to 4 fold increase above control hemisphere	Progressive loss of DA neurons, swelling and progressive degeneration of axons	Deficits in motor function	Reduced expression of TH, VMAT2, and DAT
(Lundblad et al. 2012)	Rat: AAV (with and without WPRE enhancer) overexpression of human α -synuclein	2 to 4 fold increase above control hemisphere	Degenerative changes in SN and striatum with reduced striatal innervation	Aggregates and axonal swellings in striatum	Early signs of progressive deficits in DA release, uptake, and DAT dysfunction

(Nuber et al. 2013)	Rat: BAC overexpression of human α -synuclein	2-3 fold of control rat brain	Cell loss in SN and striatum	Insoluble proteinase K-resistant fibres (mainly in striatum), locomotor decline.	Reduced striatal dopamine content.
(Janezic et al. 2013)	Mouse: BAC overexpression of human α -synuclein on <i>snca</i> $-/-$ background	1.8 fold of WT mouse brain (5-6 fold human brain), 1 fold human brain	Age-dependant cell loss in SN	Age dependent motor impairments. Phenotype not dependent on overt protein aggregation.	Deficit of evoked DA release in dorsal striatum precedes motor effects and cell loss. Altered distribution of DA vesicles in dorsal striatum. Age dependent reduction in DA neuron firing rate

Abbreviations: AAV (adeno-associated virus), BAC (bacterial artificial chromosome), dopamine (DA), dopamine transported (DAT), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), PDGF (platelet-derived growth factor), PrP (Prion protein promoter), WPRE (woodchuck hepatitis post-transcriptional regulatory element), TG (Transgenic), nTG, (non-Transgenic)

1.6.2 α -Synuclein and DA homeostasis

In addition to affecting synapse function, α -synuclein is also involved in DA homeostasis. The activity of TH, the rate-limiting enzyme for DA synthesis, is reduced when α -synuclein is overexpressed in vitro (Gao et al., 2007; Perez et al., 2002). Similarly, the mRNA and protein levels of TH are also reduced when α -synuclein is overexpressed (Baptista et al., 2003; Yu et al., 2004), and α -synuclein has been shown to promote activity of protein phosphatase A2 which deactivates TH by dephosphorylating a key serine residue (Peng et al., 2005). Furthermore, α -synuclein overexpression in vitro has also been shown to reduce the activity of AADC, another enzyme involved in DA production (Tehrani et al., 2006). Paradoxically, the negative effect that α -synuclein has on TH and AADC does however not mean that a lack of α -synuclein will increase overall DA content in vivo. In fact, several studies have shown that DA content is either decreased or unaltered in α -synuclein KO mice (Abeliovich et al., 2000; Robertson et al., 2004; Schlüter et al., 2003). The intensity of TH immunostaining in the striatum is also unchanged in $\alpha\beta\gamma$ -synuclein triple KO mice (Anwar et al., 2011). Taken together, these observations suggest that the effect of α -synuclein on DA production is buffered by other factors in vivo, and that α -synuclein does not play a major role in regulating DA content.

In addition to influencing DA production, α -synuclein can also play a role in the compartmentalisation of intracellular DA. In adrenal cells, α -synuclein overexpression was associated with changes to the vesicular pH gradients, which is important for correct VMAT2 functioning. Increase in cytoplasmic catecholamine levels were also detected (Mosharov et al., 2006). Decreased VMAT 2 has been observed in human DA cells, derived from a mesencephalic cell line, and overexpressing the A53T mutant form of α -synuclein

(Lotharius and Brundin, 2002; Lotharius et al., 2002), suggesting that α -synuclein can influence levels of VMAT 2. These findings are also in line with increased VMAT2 observed in SH-SY5Y human neuroblastoma cells with reduced α -synuclein (Fountaine et al., 2008). However, the reduced α -synuclein observed by Fountaine et al also prevented MPP+ induced superoxide production in a manner unrelated to the increase in VMAT2, which suggests that α -synuclein can have more than one mode of action and that not all of its effects are linked to pathology in the same way (Fountaine et al., 2008).

In addition to influencing DA production and DA localisation, α -synuclein can also play a role in regulating DA uptake through DAT. An investigation in neuroblastoma cell lines found that α -synuclein knockdown can reduce the amount of DAT at the cell surface, therefore preventing against MPTP toxicity (Fountaine and Wade-Martins, 2007). In addition to this, α -synuclein KO mice are more resistant to MPTP toxicity than WT controls (Dauer et al., 2002; Robertson et al., 2004; Schlüter et al., 2003), suggesting that the effects of α -synuclein on DAT are also possible *in vivo*. Another investigation in α -synuclein KO mice revealed a reduced locomotor response to amphetamine, a drug that causes DA release by leaking out of the DAT, which also suggests a reduced availability of surface DAT in α -synuclein KO mice (Abeliovich et al., 2000; Rodriguez-Menchaca et al., 2012). Interestingly, overexpressing α -synuclein also attenuates the activity of the noradrenaline and 5-HT transporters (Wersinger et al., 2006a, 2006b), suggesting that its function at DAT is not the foundation for α -synuclein induced selective degeneration of DA neurons.

Taken together, the observations here shed light on various different ways that α -synuclein can influence DA homeostasis, and suggest that normal α -synuclein activity is necessary for DA neurons to function optimally.

1.6.3 α -Synuclein and other protein interactions

Although some interesting themes can be drawn from observations of α -synuclein regulating synapse function and DA homeostasis, there are also observations of α -synuclein interacting with a large number of other proteins and pathways not specifically related to DA synapse function.

It has been suggested that α -synuclein plays a role in neuronal plasticity since the highest levels of α -synuclein, in both human and rodent brains, has been observed during the embryo development period of intense plasticity, which is just after synaptogenesis (Galvin et al., 2001; Hsu et al., 1998). Furthermore, α -synuclein has been shown to influence the development of DA neurons in mouse SNc (Garcia-Reitboeck et al., 2013), suggesting a specific role for α -synuclein in these neurons. It has also been observed that α -synuclein can interact with a range of cytoskeletal proteins such as Tau (Jensen et al., 1999), MAP1B (Jensen et al., 2000), MAP2 (D'Andrea et al., 2001), tubulin (Payton et al., 2001), actin (Sousa et al., 2009) and Synphilin-1 (Engelender et al., 1999). Furthermore, hippocampal neurons from mice that lack α -, β -, and γ -synuclein, have a ~30% reduction in the size of excitatory synapses (Greten-Harrison et al., 2010), which supports the idea that α -synuclein can be involved in neuronal plasticity.

It has also been observed that γ -synuclein, another member of the synuclein family, can play a functional role in fatty acid metabolism, and even provide partial protection from

high-fat diet induced obesity (Millership et al., 2012). Furthermore, α -Synuclein shares some homology with class A2-apolipoproteins, which are involved in lipid transport (Clayton and George, 1999; Uéda et al., 1993), and cytosolic fatty acid binding motifs in FABPs, which interact with fatty acids (Sharon et al., 2001). Two studies in human embryonic kidney and ovarian cell lines found that α -synuclein physically binds and inhibits phospholipase D2, a membrane bound enzyme that hydrolyses phospholipids (Ahn et al., 2002; Jenco et al., 1998). However, this observation could not be replicated in yeast or cell free assays, so it has been suggested that α -synuclein influences phospholipase D2 indirectly through endoplasmic reticulum-related stress (Rappley et al., 2009). A small number of investigations have revealed that α -synuclein can interact with 14-3-3 chaperone proteins, MAPKK, and protein kinase C, which suggests a role in intracellular signalling, although no major role for α -synuclein in intracellular signalling cascades has been established (Aoki and Li, 2011; Iwata et al., 2001; Ostrerova et al., 1999).

Aggregation of α -synuclein has been shown to play a large role in PD. Fibrillar forms of α -synuclein are mostly detected in Lewy bodies, but various low- and high molecular weight oligomeric α -synuclein species have been detected throughout affected cells, including in axon terminals (Baba et al., 1998; Lundblad et al., 2012; Tsigelny et al., 2008). Protein aggregates observed in the brains of PD patients have to some extent been recreated in rodent models. Rats that overexpress A30P, A53T, or WT forms of human α -synuclein under viral promoters have revealed α -synuclein positive inclusions in cell bodies and axon terminals, along with caspase activation and cell death (Lo Bianco et al., 2002; Kirik et al., 2002; Yamada et al., 2004). Mice expressing A53T or WT α -synuclein under Thy-1 or prion

promoters revealed pathological inclusions of α -synuclein and ubiquitin, and also α -synuclein accumulation in synapses, neurons and glial cells throughout the brain (Lee et al., 2002a; Rockenstein et al., 2002; Unger et al., 2006). More recently, rats that express mutant α -synuclein through a lentiviral vector displayed a greater loss of SNc TH positive neurons when α -synuclein was mainly in the form of membrane-associated oligomers, rather than rapid-forming insoluble fibrils, which suggests that the α -synuclein soluble oligomeric species are more toxic than large aggregates (Winner et al., 2011a).

Aggregation has however not been reported in all rodent models of α -synuclein overexpression, which suggest that protein aggregation might not be the only way for α -synuclein to cause cell loss and dysfunction. For example, overexpressing WT α -synuclein from an adenoviral vector in rats caused changes to K⁺ evoked DA release with subsequent aggregate formation, demonstrating that other forms of cell dysfunction might precede α -synuclein aggregation (Lundblad et al., 2012).

Aggregates of α -synuclein can spread in a manner similar to the prion protein (Alvarez-Erviti et al., 2011; Emmanouilidou et al., 2010), and can damage the cells either by displacing other structures (Spillantini et al., 1997, 1998), or by exerting other toxic effects (Conway et al., 2000; Tanaka et al., 2004; Tompkins and Hill, 1997). The full extent of mechanisms by which α -synuclein aggregation leads to cell death remains uncertain, but, α -synuclein aggregates have been shown capable of hindering the ubiquitin proteasome system and autophagy lysosomal pathway (Cuervo et al., 2004; Ebrahimi-Fakhari et al., 2012), blocking vesicle trafficking between endoplasmic reticulum and Golgi (Cooper et al., 2006; Gosavi et al., 2002), and disrupting microtubules (Alim et al., 2004).

In addition to pathology that involves protein aggregation, other pathways that involve oxidative stress, mitochondrial dysfunction, and apoptosis can also cause cell death during PD. Lewy body pathology has been associated with mitochondrial DNA damage in PD patients (Müller et al., 2013), and pathogenic mutations of α -synuclein in transgenic mice have been shown to cause mitochondrial degeneration and cell death (Martin et al., 2006). These findings suggest that α -synuclein can cause mitochondrial dysfunction and cell death. Furthermore, it has been shown *in vitro* that α -synuclein can lead to inhibition of mitochondrial complex I (Devi et al., 2008; Chinta et al., 2010), which suggests a possible mechanism by which mitochondrial dysfunction may be caused. However, roles for elevated intracellular Ca^{2+} have also been shown to cause mitochondrial dysfunction (Dryanovski et al., 2013; Melachroinou et al., 2013), and yet other mechanisms that involve oxidative stress through nitric oxide have also been described (Fountaine et al., 2008). Mitochondrial dysfunction can lead to apoptosis, through the release of cytochrome C, in an aggregation-independent manner (Gao et al., 2001; Wang and Youle, 2009). Furthermore, it has been shown that α -synuclein can cause apoptotic cell loss in the absence of inclusion formation (Saha et al., 2000).

Taken together, the data mentioned here suggest that α -synuclein can to some extent lead to cell loss through multiple mechanisms, such as protein aggregation, mitochondrial dysfunction, and apoptosis. The exact pathways underlying these mechanisms are however incompletely understood and, being the subject of on-going investigation, will be further discussed in Chapter 7.

In spite of the vast number of proposed roles and interactions, a loss of α -synuclein can be compensated for by β - and γ -synuclein, suggesting some functional redundancy (Senior et al., 2008). Furthermore, none of the proteins in the synuclein family are essential for neuronal survival or viability of the organism (Anwar et al., 2011). The role of α -synuclein therefore seems to be more along the lines of regulating dynamics of presynaptic vesicles in a way that is not crucial to all cells.

Part of this thesis will involve characterizing evoked monoamine release in mice that express different levels of human α -synuclein. More about the rationale for using BAC transgenic mice will be discussed in the methods section (Chapter 2), along with further details on the model.

1.7. Structure, expression, and function of *LRRK2*

The discovery of leucine rich repeat kinase 2 (*LRRK2*) stemmed from several families being identified with inherited forms of PD. A genome wide linkage analysis study published in 2002 identified the PARK8 locus on chromosome 12p11.2-q13.1 (Funayama et al., 2002), which was more recently found to be caused by missense mutations in *LRRK2* (Paisan-Ruiz et al., 2009; Zimprich et al., 2004).

The *LRRK2* gene codes for a 280 kDa protein known as dardarin, but often also referred to as *LRRK2*. *LRRK2* has 144 kbp, which code for 2527 amino acids over 51 exons (Dachsel and Farrer, 2010). *LRRK2* is part of the *LRRK* family and homologous to *LRRK1*, believed to have derived from a common ancestor gene, although no significant association between *LRRK1* and PD has been found to date (Haugarvoll et al., 2007; Taylor et al., 2007). Figure

1.2 shows the primary structure of LRRK2 including all the identified domains and some important pathogenic mutations. Two notable enzymatic domains of LRRK2 are the kinase domain and the GTPase domain, which has been shown by some *in vitro* studies to regulate kinase activity (Guo et al., 2007; Li et al., 2007). The kinase domain is flanked by protein interacting domains, including the leucine rich repeat domain near the N-terminal, and a WD40 domain near the C-terminal (Kett and Dauer, 2012; Mata et al., 2006).

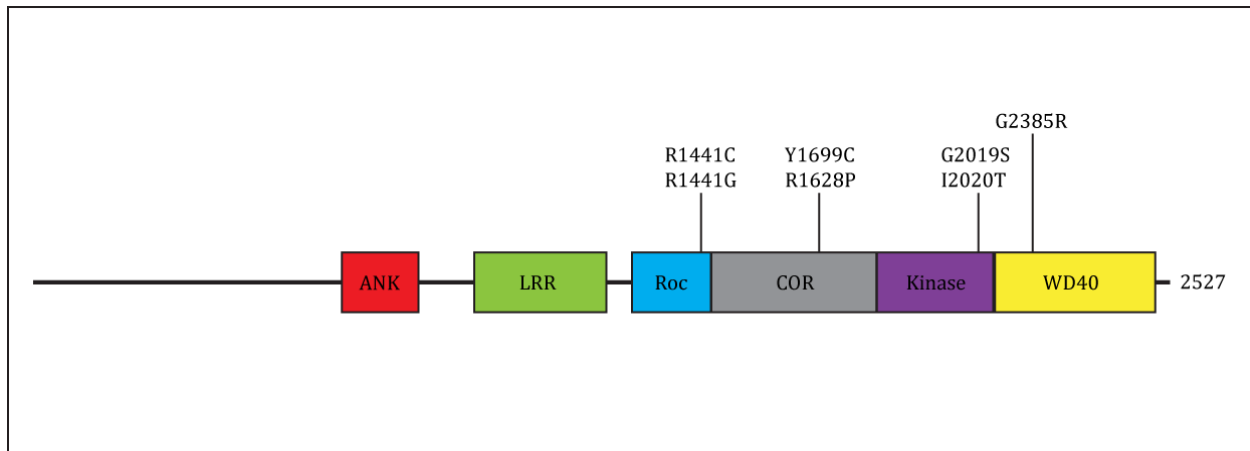


Figure 1.2 Simplified diagram adapted from (Mata et al., 2006) shows primary structure of LRRK2 protein with notable domains in coloured boxes and locations of confirmed pathogenic substitutions (Dachsel and Farrer, 2010). Abbreviations: ANK, ankyrin repeat region; COR C-terminal of Ras; LRR, leucine-rich repeat; Roc, Ras of complex (GTPase).

The large structure of LRRK2 provides technical difficulties with resolving the protein structure or observing its native conformation (Webber and West, 2009). Various studies have shown that LRRK2 functions as a homodimer, and can also be found in other oligomeric forms (Deng et al., 2008, 2008), although a microscopy study in Chinese hamster ovary cells revealed that LRRK2 appears mostly as a monomer in the cytosol and a dimer at the cell membrane (James et al., 2012). An *in vitro* study with human LRRK2 revealed that disruption of GTPase or kinase activity promotes oligomer formation and reduced dimer formation, suggesting a possible link between LRRK2 function and protein aggregation (Sen et al., 2009).

LRRK2 is not expressed during the earliest stages of development in mice (Biskup et al., 2007), but can be found in various tissues of adult mice with particular abundance in the brain, lungs, kidney, spleen, and leucocytes. Expression patterns for *LRRK1* and *LRRK2* have been found to overlap in some mouse tissues, such as the lungs, heart, and lymph nodes (Biskup et al., 2007), but in the adult mouse brain, strongest expression of *LRRK2* is in the dorsal striatum and cortex, whereas *LRRK1* expression is relatively restricted to the olfactory bulb (Giesert et al., 2013). LRRK2 expression has also been found in the olfactory tubercle, nucleus accumbens, hippocampus, cerebellum, and SN (Biskup et al., 2006; Greggio et al., 2006; Higashi et al., 2007; Miklossy et al., 2006). The intracellular localisation of LRRK2 is diffuse in transfected cells (Gloeckner et al., 2006; Greggio et al., 2006; Smith et al., 2006). Although LRRK2 has no transmembrane domain or nuclear-targeting sequences (West et al., 2005), subcellular fractionation has revealed it associated with membranous and microsomal fractions (Gloeckner et al., 2006; Hatano et al., 2007; West et al., 2005), suggesting loose interactions with membranes. Furthermore, confocal colocalisation and

ultrastructural analysis reveal LRRK2 localised with lysosomes, endosomes, transport vesicles, and mitochondria (Biskup et al., 2006).

LRRK2 was identified as a locus for PD in 2002 (Funayama et al., 2002), and has since produced various rodent models that recapitulate some features of PD (see Table 1.3 for overview). The following sections briefly summarise some of the main functions of LRRK2, along with the role of mutant LRRK2 in PD pathophysiology.

Table 1.3 summarises recent literature on rodent and cell models that express the G2019S and R1441C mutant forms of LRRK2.

Reference	Species & promoter	Protein expression level	Cell death	Aggregation/ pathology	Synapse dysfunction
(Greggio et al. 2006)	Primary rat cortical neuron culture: G2910S, R1441C, kinase 'dead' variants	not specified		Both: formed cytoplasmic inclusion bodies (but not kinase dead versions)	
(Tong et al. 2009)	Mouse: knock in of R1441C at exon 31, & cultured chromaffin cells	endogenous	No DA cell death for up to 24 months	No protein aggregation for up to 24 months, but reduction in amphetamine-induced locomotor activity, impaired reduction in locomotor activity by QP	No changes to striatal DA content over 24 months, but reduced catecholamine release from cultured chromaffin cells
(Y. Li et al. 2009)	Mouse: BAC expression of human R1441C and WT LRRK2	5 – 10 fold the level of endogenous mouse LRRK2	In R1441C: No cell death in VTA or SNc at 10 months, but decreased TH-positive dendrites in SNc	In R1441C: Age-dependent progressive motor deficits starting at 12 months (reversed by L-DOPA), dystrophic DA neurites in striatum, aggregation of hyper-phosphorylated Tau	In R1441C: Reduced striatal [DA] _o detected from microdialysis in vivo in the presence of a DAT blocker (nomifensine)
(Lin et al. 2009)	Mouse: tetracycline inducible expression of WT & G2019S LRRK2, and A53T α -synuclein under CaMKII promoter	5 – 16 fold the level of endogenous expression for LRRK2 and 30 fold for α -synuclein	G2019S: no cell death or neuronal pathology. A53T: progressive cell death in cortex and striatum. Double-mutant: early inflammation and accelerated cell loss	G2019S: no motor phenotype. A53T: early motor phenotype. Double mutant, enhanced aggregation and disruption of Golgi trafficking	

(X. Li et al. 2010)	Mouse: BAC expression of human G2019S and WT LRRK2	For WT and G2019S: about 6 fold the level of endogenous LRRK2	No cell death in SNc or loss of nigrostriatal terminals	WT: hyperactive and enhanced motor performance. G2019S: increase in phosphorylated Tau	WT: Elevated striatal DA release. G2019S: age dependent decrease in DA content, decrease in DA release and uptake
(Winner et al. 2011)	Mouse: BAC expression of human G2019S LRRK2	3 fold the level of endogenous LRRK2	Decrease in proliferation of new cells in hippocampus and SVZ	Reduced dendritic arborisation and fewer spines in newly generated neurons	
(Dusonchet et al. 2011)	Rat: AAV (synapsin 1 promoter) human WT and G2019S LRRK2	overall 2 fold the level of endogenous LRRK2 (but only 1/3 of cells are successfully transfected)	WT: no significant neuronal loss. G2019S: progressive SNc cell loss	Both: found in high molecular weight complexes, no α -synuclein aggregation	
(Melrose et al. 2010)	Mouse: BAC expression of human G2019S LRRK2	2.5 – to 3.5 fold the level of endogenous LRRK2	Both: no difference in SNc TH +ve cell counts	G2019S: increased phosphorylated Tau and microglial activation, faster walking speed	Both: no changes to striatal DA content, lower baseline striatal DA (<i>in vivo</i> microdialysis) which is restored by amphetamine challenge, trend towards increased striatal DA receptors
(Ramonet et al. 2011)	Mouse: PDGF expression of human G2019S and R1441C	G2019S ~ 2.7 fold the level of endogenous LRRK2, R1441C at lower levels than G2019S	G2019S: Progressive cell loss in SNc R1441C: no cell loss	G2019S: progressive abnormal autophagy and mitochondria, reduced neurite growth in cultured neurons, normal locomotor activity. R1441C: Abnormal autophagy, reduced locomotor activity	G2019S: normal striatal DA content. R1441C reduced striatal DA content.

(Zhou et al. 2011)	Rat: doxycycline inducible expression of G2019S LRRK2	Not Specified	No DA cell loss	Increased locomotor activity	Impaired DAT activity (<i>in vivo</i> microdialysis), no changes to DA content
(Chen et al. 2012)	Mouse: PDGF expression of human WT and G2019S LRRK2, HEK 293	Not specified, WT and G2901C at similar levels	G2019S: progressive degeneration of SNc TH +ve neurons – coupled with increased phosphorylation of MKK4 pathway (upregulates JNK, c-Jun, BiM, FAsL, Caspase 9 & 3)	G2019S: no α -synuclein aggregation, increased phosphorylated Tau, progressive motor deficit – responds to L-DOPA	G2019S: decreased DAT function
(Herzig et al. 2012)	Mouse: Thy1 expression of human WT and G2910S LRRK2. Thy1 expression of human A53T α -synuclein	Not specified, no expression in SNc TH +ve neurons		G2019S: No motor deficits, no α -synuclein or Tau aggregation. A53T: severe motor phenotypes, microgliosis in hindbrain, α -synuclein aggregation. Both: motor phenotype and microgliosis no worse than A53T alone.	

Abbreviations: AAV (adeno-associated virus), AD (autosomal dominant), AR (autosomal recessive), BAC (bacterial artificial chromosome), dopamine (DA), dopamine transported (DAT), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), EPSC (excitatory post synaptic current), IPSC (inhibitory post synaptic current), IR (immunoreactive), LOPD (late onset Parkinson's disease), PDGF (platelet-derived growth factor), PrP (Prion protein promoter), WPRE (woodchuck hepatitis post-transcriptional regulatory element), TG (Transgenic), nTG, (non-Transgenic)

1.7.1 LRRK2 function at the synapse

Changes to neurotransmission have been observed in rodents that express pathological mutant forms of LRRK2 (Li et al., 2010; Tong et al., 2009), and understanding the role of LRRK2 at presynaptic terminals can therefore contribute to understanding its role as a risk factor for PD. The physiological role of LRRK2 at the synapse has mainly been elucidated by two investigations in particular that looked at the effect of changing LRRK2 activity in primary neuron cultures. Silencing LRRK2 activity in mouse cortical neuron cultures, through RNA interference, resulted in substantially increased EPSPs evoked by presynaptic depolarisation (Piccoli et al., 2011). These data suggests that LRRK2 can play a role in the presynaptic control of neurotransmitter release. LRRK2 was found to co-localise with Rab5b, a small GTPase, at synaptic vesicles (Shin et al., 2008), and has also been shown to directly interact with Rab5B, synapsin I and a synaptic vesicle associated ATPase called NSF, suggesting the LRRK2 acts by working in conjunction with other presynaptic proteins. Electron microscope imaging revealed that LRRK2 silencing causes fewer vesicles in cortical neuron cultures to be docked at the active zone, which can be explained by changes to the ratio of exocytosis to endocytosis (Piccoli et al., 2011). This observation is in line with the increased EPSPs observed in post-synaptic neurons, which suggests a reduced rate of endocytosis of neurotransmitters. Moreover, the rate of synapto-pHluorin quenching in primary hippocampal cultures, which depends on the rate of endocytosis, is reduced with LRRK2 silencing (Shin et al., 2008). This finding is in conjunction with immunostaining in primary cortical cultures, which revealed that LRRK2 silencing increased synaptic vesicle exposure to synaptotagmin. Together these findings suggest that reduced LRRK2 activity causes a reduced rate of endocytosis at the synaptic terminal

(Dihanich and Manzoni, 2011). The exact mechanism by which LRRK2 influences synaptic vesicle dynamics remains unknown, but it has been proposed that LRRK2 acts through phosphorylation of actin or synapsin 1A (Belluzzi et al., 2012). It might however also be possible that LRRK2 provides a scaffold for other protein interactions that regulate synaptic vesicle mobilisation.

Investigations in mice that express the G2019S mutant form of *LRRK2* in a *bacterial artificial chromosome* (BAC) transgene showed lower baseline $[DA]_o$ detected by microdialysis, an age-dependent decrease in striatal DA content, along with changes to DA uptake, and evoked release detected by FCV (Li et al., 2010; Melrose et al., 2010).

Furthermore, changes in striatal DA content and release were found in the absence of cell death in the SNc, or overt motor phenotypes, which suggests that presynaptic changes to DA neurons may be one of the earliest stages of *LRRK2* pathology. Another rat model that expresses the G2019S mutant form of *LRRK2* under a doxycycline inducible promoter found impaired DAT activity, and a model that used a platelet-derived growth factor (PDGF) promoter found progressive degeneration of the SNc (Chen et al., 2012; Zhou et al., 2011).

The effects of the R1441C mutation on striatal DA release dynamics are comparable to that of the G2019S mutation. One study that expressed the R1441C mutant form of *LRRK2* in BAC transgenic mice found reduced striatal *concentration of extracellular DA* $[DA]_o$ detected from microdialysis *in vivo* (Li et al., 2009). Another study that expressed the R1441C mutation in mice under a PDGF promoter, and reported reduced striatal DA content (Ramonet et al., 2011). Expressing the R1441C mutant in cultured chromaffin cells

also reduces catecholamine release (Tong et al., 2009). Taken together, these data suggest that both the G2019S and R1441C mutant forms of LRRK2 can affect the function of mesostriatal DA pathways.

1.7.2 Other protein interactions, including GTPase and kinase function of LRRK2

In addition to influencing synapse function, LRRK2 is also known for acting as a kinase and a GTPase. The kinase domain of LRRK2 shares the closest sequence homology with mixed lineage kinases (MLKs), which is part of the mitogen activated protein kinase kinase kinase (MAPKKK) family (Moore, 2008). LRRK2 kinase activity is preferential towards serine/threonine residues, but not tyrosine residues (West et al., 2007). LRRK2 autophosphorylates *in vitro* in the presence of ATP and Mg^{2+} , which requires the C-terminal domain and is inhibited by the N-terminal domain (Anand et al., 2009; Greggio and Cookson, 2009; Greggio et al., 2008). The *in vivo* autophosphorylation of LRRK2 is however still contested, as antibodies against some of the main autophosphorylation sites failed to detect LRRK2 from cell lysates (Kamikawaji et al., 2009; Webber et al., 2011). This suggests that only a small fraction of LRRK2 might be in the autophosphorylated state in cells.

Other potential substrates for LRRK2 phosphorylation include actin, β -tubulin, ezrin/radixin/moesin (ERM) proteins, tau, α -synuclein, 4EBP1, and mitogen activated protein kinase kinase proteins (MAPKK). However, finding a convincing physiological substrate for LRRK2 remains a challenge. Studies to reveal that LRRK2 phosphorylates β -tubulin, actin, and tau have all been done *in vitro* (Gillardon, 2009; Kawakami et al., 2012; Meixner et al., 2011). Mutant forms of LRRK2 have been associated with increased phosphorylated tau in mice and humans, but these studies did not confirm LRRK2 as the

primary kinase for tau (Kawakami et al., 2012; Li et al., 2010; Zimprich et al., 2004). It might therefore be that tau phosphorylation was an indirect consequence of LRRK2 activity. Similarly, LRRK2 can phosphorylate the ERM protein moesin, but only after pre-heating of moesin to unfold the C-terminal domain (Jaleel et al., 2007). A single study found that LRRK2 can phosphorylate α -synuclein in cell culture (Qing et al., 2009), and the two proteins have been found to colocalise in *in vivo* (Guerreiro et al., 2013). However, the extent to which LRRK2 contributes to α -synuclein pathology remains contested, as another study found no correlation between the number of α -synuclein-positive Lewy bodies and those also containing LRRK2 in the brains of PD patients (Guerreiro et al., 2013). Another potential substrate for LRRK2 is 4EBP1 (eukaryotic initiation factor 4E-binding protein 1), which was first suggested from a study in drosophila (Imai et al., 2008). However, an *in vitro* study showed that 4EBP is a very weak substrate compared to autophosphorylation, and that 4EBP is better phosphorylated by another kinase called MAPK14 (Kumar et al., 2010). Since LRRK2 is part of the MKKK (mitogen activated protein kinase kinase kinase) family, perhaps the closest candidates for physiological substrates are MKK (mitogen activated protein kinase kinase) 3, 4, 6, and 7 (Chen et al., 2012; Gloeckner et al., 2009; Hsu et al., 2010). One study in particular revealed that expressing the G2019S mutant form of LRRK2 in mice increased phosphorylation of the MKK4 pathway along with its downstream targets. However the activation MKK4 by other MKKKs was not ruled out in this study, so it is not certain that LRRK2 directly activates MKK4 *in vivo* (Chen et al., 2012). Therefore it has not yet been established that LRRK2 is both necessary and sufficient for activating any particular kinase pathway.

Kinase activity can be regulated by phosphorylation of Thr2031, Ser2032, and Thr2037, and removal of these residues completely impairs kinase autophosphorylation (West et al., 2007).

The LRRK2 GTPase belongs to the Ras-GTPase superfamily of GTPases, more specifically to the ROC (Ras of complex proteins) subfamily, which would normally be activated by ligand-activated tyrosine kinase receptors (McKay and Morrison, 2007). Ras family GTPases are known to function as molecular switches, and several studies have explored this possibility for LRRK2. Firstly, some studies suggest that the GTPase regulates kinase activity (Dachsel and Farrer, 2010; Ito et al., 2007), although other studies suggest that kinase activity regulates GTPase function (Greggio et al., 2009). Some studies show that kinase activity is not inhibited by presence of GDP or enhanced by presence of GTP (Liu et al., 2011; Taymans et al., 2011), suggesting that the extent to which the kinase domain of LRRK2 is influenced by GTPase activity is limited. Other potential substrates for GTPase activity include a co-chaperone called CHIP (C-terminus of heat shock protein 70 interacting protein) and disheveled homologues DVL-1/2/3, but it remains to be known whether these interactions depend on GTP (Ko et al., 2009; Sancho et al., 2009). The LRRK2 GTPase domain also interacts with α - and β - tubulin, but independently of GTP or GDP, which makes tubulins an unlikely substrate (Gandhi et al., 2008).

The GTPase function of LRRK2 is activated by ARHGEF7 *in vitro* (Haebig et al., 2010). ARFGAP1 can accelerate the GTPase activity of LRRK2 approximately 2–3-fold *in vitro*, and is also phosphorylated by LRRK2 *in vitro* (Stafa et al., 2012; Xiong et al., 2012). How these proteins interact with LRRK2 *in vivo*, is however still unknown.

In addition to functioning as a GTPase and a kinase, the large multidomain structure of LRRK2 suggests that it is involved in complex processes, likely involving other proteins. Indeed the interaction of LRRK2 with cytoskeleton proteins such as actin, tubulins and ERM proteins suggest that it may act as a scaffold for various molecular interactions. It has also been shown that LRRK2 interacts with 14-3-3 proteins (Nichols et al., 2010), suggested to play a role stabilising the structure of LRRK2 and protecting sites of autophosphorylation (Drolet et al., 2011).

LRRK2 can be degraded by chaperone-mediated autophagy, and also plays a role in regulating autophagy (Alegre-Abarrategui and Wade-Martins, 2009). Pathogenic mutant forms of LRRK2 are however poorly degraded by chaperone-mediated autophagy, and can interfere with the translocation complex, thereby hampering autophagy (Orenstein et al., 2013). Protein aggregation in PD can therefore be caused by LRRK2-mediated insults to chaperone mediated autophagy.

Since LRRK2 is a multi-function protein, different mutations affect different physiological pathways (Cookson, 2010). The G2019S mutation, for example, is the only mutation that increases kinase activity (Chen et al., 2012; Greggio and Cookson, 2009). However, G2019S, R1441C, and Y1699C mutations in LRRK2 are all associated with reduced GTPase activity (Li et al., 2007; Liu et al., 2011). In addition to causing protein aggregation, LRRK2 has also been shown to interfere with several other cellular processes. Including microglial inflammation (Moehle et al., 2012), tau phosphorylation (Li et al., 2009), disrupted vesicle trafficking (Lin et al., 2009), and reduced dendritic arborisation (Ramonet et al., 2011; Winner et al., 2011b).

Part of this thesis will involve characterizing evoked DA release in rats that express the G2019S or R1441C mutant forms of *LRRK2*. More about the rationale for using BAC transgenic rats will be discussed in the methods section (Chapter 2), along with further details on the model.

Overall, this thesis will explore the electrically-evoked release of two monoamines, namely DA and 5-HT, in transgenic rodents that carry pathogenic forms of either the SNCA or LRRK2 genes. The next chapter will explain the methods used to explore DA and 5-HT release in these rodent models. The main aims of this thesis are to:

- explore the dose-dependence of human WT α -synuclein overexpression on electrically evoked striatal DA release in mice;
- identify a candidate mechanism by which high levels of human WT α -synuclein can cause a deficit in evoked striatal DA release;
- investigate whether mice that express high levels of human WT α -synuclein, with an established deficit in evoked striatal DA release, will also exhibit changes to evoked 5-HT release in the SN;
- explore the release of electrically evoked striatal DA release in two different lines of transgenic rats, each with a mutant form of the human LRRK2 gene associated with PD.

CHAPTER 2. Methods

2.1. Overview.

For the purposes of this thesis, several lines of transgenic animals, with either a human *SNCA* or *LRRK2* BAC transgene, were used. The creation and maintenance of transgenic animals was carried out by the Oxford Parkinson's disease centre (OPDC), and a brief overview of the transgenic animals used for this thesis will be provided here. Details about the handling of the animals prior to experiments and preparation of experimental tissue will also be discussed. Two main approaches were used to investigate monoamine release in BAC transgenic rodents. Firstly, fast-scan cyclic voltammetry (FCV) was used to detect extracellular concentrations of evoked DA and 5-HT, after local electrical stimulation of acute brain slices. Secondly, high performance liquid chromatography (HPLC) was used to determine overall content of electroactive compounds in specific brain regions.

2.2. The use of transgenic rodent models to explore monoamine release

The investigations performed for this thesis pertain to developing a greater understanding about PD, which is a human disease. PD has been investigated in humans through various approaches, such as post mortem brain tissue analysis (Braak et al., 2002), and positron emission tomography (PET) imaging of PD patients (Brooks, 2010). However, only indirect methods of measurement can be widely and ethically used in humans, which presents limitations to the type of investigations that can be performed. Therefore, in order to specifically explore presynaptic factors that control DA and 5-HT release, an animal model must be used.

The animals investigated here will be transgenic models and have several advantages over pharmacological models, which make use of toxins that cause PD-like symptoms (Hoffman et al., 1997). For example, intrastriatal 6-OHDA injections in rats cause loss of SNc DA neurons, together with a change in basal ganglia activity in favour of the indirect pathway, and concomitant motor impairments (Galeffi et al., 2003; Lindner et al., 1999).

Pharmacological models have several practical advantages, such as a reduced cost and production time in comparison to transgenic models, and these models can be very useful for investigating how surviving DA neurons function after severe cell loss. The process of cell death is however relatively quick, and DA levels can be depleted in a matter of days (Faull and Laverty, 1969), which limits the utility of this model for studying the early stages of PD, and the systematic progression of the disease. MPTP and 6-hydroxydopamine (6-OHDA) are currently the most frequently used Parkinsonian toxins in animal models (Beal, 2001; Lindner et al., 1999; Przedborski et al., 2001), and when administered in small doses

over a prolonged period of time, MPTP can cause chronic degeneration of DA neurons (Bezard et al., 1997; Schneider and Roeltgen, 1993). However, unlike most human PD cases, the neurodegeneration in animal models due to MPTP, is relatively restricted to DA neurons (Braak et al., 2003; Dauer and Przedborski, 2003; Forno et al., 1993). Unlike acute pharmacological models, animals with PD-related transgenes can develop progressive changes with a range of affected neurons (Eslamboli et al., 2007; Janezic et al., 2013; Nemani et al., 2010). Moreover, since the transgenes like SNCA and LRRK2 are linked to PD in humans, exploring mechanisms by which the protein-products of mutant genes contribute to PD pathophysiology, may provide a better understanding of the underlying causes of PD.

The animals used for investigations here will be the C57BL/6 strain of mouse (*Mus musculus*) and the Sprague Dawley strain of rat (*Rattus norvegicus*). Rodent animal models have the advantage over simpler organisms of having DA and 5-HT systems that more closely model human DA and 5-HT systems. Rodent animal models also have at least two advantages over higher organisms, such as primates, for laboratory use. Firstly, using rodents have fewer ethical concerns and less practical restrictions enforced by the Animal Scientific Procedures act. Secondly, genetic manipulation techniques for rodents are relatively advanced in comparison to higher organisms (Eslamboli et al., 2007; Melrose et al., 2010).

The transgenic rodent models used for this thesis make use of a bacterial artificial chromosome (BAC) vector, which allows for insertion of the entire transgene and all adjacent regulatory elements. BAC transgenic models have several advantages over other

transgenic models. Unlike models that use the TH or Thy-1 promoters, a BAC transgene is expressed under regulation of the relevant endogenous promoter element, which allows for a gene to be specifically expressed in a physiological pattern. In contrast, the TH promoter limits expression of the transgene to cells that express TH (Thiruchelvam et al., 2004), and the AAV promoter limits expression to the area surrounding the injection site (Yamada et al., 2004). The PDGF promoter does not express α -synuclein in the SNc (Rockenstein et al., 2002), and although the Thy-1 and PrP promoters lead to wide expression in areas including the SNc (Lee et al., 2002a; Rockenstein et al., 2002), a purely physiological expression pattern cannot be guaranteed. In addition to the expression pattern, BAC transgenic animals can consistently produce generations of animals with the same level of gene expression (Janezic et al., 2013), which cannot be achieved as accurately through injection of viral vectors (Lundblad et al., 2012; Yamada et al., 2004).

2.2.1 Mice that express human SNCA on a mouse Snca -/- background

The *Oxford Parkinson's Disease Centre* (OPDC) consortium developed three lines of BAC transgenic mice, each expressing human α -synuclein at different levels in a range that is relevant to the brains of PD patients who carry *SNCA* gene multiplications. The generation of mice described here was performed entirely by the OPDC, and did not form part of this project.

Transgenic lines were generated by pronuclear injection of BAC DNA (performed by Charles River, UK), containing the complete 111 kb human WT *SNCA* gene locus, including all 6 exons together with 18-kb upstream- and 6-kb downstream flanking elements, into C57BL/6 mice (Janezic et al., 2013). Three different mouse lines were identified, and for

the purpose of this thesis, the three lines will be referred to as **SNCA-A**, **SNCA-B**, and **SNCA-C**. The SNCA-A line of mice has levels of α -synuclein no higher than the human brain (Figure 2.1). Brain levels of α -synuclein in SNCA-B mice are about 2-3-fold that of humans (Figure 2.1), and brain levels of α -synuclein in SNCA-C mice are about 5-6-fold of humans (Figure 2.1) (Janezic et al., 2013).

Each BAC transgenic line was backcrossed onto an *Snca* $-/-$ background (Abeliovich et al., 2000) for nine generations to establish transgenic lines expressing human WT *SNCA* on an *Snca* $-/-$ background. Full-length integration of the human *SNCA* BAC transgene was confirmed in each line by PCR of all six exons, and fluorescence in situ hybridisation was used to observe a single integration site for each line. Quantitative western blot analysis was used to confirm that levels of neither β -synuclein nor γ -synuclein were affected. Expression of the *SNCA* transgene was confirmed by immunohistochemistry to have the same pattern as C57BL/6 mice in striatum SNc, VTA, Hippocampus, Cortex, and dorsal Raphe (Janezic et al., 2013).

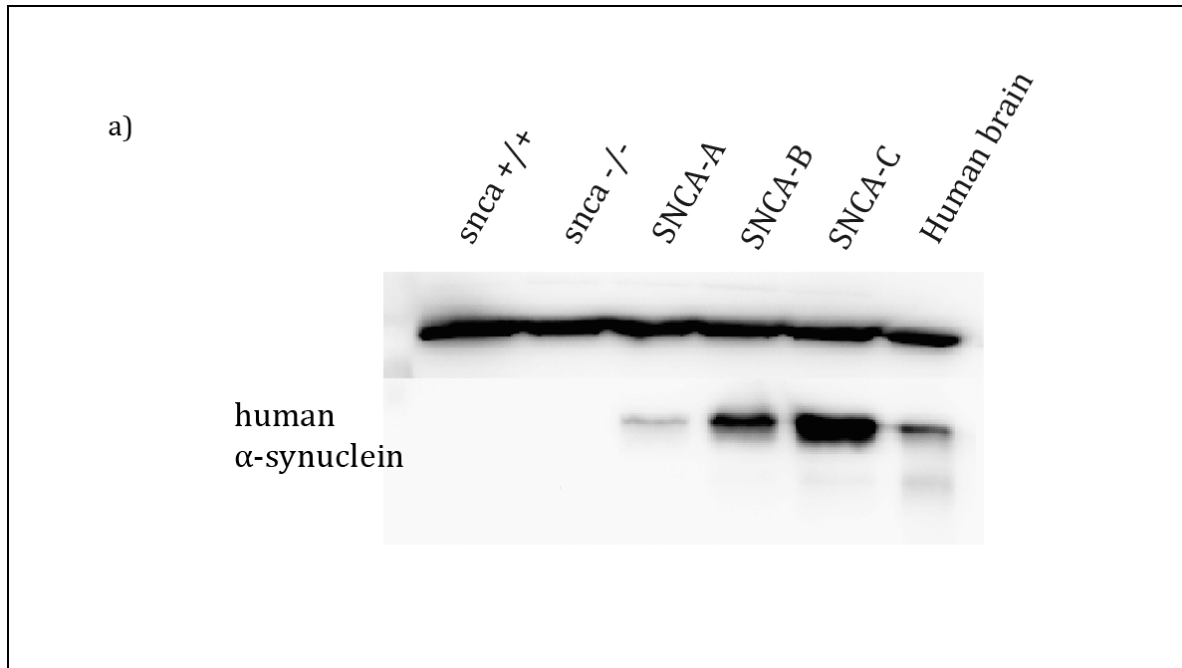


Figure 2.1 Quantitative western blots of striatal and whole brain tissue. Performed by Sabina Anwar, Steve Senior, and Stephanie Janezic of the OPDC consortium, University of Oxford. Identical ratios of α -synuclein between the mouse lines were found in both striatal and full brain homogenate (unpublished observation), and was compared to human striatum a) Human α -synuclein is not present in C57BL/6 (*Snca +/+*) mice or *Snca -/-* mice. Human α -synuclein is however present in SNCA-A, SNCA-B, SNCA-C mice, and human brain tissue. Levels of α -synuclein in SNCA-A, -B, and -C mice relative to human brain tissue is approximately <1 (less than one), 2-3, and 5-6 fold respectively.

2.2.2 Rats that express human *LRRK2* on a *lrrk2* *+/+* background

The generation of rats described here was performed entirely by the OPDC, and did not form part of this project. Several lines of rats that express human *LRRK2* were generated through pronuclear injection of BAC DNA, containing the complete 144 kb human WT *LRRK2* gene locus including all 51 exons together with flanking elements and a YPet fluorescent tag, into pure Sprague Dawley oocytes (Javier Alegre-Aberrategui, Max Sloan, personal communication). Two of the resulting lines will be the focus of this chapter.

One line of BAC transgenic rats expresses the G2019S mutant form of *LRRK2*, here named **G2019S**, and the other line expresses the R1441C mutant form of *LRRK2*, here named **R1441C**. Brain levels of *LRRK2* in G2019S rats are about 5 fold that of non-transgenic (nTG) rats. Brain levels of *LRRK2* in R1441C rats are about 2.5 fold that of nTG rats (Figure 2.2; Max Sloan, personal communication). Each BAC transgenic line was bred to have hemizygous expression of a human *LRRK2*, and a single insertion site was confirmed for each line using fluorescence *in situ* hybridisation by Max Sloan. The expression of human *LRRK2* was also confirmed in the cortex, hippocampus, cerebellum, striatum, and substantia nigra (Max Sloan, personal communication).

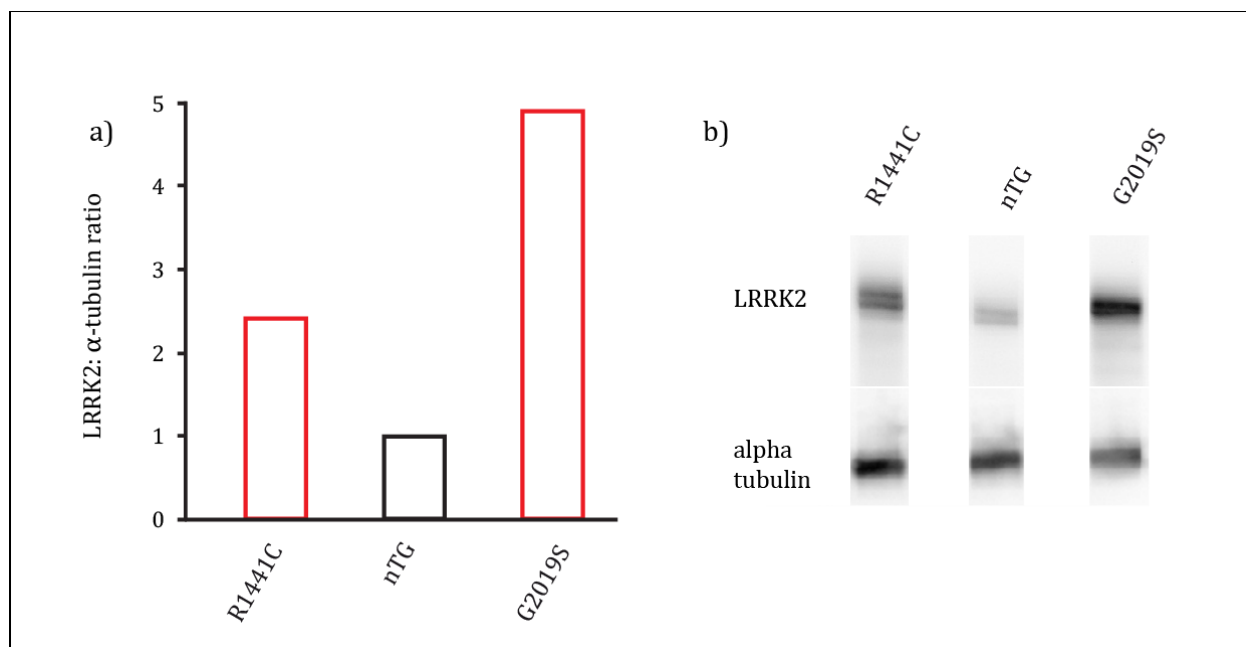


Figure 2.2 Quantitative western blots of brain tissue from LRRK2 rats. Performed by Max Sloan of the OPDC consortium, University of Oxford. a) LRRK2: α -tubulin ratio of R1441C and G2019S rats is about 2.5 and 5 fold that of non-transgenic Sprague Dawley (nTG) rats respectively, as measured by antibody that detects both human and rat LRRK2. b) Western blots of LRRK2 and alpha tubulin in G2019S, R1441C, and nTG rats.

2.2.3 Using acute coronal brain sections as a model system

In this thesis, acute coronal brain slices were used as a model system to monitor extracellular concentrations of evoked DA and 5-HT after local electrical stimulation. Using acute brain slices for this purpose has several advantages over other popular models systems, such as *in vivo* brain tissue and neuronal cultures. Working with acute coronal brain slices has an advantage over *in vivo* measurements, by being more accessible for precise experimental manipulation. For example, the temperature of the tissue, ion concentrations in recording solution, and concentration of applied drugs can be precisely controlled in acute slices. Furthermore, placing electrodes in acute brain slices provides fewer practical challenges than doing so *in vivo*, making it easier to explore specific brain regions.

Using neuronal cultures or acute slices have a shared advantage over *in vivo* tissue, in that the axon terminals have been severed from the cell body, which allows for specific factors contributing to neurotransmitter release to be separated from factors like pacemaker neuronal activity. However, acute slice preparations have the advantage over neuronal cultures that the tissue developed in an intact physiological environment, which contained all physiological growth factors and network connections. Acute slices may therefore more closely model neuron function in the intact brain compared to neuronal cultures.

The acute slices used in this thesis had a thickness of 300 μm , which allows the DA or 5-HT terminals to maintain local interactions with other neurons, dendrites, and synaptic connections (Cragg et al., 1997b; Threlfell et al., 2010b), and also allowing enough oxidation to keep the tissue active for several hours (Bull et al., 1990; Exley et al., 2007).

2.3. Fast-scan cyclic voltammetry

2.3.1 Principles behind FCV

Some monoamine neurotransmitters, such as DA and 5-HT, are electroactive compounds. The correct electromotive force in an electrode can therefore stimulate a redox reaction in these molecules by inducing a transfer of electrons. A carbon fibre microelectrode (CFM), exposed to extrasynaptic DA or 5-HT, can stimulate a redox reaction with a measurable current. This process is illustrated in Figure 2.3.

A potential difference is placed across a carbon fibre microelectrode (CFM), which periodically cycles from -0.7 V to +1.3 V and back to -0.7 V (vs. Ag/AgCl), at a rate of 800 V/s. When the electrode potential rises towards +1.3 V, electroactive compounds like DA and 5-HT are oxidised, and electrons are transferred to the electrode (Figure 2.3, a). As the electrode potential drops back to -0.7 V, the oxidised compounds are reduced, and electrons transfer back from the recording electrode to DA or 5-HT. The flow of electrons between the recording electrode and an electroactive compound can be measured as an electrical current in the CFM. The same amount of electrons are transferred during every redox reaction of a particular compound, which means that the current produced is therefore directly proportional to the concentration of the respective compound (Stamford, 1990; Stamford et al., 1988). Therefore, the extracellular concentration of either DA ($[DA]_o$) or 5-HT ($[5-HT]_o$) can be determined by comparing the amplitude of the oxidation current with a calibration concentration.

The oxidation/reduction peaks for any electroactive compound will exist at distinct electrode potential values. The electronegativity and diffusion kinetics of the molecule will

also influence the ratio between oxidation and reduction peaks. Electroactive molecules, such as DA and 5-HT, can therefore be distinguished from each other by the analysing the recorded voltammogram (Figure 2.3, b). 5-HT and DA both have the same oxidation potential (+500 to +650 mV), but different reduction potentials (- 200 mV for DA, but -20 mV and -670 mV for 5-HT). Voltammograms are recorded at 8 Hz, allowing for $[DA]_o$ and $[5-HT]_o$ to be monitored in real time (Figure 2.3 c,d) (Bull et al., 1990; Stamford et al., 1988).

For the purpose of this thesis, FCV was chosen for the high temporal resolution, relatively small electrode size, and the electrochemical species identification. Some methods for detecting extracellular evoked monoamines, such as microdialysis or the use of radiolabelled neurotransmitters, collect neurotransmitters over a timescale of minutes. This does not allow for real time measurements of monoamine release and uptake, and is therefore limited in the information that can be gathered about neurotransmitter release mechanisms. FCV detects DA and 5-HT at a frequency of 8 Hz, which allows for real-time measurement of neurotransmitter release and uptake, including uptake kinetics.

Microdialysis also uses relatively large probes, often being over 300 μm in diameter (Murillo-Rodríguez et al., 2013), which can cause substantial tissue damage in comparison to the CFM used for FCV, which is only 7-8 μm in diameter. Other electrochemical detection techniques, such as constant-potential amperometry, also make use of small electrodes (Lundblad et al., 2012). However, the electrochemical signal obtained from amperometry cannot be used to distinguish between DA and 5-HT, which makes such a technique unsuitable for this thesis where some experiments had a risk of 5-HT detection signals being contaminated by DA.

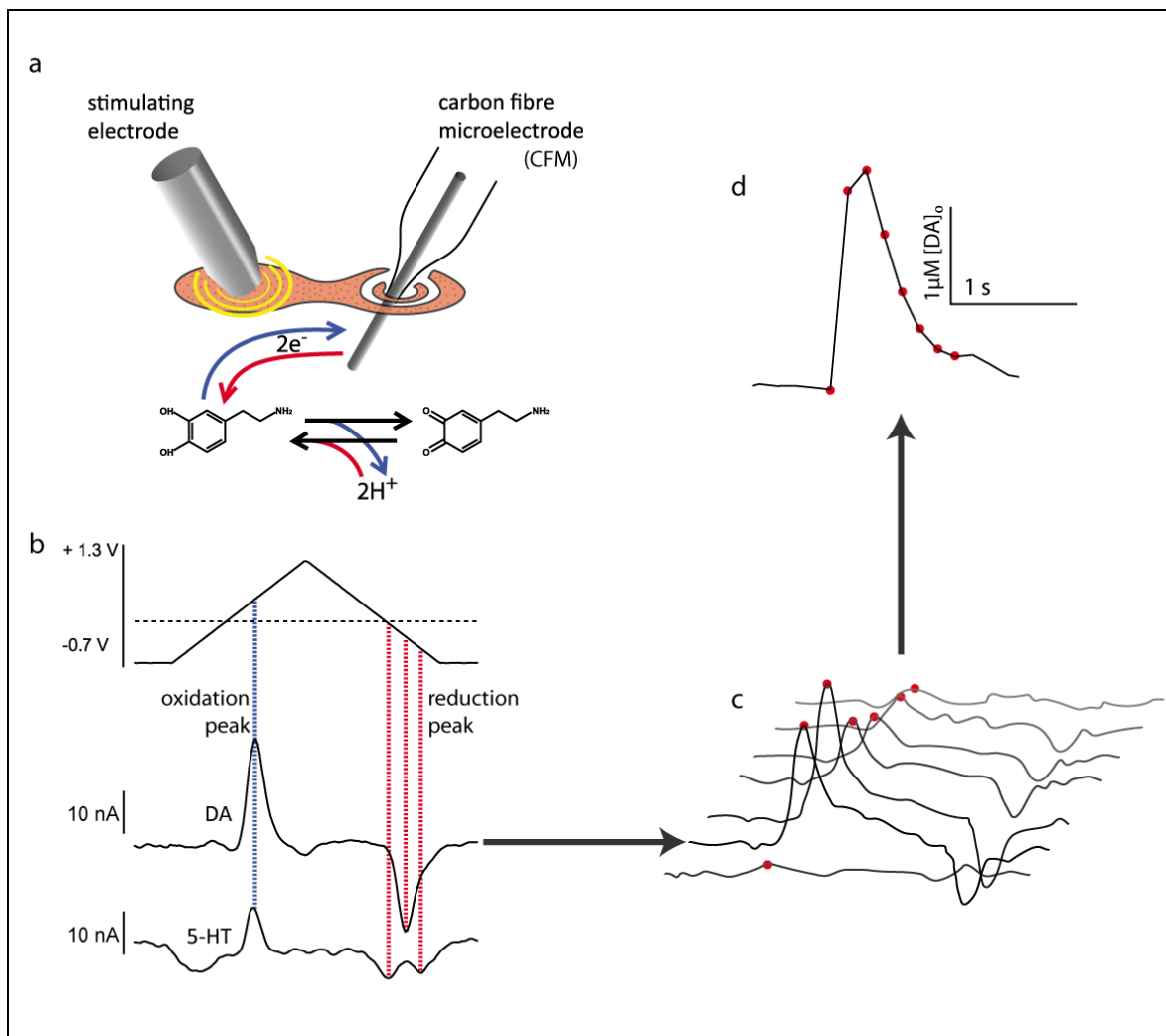


Figure 2.3: Using FCV to detect evoked [DA]_o or [5-HT]_o in real time. a) A stimulating electrode evokes monoamine release. A CFM is inserted inside the tissue. Monoamines (DA in the diagram) are oxidised (blue arrows) and reduced (red arrows) by the cyclic change in potential difference across the CFM. b) The redox reactions cause flow of an electrical current in the CFM, which can be seen at oxidation peaks (blue line) and reduction peaks (red lines) on voltammograms for DA and 5-HT. c) Voltammograms are recorded at intervals of 0.125 s, showing transient oxidation and reduction peaks caused by evoked striatal [DA]_o. d) The peak value at the oxidation potential is extracted on each voltammogram (red dots), and plotted over time. The magnitude of the oxidation peak is proportional to the concentration of that monoamine.

2.3.2 Preparation and incubation of mouse brain tissue

All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and approved by the local ethical review panel at the Department of Physiology, Anatomy and Genetics, University of Oxford. All mice were generated and maintained by the OPDC. Mice were exposed to 12 h light/dark cycles, and given food and water *ad libitum*. Mice were transferred to a holding area at least 48h before experiments. *SNCA* BAC transgenic mice were always compared experimentally to *Snca* $-/-$ controls of the same sex, and at the same age. Evoked $[DA]_o$ in *Snca* $-/-$ mice has been shown to be no different from WT C57BL/6 mice (Senior et al., 2008).

Tissue samples were prepared as done previously (Janezic et al., 2013; Threlfell et al., 2010b) For experiments reported in Chapter 3 and 5, brain slices from one animal that expresses human α -synuclein, as well as a *Snca* $-/-$ control, were prepared in immediate succession and in randomised order on the same day. For experiments reported in Chapter 4, only one brain was used per day. Mice were killed by cervical dislocation, with the brain removed over ice, and placed in ice cold Hepes-buffered artificial cerebrospinal fluid (HaCSF), which consisted of 120 mM NaCl, 5 mM KCl, 20 mM NaHCO₃, 6.7 mM Hepes acid, 3.3 mM Hepes salt, 2 mM CaCl₂, 2 mM MgSO₄, 1.2 mM KH₂PO₄, and 10 mM glucose and saturated with 95% O₂/5% CO₂. Coronal slices (300 μ m) of striatum and midbrain were sectioned using a vibratome (VT 1200S; Leica), and slices were then left to incubate for 1 h in HaCSF at room temperature. Slices were transferred into the recording chamber and maintained in artificial cerebrospinal fluid (aCSF) at a flow rate of $\sim$ 1.5 ml/min. Slices were left to recover in the recording chamber for at least 30 min before recordings started.

For all experiments that recorded DA in mice, aCSF was made up of 124 mM NaCl, 3.8 mM KCl, 26 mM NaHCO₃, 2.4 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose and saturated with 95% O₂/5% CO₂. This aCSF historically contains a total of 5 mM K⁺, and was therefore maintained throughout experiments that recorded DA in mice. However, for experiments that recorded 5-HT in mice, or DA in rats, a more physiologically relevant K⁺ concentration of 3.3 mM was used (Jones and Keep, 1987). Therefore, for all experiments that recorded [DA]_o in rats or [5-HT]_o in mice, aCSF was made up of 124 mM NaCl, 2.1 mM KCl, 26 mM NaHCO₃, 2.4 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose and saturated with 95% O₂/5% CO₂.

2.3.3 Preparation and incubation of rat brain tissue

All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and approved by the local ethical review panel at the Department of Physiology, Anatomy and Genetics, University of Oxford. As with the mice, all rats were generated and maintained by the OPDC. Rats were also exposed to 12 h light/dark cycles, and given food and water *ad libitum*. Rats were transferred to a holding area at least 48h before experiments. All rats used for this thesis were male. *LRRK2* transgenic rats were always paired with nTG controls, carrying WT rat *lrrk2*, of the same age and sex.

Brain slices from one animal that expresses human LRRK2, as well as a *Lrrk2* *+/+* control, were prepared in immediate succession and in randomised order on the same day. Some of the aged rats were very large (weighing up to 800 g), so a tissue preparing protocol were adjusted from previous methods (Bull et al., 1990; Janezic et al., 2013). Rats were

anaesthetised either by halothane (5%, 5 l/min O₂) or isoflourane (4%, 5l/min O₂), and decapitated. The brain was removed over ice and placed in ice cold HaCSF. The brain was bisected into two hemispheres to fit the slices in the recording chamber. Coronal slices (300 µm) of striatum were sectioned using a vibratome (VT 1200S; Leica), and slices were then left to incubate for 1 h in HaCSF at room temperature. Slices were transferred into the recording chamber and maintained in aCSF at a flow rate of ~ 1.5 ml/min. Slices were left to recover in the recording chamber for at least 30 min before recordings started.

2.3.4 Manufacturing of recording electrodes

The CFM recording electrodes used for FCV were fabricated in house as done previously (Janezic et al., 2013; Threlfell et al., 2010b). A carbon fibre thread of 7-8 µm diameter (Grade 34-700, epoxy free, Goodfellow, Cambs) was pulled through a borosilicate capillary of 2 mm diameter (GC200F-10 Harvard Apparatus Limited, Kent). Threading was performed in acetone for ease of insertion. After threading, the capillary was drained of acetone by gentle shaking, and was pulled by an electrode puller (Narishige, Japan). The carbon fibre thread was trimmed with a pair of micro-scissors, and was then cut to a length of 50 µm to 100 µm under a 10x objective, using a razor blade (Wilkinson Sword Classic). The carbon fibre is connected to fine copper wire, through silver epoxy glue (RS Components, Northants), and was held in place by cyanoacrylate glue sealing the open end of the tube.

2.3.5 Equipment setup, electrode placement and tissue stimulation

The equipment set up for FCV experiments is illustrated in Figure 2.4, and was performed as done previously (Cragg et al., 1997b; Janezic et al., 2013). Brain slices were transferred

using a glass Pasteur pipette (Volac, London) to the recording chamber with aCSF at 32°C flowing through at ~1.5 ml/min. The stimulating electrode is a concentric bipolar Pt/Ir electrode, with a tip 25 µm in diameter, which was placed on top of the tissue through a micromanipulator. The CFM was placed inside the tissue with a micromanipulator, at a depth of ~100 µm, and ~150 µm away from the stimulating electrode. A new CFM was used each day, because repeated use can cause electrode fouling through tissue build-up on the surface, which leads to a delayed response time.

The CFM was connected to a Millar voltammeter (Julian Millar, Barts and The London School of Medicine and Dentistry, London, UK) and an oscilloscope (Figure 2.4, a-b). A scanning waveform was run across the CFM by the voltammeter from, and the resulting voltammogram was recorded on a computer through WCP (Strathclyde Whole Cell Program software, University of Strathclyde, Glasgow, Scotland) or Axo Scope (Molecular devices, MDS Analytical technologies) at a frequency of 8 Hz. Since the scanning rate of each waveform is at 800 V/s, it means that each scan takes only 5 ms to record. The electrode is switched out of circuit between scans (Millar and Barnett, 1988; Millar and Williams, 1991)

An electrical pulse generator provided electrical pulses to the stimulating electrode (Figure 2.4, c). The stimulating electrode was a surface concentric bipolar electrode 25 µm diameter Pt/Ir; FHC, Bowdoinham, ME, USA). Typically, electrical pulses were delivered to the tissue for 200 µs at a perimaximal current of 0.6 mA as done previously (Threlfell et al., 2010b). Electrical stimulation at the tissue leads to the release of neurotransmitters, such as DA and 5-HT, which can be detected at the CFM. Electrical stimulations were delivered

when the CFM was switched out of circuit, which avoided stimulation pulses appearing on the voltammogram.

Acute brain slices require time to recover in between stimulation protocols. Therefore, electrical stimulations were separated by 2.5 min in experiments that sampled striatal DA release and done previously (Exley et al., 2007; Janezic et al., 2013). Electrical stimulations were however separated by 5 min in experiments that sampled 5-HT release in the SNr, as 5-HT release typically takes longer to recover (Jennings et al., 2010; Threlfell et al., 2010a).

At the end of each experiment, electrode calibrations were carried out with 2 μ M DA and/or 0.5 μ M 5-HT in aCSF at 32°C. Stock solutions of DA and 5-HT for calibration were 2.5 mM and 1.25 mM in 0.1 M HClO₄ respectively, and were kept at 4°C. Calibration was performed after the experiment, rather than before the experiment, because post-experiment calibration takes into account the electrode deterioration caused by insertion into the tissue.

2.3.6 Use of drugs and concentrations

Cocaine was purchased from Sigma-Aldrich, dissolved to 10 mM in dH₂O for storage, and bath applied to the acute brain slices at 5 μ M. DH β E was also obtained from Sigma-Aldrich, and bath applied at 1 μ M, made up from a stock solution of 1 mM in dH₂O. Quinpirole (QP) was purchased from Tocris Bioscience, dissolved in dH₂O at 10 mM for storage, and bath applied at 10 nM, 30 nM, 100 nM, 300 nM, 1 μ M, and 5 μ M. Stock solutions of all drugs were kept at -20 °C.

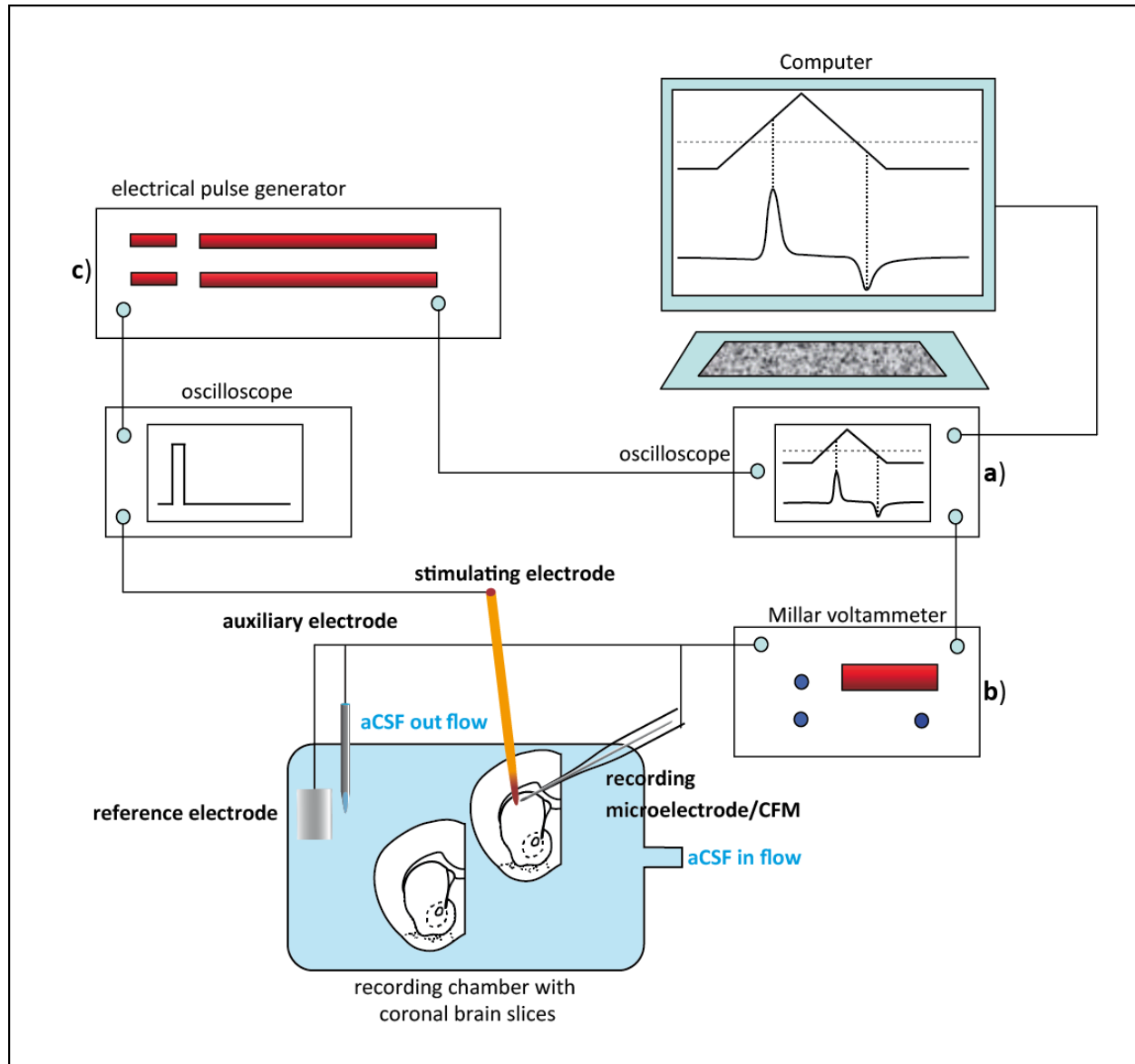


Figure 2.4: Equipment setup for FCV experiments. Brain slices are perfused with aCSF in the recording chamber. The CFM is connected to an oscilloscope (a) and the computer through a Millar voltammeter (b). The Millar voltammeter also connects to an auxiliary electrode (outflow pipe), and an Ag/AgCl reference electrode. An electrical pulse generator provides pulses to the stimulating electrode. An oscilloscope connects between the pulse generator and stimulating electrode to confirm the number of electrical pulses sent. The Millar voltammeter connects to the electrical pulse generator through the oscilloscope (a) to ensure that electrical stimulation pulses are only generated when the CFM is out of circuit.

2.4. High-performance liquid chromatography

HPLC with electrochemical detection is a well-established method for quantifying electroactive compounds (Musso et al., 1990; Reinhoud et al., 2013), which makes it the ideal technique for sampling the content of neurotransmitters like DA. HPLC involves passing samples at high pressure through a chromatography column that separates molecules based on interactions with beads inside the column. The electrochemical compounds, such as DA or 5-HT, are detected at an electrode passing out of the column, and can be identified by comparing the time of elution to a calibration standard. HPLC of tissue punches reports on the content of a neurotransmitter inside the tissue, which is independent of neurotransmitter release, and can therefore compliment FCV recordings.

2.4.1 Storage and preparation of tissue for HPLC

Samples for HPLC were taken from the same slices used for FCV as done previously (Anwar et al., 2011; Janezic et al., 2013). For each mouse coronal section, two circular punches were taken (one in each hemisphere) from the dorsal striatum (diameter 2mm), and two were also taken from the ventral striatum (diameter 1.2 mm). Both punches from each region in a brain slice were stored together in an eppendorf tube containing 200 μ l HClO₄ (0.1M). Punches from different regions and genotypes were stored in separate tubes. Eppendorf tubes containing tissue punches were kept at -80°C until the day of the assay.

For each rat coronal section, four circular punches were taken (two in each hemisphere) from the dorsal striatum (diameter 2mm per punch). Two circular punches were taken (one in each hemisphere) from the ventral striatum (diameter 2 mm). All the punches from

each region in a brain slice were stored together in an eppendorf tube containing 400 μ l HClO_4 (0.1M).

2.4.2 Equipment setup and protocol

HPLC samples were processed as done previously (Anwar et al., 2011; Janezic et al., 2013). Samples were thawed on the day of the assay and homogenised on ice through sonication. Samples were centrifuged at 13 000 rpm for 15 min at 4 °C, and the supernatant was diluted in 0.1 M HClO_4 to ensure that the concentration of electroactive monoamines would be within the detection range of the electrode. The pellet was discarded. Samples from mouse CPu underwent a 1:10 dilution. Samples from mouse NAc were not diluted. Samples from rat CPu underwent a 1:5 dilution. Samples from rat NAc underwent a 1:2 dilution. Mobile phase used for HPLC contained 13% methanol, 0.8 mM EDTA, 0.5 mM 1-octanesulfonic acid, 0.12 M NaH_2PO_4 . Mobile phase was buffered to between pH 3.5 and pH 4.6, depending on the equipment calibration.

Samples of 50 μ l were injected into the column using an autosampler. Samples flowed at 1 ml/min through a Microsorb C18 reverse-phase column (Varian or Agilent). DA, and its respective metabolites were detected using a Decade II SDS electrochemical detector with a glassy carbon working electrode (Decade II, SDC, Antec, Leyden), set at + 0.7 V with respect to a Ag/AgCl reference electrode. Most samples ran for 15 – 20 min depending on the settings of the equipment. Like with FCV, the concentration of a monoamine is proportional to the peak amplitude of the elution peak (Anwar et al., 2011; Janezic et al., 2013).

2.4.3 Electrochemical identification and measurement

Stock solutions of DOPAC, DA, NA, 5-HIAA, and HVA were made at 1mM in 0.1 M HClO₄. A dilute sample (1:1000 dilution) was taken from each stock solution and ran through the HPLC machine at 5 pmol. The pH was adjusted before each assay to maximise the time between the elution of individual compounds, and therefore avoid overlapping elution peaks. The elution time of different compounds varied with pH and other conditions, but DA normally eluted at about 10 min. The DA peak was by far the most prominent, and for the majority of tissue samples, the only detectable peak was DA. The peak height of the electrochemical detection signal was measured rather than the area under the curve, as this minimises the risk of interference when two peaks overlap at the base. The running time was adjusted to keep DA elution peaks as thin as possible, and thereby maximise accuracy of the height measurement.

Each assay was run with a mixed standard to identify different compounds, and for calibration purposes. Mixed standards were run at the beginning and end of each assay. The decrease in peak amplitude of DA was typically about 5% over an 8h assay, which processed 24 samples. To minimise the effect of sample degradation on the outcome of results, samples from different genotypes were run in random order, and corresponding samples from matching pairs were run in immediate succession.

The final measurement of DA was given with respect to the volume of tissue sampled (DA/mm³), as calculated from the width and diameter of tissue punches. Preliminary experiments attempted to use a BSA assay to normalise DA measurements to protein

content, however, the available equipment was not suitable for the small protein concentrations used and volume is therefore a more reliable measure.

2.5. Experimental design, data processing, and statistical analysis

For experiments where differences in monoamine release was explored between two genotypes, such as those performed in Chapters 3, 5, and 6, each used three to eight pairs of animals. For these experiments a single pair of animals was used per day, and brain slices from both animals were sampled, in approximately matching locations, and in the same bath. For experiments in the striatum, naïve sites were sampled to minimise the effect of site decline. The sites in corresponding locations in different brain slices were sampled in immediate succession. For experiments in the SNr, sites were in too close proximity to ensure naïve release, so all the data was pooled together. Variation in the concentration of DA released is similar between different sites or slices of the same animal, and the same sites of different animals. Therefore, two to three slices were sampled in each animal to increase the number of observations and minimise the number of animals used.

For experiments that used pharmacological tools to investigate the underlying mechanisms of genotype-specific changes to DA release, such as those described in Chapter 4, each used three to five pairs of animals. With these experiments however, only a single animal was tested per day. Data from pharmacological experiments were normalised to release under control conditions on that day, which minimised variation between days. Each stimulation protocol was repeated three times in every site. Two slices were sampled in each animal to increase the number of observations and minimise the number of animals used.

All experiments were performed blind with respect to the genotype of the animal. The ear code, order date, and cage number was recorded and matched to a database after the first stage of data processing.

Data was processed with Microsoft Excel, and statistical tests were performed using Graph Pad Prism 6 (Version 6.02, Graph Pad Software Inc.), and a web browser interface of R (http://www.wessa.net/rwasp_varia1.wasp). For each set of experiments, data from all pairs of animals were pooled together and explored through scatter plots, box and whisker graphs, and histograms. Data distribution was explored using D'Agostino-Pearson omnibus normality test, Shapiro-Wilk normality test, and also by looking at histograms and Q-Q plots. Normally distributed data was analysed by 2way-ANOVA, or t-test if no interaction was found. Data with non-normal distributions were analysed by Mann-Whitney, which compares ranks, and Kolmogorov-Smirnov, which compares cumulative distributions of the data.

2.6. Concluding remarks

The aim of this thesis is to gain a better understanding of presynaptic dysfunction during the early stages of PD development. The BAC transgenic rodent models used here are appropriate for investigating the early stages of PD development in affected sub-populations of neurons. Using FCV in acute coronal brain sections is the most appropriate technique for sampling evoked $[DA]_o$ and $[5-HT]_o$, and further exploring presynaptic mechanisms that may affect neurotransmission. HPLC is appropriate for sampling monoamine content, and thereby providing further information about factors that may affect the amount of neurotransmitter released. Taken together these techniques can be used to detect changes to the amount of evoked DA and 5-HT neurotransmission, and also narrow down the potential underlying mechanisms.

CHAPTER 3. Human α -synuclein causes a deficit of evoked striatal DA release in mice, which is region-specific and expression level-dependent.

3.1. Introduction

Previous investigations (see Chapter 1 for overview) have revealed that genetic factors such as duplication or triplication of the SNCA gene locus, which codes for the protein α -synuclein, are known to cause PD (Chartier-Harlin et al., 2004; Singleton et al., 2003). Current transgenic mouse models of α -synuclein overexpression have to some extent been able to recapitulate different aspects of PD-like pathology (see Table 1.2), where changes to neurotransmission, including a reduction in striatal DA release, often precedes other phenotypes associated with PD (Gaugler et al., 2012; Janezic et al., 2013; Lundblad et al., 2012). Furthermore, changes to neurotransmission in models that overexpress α -synuclein are associated with dysfunction of presynaptic vesicle dynamics (Garcia-Reitböck et al., 2010; Janezic et al., 2013; Larsen et al., 2006; Nemani et al., 2010). In accordance with these findings, α -synuclein can be localised at synaptic boutons, and associated with presynaptic vesicles (Clayton and George, 1999; Kahle et al., 2000; Lee et al., 2008; Zhang et al., 2008). It has been suggested that α -synuclein directly interacts with vesicle membranes to regulate synaptic vesicle recycling (Abeliovich et al., 2000; Murphy et al., 2000; Sharon et al., 2001).

The presynaptic dysfunction caused by α -synuclein overexpression often precedes protein aggregation and cell death (Tofaris et al., 2006; Garcia-Reitböck et al., 2010; Nemani et al., 2010; Janezic et al., 2013). Therefore, exploring the underlying mechanisms of presynaptic dysfunction may contribute to developing a deeper understanding of PD pathogenesis.

However, current transgenic models of α -synuclein overexpression often express α -synuclein in high quantities that may not be physiologically relevant, and through promoter elements that do not recapitulate an endogenous expression pattern (Lam et al., 2011; Lundblad et al., 2012; Nemani et al., 2010). Further investigation is therefore needed to understand how DA release and synapse function are affected in transgenic models that represent overexpression of α -synuclein at levels and anatomical distributions comparable to human PD.

The OPDC consortium has generated three lines of *SNCA* BAC transgenic mice, here named *SNCA-A*, *SNCA-B*, and *SNCA-C* (see section 2.2.1 for overview). All three lines express human α -synuclein on a mouse *Snc**a* $-/-$ background. The **SNCA-A** line of mice has brain levels of α -synuclein no higher than the human brain. Brain levels of α -synuclein in **SNCA-B** mice are about 2-3-fold that of humans, and levels of α -synuclein in **SNCA-C** mice are about 5-6-fold of humans (Figure 2.1) (Janezic et al., 2013). The difference in the level of brain α -synuclein between *SNCA-B/C* and human brain is comparable to that between PD patients with *SNCA* gene multiplications and healthy controls (Eriksen et al., 2005). The level of α -synuclein expression in *SNCA-C* mice therefore more accurately reflects the level of α -synuclein expressed in PD patients with *SNCA* multiplications than previous studies that use foreign promoter elements (Lee et al., 2002a; Rockenstein et al., 2002).

Studies in this laboratory have revealed that the *SNCA-C* line has a ~30% reduction in evoked $[DA]_o$ from acute striatal slices, compared to *Snc**a* $-/-$ controls (Janezic et al., 2013). However, it remains unknown whether this reduction is simply due to the presence of human α -synuclein rather than its high level of expression, and whether the reduction in

evoked $[DA]_o$ depends on the dose of human α -synuclein. In this chapter, DA release in SNCA-C mice is firstly explored to reproduce earlier work, and to act as a benchmark for subsequent results. Evoked $[DA]_o$ will be sampled by FCV at a CFM in acute striatal slices. Where a deficit in DA release is found, investigations will proceed to determine whether such a deficit is due to the presence of the human α -synuclein, or whether it depends on the overexpression of human α -synuclein. If a high level of α -synuclein expression is required to produce changes in DA release, then a deficit in evoked $[DA]_o$ will not be expected in SNCA-A mice.

In addition to this, a dose-dependence in the effects of α -synuclein will be explored. People with *SNCA* triplications have shown higher levels of cortical α -synuclein, earlier disease onset, and more severe symptoms than people with *SNCA* duplications, suggesting a dose-dependence for the pathophysiology of α -synuclein in humans (Eriksen et al., 2005; Farrer et al., 2004). If the same dose-dependence exists for the effects of human α -synuclein in mice, then the DA release deficit in SNCA-C mice would be expected to be greater than SNCA-B mice. Therefore, to explore whether a dose-dependence exists for the effects of human α -synuclein in mice, evoked $[DA]_o$ will also be sampled in SNCA-B mice.

3.2. Methods

Unless otherwise specified, experimental procedures were as described in Chapter 2.

3.2.1 Electrical stimulation and FCV

Figure 3.1 shows the location of FCV sampling sites. Sampling sites were not specifically aimed at either striosome or matrix compartments. Each site received a single pulse electrical stimulation, and a four-pulse train at 100 Hz.

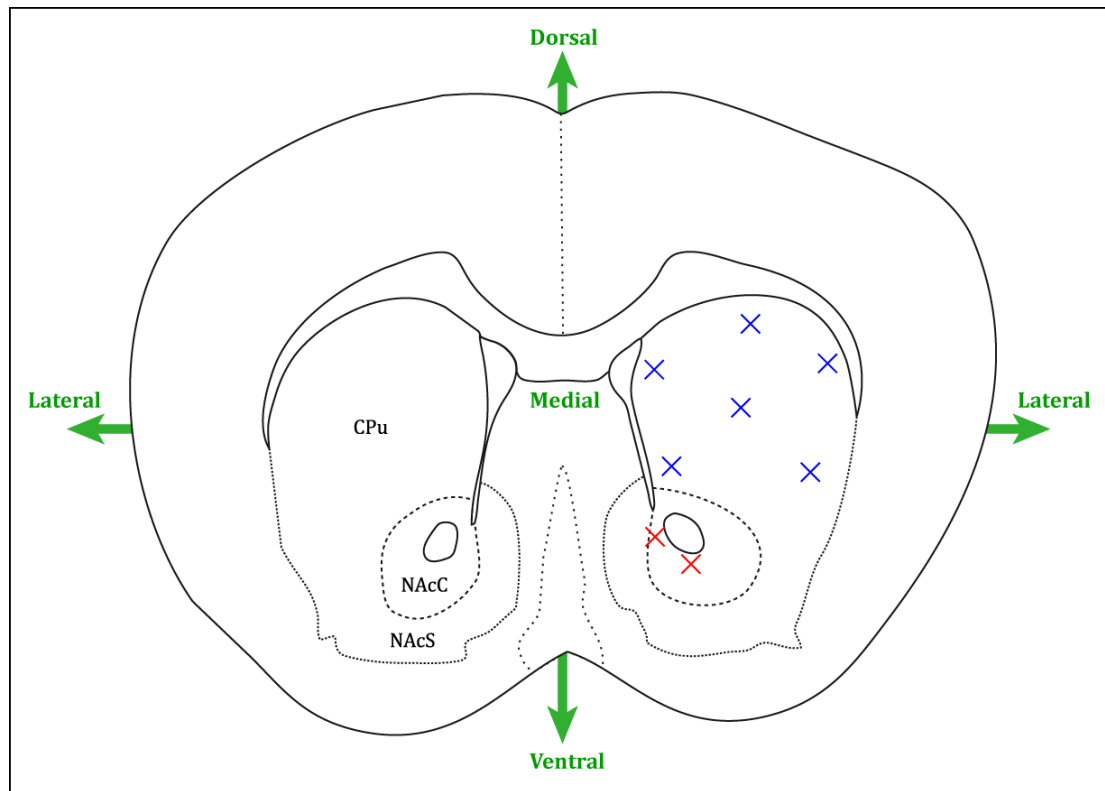


Figure 3.1: Coronal mouse brain section sampled by FCV. Evoked DA release was sampled in acute coronal sections of mouse striatum at Bregma 1.1 mm. Due to the variation of DA release throughout the striatum, evoked $[DA]_o$ was sampled at 8 different sites in the CPu (6 blue sites) NAc (2 red sites). Individual sites were sampled in random order, with corresponding sites from α -synuclein expressing- and *Snc α* ^{-/-} mice sampled in immediate succession, and in the same bath. The different sites were approximately matched in all slices to control for regional variation, and to allow for sub regional analysis. Green arrows show dorsal-ventral, and lateral-medial axis. Diagram adapted from (Franklin and Paxinos, 2007).

3.3. Results

3.3.1 SNCA-C mice have a significant deficit in evoked [DA]_o in the dorsal striatum compared to Snca -/- controls.

Earlier work in this lab revealed that the SNCA-C line has a ~30% reduction in DA release (Janezic et al., 2013) at ages 3, 12, and 18 months. Before expanding on this work, evoked [DA]_o was sampled in a smaller population (3 pairs of animals rather than 5, and only at 3 months) to confirm the results previously obtained by the OPDC, and as a basis for further investigation. Electrically evoked [DA]_o was detected by FCV, and the voltammogram of evoked DA showed oxidation and reduction peaks at the same potential as 2 μ M DA used for calibration (Figure 3.2). Evoked [DA]_o was sampled at eight distinct sites in the striatum to explore how α -synuclein overexpression affects different regions as done previously (Janezic et al., 2013).

An electrically evoked monoamine signal was detected at each of the sites sampled (Figure 3.2 a). The monoamine voltammogram fitted the electrochemical profile of DA (Figure 3.2 b). In both genotypes, mean [DA]_o profiles vs. time show some regional variation with larger profiles in dorsal than ventral sites. All regions show a consistent trend towards lower evoked [DA]_o from SNCA-C mice than *Snca* -/- controls. Pooling data of mean [DA]_o profiles from individual sites into either CPu or NAc regions, (blue crosses and red crosses respectively in figure 3.2) as done previously (Anwar et al., 2011; Janezic et al., 2013), shows that peak evoked [DA]_o in the CPu (but not NAc) of three-month old SNCA-C mice is significantly lower than *Snca* -/- controls by ~30% (Figure 3.3 a-d). These data confirms previous findings from a larger dataset (Janezic et al., 2013), which showed a similar deficit of evoked [DA]_o in SNCA-C mice vs. *Snca* -/- controls.

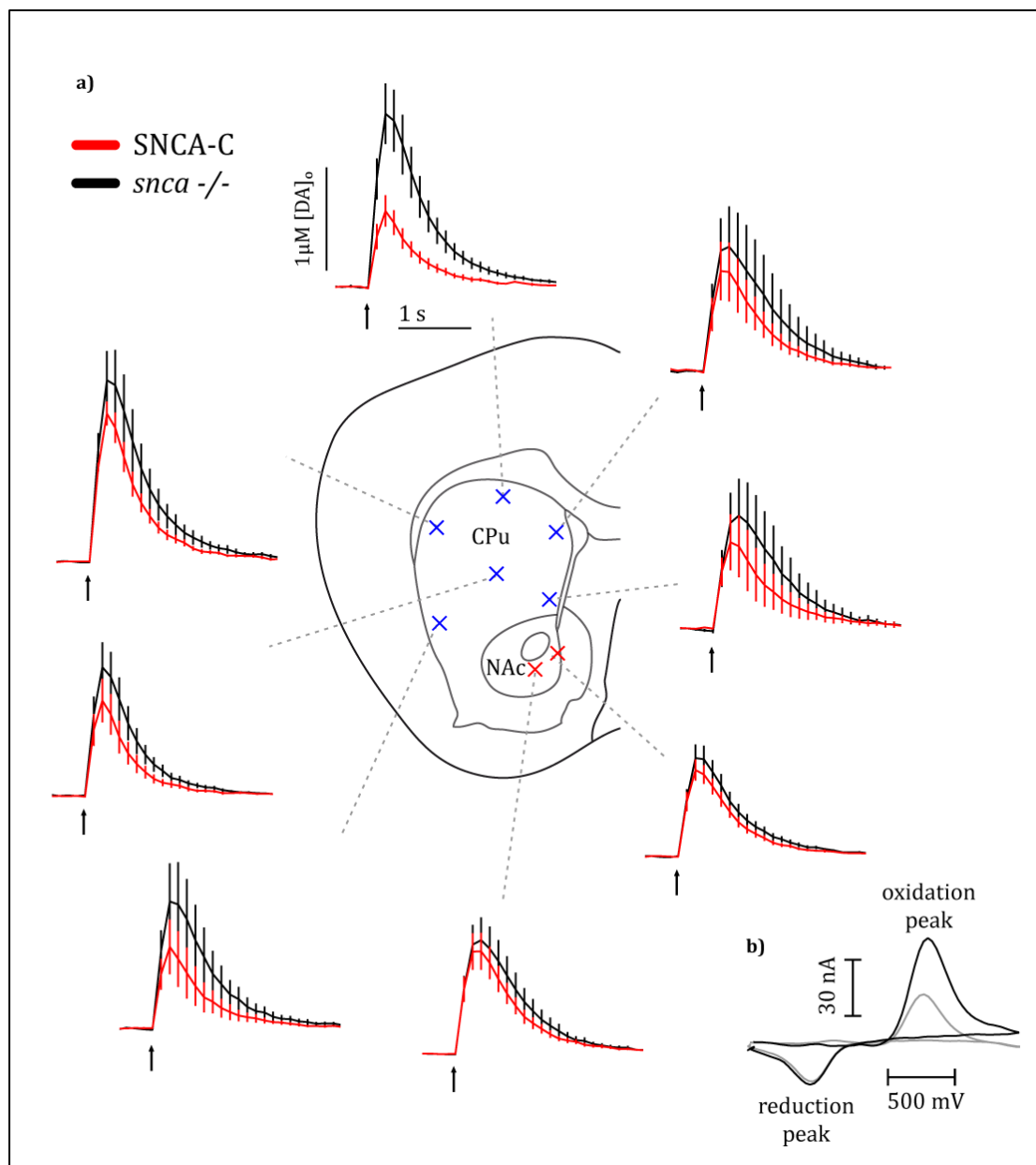


Figure 3.2: Evoked [DA]_o from SNCA-C and *Snca* -/- mice at 3 months. Mean [DA]_o profiles vs. time (mean ± SEM) following single pulse stimulation (↑) at eight different sites in the striatum (a) in SNCA-C vs. *Snca* -/- controls. Data from the top six sites (blue crosses) in the CPu were pooled together, and the bottom two sites (red crosses), which are in the NAc, were also pooled together. The pooled data is displayed in Figure 3.3. Typical voltammogram (b) of evoked DA (black) showed oxidation and reduction peaks at the same potential as 2 μM DA used for calibration (grey).

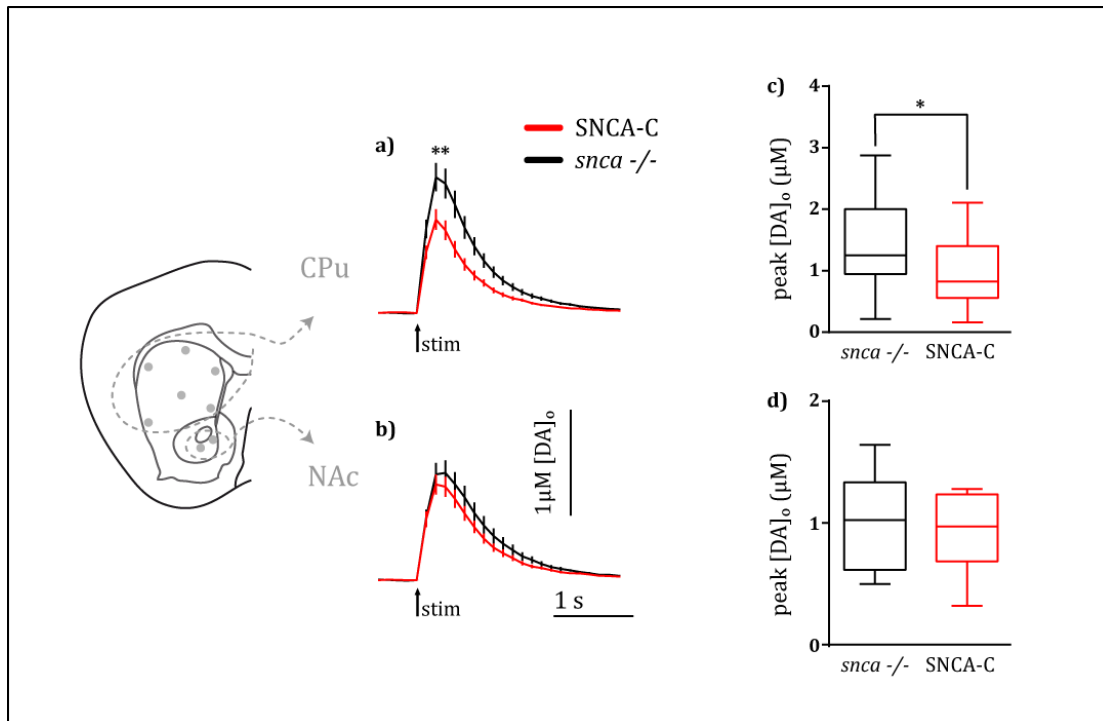


Figure 3.3: Pooled data of evoked $[DA]_0$ from SNCA-C and *Snca* $-/-$ mice at 3 months.. Mean $[DA]_0$ profiles vs. time ($\pm$ SEM) following single pulse stimulation ($\uparrow$) in CPu (a) and NAc (b). Mean peak $[DA]_0$ from SNCA-C is about 30% lower than *Snca* $-/-$ controls in CPu ($P < 0.01$, unpaired two-tailed t test, $n = 31$) but not NAc ($P > 0.05$, unpaired two-tailed t test, $n = 11$). Distribution of mean peak evoked $[DA]_0$ was assumed to be normally distributed after not failing D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test, and appearing normally distributed on Q-Q plot. Box and whiskers plots of peak evoked $[DA]_0$ in CPu (c) and NAc (d) showing median, interquartile range, along with lowest- and highest observations. Peak evoked $[DA]_0$ from SNCA-C mice is ranked significantly lower in CPu ($P < 0.05$, Two-tailed Mann-Whitney, $n = 31$), but not NAc ($P > 0.05$, Two-tailed Mann-Whitney, $n = 11$).

3.3.2 SNCA-A mice at 18 months show no deficit in evoked striatal [DA]_o or striatal DA content compared to *Snca* ^{-/-} controls.

Data presented earlier in this chapter (section 3.3.1) show that there is a deficit of evoked [DA]_o in SNCA-C mice compared to *Snca* ^{-/-} controls. However whether this deficit is due to the presence of human α -synuclein, or α -synuclein being expressed above levels found in human brain remains unknown. Previous efforts explored this question by investigating whether deficits in evoked [DA]_o could also occur in the SNCA-A line of mice, which expresses human α -synuclein at levels not exceeding that of the human brain. These studies found no difference in evoked striatal [DA]_o between three-month old SNCA-A mice and age-matched *Snca* ^{-/-} controls (Janezic et al., 2013). However, PD is a progressive disease (Lees et al., 2009; Thiruchelvam et al., 2004; Wu et al., 2010), and it therefore remains unknown whether the presence of α -synuclein expressed at the same levels as human brain could develop changes in evoked [DA]_o with age.

To investigate whether the DA release deficit can also develop in SNCA-A mice, evoked [DA]_o was explored in SNCA-A mice at 18-months. FCV was used to sample evoked [DA]_o at eight distinct sites in the striatum to explore changes to evoked [DA]_o in different regions. To investigate whether any differences in DA content will develop between SNCA-A mice and *Snca* ^{-/-} controls, striatal DA content was also measured by HPLC with electrochemical detection.

Electrically evoked DA was detected at each of the sites sampled (Figure 3.4, a). Mean profiles of evoked [DA]_o vs. time show some regional variation within each genotype, with larger profiles in dorsal- than ventral sites. Mean profiles of evoked [DA]_o vs. time (Figure 3.4 a) show no overall trend towards a difference of evoked [DA]_o in SNCA-A mice

compared to *Snca* $-/-$ controls, and both genotypes have overlapping $[DA]_o$ profiles at the majority of sites. In contrast to the rest of the profiles, a site in the ventromedial CPu and a site medial of the anterior commissure (AC) do however show $[DA]_o$ profiles with higher peaks in SNCA-A mice than controls. The difference in each of these areas is however not statistically significant. Combining transients from individual sites in CPu or NAc regions as done previously (Anwar et al., 2011; Janezic et al., 2013), shows that the peak evoked $[DA]_o$ in 18-month old SNCA-A mice and *Snca* $-/-$ controls have a very similar distributions, with no significant difference between them (Figure 3.5). There is also no significant difference in the DA content of SNCA-A mice vs. *Snca* $-/-$ controls.

These data reveal that mice expressing human α -synuclein at a level comparable to human brain tissue do not have any progressive changes to evoked $[DA]_o$. The data presented here therefore suggests that the deficit in evoked $[DA]_o$ reported in SNCA-C mice (section 3.3.1) is not due to the presence of human α -synuclein, but rather because α -synuclein is expressed above physiological levels found in the human brain.

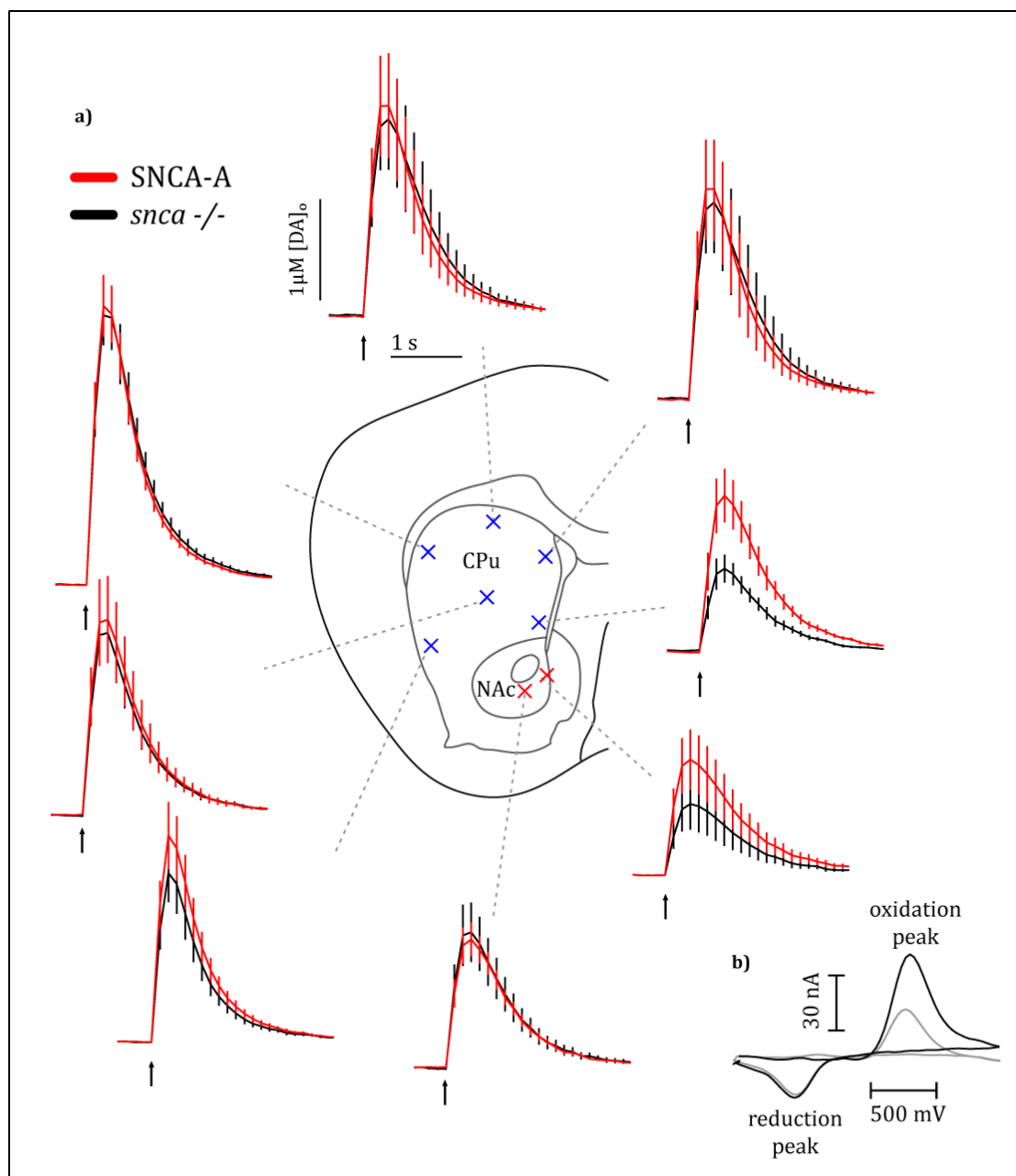


Figure 3.4: Evoked [DA]_o from SNCA-A and *Snca* -/- mice at 18 months. a) Mean [DA]_o profiles vs. time (mean ± SEM) following single pulse stimulation (↑) in SNCA-A vs. *Snca* -/- controls. The top six sites form the CPu (blue crosses), and the bottom two sites form the NAc (red crosses) in Figure 3.5. Typical voltammogram (b) of evoked DA (black) showed oxidation and reduction peaks at the same potential as 2 μM DA used for calibration (grey). Peak evoked [DA]_o in ventromedial CPu and medial of NAc sites are not distributed normally, and the differences between SNCA-A and *Snca* -/- controls in this area each of these areas are not statistically significant ($P > 0.05$, Mann-Whitney or Kolmogorov-Smirnov, $n = 8$).

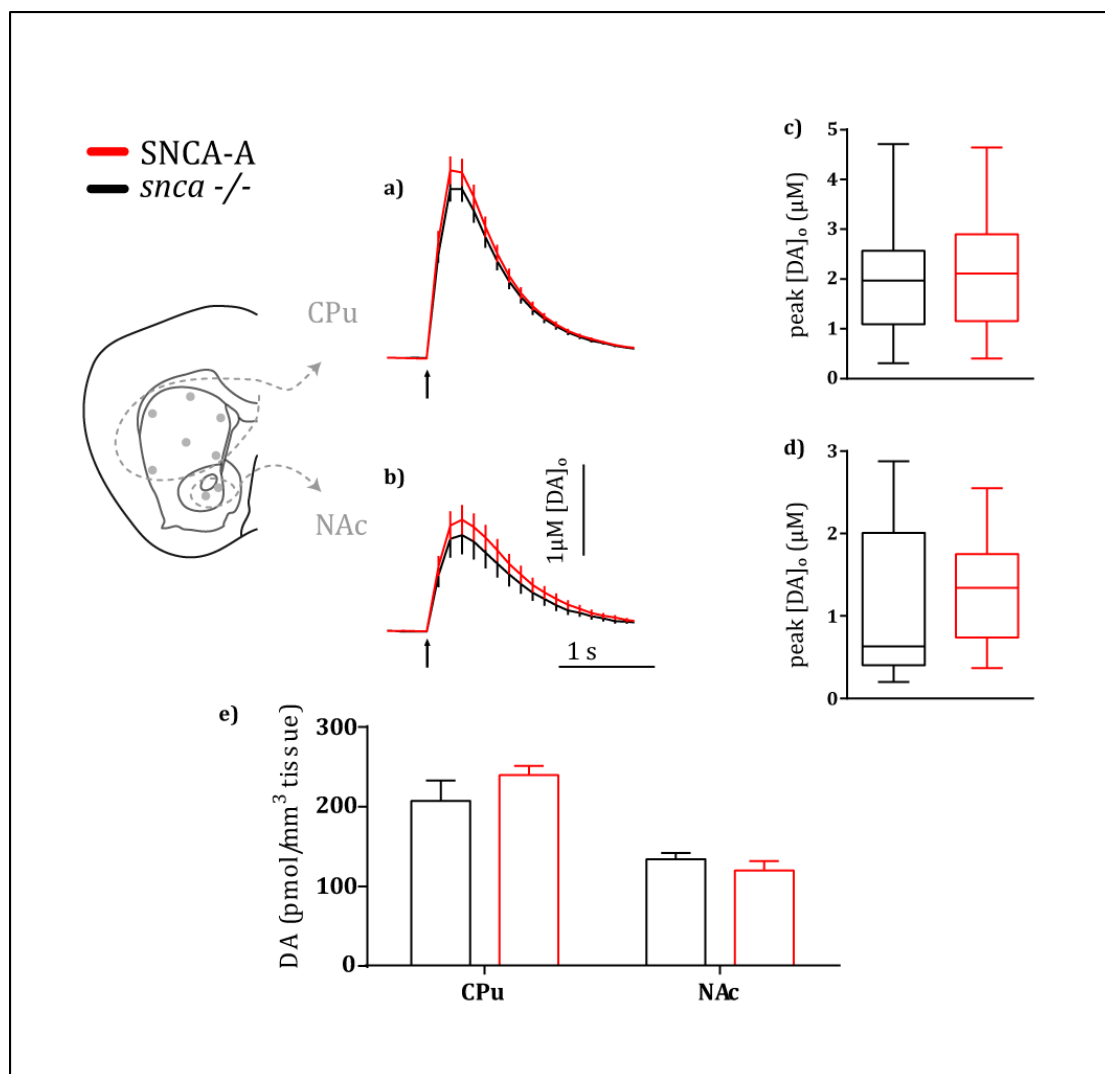


Figure 3.5. : Pooled data of evoked $[DA]_0$ from SNCA-A and *Snca* $-/-$ mice at 18 months. α -synuclein expression at the same levels as human brain has no effect on evoked $[DA]_0$ in SNCA-A mice compared to *Snca* $-/-$ controls. Mean $[DA]_0$ profiles vs. time ($\pm$ SEM) following single pulse stimulation ($\uparrow$) in CPu (a) and NAc (b). Data was assumed to have non-normal distribution after failing Shapiro-Wilk normality test and appearing not normally distributed on a histogram and Q-Q plot. Mean peak $[DA]_0$ from SNCA-A is not significantly different from *Snca* $-/-$ controls in either CPu ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 47$) or NAc ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 15$). Box and whiskers plots of peak evoked $[DA]_0$ in CPu (c) and NAc (d) showing median, interquartile range, along with lowest-and highest observations. HPLC with electrochemical detection (e) reveals no differences in DA content between SNCA-A and *Snca* $-/-$ in either CPu or NAc ($P > 0.05$, t-test, $n = 7$)

3.3.3 SNCA-B mice show no deficit in evoked striatal [DA]_o or striatal DA content compared to *Snca* ^{-/-} controls.

The previous two sections of this chapter reported that expressing human α -synuclein above the level found in the human brain causes a ~30% deficit of evoked [DA]_o in the CPu, but not NAc of SNCA-C mice vs. *snca* ^{-/-} controls. However, it is still unknown to what extent there might be a gene dose effect similar to that in patients with *SNCA* duplications and triplications (Eriksen et al., 2005).

To further explore the dose dependent effects of α -synuclein overexpression in SNCA-C mice, evoked striatal [DA]_o was investigated in SNCA-B mice, which express human α -synuclein on a *Snca* ^{-/-} background at ~2-3 times the levels of human brain tissue (about half the level of SNCA-C, see Figure 2.1). To determine whether there is a DA release deficit in SNCA-B mice, evoked [DA]_o was sampled in SNCA-B mice vs. *Snca* ^{-/-} controls using FCV at a CFM. Evoked [DA]_o was sampled at eight distinct sites in the striatum to investigate any region-specific changes to DA release. Experiments were carried out on mice at 3 months and 18 months to explore whether changes to evoked [DA]_o might develop with age.

At 3 months (Figure 3.6 a) electrically evoked DA was detected at each of the eight sites sampled. The monoamine voltammogram fitted the electrochemical profile of DA (Figure 3.6 b). Mean profiles of evoked [DA]_o vs. time show some regional variation within each genotype, with larger profiles in dorsal- than ventral sites. Mean profiles of evoked [DA]_o vs. time from both genotypes are overlapping at the majority of sites (Figure 3.6).

At 18 months, (Figure 3.7 a) electrically evoked DA was detected at each of the eight sites sampled. Mean profiles of evoked [DA]_o vs. time show some regional variation within each

genotype, with larger profiles in dorsal- than ventral sites. Unlike at 3 months, mean $[DA]_o$ profiles vs. time are not overlapping at all sites at age 18 months, but no significant difference in evoked $[DA]_o$ between SNCA-B mice and *Snca* $-/-$ controls (Figure 3.7) were found at any of the sites. Combining transients from individual sites in CPu or NAc regions as done previously (Anwar et al., 2011; Janezic et al., 2013), reveals that evoked $[DA]_o$ in both 3- and 18-month old SNCA-B are not significantly different from *Snca* $-/-$ controls (Figure 3.8 a-f).

HPLC with electrochemical detection was used to explore whether SNCA-B mice have any changes to DA content (Figure 3.9). DA content per mm^3 of striatal tissue was about two fold higher in CPu than NAc. DA content was similar between all animals at 3- and 18 months, and no significant differences were found between SNCA-B and *Snca* $-/-$ controls.

The deficit in evoked $[DA]_o$ release observed in SNCA-C mice, but not in SNCA-B mice, suggesting that the effect of α -synuclein on DA release depends on a relatively high dose of α -synuclein.

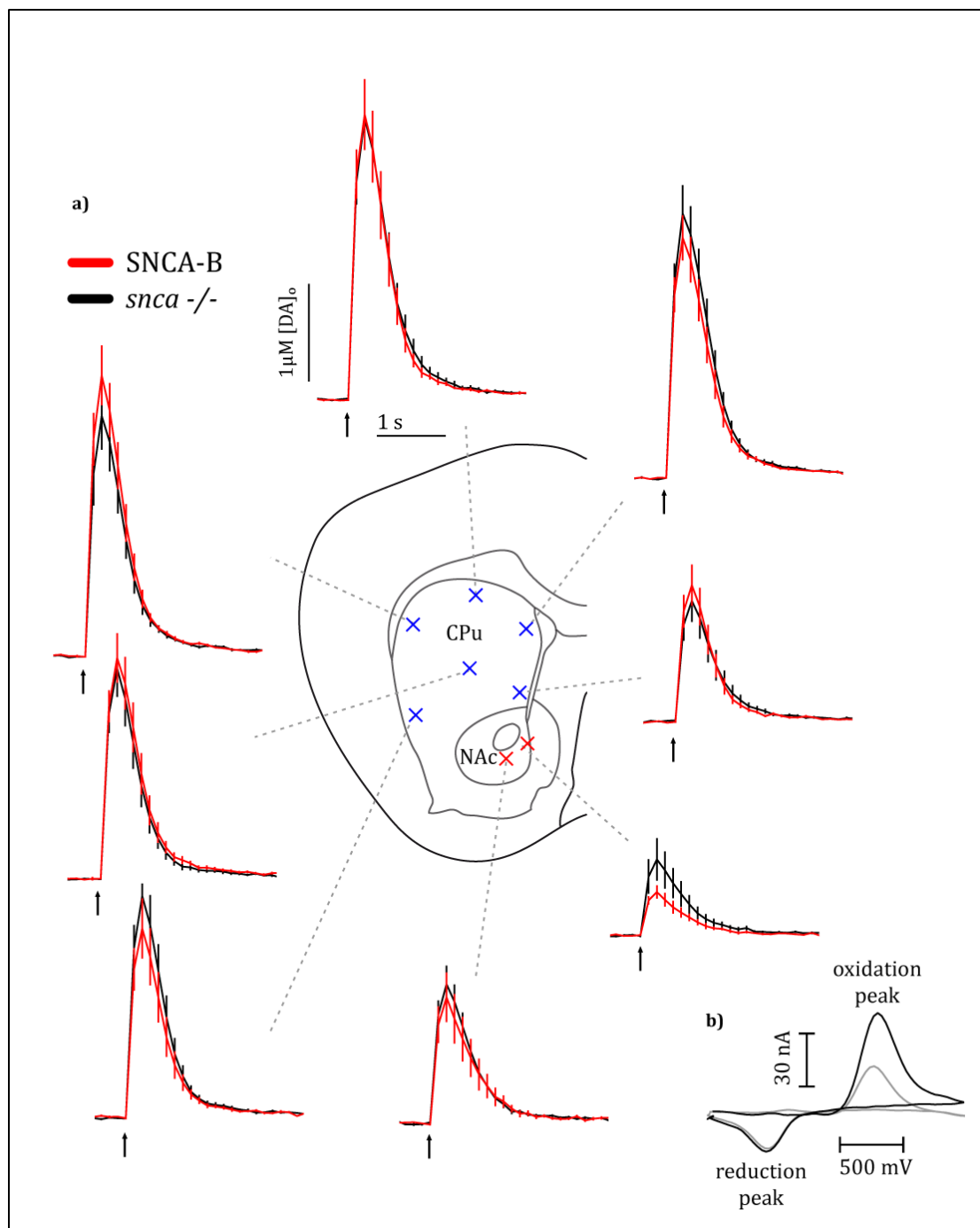


Figure 3.6: Evoked [DA]_o from SNCA-B and *Snca* -/- mice at 3 months. Mean [DA]_o profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in SNCA-B vs. *Snca* -/- controls at age 3 months. The top six sites form the CPu (blue crosses), and the bottom two sites form the NAc (red crosses) in Figure 3.8. Typical voltammogram (b) of evoked DA (black) showed oxidation and reduction peaks at the same potential as 2 μ M DA used for calibration (grey).

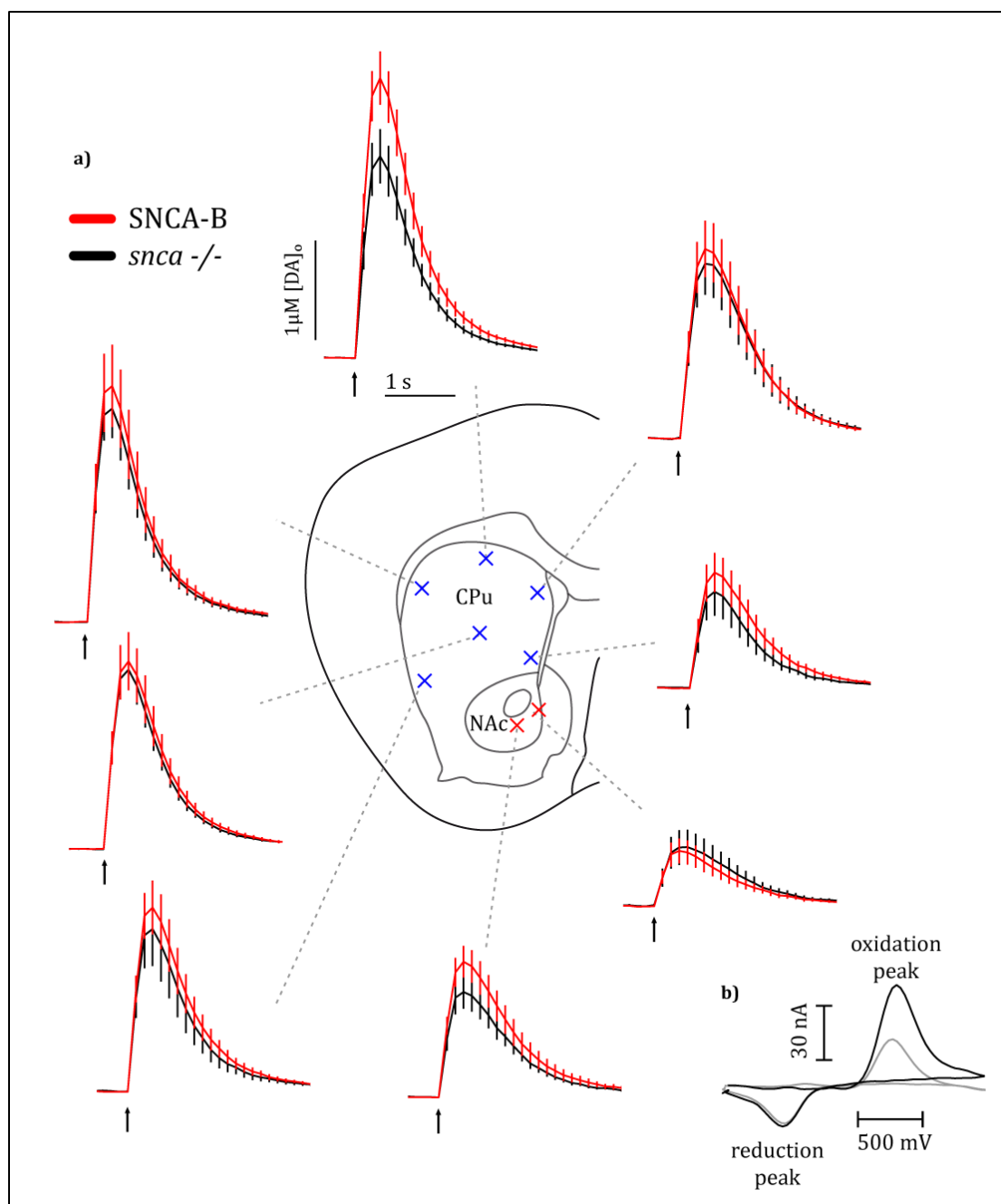


Figure 3.7: Evoked [DA]_o from SNCA-B and *Snca* -/- mice at 18 months. Mean [DA]_o profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in SNCA-B vs. *Snca* -/- controls at age 18 months. The top six sites form the CPu (blue crosses), and the bottom two sites form the NAc (red crosses) in Figure 3.8. Typical voltammogram (b) of evoked DA (black) showed oxidation and reduction peaks at the same potential as 2 μ M DA used for calibration (grey).

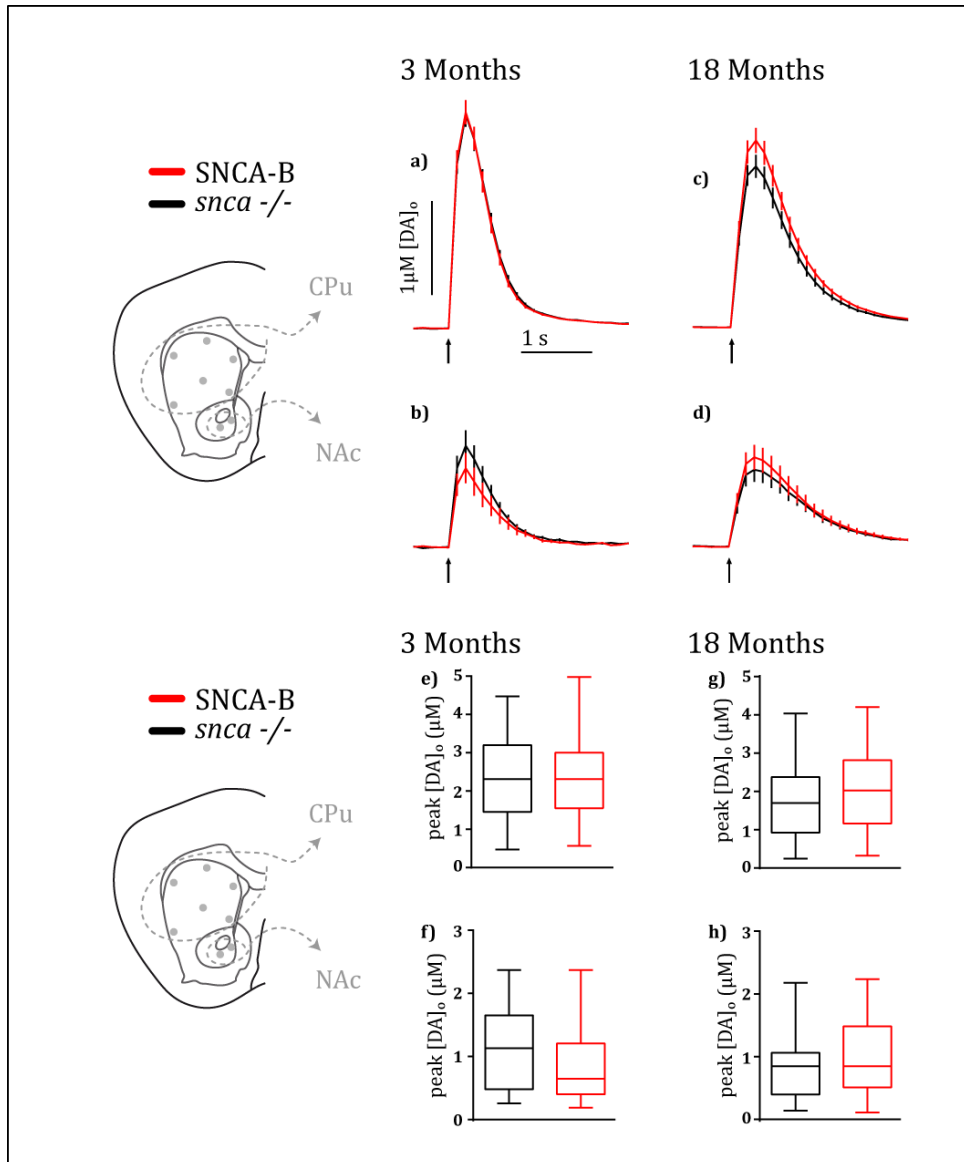


Figure 3.8. : Pooled data of evoked [DA]_o from SNCA-B and *Snca* -/- mice at 3, and 18 months. Mean [DA]_o profiles vs. time (± SEM) following single pulse stimulation (↑) in CPu (a,c) and NAc (b,d) of 3-month- (a,b) and 18-month old (c,d) mice. Box and whiskers plots of peak evoked [DA]_o in CPu (e,g) and NAc (f,h), of mice at 3 months (e,f) and 18 months (g,h), showing median, interquartile range, along with lowest and highest observations. Peak evoked [DA]_o is not significantly different between SNCA-B mice and *Snca* -/- controls at age 3 months in CPu ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 60$), age 3 months in NAc ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 17$), age 18 months in CPu ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 60$) or age 18 months in NAc ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 20$). Data was assumed to have non-normal distribution after failing D'Agostino & Pearson omnibus- and Shapiro-Wilk normality tests and appearing not normally distributed on a histogram and Q-Q plot.

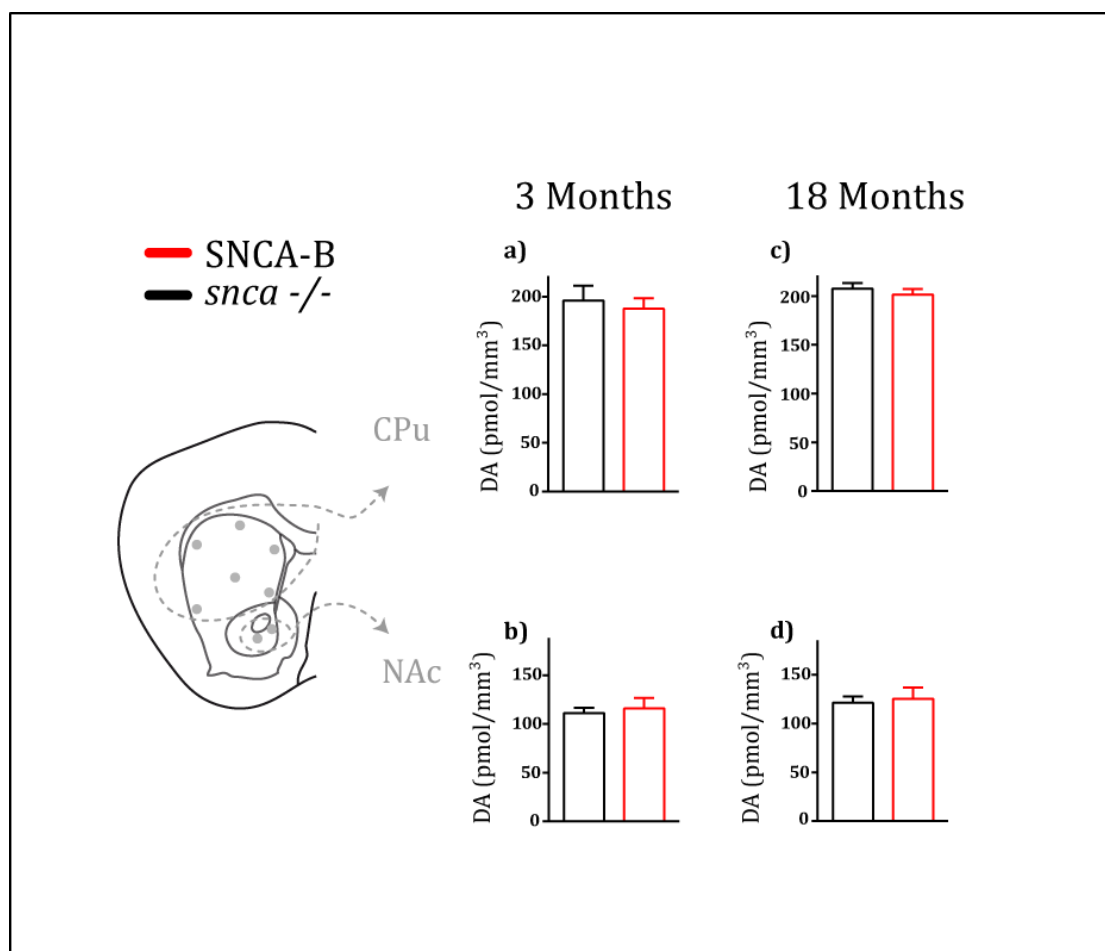


Figure 3.9: DA content in SNCA-B and *Snca* ^{-/-} mice at 3, and 18 months. HPLC with electrochemical detection shows DA content in CPu (a,c) and NAc (b,d) of 3-month- (e,b) and 18-month old (c,d) mice. Data was assumed to have non-normal distribution after failing D'Agostino & Pearson omnibus- and Shapiro-Wilk normality tests, and appearing not normally distributed on a histogram and Q-Q plot. There are no significant differences in DA content between SNCA-A and *Snca* ^{-/-} in either CPu ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 10$), age 3 months in NAc ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 9$), age 18 months in CPu ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 8$) or age 18 months in NAc ($P > 0.05$, unpaired two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 8$).

3.4. Discussion

The data presented in this chapter reproduced earlier findings that electrically evoked [DA]_o is reduced by ~30% in the CPu of SNCA-C mice, which express human α -synuclein at levels 5-6 fold of human brain tissue. It was subsequently shown here that SNCA-A mice, which express human α -synuclein at a comparable level to human brain tissue, do not develop changes to evoked CPu [DA]_o. These data suggest that the deficit of evoked [DA]_o seen in SNCA-C mice, is due to the expression level of α -synuclein, and not just the presence of human α -synuclein. It was then shown that SNCA-B mice, which express α -synuclein at levels 2-3 fold of human brain, develop no changes in evoked striatal [DA]_o. Taken together, these data suggest that the deficit of evoked [DA]_o seen in SNCA-C mice is dependent on the relatively high level of α -synuclein expression.

The deficit of evoked [DA]_o in SNCA-C vs. *Snc*a -/- is only present in the CPu, and not the NAc (Figure 3.3.). The NAc however only includes two recording sites (Figure 3.2) and data from the NAc therefore has less statistical power than data from the CPu. Having said that, a power calculation estimated that three fold the number of observations obtained in the CPu would be required to reach $p < 0.05$ from a t-test in the NAc (G*Power, data not shown). It is therefore unlikely that a significant deficit in DA release in the NAc of SNCA-C mice will arise, even if the number of observations in the NAc were increased to match the CPu. In addition to this, observing a deficit of evoked [DA]_o in the CPu and not the NAc, which are primarily innervated by DA neurons from the SNc and VTA respectively, is in line with clinical observations that the SNc is more prone to be affected during PD (Gibb and Lees, 1991).

The three lines of mice characterised here are from the same genetic background, with the only major difference being the level of human α -synuclein expressed. Therefore, the changes to evoked [DA]_o reported in SNCA-A, -B, and -C mice is unique in the way it reflects on the dose-dependent effects of α -synuclein expression on DA synapse function.

Only one other study has been able to directly compare evoked [DA]_o from different levels of α -synuclein expressed either with or without a WPRE enhancer element (Lundblad et al., 2012). This study however used a viral vector injected directly into the SNc, which leads to a rapid decline in DA release, and therefore doesn't allow for a progressive phenotype to be studied in aged animals. The data presented here is however in line with reported findings from Lundblad *et al.* suggesting that unmutated human α -synuclein has negatively modulates DA release in a dose-dependent manner.

Other studies do not explore the dose dependence of α -synuclein affecting neurotransmitter release, and also utilised such a wide range of organisms, promoter elements, and methods of detecting DA release, that the dose of α -synuclein expression cannot be compared between different investigations. For example, no early changes were seen to amphetamine evoked [DA]_o in mice that expressed human α -synuclein under the Thy-1 promoter at levels 5-10 fold of human brain tissue (Lam et al., 2011). On the other hand, early changes was seen to K⁺ evoked [DA]_o in rats that express human α -synuclein under the AAV promoter at levels 2-3 fold of the control hemisphere (Gaugler et al., 2012). If only the results on DA release are taken into account, then it would suggest that α -synuclein changes DA release in some cases, but not others. Comparing the effects of α -synuclein on DA release between these two experiments is however not feasible because

they make use of different promoters, model organisms, and [DA]_o recording techniques. Comparisons between such studies can therefore not accurately report on the dose dependence of α -synuclein expression on evoked [DA]_o.

The findings presented here are broadly in agreement with other studies, which have shown that α -synuclein negatively modulates catecholamine release in PC12 chromaffin cells (Larsen et al., 2006), DA release in the rodent striatum (Garcia-Reitböck et al., 2010; Lam et al., 2011; Lundblad et al., 2012), and glutamate release in mouse hippocampus (Nemani et al., 2010). Furthermore knocking out α -, β -, and γ -, synuclein in mice leads to increased amounts of evoked striatal DA release (Anwar et al., 2011; Senior et al., 2008). Taken together these data point towards an inverse relationship between the dose of α -synuclein and the amount of neurotransmitter released from the affected system.

In contrast to the data presented here, there is one study that reported increased tonic striatal [DA]_o in 6-month old mice expressing human α -synuclein under the Thy-1 promoter (Lam et al., 2011). However, striatal [DA]_o under tonic release conditions is influenced by factors like DA neuron firing rate and D2 autoreceptor activation, which do not play a role in [DA]_o evoked from a single electrical pulse in acute brain slices. The discrepancy in finding reported from SNCA-C mice here vs. Thy-1 mice by Lam *et al.* could therefore be due to increased DA firing rate or reduced D2 autoreceptor function in Thy-1 mice, and certainly deserves further investigation. Moreover Thy-1 α -synuclein overexpressing mice characterised by Lam *et al.* exhibit a progressive decrease of tonic striatal [DA]_o with age, and a smaller reserve pool of synaptic vesicles in primary cortical

cultures (Rockenstein et al., 2002), which is in line with deficits of evoked $[DA]_o$ observed in SNCA-C mice.

The exact mechanism by which α -synuclein overexpression causes a reduction of evoked $[DA]_o$ in SNCA-C mice remains unknown, but it is independent of changes to striatal DA content and the rate of DA uptake (Janezic et al., 2013), which suggests changes to the releasability of DA synaptic terminals. The mechanism by which α -synuclein expression affects the releasability of DA in SNCA-C mice will be explored in the next chapter.

CHAPTER 4. DA release in SNCA-C mice has a normal response to D₂ autoreceptor activation, but increased sensitivity to a vesicle mobilising agent.

4.1. Introduction

In Chapter 3, electrically evoked striatal [DA]_o from SNCA-C mice was found to be about ~30% lower than *Snca* ^{-/-} controls. It was also shown in Chapter 3, that the deficit of evoked [DA]_o in SNCA-C mice is not just due to the presence of human α -synuclein, but that it depends on the relatively high level of α -synuclein expression. Understanding the underlying mechanisms by which human α -synuclein expression in SNCA-C mice cause a deficit in evoked striatal [DA]_o may be relevant to understanding the earliest stages of PD, and will be the main focus of this chapter.

At age 3 months, the deficit of evoked [DA]_o in SNCA-C mice is not accompanied by cell death, changes to DA content, or changes to the rate of DA uptake (Janezic et al., 2013). These observations suggest that the deficit of evoked [DA]_o is due to changes in releasability of DA at a presynaptic level. This idea is in agreement with other studies that found changes to neurotransmission, in animals overexpressing α -synuclein, precede protein aggregation, cell death, and changes to DA content (Lundblad et al., 2012; Nuber et al., 2013; Tofaris et al., 2006; Wu et al., 2010).

The releasability of DA is regulated at a presynaptic level by several factors, ranging from plasma membrane receptors and ion channels to vesicular mechanisms and SNARE machinery. Some of these factors may be candidate mechanisms through which α -synuclein affects DA release, and will be introduced here in more detail.

Extracellular striatal ACh, released by tonically active cholinergic interneurons, has been known to affect DA release by activating presynaptic $\beta 2$ nicotinic ACh receptors ($\beta 2$ nAChRs) (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). It may therefore be that changes to either extracellular ACh concentration, or the activation of nAChRs, can explain the deficit of evoked $[DA]_o$ in SNCA-C mice. However, the deficit of evoked $[DA]_o$ in SNCA-C mice remains in the presence of DH β E, a $\beta 2$ nAChR antagonist, and the frequency-dependence of evoked $[DA]_o$ in SNCA-C mice and *Snca* $-/-$ controls are not different under nAChR blockade (Janezic et al., 2013). These data suggest that nAChR activity is not involved in the DA release deficit caused by α -synuclein overexpression in SNCA-C mice.

The releasability of DA is under regulation from presynaptic D₂ autoreceptors, which activate the Kv1 family of voltage gated potassium channels to increase K⁺ conductance, and therefore causing hyperpolarisation (Cragg and Greenfield, 1997; Fulton et al., 2011; Martel et al., 2011). Increased sensitivity of D₂ autoreceptors, or constitutive activation of Kv1 channels, may therefore underlie the reduced DA releasability in SNCA-C mice. This mechanism has not yet been investigated in SNCA-C mice, and will be discussed in more detail later in this chapter.

Ca²⁺ triggers synaptic vesicle fusion with the plasma membrane, which is a key step in DA release (Malva et al., 1994; Vrljic et al., 2010). Therefore, mechanisms related to Ca²⁺ dependent exocytosis, activity at Ca²⁺ binding domains on proteins like synaptotagmin, or buffering of intracellular Ca²⁺, may all play a role in mediating the effects of α -synuclein overexpression on DA releasability. However, evoked $[DA]_o$ from SNCA-C and *Snca* $-/-$ mice

have the same response to changes in $[Ca^{2+}]_o$ (Janezic et al., 2013), which suggests that Ca^{2+} dependent mechanisms do not play a role in mediating the effects of α -synuclein overexpression on DA releasability.

An electron microscopy investigation found increased vesicle clustering in synaptic axonal profiles of nigrostriatal DA neurons in SNCA-C mice (Janezic et al., 2013). The amount of DA release depends on mobilisation of presynaptic DA-containing vesicles to the cell membrane (Venton et al., 2006). An increased clustering of presynaptic vesicles suggests these vesicles also have a reduced mobility, which may explain the reduced DA releasability in SNCA-C mice. This mechanism has not been further investigated in SNCA-C mice, and will be discussed in more detail later in this chapter.

The SNARE machinery is responsible for fusing vesicles with the membrane, and changes to the availability of functional SNARE complexes could affect the amount of neurotransmitter released. Furthermore, it has been shown that α -synuclein can interact with the SNARE machinery in mouse brain homogenates (Burré et al., 2010; Chandra et al., 2005), and changes to the SNARE machinery may therefore underlie the reduced DA releasability in SNCA-C mice. However, SNCA-C mice have no notable changes in the striatum to coimmunoprecipitation of the SNARE complex proteins, SNAP25 and VAMP2, or total levels of complexin I and II (Janezic et al., 2013). These data suggest that changes to SNARE machinery do not play a role in mediating the effects of α -synuclein overexpression on DA releasability.

Several of the candidate mechanisms mentioned here have been tested before, and were unrelated to the DA release deficit in SNCA-C mice. The focus of this chapter will however

be on two candidate mechanisms that have not yet been sufficiently explored. The first candidate mechanism to be explored in this chapter is activation of the DA D₂ autoreceptor pathway. Several studies have suggested changes to the activation and function of D₂ receptors in mice that overexpress α -synuclein, but it remains unknown whether there are any changes to activity of presynaptic D₂ autoreceptors in SNCA-C mice. The D₂ selective agonist, *quinpirole* (QP), and the D₂ selective antagonist, sulpiride, had opposite effects in mice that overexpress α -synuclein vs. WT controls, on EPSCs of striatal MSN (Lam et al., 2011). This suggests that α -synuclein overexpression may change DA receptor activity in a complex manner. Overexpressing the A53T mutant form of α -synuclein causes an overall up-regulation of striatal D₁ and D₂ receptors (Kurz et al., 2010). Moreover, α -synuclein can enhance the effect transfected D₂ receptor signalling on adenylate cyclase in Chinese hamster ovary cells (Jae Kim et al., 2006). Taken together these data suggest that α -synuclein overexpression can affect the function of D₂ receptors. Possible mechanisms by which α -synuclein overexpression may affect activation of the D₂ autoreceptor pathway include upregulation of presynaptic D₂ autoreceptors, an enhanced coupling between D₂ receptors and Kv1 channels, or a ligand-independent constitutive activity of Kv1 channels that could cause reduced DA releasability. The first part of this chapter explores possible changes to striatal D₂ autoreceptor function in SNCA-C mice by observing the response of evoked [DA]_o to increasing concentrations of QP. QP is a D₂ receptor antagonist, and enhanced activity of the D₂ receptor signalling pathway can be expected to change the I_{c50} value or hill slope of a QP dose response curve.

The second candidate mechanism to be explored in this chapter is a reduction in the mobility of presynaptic vesicles. In addition to the electron microscopy investigation that found increased vesicle clustering in SNCA-C mice, several other findings suggest that α -synuclein might affect the distribution of presynaptic vesicles. α -Synuclein can be localised with synaptic vesicles (Kahle et al., 2000; Lee et al., 2008; Zhang et al., 2008), and overexpressing α -synuclein in mice causes a reduction in synaptic proteins such as synapsin 1 and synaptobrevin 2 (Nemani et al., 2010; Scott et al., 2010). Adenoviral overexpression of WT α -synuclein in rats reduced synaptic vesicle density in striatal DA neurons, along with reducing striatal DA release (Gaugler et al., 2012). Prion promoter overexpression of WT human α -synuclein has been shown to cause reduced vesicle density at the active zone, reduced clustering after endocytosis, reduction of readily releasable pool, and impaired exocytosis of glutamate in acute hippocampal slices (Nemani et al., 2010). Overexpressing α -synuclein under a PDGF promoter in primary mouse cultures inhibits intersynaptic trafficking of vesicles, which leads to a smaller reserve pool of vesicles (Scott and Roy, 2012). Taken together, these data suggests that α -synuclein can decrease the mobilisation of presynaptic DA vesicles, and therefore reduce DA releasability. However, very little has been done to date to explore a functional deficit in the mobility of presynaptic vesicles. The second part of this chapter will therefore explore whether evoked $[DA]_o$ in SNCA-C mice involves a reduction in the mobility of presynaptic vesicles. This will be done by comparing the effect of cocaine on evoked $[DA]_o$ between SNCA-C mice and *Snca*^{-/-} controls. Although, cocaine is well known for its effects at the DAT (Jones et al., 1996), it has also been shown to increase mobilisation of DA-containing vesicles. Cocaine has been shown to enhance electrically evoked $[DA]_o$ after the readily releasable pool of DA vesicles

was depleted by α -methyl-p-tyrosine, suggesting that a reserve pool of vesicles is mobilised (Venton et al., 2006). The ability of cocaine to enhance DA release is also impaired in mice with reduced synaptic vesicle reserve pools (Kile et al., 2010; Venton et al., 2006). Taken together, these data suggest that cocaine can enhance evoked $[DA]_o$ by mobilising a reserve pool of vesicles. If the deficit of DA releasability in SNCA-C mice is due to reduced presynaptic vesicle mobilisation, then evoked $[DA]_o$ from these mice may be more sensitive to the vesicle mobilising properties of cocaine.

4.2. Methods

Unless otherwise specified, experimental procedures were as described in Chapter 2.

4.2.1 Electrical stimulation and FCV

Figure 4.1 shows the locations of FCV recording sites for this chapter. For experiments involving QP and DH β E, [DA]_o was sampled in the dorsomedial part of the CPu, but not on the NAc (Figure 4.1). For experiments involving cocaine, evoked [DA]_o was sampled in NAc and a dorsomedial part of the CPu. Sites in the dorsal and ventral striatum were picked to report from areas primarily innervated by the SNc and VTA respectively.

Each site received a range of electrical stimulations, from a single pulse to a range of pulses at different frequencies. For experiments involving DH β E and QP, three-pulse (3p) or nine-pulse (9p) train stimulations were delivered at 100 Hz. Single pulse electrical stimulations evoke very little [DA]_o in the presence of 1 μ M DH β E and 5 μ M QP, which make the DA oxidation peaks difficult to distinguish from noise. Therefore 3p or 9p train stimulations at 100 Hz were used to increase the likelihood of obtaining a measurable signal under all conditions. For experiments involving cocaine, four-pulse train stimulations were delivered to report on the cumulative effects of DA release under DAT blockade. Stimulation frequencies of 5, 10, 25, and 100 Hz were chosen to obtain a full range of physiological activation patterns. Each stimulation protocol was performed three times in a randomised order under drug-free conditions, and in the presence of cocaine.

4.2.2 Experimental design and use of drugs

Some of the experiments described in this chapter compare the effect of pharmacological agents, such as quinpirole or cocaine, between two genotypes. Adding these drugs each have an effect on DA release and therefore introduce a second variable (in addition to the difference in genotype). To account for this added variable, the profiles of $[DA]_o$ evoked in the presence of a drug were normalised. This means that each $[DA]_o$ value of every profile recorded in the presence of a drug, was divided by the peak value of an average $[DA]_o$ profile recorded in same site of the same animal, and under a control condition (Figure 4.2a,b 4.3 and 4.6). The resulting normalised profiles in Figures 4.2, 4.3, and 4.6 therefore show the effect of adding a drug on $[DA]_o$ rather than the absolute concentration.

For the experiments reported in Figures 4.2, 4.3, and 4.6, the absolute $[DA]_o$ is not compared between genotypes, because each of these experiments collected data from a single site specifically selected for releasing consistent levels of evoked DA, rather than randomly recording from a naïve site. The absolute $[DA]_o$ recorded from such experiments can therefore not be meaningfully compared in the same way as the data reported in Chapter 3.3.1, which were obtained from unbiased sampling at multiple sites.

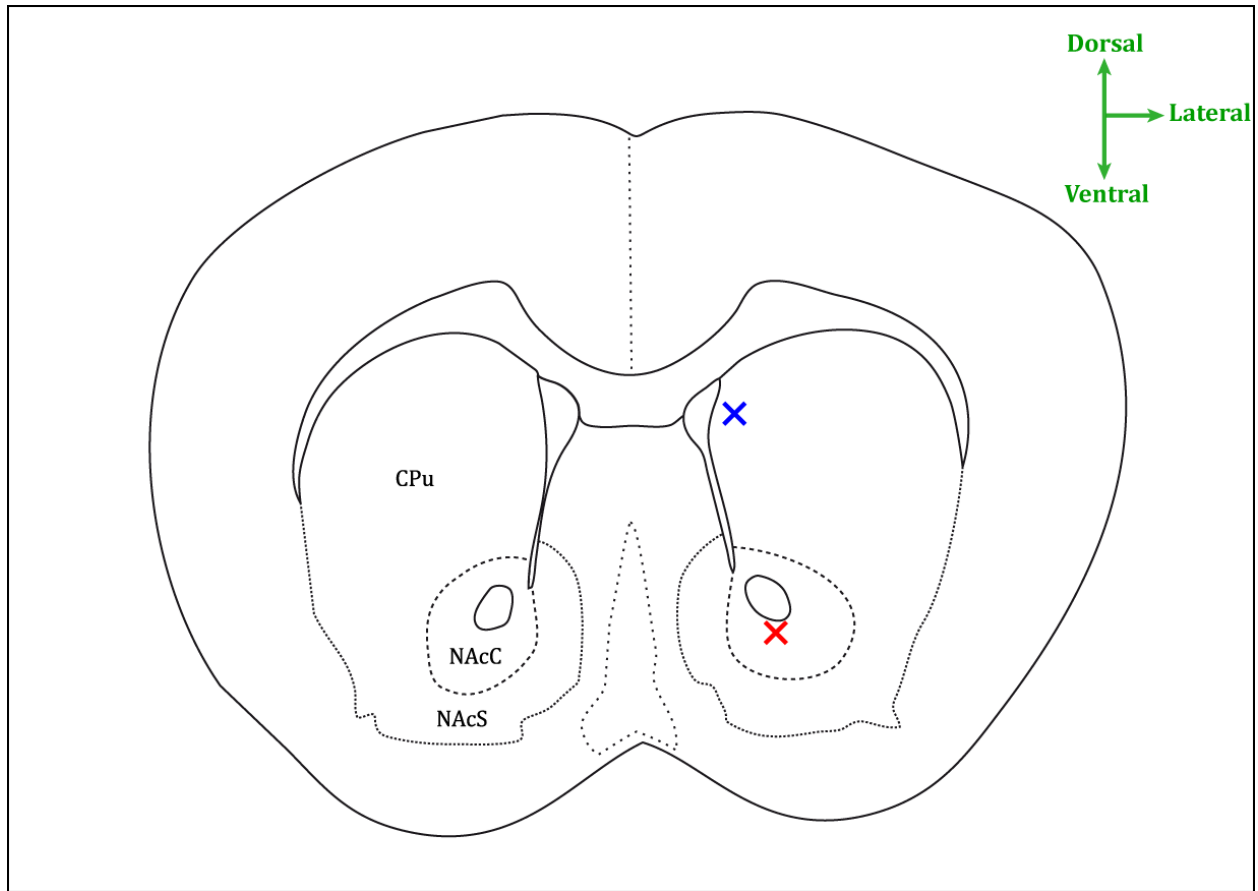


Figure 4.1 Coronal mouse brain section sampled by FCV. Evoked DA release was sampled in acute coronal sections of mouse striatum at Bregma 1.1 mm. The area marked *CPu* is the caudate putamen, the areas marked *NAcC* and *NAcS* are respectively the core and shell of the nucleus accumbens. Evoked $[DA]_o$ was sampled in up to 2 different sites in the CPu (blue) NAc (red). The different sites were approximately matched in all slices to control for regional variation. Green arrows show dorsal-ventral, and lateral-medial axis. Diagram adapted from (Franklin and Paxinos, 2007).

4.3. Results

4.3.1 Deficit of evoked [DA]_o in SNCA-C mice is independent of changes to activation of D₂ autoreceptors.

To explore possible changes to striatal D₂ autoreceptor function in SNCA-C mice, QP was bath-applied in increasing concentrations (from 10 nM to 5 μ M) onto acute striatal slices of SNCA-C and *Snca* ^{-/-} mice at age three months, and FCV was used to detect evoked [DA]_o in the CPu. Experiments were performed in 1 μ M DH β E, to inhibit the effect of nAChR activation on DA release, and thereby prevent indirect effects of D₂ activation at striatal cholinergic interneurons (Alcantara et al., 2003; Ikarashi et al., 1997).

DA was evoked from 3p train stimulations at 100 Hz in 1 μ M DH β E (control), and in 1 μ M DH β E + QP, ranging at concentrations of 10 nM, 30 nM, 100 nM, 300 nM, 1 μ M, and 5 μ M (Figure 4.2 a). Evoked [DA]_o was progressively reduced by increasing concentrations of QP, and up to 85-90% in the presence of 5 μ M QP. The reduction in evoked [DA]_o due to adding QP can be explained by the activation of presynaptic D₂ autoreceptors (Cragg and Greenfield, 1997). DA was also evoked from 9p train stimulations in 1 μ M DH β E, and in 1 μ M DH β E + QP, ranging from concentrations of 10 nM to 5 μ M (Figure 4.2 b). Evoked [DA]_o was also progressively reduced by increasing concentrations of QP, and up to 87-91% in the presence of 5 μ M QP. The reduction in evoked [DA]_o from both SNCA-C and *Snca* ^{-/-} mice were at roughly to the same extent, suggesting no significant difference in the effect of D₂ activation between the two genotypes.

The change to mean peak [DA]_o evoked from 3p approaches 100% at around 1 μ M for both SNCA-C and *Snca* ^{-/-} mice (Figure 4.2, c). The percentage effect at each concentration of QP

on mean evoked $[DA]_o$ is similar between genotypes, with overlapping error bars at low and high concentrations of QP. Sigmoidal curve fits for the % effect of QP on $[DA]_o$ evoked from 3p have a $LogIC_{50}$ of 1.513 and Hill slope of 0.923 for *Snca* $-/-$, and $LogIC_{50}$ of 1.342 and Hill slope of 1.155 for SNCA-C. The $LogIC_{50}$ values have non-overlapping 95% confidence intervals, but there is no significant difference between the Hill Slope values.

The change to mean peak $[DA]_o$ evoked from 9p also approaches 100% at around 1 μM for both SNCA-C and *Snca* $-/-$ mice (Figure 4.2, d). The percentage effect at each concentration of QP on mean evoked $[DA]_o$ is similar between genotypes, with overlapping error bars at low and high concentrations of QP. Sigmoidal curve fits for the % effect of QP on $[DA]_o$ evoked from 9p have a $LogIC_{50}$ of 1.614 and Hill slope of 0.868 for *Snca* $-/-$, and $LogIC_{50}$ of 1.413 and Hill slope of 1.048 for SNCA-C. The $LogIC_{50}$ values have non-overlapping 95% confidence intervals, but there is no significant difference between the Hill Slopes.

Since the QP was added in rising concentrations over a three-hour period, the decrease in evoked $[DA]_o$ from increasing concentrations of QP, at either 3p or 9p stimulations, can also be explained by a decline in the slice viability over time. It may therefore be possible for the IC_{50} or Hill slope values in *Figure 4.2 c* and *Figure 4.2 d* to be influenced by a reduction in slice viability. To obtain a measure of D_2 activation independent of changes to slice viability over time, the ratios of peak evoked $[DA]_o$ from 9p vs. 3p stimulations (9p:3p ratio) were calculated. The 9p:3p ratio increased with the concentration of bath applied of QP (Figure 4.2 e), however, the 9p:3p ratio for SNCA-C mice decreased at 5 μM QP, possibly because evoked $[DA]_o$ from 3p stimulation approached the CFM detection limit of ~ 30 nM (Cragg et al., 1997b). Therefore, the 9p:3p ratio value at 5 μM QP was discarded, and the percentage

effect of the rest of the values were plotted with a sigmoidal curve (Figure 4.2 f). The percentage effect 9p:3p ratios for SNCA-C and *Snca* ^{-/-} mice are similar with overlapping error bars. Sigmoidal curve fits for the % effect 9p:3p ratio have a LogIC₅₀ of 2.022 and Hill slope of 1.086 for *Snca* ^{-/-} mice, and a LogIC₅₀ of 2.35 and Hill slope of 1.23, for SNCA-C mice. The LogIC₅₀ values have overlapping 95% confidence intervals, and there is no significant difference between the Hill Slope values.

These data reveal no difference in the IC₅₀ or Hill slope of the 9p:3p ratio for a QP dose response, which suggests that there are no changes to presynaptic D₂ autoreceptor function in the CPu of SNCA-C mice. Since no significant difference was found in the CPu of SNCA-C mice, the experiment was not performed in the NAc.

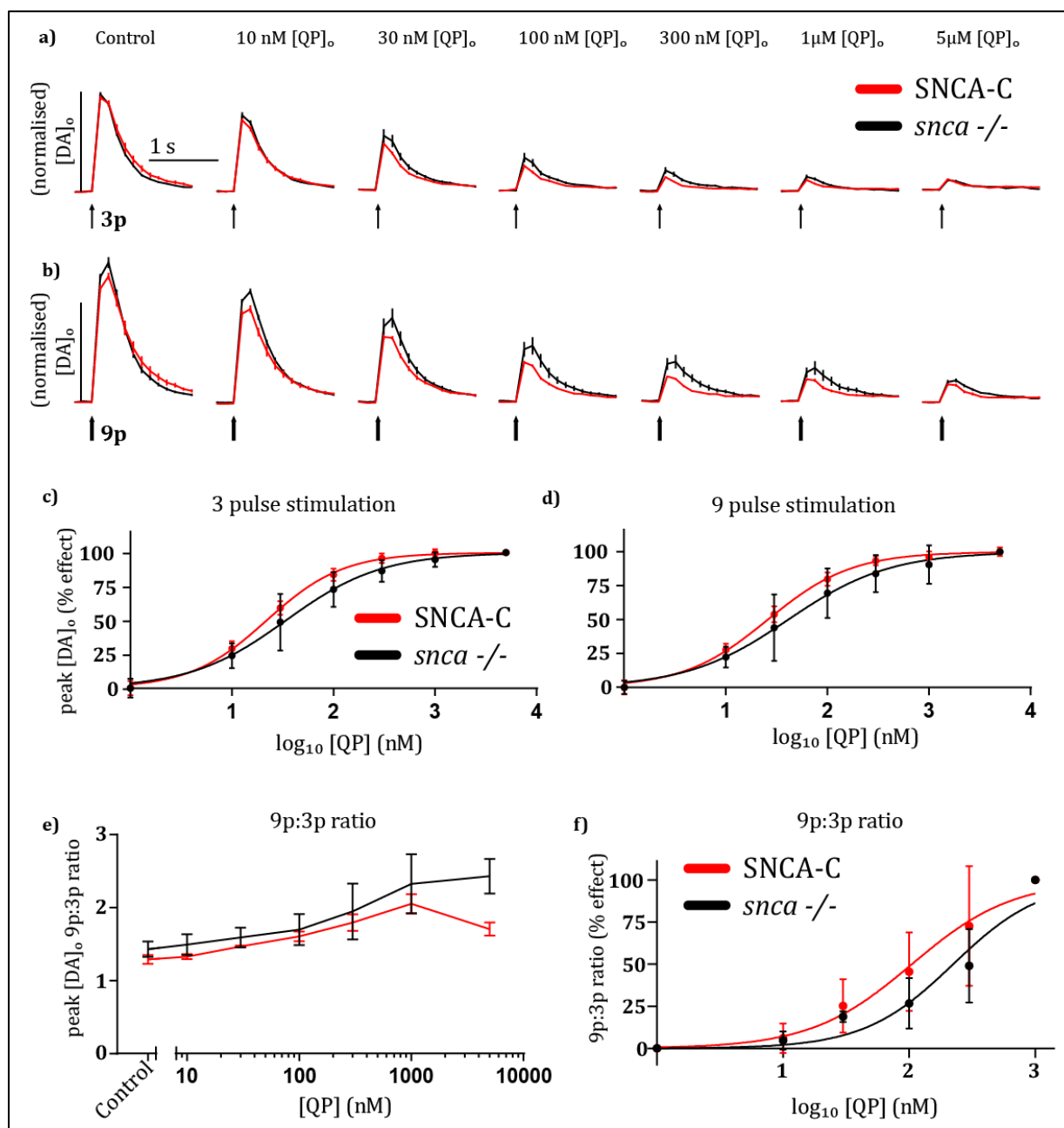


Figure 4.2. Dose response of QP on evoked [DA]₀ in SNCA-C and *Snca* -/-. a&b) Mean profiles of evoked [DA]₀ from 3p (a) and 9p (b) stimulations in 1 μM DHβE (control) and increasing concentrations of QP from SNCA-C and *Snca* -/- mice. To isolate the effect of quinpirole, the evoked [DA]₀ of all profiles are normalised to peak [DA]₀ evoked by 3p under 'Control' conditions. c&d) Percentage of full effect on mean peak [DA]₀ from 3p (c) and 9p (d) stimulations in 1 μM DHβE (control) and increasing concentrations of QP from SNCA-C and *Snca* -/- mice. Sigmoidal curve fit of 3p stimulations (c) has non-overlapping 95% confidence intervals of LogIC₅₀ (1.310 to 1.373 for SNCA-C and 1.445 to 1.618 for *Snca* -/-, n=9), and overlapping 95% confidence intervals for Hill slope (1.059 to 1.251 for SNCA-

C and 0.759 to 1.089 for *Snca* $-/-$, n=9). Sigmoidal curve fit of 9p stimulations (d) has non-overlapping 95% confidence intervals of LogIC_{50} (1.378 to 1.449 for SNCA-C and 1.498 to 1.730 for *Snca* $-/-$, n=9), and overlapping 95% confidence intervals for Hill slope (0.959 to 1.136 for SNCA-C and 0.6732 to 1.064 for *Snca* $-/-$, n=9). e) 9p:3p ratio of evoked $[\text{DA}]_o$ in 1 μM DH β E and increasing concentrations of QP. f) Percentage of full effect on 9p:3p ratio in SNCA-C and *Snca* $-/-$ mice. Non-linear fit curves of 9p:3p ratios for SNCA-C and *Snca* $-/-$ have overlapping 95% confidence intervals of LogIC_{50} (2.184 to 2.515 for SNCA-C and 1.794 to 2.25 for *Snca* $-/-$, n=9), and also for Hill slope (0.6857 to 1.773 for SNCA-C and 0.5144 to 1.658 for *Snca* $-/-$, n=9).

4.3.2 Deficit of evoked [DA]_o in SNCA-C mice is accompanied by an increased effect of cocaine on DA releasability.

To test whether DA release in SNCA-C mice is more sensitive to a vesicle mobilising agent, evoked [DA]_o was compared, in the presence of 5 μM cocaine, between 3-month old SNCA-C mice and age-matched *Snca* ^{-/-} controls. [DA]_o was evoked by single pulse stimulations and four-pulse trains at frequencies of 5, 10, 25, and 100 Hz to allow for summation of the [DA]_o signal in a range of physiologically relevant stimulation frequencies. DA was sampled using FCV in the dorsomedial region of the CPu and the NAc to also explore regional variation in the response to cocaine.

Evoked [DA] was detected in the CPu from all stimulation frequencies (Figure 4.3 a).

Application of 5 μM cocaine increased the peak amplitude of [DA]_o signals from all stimulation frequencies in both genotypes. Application of cocaine also reduced the decay rate of [DA]_o signals in both genotypes. There was a general trend of the peak [DA]_o signals in SNCA-C mice increasing more than *Snca* ^{-/-} controls from the application of 5 μM cocaine (Figure 4.3 a). The mean and median percentage increase in peak [DA]_o, due to cocaine application, was about two-fold to three-fold greater in SNCA-C mice than *Snca* ^{-/-} controls at 4-pulse stimulations of 5, 10, and 25 Hz (Figure 4.4 a,c).

Evoked [DA]_o was detected in the NAc at all stimulation frequencies (Figure 4.3 b).

Application of 5 μM cocaine increased the peak amplitude of [DA]_o signals from all stimulation frequencies in both genotypes. Application of cocaine also reduced the decay rate of [DA]_o signals in both genotypes. In the NAc, evoked [DA]_o signals of both SNCA-C and *Snca* ^{-/-} mice increased to the same extent with mean [DA]_o profiles overlapping. The

percentage increase in peak $[DA]_o$ due to cocaine was similar for both SNCA-C mice and *Snca* $-/-$ controls at all stimulation frequencies (Figure 4.4 b,d).

The observation that cocaine caused a greater increase in evoked $[DA]_o$ from the CPu of SNCA-C mice than *Snca* $-/-$ controls might be the product of three distinct mechanisms. Firstly, it has been suggested that cocaine increases DA releasability through changes to Ca^{2+} dependence of DA release (Han et al., 2002; Kile et al., 2010), but it is an unlikely explanation here, because Ca^{2+} dependence of evoked $[DA]_o$ in SNCA-C mice was previously found to be the same as *Snca* $-/-$ controls (Janezic et al., 2013). Secondly, it may be that cocaine has a greater inhibitory effect on the rate of DA uptake in SNCA-C mice. α -Synuclein has been shown to bind DAT (Lee et al., 2001), and overexpressing α -synuclein may therefore affect the way that cocaine inhibits DA uptake. Thirdly, it may be that presynaptic DA terminals in SNCA-C mice are more sensitive to the vesicle mobilisation properties of cocaine, due to deficits in vesicle mobility (Kile et al., 2010; Venton et al., 2006).

To determine whether cocaine has different effects on the rate of DA uptake between SNCA-C and *Snca* $-/-$ mice, the reduction slope of mean $[DA]_o$ profiles, which depends on the rate of DA uptake (Anwar et al., 2011; Janezic et al., 2013), can be compared between genotypes. To explore whether the enhanced effect of cocaine on evoked $[DA]_o$ was due to a reduction in DA uptake, the reduction slopes of mean $[DA]_o$ were compared (Figure 4.5 a-c). The uptake of extracellular DA by DAT is dependent on DA concentration, and the rate of uptake for the two genotypes therefore has to be compared in the same range of concentrations. The reduction slopes of mean $[DA]_o$ profiles from 5, 10, and 25Hz stimulations in the CPu are very similar for SNCA-C and *Snca* $-/-$, with the linear phase of

the two slopes running parallel. These data show that the enhanced effect of cocaine on evoked $[DA]_o$ in the CPu of SNCA-C mice, is not due to a reduction in DA uptake.

The data presented here show that DA releasability in the CPu of SNCA-C mice is more sensitive to the vesicle mobilisation properties of cocaine. Taken together with previous observations, these data reveal that the reduction in DA releasability in SNCA-C mice is accompanied by increased sensitivity to a vesicle mobilising agent, therefore suggesting that the reduction of DA releasability may be due to deficits in vesicle mobilisation.

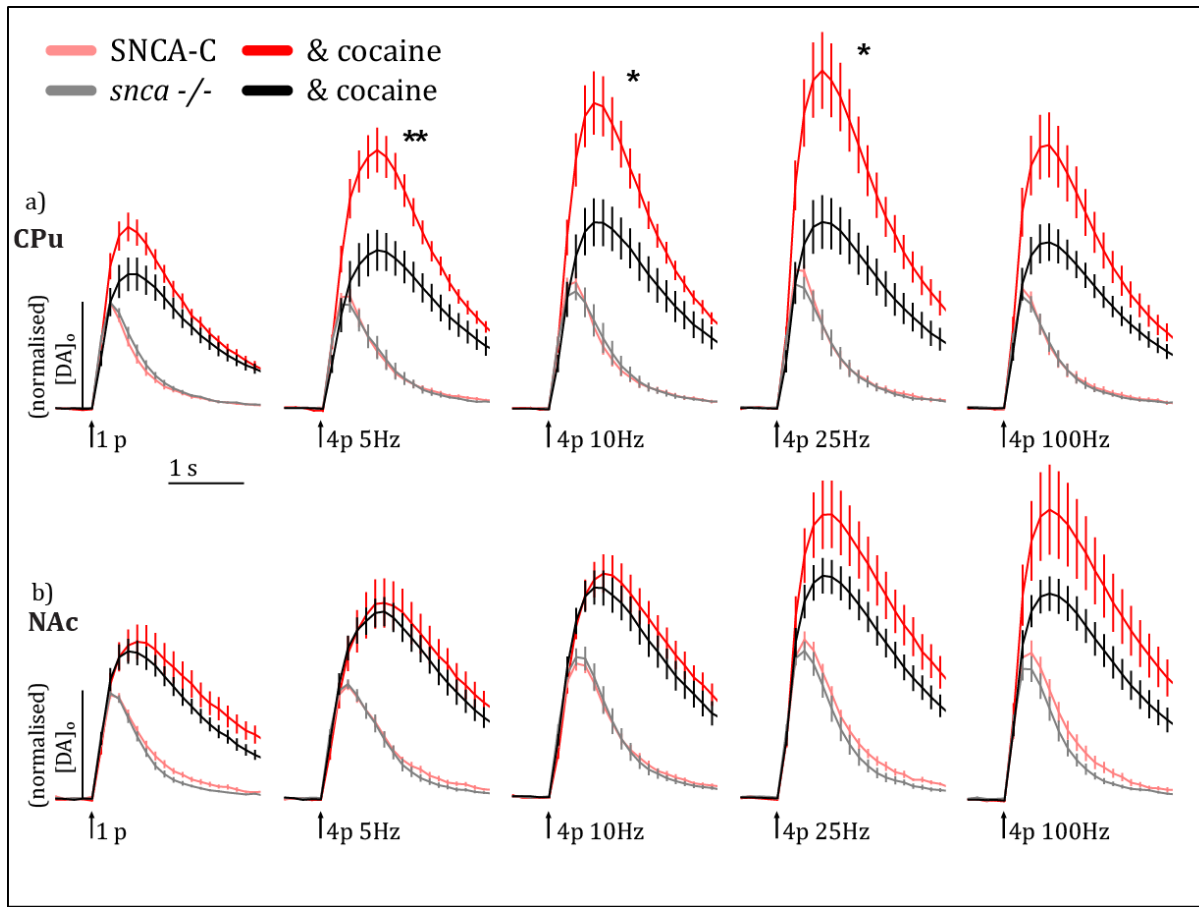


Figure 4.3 Evoked $[DA]_0$ from SNCA-C and *Snca* $-/-$ mice in aCSF and 5 μ M cocaine. a&b) Profiles of mean $[DA]_0$ ($\pm$ SEM) vs. time in CPu (a) and NAc (b) of SNCA-C mice and *Snca* $-/-$ controls. The evoked $[DA]_0$ of all profiles were normalised to peak $[DA]_0$ evoked by 1p in the absence of cocaine in that respective site. $[DA]_0$ was evoked by either a single pulse (1p) or a 4-pulse train (4p) stimulation at different frequencies (5, 10, 25, 100Hz). Semi-transparent profiles are in control aCSF conditions, and full-colour profiles are in the presence of 5 μ M cocaine.

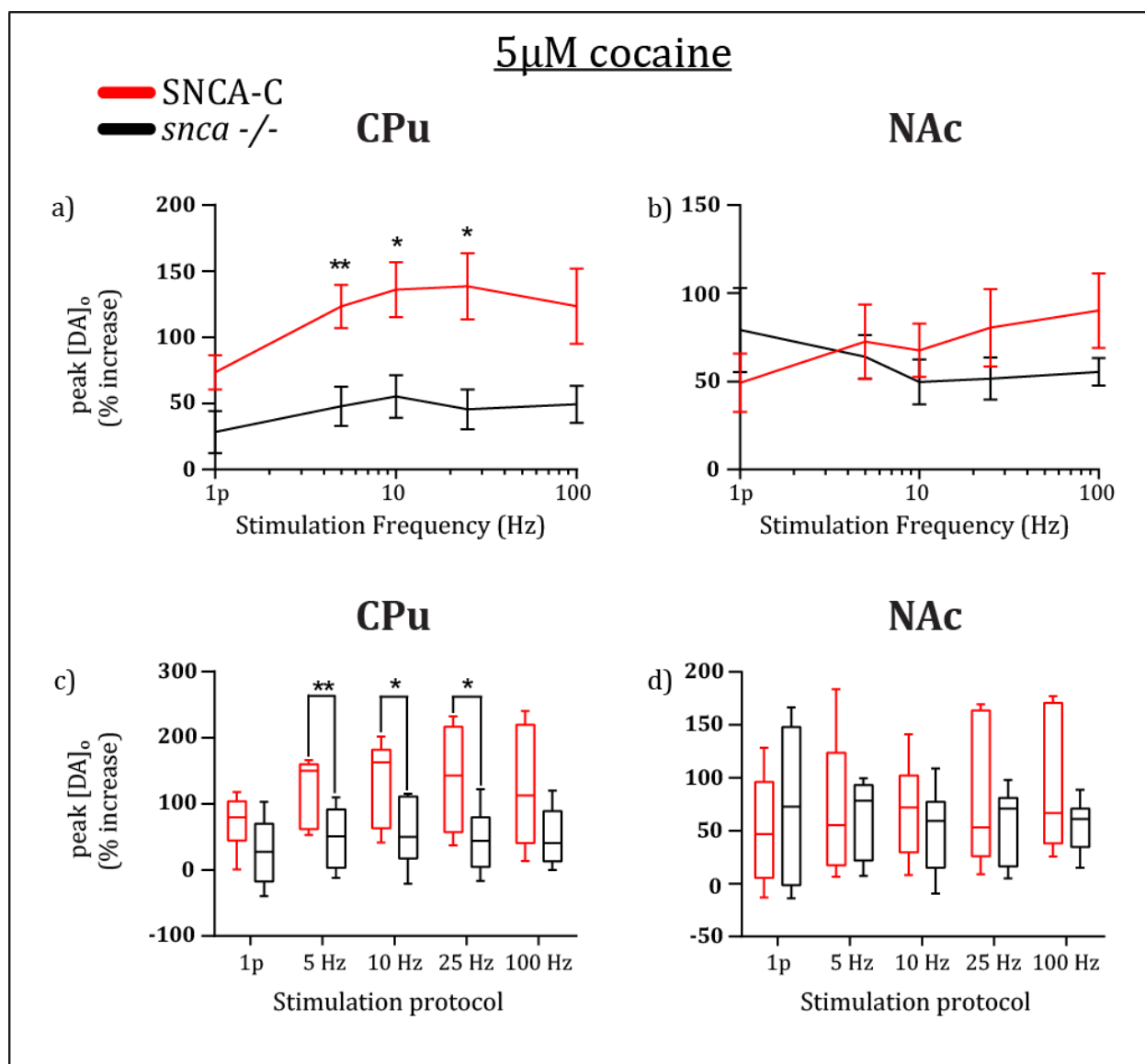


Figure 4.4: Percentage difference in peak [DA]_o due to 5 μ M cocaine. a&b) Frequency response graphs showing mean percentage increase of peak [DA]_o and SEM of SNCA-C and *Snca* -/- mice. c&d) Box and whiskers plots, showing median, interquartile range, and range of percentage increase of peak [DA]_o after application of 5 μ M cocaine. The percentage increase is significantly higher in the CPu (c) of SNCA-C than *Snca* -/- mice after 4-pulse train stimulations of 5-25 Hz (* $P < 0.05$, ** $p < 0.01$, Two-tailed Mann-Whitney, $n = 9$). No significant difference in percentage increase was found at another frequency of the CPu, or in the NAc (d) ($P > 0.05$, Two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 9$).

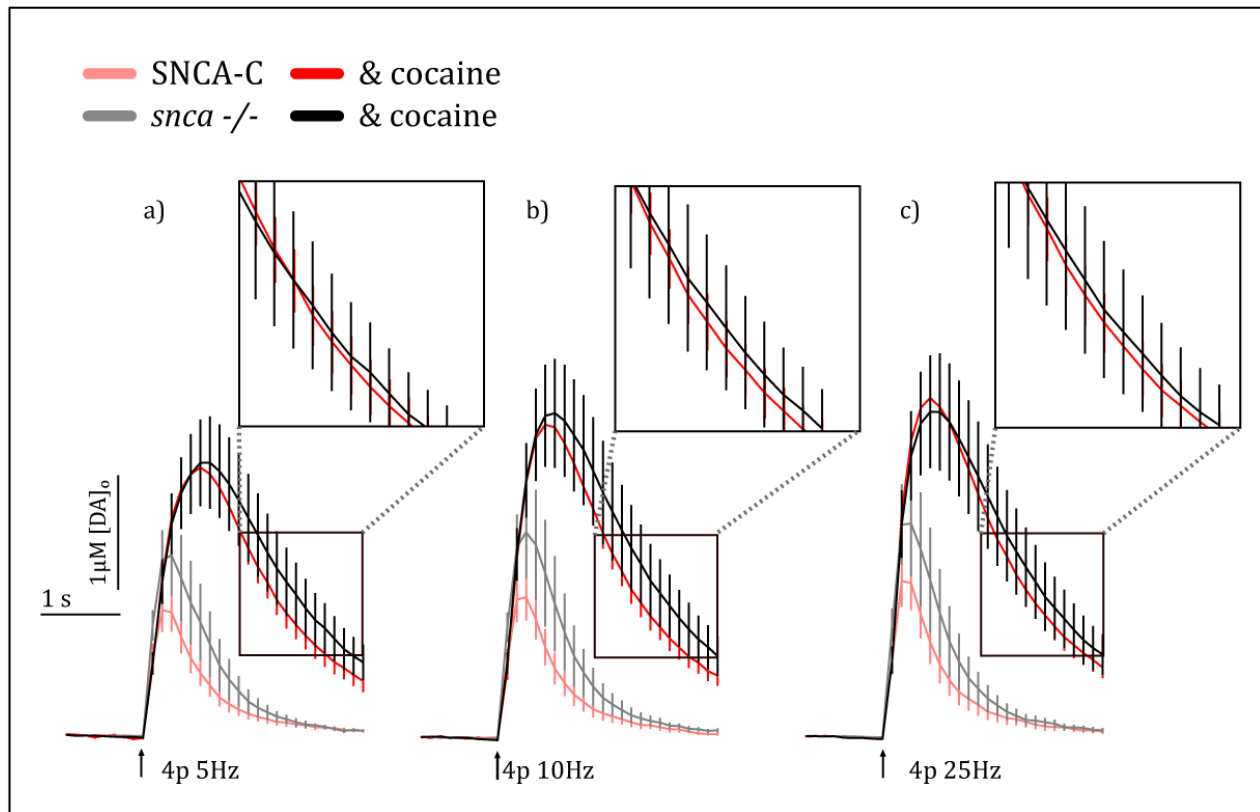


Figure 4.5 Evoked $[DA]_0$ from CPu of SNCA-C and *Snca* $-/-$ mice in aCSF and 5 μ M cocaine. Profiles of mean $[DA]_0$ ($\pm$ SEM) vs. time in CPu of SNCA-C mice and *Snca* $-/-$ controls. $[DA]_0$ was evoked by 4p train stimulations at 5 Hz (a), 10 Hz (b), and 25 Hz (c). Semi-transparent profiles are in control aCSF conditions, and full-colour profiles are in the presence of 5 μ M cocaine. The inset panels have been shifted in time along the x-axis, so that $[DA]_0$ is matching at each time point.

4.3.3 The increased effect of cocaine on DA releasability in SNCA-C mice vs. Snca -/- controls is not observed in SNCA-B mice.

Evoked DA releasability is markedly reduced in SNCA-C mice, which express human α -synuclein at levels 5-6 fold of human brain tissue (Chapter 3.3.1; (Janezic et al., 2013)). This deficit is not seen in SNCA-B mice, which express human α -synuclein at levels 2-3 fold of human brain tissue, therefore, the effect of α -synuclein on DA releasability depends on the dose of α -synuclein expression (Chapter 3.3.3). Thus far, the mechanism by which α -synuclein reduces DA releasability has been largely unknown. Findings from *section 4.2.3* revealed that evoked $[DA]_o$ in SNCA-C mice has an increased sensitivity to cocaine, which suggests that the reduction of DA releasability may be due to deficits in vesicle mobilisation. However, it remains unknown whether the effect of cocaine on SNCA-C mice is unique to a line with relatively high levels of α -synuclein expression, and to conditions where DA releasability is compromised. If α -synuclein overexpression in SNCA-C mice attenuates DA releasability by reducing the mobility of presynaptic vesicle pools, then an increased sensitivity to the vesicle mobilising properties of cocaine should be expected only in SNCA-C mice, and not in SNCA-B mice.

To further explore the sensitivity to cocaine seen in SNCA-C mice, evoked $[DA]_o$ was sampled in the presence of 5 μ M cocaine in both the NAc and CPu of 3-month old SNCA-B mice. $[DA]_o$ was evoked from single pulse stimulations and four-pulse trains at different frequencies (5-25Hz) in the dorsomedial region of the CPu and the NAc, and was detected by FCV.

Evoked [DA]_o was detected in the CPu from all stimulation frequencies (Figure 4.6 a).

Application of 5 μ M cocaine increased the peak amplitude of [DA]_o signals from all stimulation frequencies in both genotypes. Application of cocaine also reduced the decay rate of [DA]_o signals in both genotypes. Evoked [DA]_o signals of both SNCA-B and *Snca* ^{-/-} mice increased to the same extent with mean [DA]_o profiles overlapping. The median percentage increase in peak [DA]_o due to cocaine was similar for both SNCA-C mice and *Snca* ^{-/-} controls at all stimulation frequencies (Figure 4.7 a,c).

Evoked [DA]_o was detected in the NAc at all stimulation frequencies (Figure 4.6 b).

Application of 5 μ M cocaine increased the peak amplitude of [DA]_o signals from all stimulation frequencies in both genotypes. Application of cocaine also reduced the decay rate of [DA]_o signals in both genotypes. In the NAc, evoked [DA]_o signals of both SNCA-B and *Snca* ^{-/-} mice increased to the same extent. The percentage increase in peak [DA]_o due to cocaine was similar for both SNCA-B mice and *Snca* ^{-/-} controls at all stimulation frequencies (Figure 4.7 b,d).

The percentage effect of cocaine on evoked [DA]_o in SNCA-B mice is not different from *Snca* ^{-/-} controls, which means that DA releasability of SNCA-B mice is no more sensitive to the vesicle mobilising properties of cocaine. The increased sensitivity to the vesicle mobilising properties of cocaine is therefore unique to SNCA-C mice, where DA releasability is compromised. Taken together, the data presented here shown that reduction of DA releasability, and deficits in vesicle mobilisation, both depend on relatively high levels of α -synuclein expression.

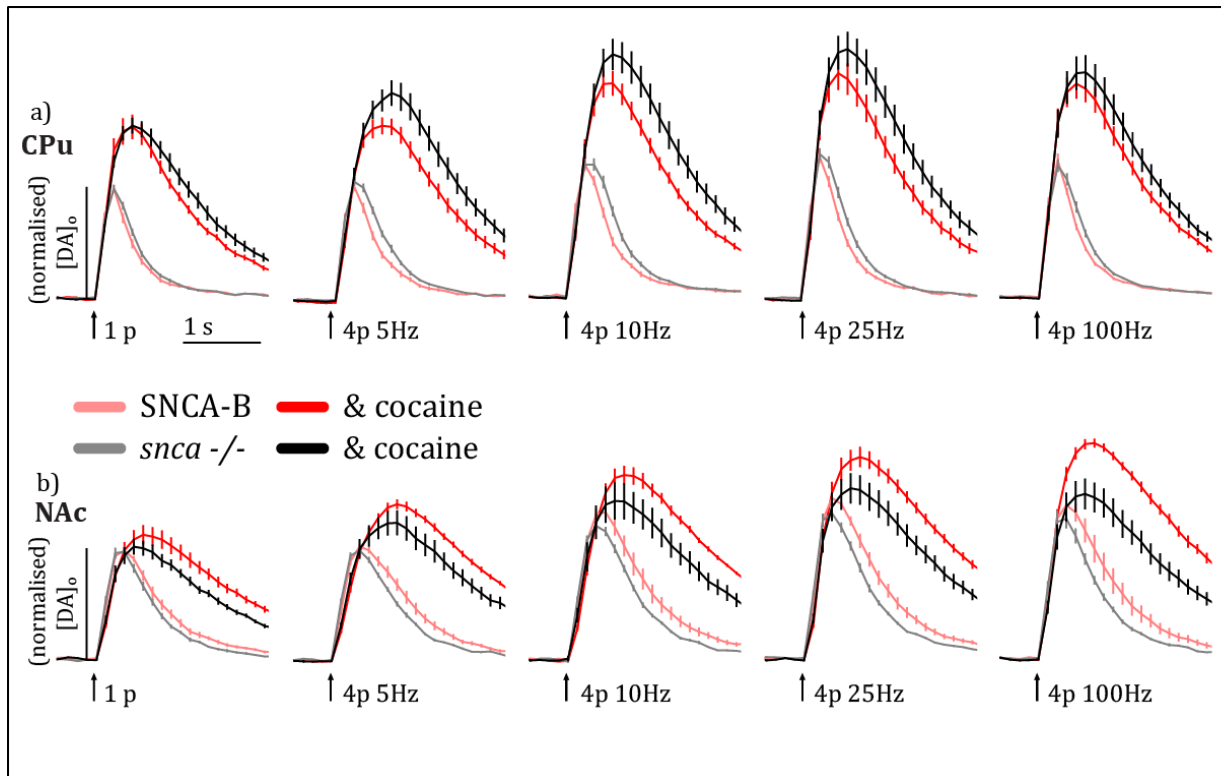


Figure 4.6: Evoked $[DA]_0$ from SNCA-C and *Snca* $-/-$ mice in aCSF and 5 μ M cocaine.

a&b) Profiles of mean $[DA]_0$ ($\pm$ SEM) vs. time in CPu (a) and NAc (b) of SNCA-B mice and *Snca* $-/-$ controls. The evoked $[DA]_0$ of all profiles were normalised to peak $[DA]_0$ evoked by 1p in the absence of cocaine in that respective site. $[DA]_0$ was evoked by either a single pulse (1p) or a 4-pulse train (4p) stimulation at different frequencies (5 Hz – 100Hz). Semi-transparent profiles are in control aCSF conditions, and full-colour profiles are in the presence of 5 μ M cocaine. c&d) Percentage increase of mean peak $[DA]_0$ after application of 5 μ M cocaine. No significant difference in percentage increase was found at any frequency of in either CPu or NAc (d) ($P > 0.05$, Two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n=9$).

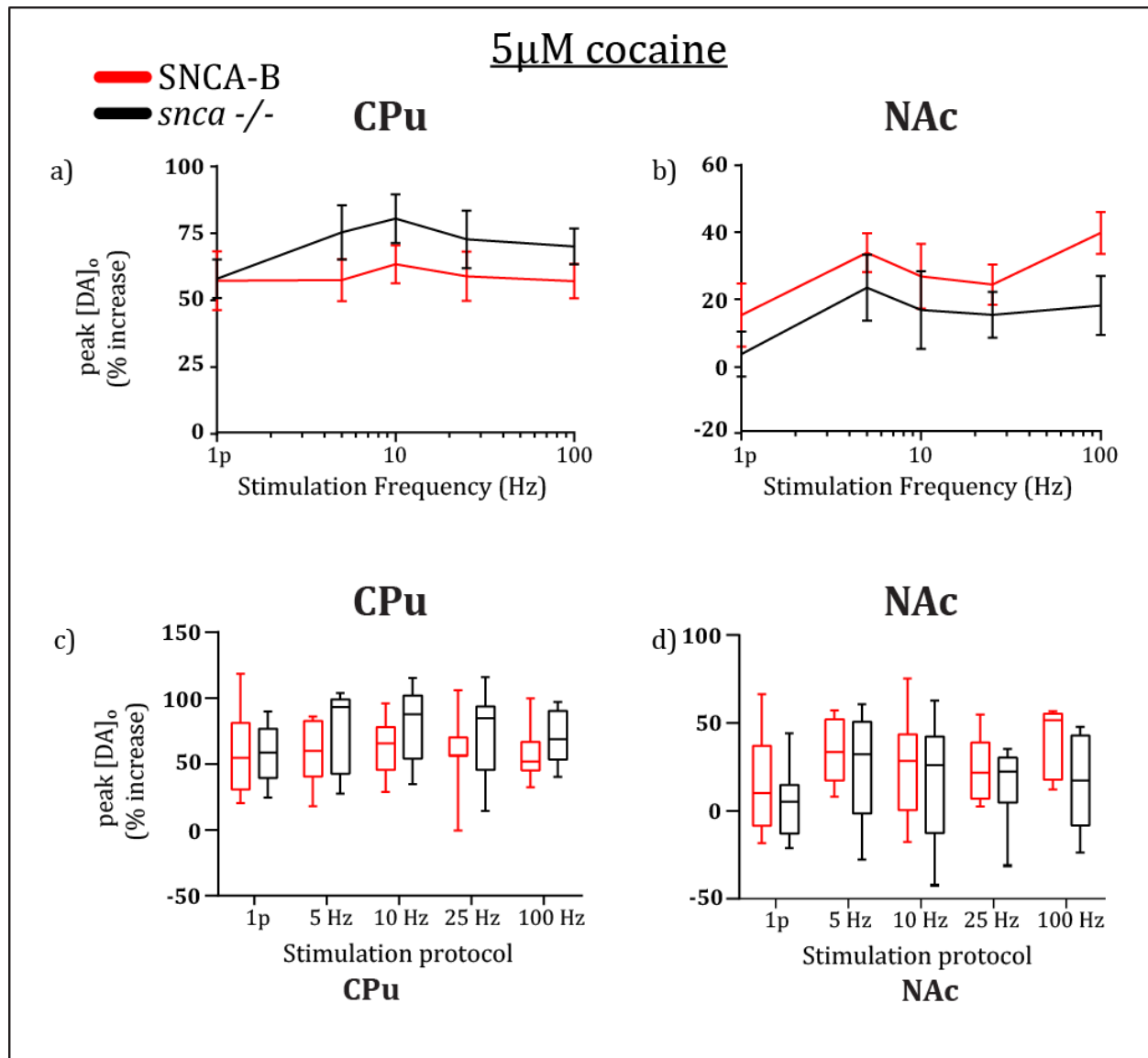


Figure 4.7: Percentage difference in peak [DA]_₀ due to 5 μ M cocaine. a&b) Frequency response graphs showing mean percentage increase of peak [DA]_₀ and SEM of SNCA-B and *Snca* $-/-$ mice. c&d) Box and whiskers plots, showing median, interquartile range, and range of percentage increase of peak [DA]_₀ after application of 5 μ M cocaine. The percentage increase is not different in the CPu (c) of SNCA-B than *Snca* $-/-$ mice after 4-pulse train stimulations of 5-25 Hz ($p > 0.05$, Two-tailed Mann-Whitney, $n = 9$). No significant difference in percentage increase was found at another frequency of the CPu, or in the NAc (d) ($P > 0.05$, Two-tailed Mann-Whitney and Kolmogorov-Smirnov, $n = 9$).

4.4. Discussion

This chapter set out to explore two candidate mechanisms by which the high level of α -synuclein expression in SNCA-C mice causes a deficit of evoked striatal $[DA]_o$. Several mechanisms have previously been explored elsewhere, but have been unable account for the changes in evoked $[DA]_o$ seen in SNCA-C mice. The first mechanism explored in this chapter was presynaptic D_2 autoreceptor function, which acts to suppress DA releasability. It was shown here that evoked $[DA]_o$ in both SNCA-C mice and *Snca* $-/-$ controls have the same responsiveness to D_2 autoreceptor activation by QP (Figure 4.2). The 9p:3p ratio at rising concentrations of QP was used to create a dose response curve. The 9p:3p ratios from SNCA-C and *Snca* $-/-$ mice had overlapping error bars at each QP concentration, and the dose response curves was no different between the two genotypes. The data presented in this chapter revealed no changes to the D_2 autoreceptor regulation of DA in SNCA-C mice, which suggests that the deficit of DA releasability in these mice is not mediated by a difference in presynaptic D_2 autoreceptor function.

A previous investigation in mice that overexpress human α -synuclein under control of the Thy-1 or prion promoters found altered striatal D_2 function compared to controls (Kurz et al., 2010; Lam et al., 2011). However, these studies only reported on D_2 receptors from MSNs, and may therefore not apply to presynaptic D_2 autoreceptors located on DA synapses. It might be that the changes to D_2 receptors on striatal MSNs are a downstream effect of changes to DA signalling, in which case, D_2 autoreceptors could remain unaffected.

The second potential mechanism to be explored in this chapter is a reduction in the mobility of presynaptic vesicles. Cocaine was used to explore whether DA release in SNCA-

C mice is more sensitive to a vesicle mobilising agent. Data in this chapter reveal that cocaine has a greater effect on evoked [DA]_o in SNCA-C, but not SNCA-B mice, compared to *Snca* -/- controls (Figures 4.3, 4.5). The greater effect of cocaine on SNCA-C mice was not due to DAT inhibition (Figure 4.5), suggesting that DA release in SNCA-C mice is more sensitive to the vesicle mobilising properties of cocaine than *Snca* -/- controls. Since SNCA-C mice express higher levels of α -synuclein than SNCA-B mice, these data suggest that the increased sensitivity to the effects of cocaine depend on a relatively high level of α -synuclein. Furthermore, the increased sensitivity of evoked [DA]_o to cocaine is confined to the CPu, which also showed a deficit in evoked [DA]_o, suggesting that this effect is unique to the subpopulation of DA neurons most affected by PD.

The data presented here reveals that overexpression of α -synuclein causes a deficit in releasability of evoked [DA]_o when no cocaine is present, but a greater enhancement of releasability due to the vesicle mobilising properties of cocaine. These data therefore suggest that reduction of DA releasability in SNCA-C mice may be due to deficits in vesicle mobilisation.

Previous reports from an electron microscopy investigation revealed increased vesicle clustering, in synaptic axonal profiles of SNCA-C mice (Janezic et al., 2013), which suggests a reduction in vesicle mobility. The findings presented here provide functional support for the previous findings described by *Janezic et al.*, to further suggest that the level of α -synuclein expression in SNCA-C mice reduces the mobility of presynaptic DA vesicles.

The idea that overexpression of α -synuclein reduces presynaptic vesicle mobility is also in agreement with other observations. Expressing high levels of α -synuclein has been associated with reduced intersynaptic trafficking of vesicles (Scott and Roy, 2012), and α -synuclein overexpression in hippocampal neurons reduces both clustering of presynaptic vesicles at the active zone and the size of the recycling pool (Nemani et al., 2010).

Furthermore, an investigation in hippocampal slices of mice that lack α -synuclein revealed normal transmission of docked vesicle pools, but impaired transmission of reserve pools (Cabin et al., 2002). Taken together these data support the idea that excess α -synuclein impairs the mobilisation of presynaptic vesicle pools.

The exact mechanism by which cocaine affects synaptic vesicle mobility remains incompletely understood. It has been shown previously that the effect of cocaine on basal levels of striatal $[DA]_o$ is diminished in DAT KO mice *in vivo* (Rocha et al., 1998), suggesting that the effects of cocaine on $[DA]_o$ depend on the presence of DAT. However, tyrosine hydroxylase availability and striatal DA release is substantially reduced in DAT KO mice (Giros et al., 1996), which suggests that DAT KO mice have wider effects that may not directly relate to physiological DAT function. Furthermore, DAT KO mice still develop a conditioned place preference for cocaine (Sora et al., 1998), and have been shown to self-administer cocaine (Rocha et al., 1998), suggesting that not all the actions of cocaine depend on DAT. Unfortunately, investigations of cocaine in DAT KO mice report on basal $[DA]_o$, and not electrically evoked $[DA]_o$ as sampled in this thesis (Giros et al., 1996; Rocha et al., 1998). It therefore remains unknown whether the effect of cocaine on DA release is directly mediated through DAT.

Interestingly, the effects of cocaine on electrically evoked $[DA]_o$ relies on the presence of synapsins (Venton et al., 2006). Synapsins bind presynaptic vesicles to influence their availability for exocytosis (Rosahl et al., 1995; Gitler et al., 2004). Knocking out synapsins has been shown to increase evoked $[DA]_o$ release in the dorsal striatum (Kile et al., 2010), and also greatly reduce the vesicle mobilising effects of cocaine (Venton et al., 2006). Therefore it is currently not known to what extent the effect of cocaine on DA releasability relies on a direct interaction between cocaine and DAT, or whether the effect of cocaine is perhaps mediated through a DAT- independent mechanism (Beuming et al., 2008; Kile et al., 2010; Rocha et al., 1998). However, the effect of cocaine on DA releasability is likely mediated by a synapsin-dependent mechanism (Kile et al., 2010; Venton et al., 2006).

The data presented here suggest that the DA release deficit in SNCA-C mice is accompanied by a reduction in presynaptic vesicle mobility, which can at least in part be rescued by synapsin-dependent vesicle mobilisation. However, whether the DA release deficit in SNCA-C mice is exclusively the result of a reduction in presynaptic vesicle mobility, is in need of further investigation. If the DA release deficit of SNCA-C mice is due to reduced vesicle mobility, then cocaine can be expected to rescue the deficit without affecting the rate of DA uptake. Future investigations could adapt the protocol used in Chapter 3 to test this hypothesis. Such an experiment might involve two stages: the first to sample evoked striatal $[DA]_o$ by FCV in both genotypes in the absence of cocaine, which will show a deficit in evoked $[DA]_o$ in SNCA-C mice as seen in section 3.3.1; and the second to sample the same slices in the same order in the presence of 5 μ M cocaine, which should reverse the DA release deficit if it is indeed due to decreased vesicle mobility.

The data presented in this chapter provides further insight in the presynaptic function of α -synuclein, and also contribute towards a greater understanding of the presynaptic dysfunction that may be present during the earliest stages of PD.

CHAPTER 5. The deficit of monoamine release in SNCA-C mice does not extend to 5-HT.

5.1. Introduction

Chapter 3 reported a deficit in evoked striatal $[DA]_o$ from SNCA-C mice, which express human α -synuclein at 5-6 fold above human brain tissue. The deficit in evoked $[DA]_o$ was independent of age, but depended on the level of α -synuclein expression. Furthermore, the deficit in evoked $[DA]_o$ was found in the dorsal striatum, which primarily receives DA innervation from the SNc, but was not found in the NAc, which primarily receives DA innervation from the VTA. This regional variation suggests that the effect of α -synuclein overexpression on evoked $[DA]_o$ is specific to a sub-population of DA neurons that are vulnerable to PD. However, α -synuclein has also been found in 5-HT neurons, and the effect of α -synuclein overexpression on 5-HT release has thus far been unexplored. This chapter will investigate the effects of α -synuclein overexpression on 5-HT release in SNCA-C mice.

Post-mortem studies have found Lewy bodies and cell loss in the *dorsal raphe nuclei* (DR) (Braak et al., 2003; Halliday et al., 1990a), suggesting that α -synuclein can accumulate in 5-HT neurons. During PD, a reduction in 5-HT levels, 5-HT related enzymes, and 5-HT markers throughout the raphe, basal ganglia and cortex have been found (Halliday et al., 1990a; Kish et al., 2008; Scatton et al., 1983), suggesting that 5-HT systems are affected during PD.

Not only are 5-HT systems affected during PD, but they have also been found to play a significant role in the symptoms of PD. 5-HT is well known for playing a role in depression (Albert and Benkelfat, 2013), which also occurs in about 40% of PD patients (Cummings,

1992). Reduced 5-HT has been found in the CSF of depressed PD patients (Mayeux et al., 1986; McCance-Katz et al., 1992), and depression in PD patients is associated with more pronounced striatal DAT loss (Hesse et al., 2008). In addition to playing a role in depression, 5-HT has also been shown to play a role in the motor symptoms of PD patients. For example, 5-HT_{1A} receptor availability in the raphe correlates with the severity of tremor in PD patients (Doder et al., 2003). 5-HT_{2A} antagonists can improve motor impairments in both PD patients (Henderson et al., 1992) and MPTP mice (Ferguson et al., 2010), suggesting that some 5-HT signalling contributes to motor symptoms in PD. Furthermore, DA production has been shown to take place in striatal 5-HT terminals (Tanaka et al., 1999) and SERT has been shown to transport DA (Larsen et al., 2011), which suggests that 5-HT neurons might release DA as a false neurotransmitter to compensate for a loss of DA signalling (Scholtissen et al., 2006; Tanaka et al., 1999). However, 5-HT signalling has also been shown to exacerbate L-DOPA induced dyskinesias (Brotchie, 2000; Kish et al., 2008).

Previous investigations into the effects of α -synuclein overexpression on 5-HT signalling have yielded mixed results. Overexpressing α -synuclein in co-transfected *Ltk* cells suppressed SERT mediated uptake of [³H]5-HT by decreasing SERT levels at the plasma membrane, suggesting that α -synuclein may have a significant influence on 5-HT neurotransmission (Wersinger et al., 2006a). An *in vitro* study found that 5-HT binds to, and stabilises intermediate aggregates of α -synuclein through direct interactions with the C-terminal domain (Falsone et al., 2011). Such data suggests that 5-HT may play a role in promoting formation and accumulation of α -synuclein aggregates. The effect of α -synuclein

on 5-HT function was however not as overt in mouse models. An investigation that knocked out both α - and β synuclein in mice revealed a decrease in striatal DA but not 5-HT content, suggesting that 5-HT content is less dependent on synuclein function than DA content (Chandra et al., 2004). In addition to this, overexpressing the A53T mutant form of α -synuclein in mice caused changes to DAT and D1 receptors, but not SERT, expression in striatum. Such data suggests that 5-HT systems are less sensitive than DA systems, to pathogenic mutations of α -synuclein (Unger et al., 2006). Taken together, these data reveal links between α -synuclein and 5-HT function, but relatively little has been explored in mouse models.

Exploring the possible effects of α -synuclein on 5-HT release can provide a greater understanding of how DA and 5-HT systems are differently affected during PD, and may also contribute to finding a mechanism by which α -synuclein affects neurotransmission. To investigate whether SNCA-C mice have any changes to 5-HT signalling, electrically evoked 5-HT release was sampled using FCV at a CFM. Human α -synuclein has been observed through immunohistochemistry in the DR of SNCA-C mice (Janezic et al., 2013), which suggests that α -synuclein is acting on 5-HT neurons in this model. The DR and MnR project 5-HT axons to the SN, which receives one of the highest 5-HT innervation densities in the brain (Corvaja et al., 1993; Lavoie and Parent, 1994). Electrically evoked 5-HT was therefore sampled in the SNr as done previously, where the input from somatodendritic DA release is minimal (Cragg et al., 1997a; Iravani and Kruk, 1997; Rice et al., 1994). If α -synuclein has the same effect on 5-HT neurons as DA neurons, then changes to 5-HT signalling could be expected in SNCA-C mice.

To explore the possibility of changes to 5-HT release developing with age, 5-HT release was sampled at 3- and 18-months. 5-HT release was evoked by electrical stimulation trains of 20 pulses (20p) and 40 pulses (40p) at 50Hz to explore changes in the response to different stimulation patterns in SNCA-C mice.

5.2. Methods

Unless otherwise specified, experimental procedures were as described in Chapter 2. Since experiments for this chapter involved the detection of both DA and 5-HT, the protocol for monoamine identification will form a substantial part of the chapter.

5.2.1 Electrical stimulation and FCV

Figure 5.1 shows the location of FCV recording sites for this chapter. Electrical stimulations were delivered in three regions across the SNr, and the extracellular concentration of 5-HT ([5-HT]_o) was detected at three sites in each region (see Figure 5.1). Electrically evoked 5-HT release in the SNr depends on stimulation frequency and pulse number, with the highest amounts released at frequencies of 30Hz– 50Hz (Jennings et al., 2010; John et al., 2006). The 5-HT signals evoked in the SNr are substantially smaller than DA signals in the striatum (Cragg et al., 1997a). This causes a reduction in the signal to noise ratio, which makes it unfeasible to study 5-HT release with the single pulse paradigm used to evoke striatal DA release. To increase the signal to noise ratio, 5-HT release in the SNr was therefore evoked from 20 pulse (20p) and 40 pulse (40p) stimulations at a frequency of 50 Hz. A perimaximal stimulation current of 0.6 mA was used. As observed previously, evoked 5-HT release in the SNr was found to be dependent on extracellular Ca²⁺ (Cragg et al., 1997a; Jennings et al., 2010; Threlfell et al., 2004).

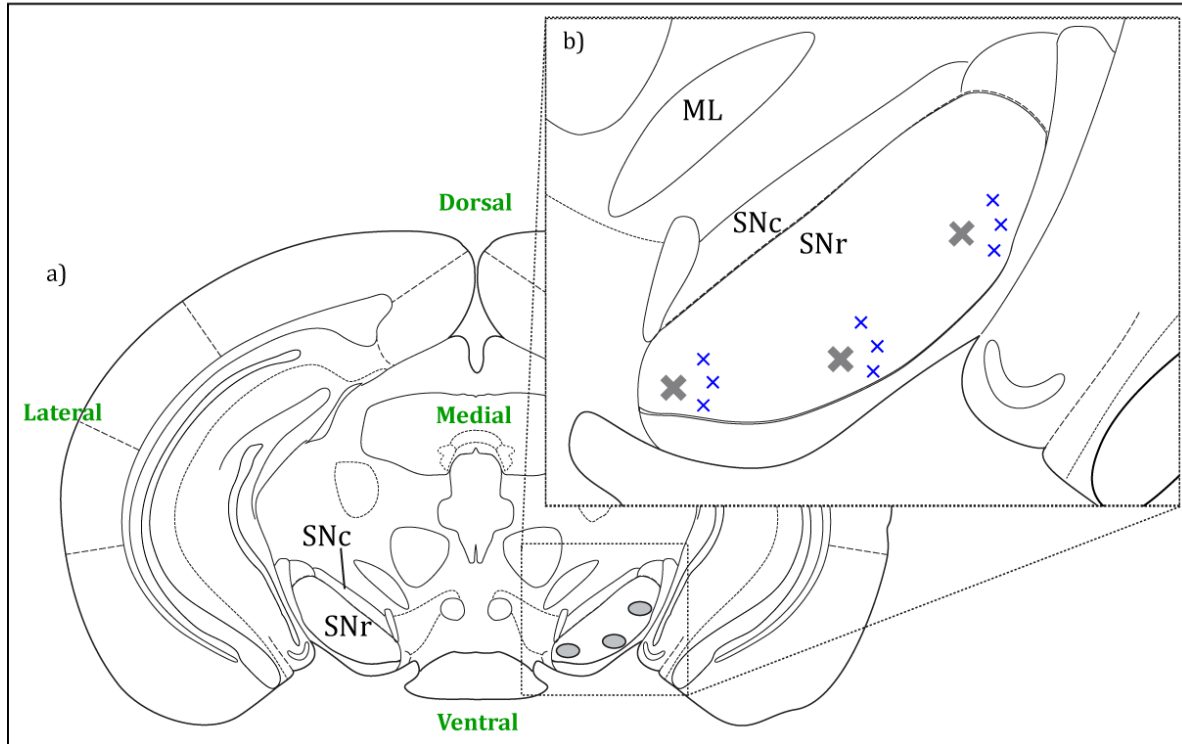


Figure 5.1: Coronal mouse brain section sampled by FCV. Evoked 5-HT release was sampled in acute coronal sections of mouse SNr at bregma -3.16 mm. Due to the variation of 5-HT release throughout the SNr, evoked $[5\text{-HT}]_o$ was sampled in 9 different sites across 3 regions along the ventral line of the SNr. The 3 regions sampled can be seen as grey areas in the full size diagram of a coronal slice (a). The stimulating electrode was only positioned once in each region, with the approximate locations shown as grey crosses in the enlarged diagram (b). In each region, the recording electrode was positioned in a random order at three sites, as shown as blue crosses in the enlarged diagram (b). Individual regions were sampled in random order, with corresponding regions from SNCA-C and *Snca* $-/-$ mice sampled in immediate succession, and in the same bath. The different sites were approximately matched in all slices to control for variation. Dorsal, ventral, lateral and medial positions indicated in green. Abbreviations in the figure are as follows: ML, medial lemniscus; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta. Diagram adapted from (Franklin and Paxinos, 2007).

5.2.2 Signal identification

Although axonal 5-HT release can be evoked in the SNr, $[5\text{-HT}]_o$ signals can often be contaminated by somatodendritic DA release (Cragg et al., 1997a). DA and 5-HT signals share the same oxidation peak on an FCV voltammogram (+500 to +650 mV), which is required to measure concentration. Therefore if DA contaminates a 5-HT signal, then the signal has to be discarded from the results. DA and 5-HT can be distinguished by their unique reduction peaks on a voltammogram (DA -200 mV; 5-HT -20 mV and -670 mV). If 5-HT is the only electroactive compound detected, then two distinct reduction peaks can be seen at the corresponding potentials (Figure 5.2, a). However, if both DA and 5-HT are present, then the reduction peaks merge into a single broad hump, or two ill-defined reduction peaks. The shape of reduction peaks can therefore be used to identify contaminated signals, as illustrated by the simulated voltammograms in Figure 5.2 (a-d).

For electrochemical signals to be positively identified as a putative 5-HT signal, at least two voltammograms were required that resembled 5-HT during a given stimulation train, with no voltammograms characteristic of DA contamination. Putative 5-HT signals were confirmed to be 5-HT during a pilot experiment, by adding 100 nM Citalopram (Figure 5.2, e). Citalopram blocks the uptake of 5-HT by inhibiting SERT, and therefore prolongs the lifetime of extracellular 5-HT. Furthermore, the lifetime of 5-HT signals were not prolonged by the DAT blocker GBR 12909 (data not shown) (Cragg et al., 1997a; Jennings et al., 2010; Threlfell et al., 2004).

In addition to potential DA contamination of 5-HT voltammograms, the low release of 5-HT means that $[5\text{-HT}]_o$ profiles were distorted by the low signal: noise ratio (Figure 5.2 f-h).

For this reason, observations with a low signal: noise ratio were discarded. To ensure that data was collected objectively, and avoid the possibility of unnecessarily excluding more data from one genotype than another, a systematic six-step method was developed. This allowed for the same exclusion criteria to be applied to all data. The six steps are summarised below:

- 1) Remove all observations with no observable oxidation peaks.
- 2) Remove observations where uncontaminated 5-HT is not identifiable throughout the entire region.
- 3) Remove observations where 5-HT is not identifiable, and putative DA signals are present in that region, even if at a neighboring site.
- 4) Remove all observations from any site where DA is present.
- 5) Remove all observations from regions with less than one observation putatively identifiable as 5-HT (a repeat observation was required for small signals).
- 6) Remove observations meeting more than two of the following three criteria:
 - A [5-HT]_o profile that is noisy or has an abnormal shape (Figure 5.2, g)
 - Peak [5-HT]_o is less than twice the amplitude of noise spikes (Figure 5.2, h)
 - Voltammogram is not identifiable as 5-HT

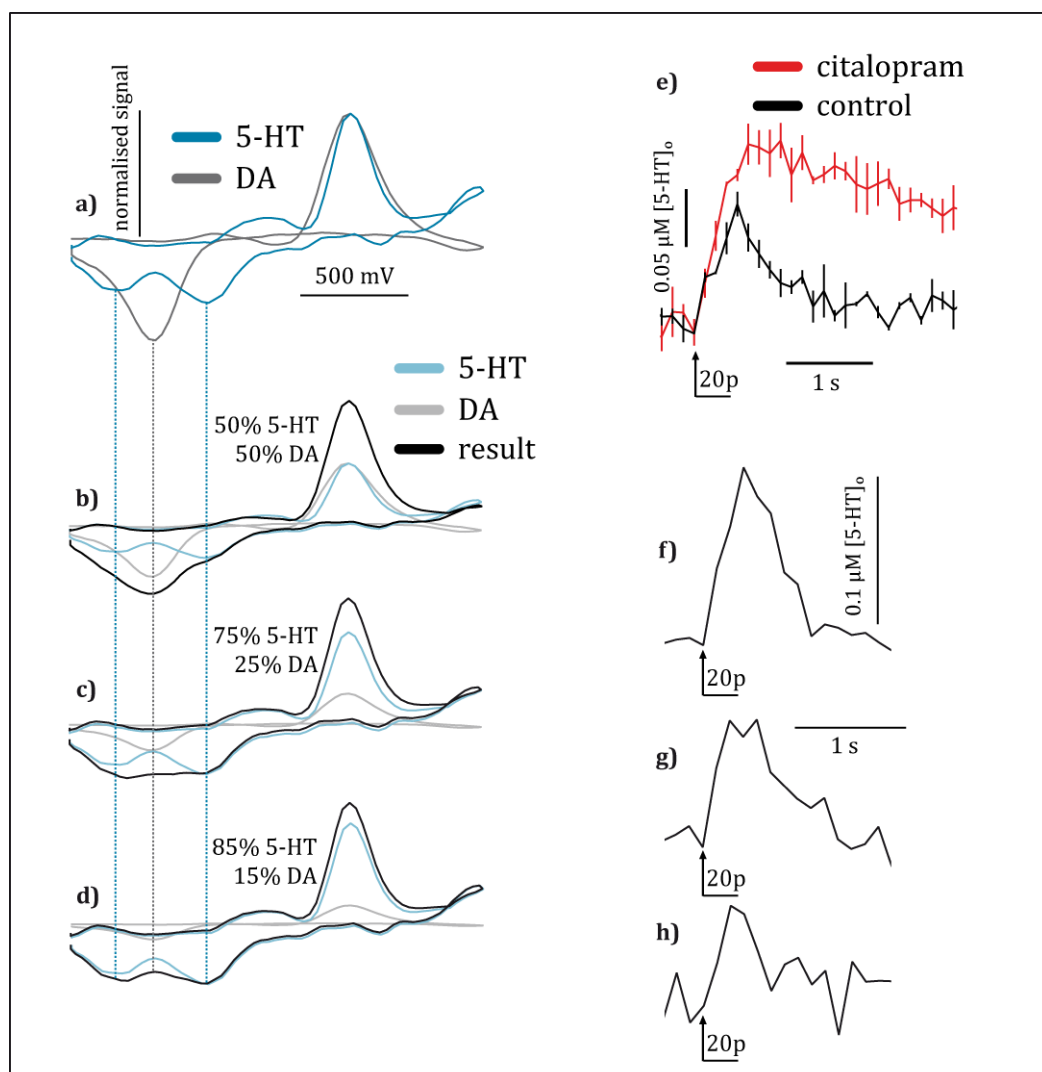


Figure 5.2: Identifying putative 5-HT signals from FCV voltammograms. a-d) Voltammograms of 5-HT and DA (derived from electrode calibrations) with normalised peak amplitudes (a). DA and 5-HT voltammograms were joined in a computer simulation to illustrate what a voltammogram can look like as a result of different contributions from DA and 5-HT towards the oxidation current. Different contributions towards the oxidation current vary from 50% 5-HT & 50% DA (b), 75% 5-HT & 25% DA (c), and 85% 5-HT & 15% DA (d). Note that electrode sensitivity is ~ 3-fold higher for 5-HT than DA (Cragg et al., 1997a), and equal contributions to the oxidation peak does therefore not indicate equal concentrations for 5-HT and DA. e) Profiles of $[5-HT]_0$ vs. time (mean $\pm$ SEM), evoked by a 20p stimulation, in aCSF and citalopram. f) Profile of evoked $[5-HT]_0$ vs. time with a well distinguished peak. g) Profile of evoked $[5-HT]_0$ vs. time with noise spikes on the peak, distorting the shape of the down-phase. h) Profile of evoked $[5-HT]_0$ vs. time with peak height approaching twice the height of noise peaks.

5.3. Results

5.3.1 Establishing a protocol to identify uncontaminated 5-HT signals, and quantify evoked $[5\text{-HT}]_o$ in the SNr

Before making a quantitative comparison of evoked $[5\text{-HT}]_o$ between SNCA-C mice and *Snca* $-/-$ controls, putative 5-HT signals contaminated with DA had to be excluded. $[5\text{-HT}]_o$ and $[DA]_o$ were evoked by either 20p or 40p at 50 Hz in the SNr of three-month old SNCA-C and *Snca* $-/-$ mice, and detected by FCV at a CFM.

The voltammograms of each recording were analysed manually and compared to the oxidation and reduction potentials of 5-HT and DA. For about 20% of recordings, no electroactive monoamines were detected after electrical stimulation. About 5% of recordings revealed a voltammogram with a monoamine oxidation peak, but poorly defined reduction peaks, which is characteristic of a mixed signal that contains 5-HT and DA (Figure 5.3, b). At about 40% of sites, putative 5-HT was detected with at least two voltammograms showing oxidation potentials at ~ 600 mV, as well as dual reduction peaks at -20 mV and -670 mV, with the current returning to zero in between peaks (Figure 5.3 c). Finally, about 35% of recordings had voltammograms with a distinct oxidation peak, indicative of monoamine release, but no identifiable reduction peaks (Figure 5.3, e). The diffusion of molecules away from the electrode means that fewer molecules are reduced than oxidised. This means that the reduction peaks are smaller than oxidation peaks, and are sometimes undetectable at low concentrations, and no electrochemical identification of the electroactive species could be made.

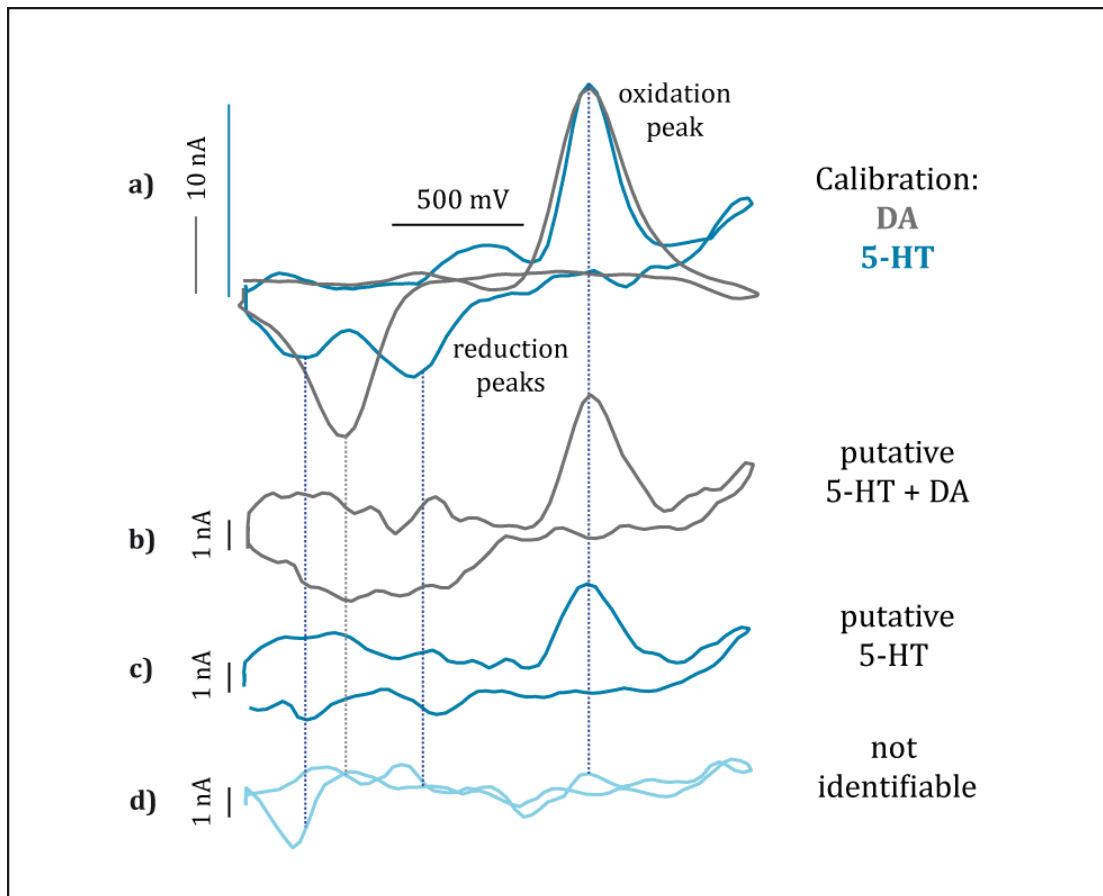


Figure 5.3: Example voltammograms of 5-HT and DA to illustrate the classification for recorded 5-HT signals. a) Calibration voltammograms of 2 μM DA (grey) and 0.5 μM 5-HT (blue). DA and 5-HT both show an oxidation peak at +500 to +650 mV on the scanning waveform. DA shows a reduction peak at -200 mV, whilst 5-HT shows a dual reduction peak at -20 mV and -670 mV. b-e) FCV voltammograms after electrical stimulation in SNr, showing characteristics of either 5-HT + DA or 5-HT release to varying degrees. b) *Putative 5-HT + DA* is characterised by a large oxidation peak, but poorly defined reduction peaks, characteristic of release from both 5-HT axons and DA dendrites. c) *Putative 5-HT* is characterised by clear oxidation and reduction peaks at potentials that correspond distinctly to 5-HT calibration. d) *Not identifiable* is characterised by a small oxidation peak at +500 – 650 mV, but no distinct reduction peak.

5.3.2 Evoked [5-HT]_o in the SNr of SNCA-C mice is no different from *Snca* -/- controls.

To investigate whether α -synuclein overexpression causes changes to 5-HT signalling, electrically evoked [5-HT]_o was sampled in the SNr of three-month old SNCA-C mice, and compared to *Snca* -/- controls. Evoked [5-HT]_o was sampled in nine sites across three regions in the SNr of acute brain slices (Figure 5.1). At each site, 5-HT was evoked first by a 20p stimulation at 50 Hz, and five minutes later by a 40p stimulation at 50 Hz.

The voltammogram of peak [5-HT]_o for each observation was either categorised as containing DA + 5-HT, 5-HT alone, an electroactive species not identifiable due to the small signal, or having no release. Different categories were plotted for both SNCA-C mice and *Snca* -/- controls into a bar chart (Figure 5.4, a). The relative distribution of categorisations is very similar for all regions, and the same for both SNCA-C and *Snca* -/- mice. These data suggest that evoked [5-HT]_o in SNCA-C and *Snca* -/- mice is released with a similar heterogeneity across the ventral part of the SNr.

Once the different observations were categorised, FCV recordings were evaluated according to the exclusion criteria in section 5.2.4. For both SNCA-C and *Snca* -/- mice, 162 observations were recorded across the SNr. For 20p stimulations in SNCA-C mice, 59% of observations were excluded by the six-step evaluation discussed in section 5.2.4.

Comparatively 46% of observations in *Snca* -/- controls were excluded for 20p stimulations. For 40p stimulations in SNCA-C mice, 62% of observations were excluded, and 57% of observations were excluded in *Snca* -/- controls.

Observations across regions were pooled together, and profiles of average [5-HT]_o vs. time were plotted. [5-HT]_o profiles of 40p stimulations had a higher peak amplitude, and lasted longer than profiles of 20p stimulations. For 20p stimulations, the average profile of evoked [5-HT]_o was similar between SNCA-C mice and *Snca* ^{-/-} controls (Figure 5.4, c). Likewise, for 40p stimulations, the average profile of evoked [5-HT]_o was also similar between SNCA-C mice and *Snca* ^{-/-} controls (Figure 5.4, d). Peak [5-HT]_o was plotted in a box and whiskers graph (Figure 5.4 e), and also in scatter plots and histograms (Figure 5.5 a-d). The median peak [5-HT]_o evoked from 20p stimulations was 0.077 μM and 0.077 μM for SNCA-C and *Snca* ^{-/-} respectively. The median peak [5-HT]_o evoked from 40p stimulations was 0.102 μM and 0.108 μM for SNCA-C and *Snca* ^{-/-} respectively. No significant differences in ranks of peak [5-HT]_o (Figure 5.4), or the distribution of [5-HT]_o (Figure 5.5) were found between SNCA-C and *Snca* ^{-/-} mice.

In contrast to the DA release deficit seen in SNCA-C mice, data presented here reveal no difference in evoked [5-HT]_o from the SNr of SNCA-C mice vs. *Snca* ^{-/-} controls. These data suggest 5-HT release is less sensitive to the high level of human α-synuclein in SNCA-C mice than striatal DA release.

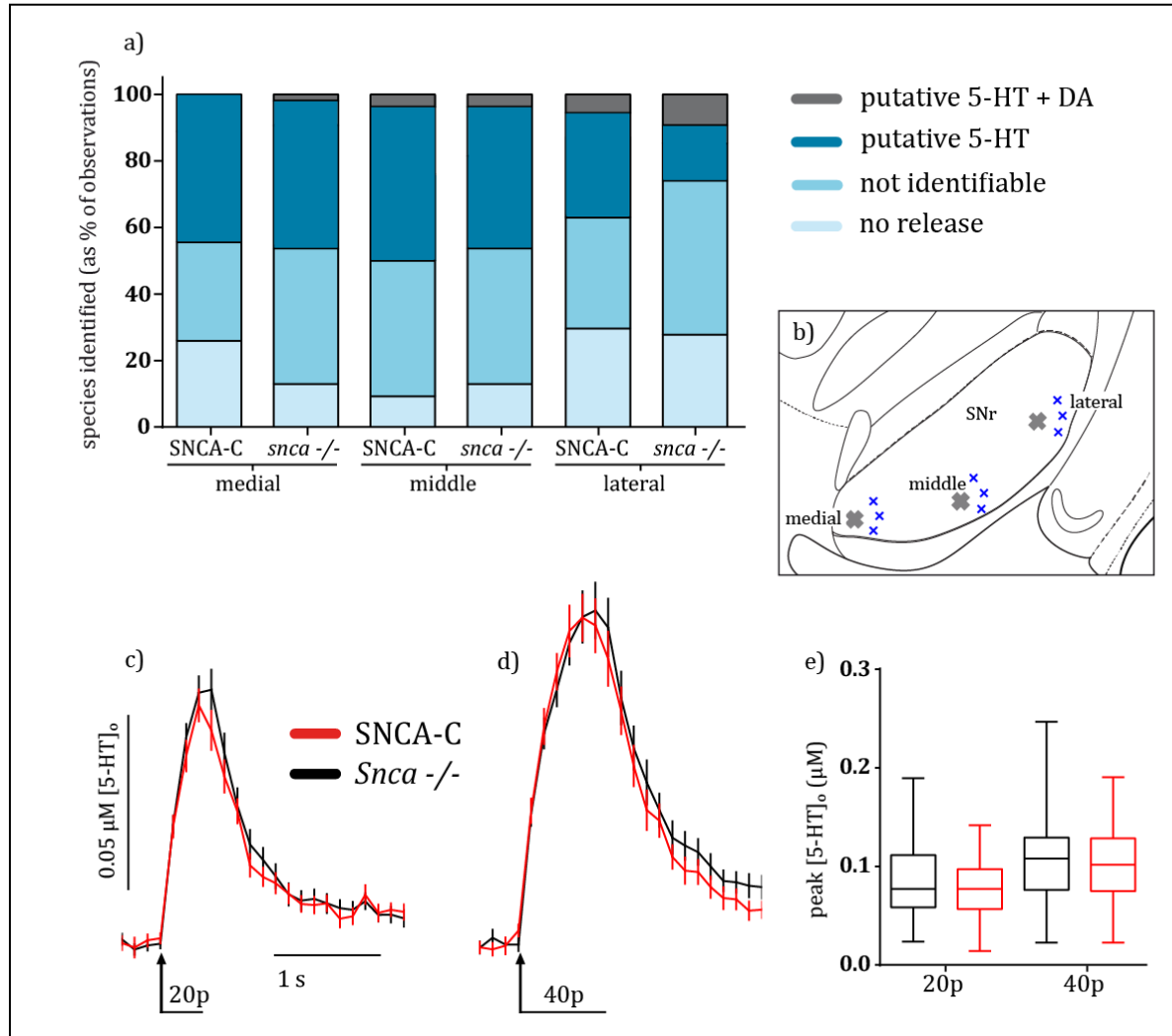


Figure 5.4: Evoked [5-HT]₀ in SNr of 3-month old SNCA-C and *Snca* -/- mice. 5-HT was evoked from SNr of SNCA-C and *Snca* -/- mice and recorded by FCV. a) Bar graphs showing how voltammograms in each region were categorised (as a percentage of total observations). Categorisations were made according to the criteria illustrated in Figure 5.3. b) Close-up diagram of the medial, middle and lateral regions on the X-axis of the bar chart (a). c) Mean [5-HT]₀ profiles vs. time (mean ± SEM) evoked from 20p stimulations in all SNr regions of SNCA-C and *Snca* -/- mice. d) Mean [5-HT]₀ profiles vs. time (mean ± SEM) evoked from 40p stimulations in all SNr regions of SNCA-C and *Snca* -/- mice. e) Box and whiskers plots of peak [5-HT]₀ evoked from 20p and 40p stimulations in all SNr regions of SNCA-C and *Snca* -/- mice. Plots show the median, interquartile range, and range. Distribution of mean peak evoked [5-HT]₀ was not assumed to be normal after failing D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test, and not appearing normally distributed on Q-Q plot. Ranks of peak [5-HT]₀ evoked from 20p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, unpaired two-tailed Mann Whitney, $n = 33-44$). Median peak [5-HT]₀ evoked from 40p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, unpaired two-tailed Mann Whitney, $n = 31-35$).

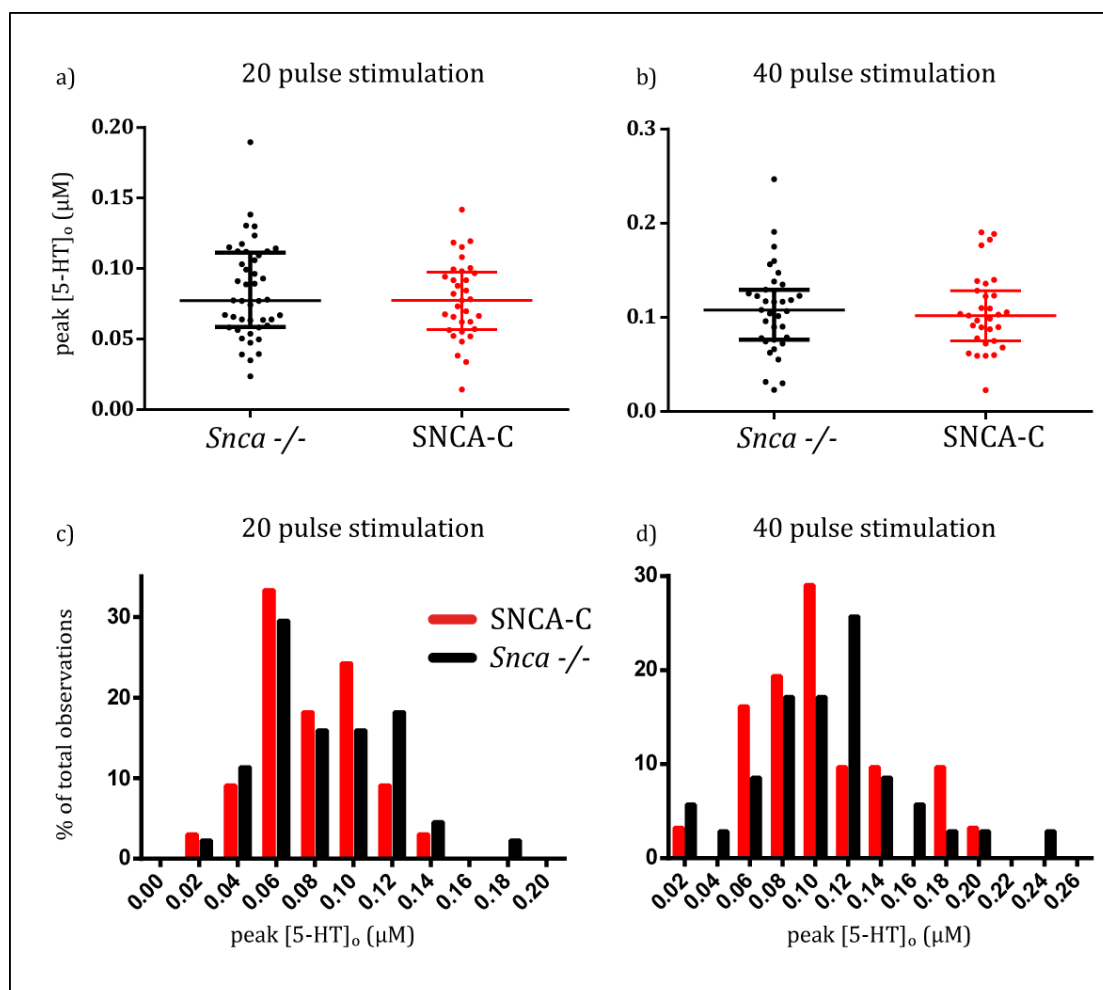


Figure 5.5: Distribution of evoked [5-HT]₀ in 3-month old SNCA-C and *Snca* -/- mice. Scatter plots show peak [5-HT]₀ (lines show median and interquartile range) of SNCA-C and *Snca* -/- mice evoked from 20p (a) and 40p (b) stimulations. Histograms show the distribution of peak [5-HT]₀ in SNCA-C and *Snca* -/- mice evoked from 20p (c) and 40p (d) stimulations. The distribution of peak [5-HT]₀ evoked from 20p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, Kolmogorov-Smirnov, $n = 33-44$). The distribution of peak [5-HT]₀ evoked from 40p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, Kolmogorov-Smirnov, $n = 31-35$).

5.3.3 SNCA-C mice do not develop any changes to evoked [5-HT]_o at 18 months.

Section 5.3.2 revealed that SNCA-C mice, at age three months, have no difference in [5-HT]_o evoked from the SNr. However, previous investigations have revealed progressive changes to monoamine transmission (Lundblad et al., 2012), and it may therefore be possible that changes in 5-HT release develop with time. To investigate whether progressive changes to 5-HT signalling develop in SNCA-C mice, electrically evoked [5-HT]_o in the SNr was sampled at 18 months. As done in section 5.3.2, [5-HT]_o was sampled in nine sites across three regions in the SNr of acute brain slices (Figure 5.1). At each site, 5-HT was evoked first by a 20p stimulation at 50 Hz, and five minutes later by a 40p stimulation at 50 Hz.

As done in section 5.3.2, the voltammograms of all recordings were categorised as containing putative signals of DA + 5-HT, 5-HT alone, not identifiable, or having no release. Different categories were plotted for both SNCA-C mice and *Snca* ^{-/-} controls into a bar chart (Figure 5.6, a). The relative distribution of categorisations is similar for all regions, and also for both SNCA-C and *Snca* ^{-/-} mice. These data suggest that, at age 18 months, evoked [5-HT]_o in SNCA-C and *Snca* ^{-/-} mice is released with a similar heterogeneity in the SNr.

Once the different recordings were categorised, they were evaluated according to the exclusion criteria in section 5.2.4. For SNCA-C mice, 83 observations were recorded, and for *Snca* ^{-/-} mice, 84 observations were recorded across the ventral SNr. For 20p stimulations in SNCA-C mice, about 34% of observations were excluded by the six-step evaluation discussed in section 5.2.4. Comparatively about 24% of observations in *Snca* ^{-/-} controls

were excluded. For 40p stimulations in SNCA-C mice, about 38% of observations were excluded, and about 27% of observations were excluded in *Snca* $-/-$ controls.

Observations across regions were pooled together, and profiles of average [5-HT]_o vs. time were plotted. [5-HT]_o profiles of 40p stimulations had a higher peak amplitude, and lasted longer than profiles of 20p stimulations. For 20p stimulations, the average profile of evoked [5-HT]_o was similar between SNCA-C mice and *Snca* $-/-$ controls (Figure 5.6, c). Likewise, for 40p stimulations, the average profile of evoked [5-HT]_o was also similar between SNCA-C mice and *Snca* $-/-$ controls (Figure 5.6, d). Peak [5-HT]_o was also plotted in a box and whiskers graph (Figure 5.6, e), as well as scatter plots and histograms (Figure 5.7 a-d). The median peak [5-HT]_o evoked from 20p stimulations was 0.068 μ M and 0.095 μ M for SNCA-C and *Snca* $-/-$ respectively. The median peak [5-HT]_o evoked from 40p stimulations was 0.081 μ M and 0.108 μ M for SNCA-C and *Snca* $-/-$ respectively. No significant differences in the ranks of peak [5-HT]_o (Figure 5.6) or the distribution of peak [5-HT]_o (Figure 5.7) were found between SNCA-C and *Snca* $-/-$ mice.

As reported in section 5.3.2, data presented here reveal no difference in evoked [5-HT]_o from the SNr of SNCA-C mice vs. *Snca* $-/-$ controls. These data show that SNCA-C mice do not develop changes in evoked [5-HT]_o over 18 months, further suggesting that 5-HT release is less sensitive than DA release to the high level of human α -synuclein in SNCA-C mice.

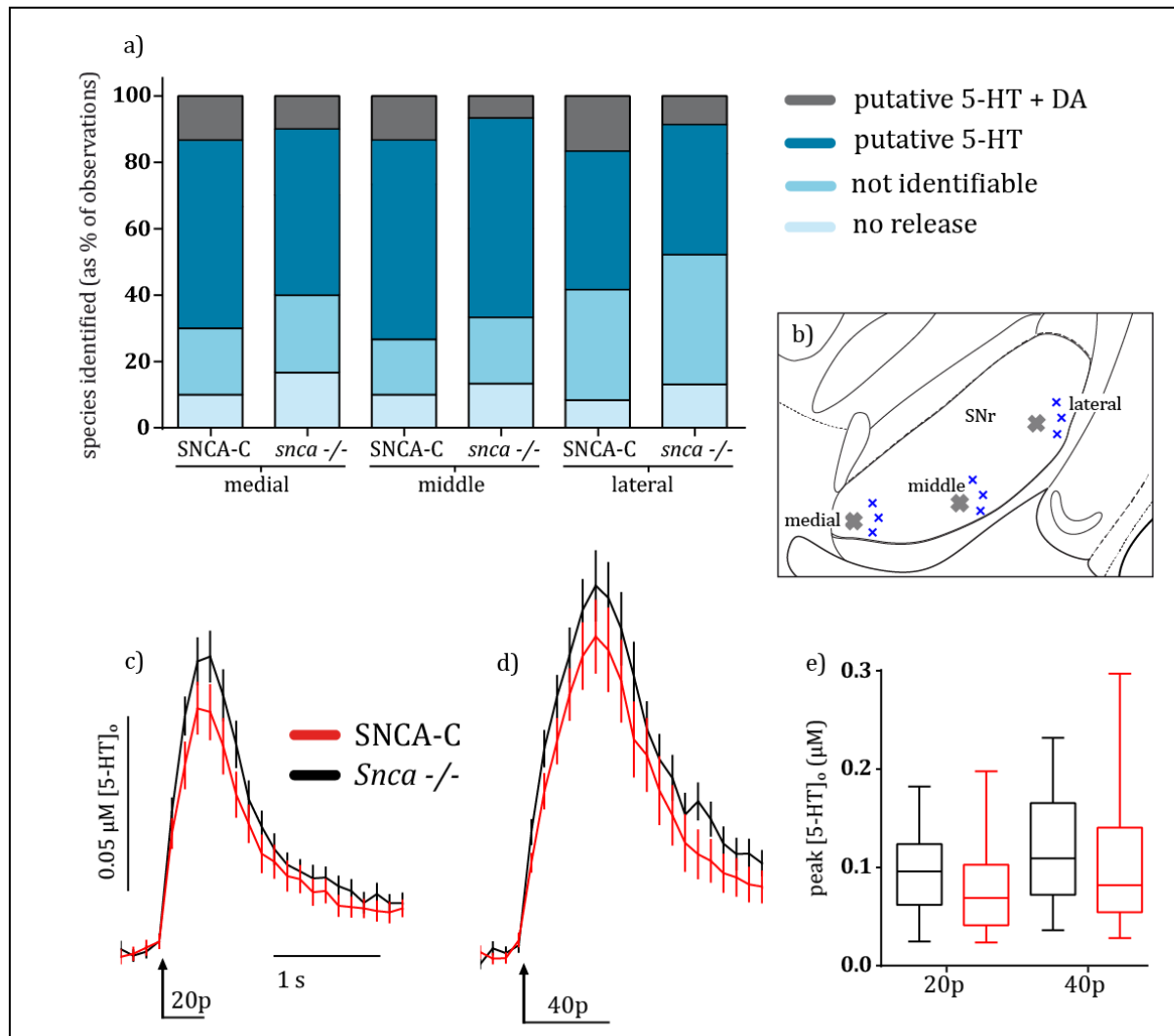


Figure 5.6: Evoked [5-HT]₀ in SNr of 18-month old SNCA-C and *Snca*^{-/-} mice. 5-HT was evoked from SNr of SNCA-C and *Snca*^{-/-} mice and recorded by FCV. a) Bar graphs showing categorisation of voltammograms in each region (% of total observations). Categorisations were made according to the criteria illustrated in Figure 5.3. b) Close-up diagram of the medial, middle and lateral regions on the X-axis of the bar chart (a). c) Mean [5-HT]₀ profiles vs. time (mean ± SEM) evoked from 20p stimulations in all SNr regions of SNCA-C and *Snca*^{-/-} mice. d) Mean [5-HT]₀ profiles vs. time (mean ± SEM) evoked from 40p stimulations in all SNr regions of SNCA-C and *Snca*^{-/-} mice. e) Box and whiskers plots of peak [5-HT]₀ evoked from 20p and 40p stimulations in all SNr regions of SNCA-C and *Snca*^{-/-} mice. Plots show the median, interquartile range, and range. Distribution of mean peak evoked [5-HT]₀ was not assumed to be normal after failing D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test, and not appearing normally distributed on Q-Q plot. Ranks of peak [5-HT]₀ evoked from 20p is no different between SNCA-C vs. *Snca*^{-/-} controls ($p > 0.05$, unpaired two-tailed Mann Whitney, $n = 28-32$). Median peak [5-HT]₀ evoked from 40p is no different between SNCA-C vs. *Snca*^{-/-} controls ($p > 0.05$, unpaired two-tailed Mann Whitney, $n = 26-31$).

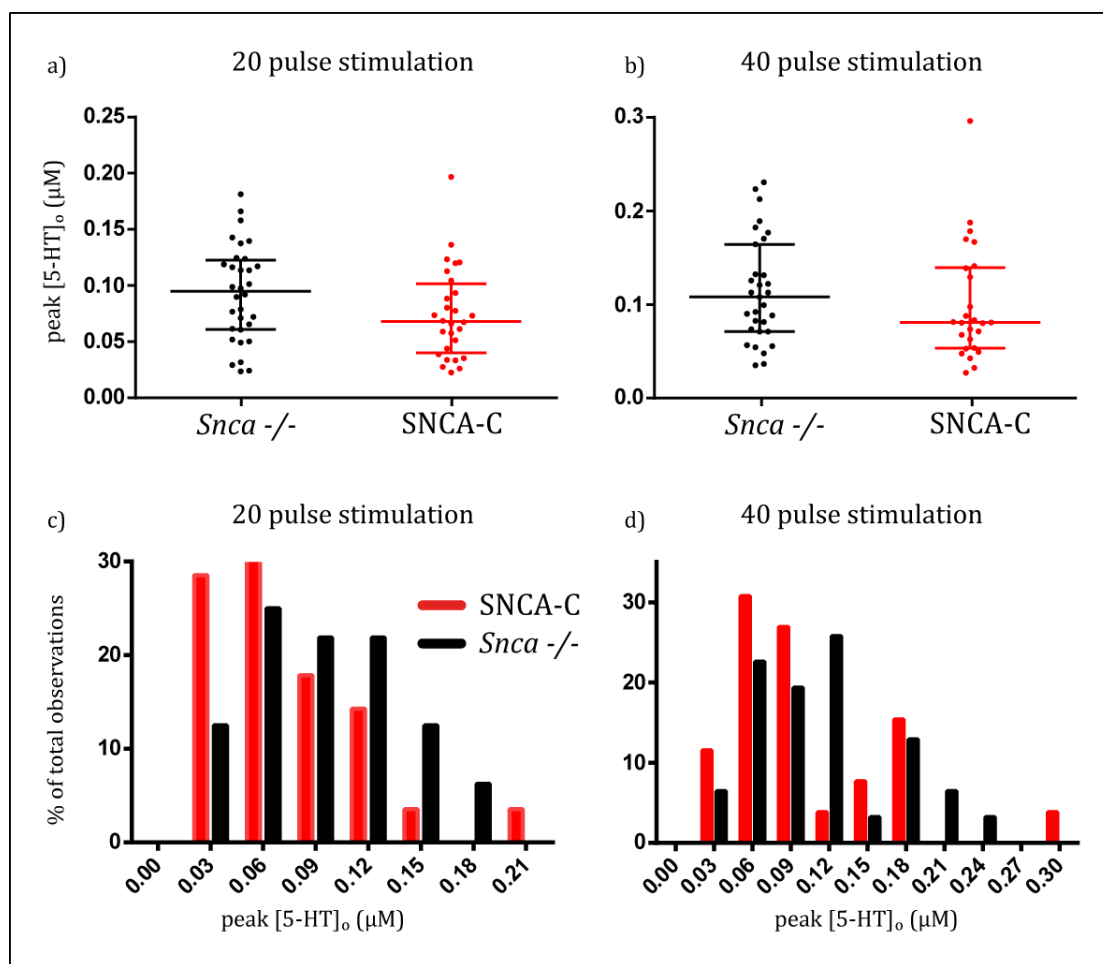


Figure 5.7: Distribution of evoked [5-HT]_o in 18-month old SNCA-C and *Snca* -/- mice. Scatter plots show peak [5-HT]_o (lines show median and interquartile range) of SNCA-C and *Snca* -/- mice evoked from 20p (a) and 40p (b) stimulations. Histograms show the distribution of peak [5-HT]_o in SNCA-C and *Snca* -/- mice evoked from 20p (c) and 40p (d) stimulations. The distribution of peak [5-HT]_o evoked from 20p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, Kolmogorov-Smirnov, $n = 28-32$). The distribution of peak [5-HT]_o evoked from 40p is no different between SNCA-C vs. *Snca* -/- controls ($p > 0.05$, Kolmogorov-Smirnov, $n = 26-31$).

5.4. Discussion

The aim of this chapter was to investigate whether SNCA-C mice have any changes to 5-HT signalling. Electrically evoked 5-HT release was sampled in the SNr of SNCA-C mice at 3 months and 18 months. However, due to the sparse innervation of 5-HT neurons in the SNr (Moukhles et al., 1997; Threlfell et al., 2004) the amount of evoked 5-HT release is very low in comparison to striatal DA. Furthermore, dendrites from SNc DA neurons extend into the SNr (Moukhles et al., 1997), and somatodendritic DA release can therefore contaminate 5-HT electrochemical signals (Cragg et al., 1997a). The potential contamination of somatodendritic DA release was minimised by sampling along the ventral line of the SNr, where DA dendritic arborisation is minimal (Moukhles et al., 1997), and also by excluding putative DA-containing signals from the final analysis.

For this study, it was important to exclude DA signals for two main reasons. Firstly, the question addressed here is specific to 5-HT release because a deficit in DA release has already been observed in SNCA-C mice. Investigating changes to 5-HT release in SNCA-C mice can provide a greater understanding of how DA and 5-HT systems are differently affected during PD, and may thereby contribute to finding a mechanism by which α -synuclein affects neurotransmission. Obtaining information from a mix of 5-HT and DA therefore interferes with the aim of the investigation. Secondly, it may be possible that the deficit in evoked axonal DA release from SNc neurons is also present in somatodendritic DA release, in which case, recording DA would provide a biased contribution to the electrochemical signal. For these reasons, a systematic approach had to be developed to ensure that only appropriately detectible, and only uncontaminated 5-HT signals were

included in the final analysis. In section 5.2.4 a six-step approach was described to exclude FCV recordings that were contaminated with DA, or had a small signal to noise ratio. This systematic approach was applied during the analysis of all observations in sections 5.3.2, and 5.3.3, where about 40% to 75% of all observations could be identified as putative 5-HT signals. Interestingly, a greater number of observations were identified as putative 5-HT signals in mice at 18 months (Figure 5.6 a) than at 3 months (Figure 5.4 a). However, data was not further compared between age groups because experiments were conducted with different electrodes in different calendar months, which can make it difficult to meaningfully compare data between ages.

It is interesting that more data was excluded from SNCA-C mice than *Snca* $-/-$ controls. For *Snca* $-/-$ and SNCA-C mice respectively, the number of observations excluded at age 3 months for 20p stimulations are 46% and 59%, and for 40p stimulations are 57% and 62%. Likewise, the number of observations excluded at age 18 months for 20p stimulations are 24% and 34%, and for 40p stimulations are 27% and 38% for *Snca* $-/-$ and SNCA-C mice respectively. The observation that more data was excluded from SNCA-C than *Snca* $-/-$ mice might indicate that a lower proportion of electrical stimulations in SNCA-C mice resulted in enough 5-HT release to cross the detection threshold, in comparison to *Snca* $-/-$ controls. In support of this, scatter plots of peak [5-HT]_o, and histograms of peak [5-HT]_o distribution, reveal a trend towards peak evoked [5-HT]_o from 18-month old SNCA-C mice being distributed at lower concentrations than *Snca* $-/-$ controls (Figure 5.7). However, no significant differences in the ranks of peak [5-HT]_o ($p > 0.05$, unpaired two-tailed Mann Whitney, $n = 26-32$) or the distribution of peak [5-HT]_o ($p > 0.05$, Kolmogorov-Smirnov,

n=26-32) were found between SNCA-C and *Snca* $-/-$ mice. Therefore, statistical analysis does not support the idea that evoked [5-HT]_o is lower in SNCA-C mice vs. *Snca* $-/-$ controls.

Evoked [5-HT]_o in the SNr was first explored at three months. Peak evoked [5-HT]_o was the same in SNCA-C mice and *Snca* $-/-$ controls, suggesting that α -synuclein overexpression does not have the same effect on the release of all monoamines. The possibility of a 5-HT release deficit developing with age was then explored by sampling evoked [5-HT]_o in SNCA-C mice at 18 months. As for three months, peak evoked [5-HT]_o at 18 months was the same in SNCA-C mice and *Snca* $-/-$ controls, suggesting that 5-HT release is unaffected in SNCA-C mice. These data show that the DA release deficit seen in the CPu of SNCA-C mice does not extend to all monoamine transmission, but rather suggests that α -synuclein overexpression preferentially affects nigrostriatal DA neurons. The conclusion presented here is further supported by the observations that evoked noradrenaline release in the BNST of SNCA-C mice is also unchanged (Janezic et al., 2013).

The findings that α -synuclein overexpression preferentially affects nigrostriatal DA neurons to a greater extent than 5-HT neurons, are in line with several previous observations in the literature. For example, 5-HT content is unaltered in α - and β -synuclein knockout mice, whereas DA content is decreased (Chandra et al., 2004). Another study, which investigated the PD-related A53T mutation of α -synuclein in mice, found changes to the expression of DAT and D₁ receptors, but not SERT (Unger et al., 2006). Taken together with these published findings, the data presented here suggest that DA neurons are more susceptible to the effect of α -synuclein overexpression than 5-HT neurons.

The idea that 5-HT neurons are less susceptible to be affected by α -synuclein overexpression than DA neurons does however need to be reconciled with two other studies, which suggest that α -synuclein can affect 5-HT neurons. Overexpressing α -synuclein in co-transfected *Ltk*-cells suppressed SERT mediated uptake of [^{3} H]5-HT by decreasing SERT levels at the plasma membrane, suggesting that α -synuclein may have a significant influence on 5-HT neurotransmission (Wersinger et al., 2006a). However, this investigation was not performed on 5-HT neurons, and therefore lacks any mechanisms that 5-HT neurons might have to compensate for α -synuclein overexpression. Another study performed *in vitro* found that 5-HT binds to, and stabilises intermediate aggregates of α -synuclein through direct interactions with the C-terminal domain (Falsone et al., 2011). However, the aggregation of α -synuclein has been described as a complex process, involving not just stabilisation of aggregates, but also seeding of aggregates and impairment of protein degradation (Colla et al., 2012; Danzer et al., 2009; Yates, 2012). Moreover, intracellular 5-HT is stored in synaptic vesicles, thereby minimising the effect on α -synuclein aggregation (Guillot and Miller, 2009). Therefore, the observation that 5-HT may contribute towards α -synuclein aggregation does not mean that α -synuclein overexpression will necessarily cause pathology in 5-HT neurons, and is not at odds with findings presented here.

Understanding why α -synuclein overexpression in SNCA-C mice affects nigrostriatal DA release, but not 5-HT release in the SNr, could contribute to elucidating the role of α -synuclein in PD pathophysiology. Immunohistochemical analyses reveal a homogenous distribution of α -synuclein across the SNc and raphe of SNCA-C mice, with an equally

homogenous distribution across the striatum and SNr (Janezic et al., 2013). This suggests the different effects of α -synuclein in DA vs. 5-HT neurons are not exclusively due to the localisation of α -synuclein. Therefore, the nigrostriatal DA release deficit in SNCA-C mice is likely due to a specific influence of α -synuclein at the synaptic terminals of these neurons. It has already been mentioned in the introduction that nigrostriatal DA neurons have a relatively large axonal arbour and high Ca^{2+} flux in comparison to other monoamine neurons (Chan et al., 2007; Matsuda, 2009). With such distinguishing characteristics, it may also be that α -synuclein has a specific effect on nigrostriatal DA neurons, which does not occur to the same extent in other neurons. A specific influence of α -synuclein on nigrostriatal DA neurons can be due to one of two factors. Firstly, it may be that nigrostriatal DA neurons have fewer processes that reduce or buffer the effects of α -synuclein. Secondly a specific influence of α -synuclein on nigrostriatal DA neurons can be due to α -synuclein has a specific effect on these neurons, which is not found in other neurons to the same extent.

Another protein that specifically affects nigrostriatal DA release, but not 5-HT release, is a member of the synapsin family called synapsin III (Kile et al., 2010). Knocking out the synapsin III gene in mice leads to a two-fold increase in striatal DA release evoked by a single electrical pulse (Kile et al., 2010), which suggests that synapsin III suppresses nigrostriatal DA release. However, knocking out synapsins does not affect the release of 5-HT in the SNr (Kile et al., 2010), or DA release in the VTA (Nicola Platt, 2012), suggesting that the effect of synapsin III is specific to nigrostriatal DA neurons. This effect of synapsin III may be of interest, because it exhibits a similar neuronal preference to the effects of α -

synuclein in SNCA-C mice. The selective effect of both synapsins and α -synuclein nigrostriatal DA release may lead to speculation that both of these proteins act through a converging mechanism. In support of such an idea, both synapsins α -synuclein have been shown to have direct interactions with GTP-Rab3 (Chen et al., 2013; Giovedì et al., 2004) , and regulate mobilisation of the reserve pool of presynaptic vesicles (Gitler et al., 2004; Nemani et al., 2010). Furthermore, overexpressing α -synuclein has lead to loss of synapsin I in cultured hippocampal neurons (Nemani et al., 2010; Scott et al., 2010), further suggesting a potential interaction between α -synuclein and synapsin. Taken together, these data provide support for the idea that α -synuclein overexpression in SNCA-C mice affects nigrostriatal DA release through a mechanism similar to synapsin III, which may involve reducing mobility of the reserve pool of presynaptic DA vesicles (Kile et al., 2010; Murphy et al., 2000; Nemani et al., 2010). Interestingly, no changes have been found in the level of synapsin I or synapsin II in the striatum of SNCA-C mice (Janezic et al., 2013), although the level of synapsin III was not tested. The data summarised here does not conclusively report whether α -synuclein and synapsins regulate striatal DA release through a shared or converging mechanism. However, the stark similarities in the effects of α -synuclein and synapsins on striatal DA release, and also the similarity in which cocaine modulates the effects of these proteins, would suggest this to be an interesting avenue for further investigation (Kile et al., 2010; Murphy et al., 2000; Venton et al., 2006).

The findings presented here may contribute to a greater understanding of the mechanism by which α -synuclein contributes to presynaptic dysfunction, which may provide a deeper insight into the early stages of PD pathophysiology. The data presented here suggests that

α -synuclein overexpression does not cause neurotransmission deficits in all systems affected by PD, but preferentially affects nigrostriatal DA release, which will be further discussed in chapter 7.

CHAPTER 6. BAC transgenic rats that express the G2019S and R1441C mutant forms of human *LRRK2* have deficits in DA release.

6.1. Introduction

Various investigations have revealed that *LRRK2* can be localised at synaptic boutons, where it may interact with endocytic machinery and presynaptic vesicles (Shin et al., 2008; Piccoli et al., 2011). Moreover, it has been suggested that *LRRK2* interacts with the cytoskeleton and synaptic vesicles to regulate neurotransmission (Piccoli et al., 2011; Belluzzi et al., 2012)

Genetic factors, such as G2019S or R1441C point mutations in the *LRRK2* gene, can contribute to PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). Although these two mutations both lead to PD, they may do so in different ways. The G2019S mutation is present in the kinase domain, and is associated with increased *LRRK2* kinase activity (Chen et al., 2012). The G2019S mutation has a high prevalence, but relatively low penetrance of only around 27% at age 70 (Goldwurm et al., 2005; Sierra et al., 2011). The R1441C mutation on the other hand is present in the GTPase domain, and is associated with reduced GTPase activity (Liu et al., 2011). The R1441C mutation is less prevalent than the G2019S mutation, especially in some areas of Europe such as Germany (Möller et al., 2008). The R1441C mutation is however more penetrant than the G2019S mutation with 70% of carriers developing PD before age 70 (Haugarvoll et al., 2008).

Studies on transgenic animals that express G2019S and R1441C mutant forms of human *LRRK2*, have to some extent been able to recapitulate different aspects of PD-like pathology (see Table 1.3), and several studies report changes to the mesostriatal DA pathways.

Investigations in mice that express the G2019S mutant form of *LRRK2* in a BAC transgene showed lower baseline [DA]_o detected by microdialysis, an age-dependent decrease in striatal DA content, along with changes to DA uptake, and evoked release detected by FCV (Li et al., 2010; Melrose et al., 2010). Furthermore, changes in striatal DA content and release were found in the absence of cell death in the SNc, or overt motor phenotypes, which suggests that presynaptic changes to DA neurons may be one of the early stages of *LRRK2* pathology. Another rat model that expresses the G2019S mutant form of *LRRK2* under a doxycycline-inducible promoter found impaired DAT activity, and a model that used a PDGF promoter found progressive degeneration of the SNc (Zhou et al., 2011; Chen et al., 2012).

The effects of the R1441C mutation on striatal DA release dynamics are comparable to that of the G2019S mutation. One study that expressed the R1441C mutant form of *LRRK2* in BAC transgenic mice found reduced striatal [DA]_o detected from microdialysis in vivo (Li et al., 2009). Another study that expressed the R1441C mutation in mice under a PDGF promoter, and reported reduced striatal DA content (Ramonet et al., 2011). Expressing the R1441C mutant in cultured chromaffin cells also reduces catecholamine release (Tong et al., 2009). Taken together, these data suggest that both the G2019S and R1441C mutations can affect the function of mesostriatal DA pathways, albeit through different effects on *LRRK2*.

Understanding how the G2019S and R1441C mutations affect DA release may help to elucidate the role of *LRRK2* in PD pathophysiology. However, some models of G2019S and R1441C *LRRK2* mutations often report on tests that use incomparable parameters (see

table 1.3). For example, mice that express the G2019S mutant form of LRRK2 under a BAC promoter showed reduced evoked [DA]_o detected from FCV (Li et al., 2010). On the other hand, mice that express the R1441C mutant form of LRRK2 under a BAC promoter showed reduced basal [DA]_o from *in vivo* microdialysis (Li et al., 2009). Both of these studies find reduced levels of striatal DA release, although the FCV study used acute striatal slices where there is no tonic neuron activity, whereas the [DA]_o recorded from the *in vivo* microdialysis study depends on neuron firing rate. The fact that these two studies used different methods to detect evoked [DA]_o, makes it difficult to make any meaningful comparison between them. For example, the FCV investigation also revealed a deficit in DA uptake for G2019S mice (Li et al., 2010), whereas microdialysis could not report on changes in DA uptake (Li et al., 2009). Differences between previous studies, such as the ones mentioned here, prevent results from different models to be accurately compared. It is therefore becoming increasingly important to generate comparable transgenic models.

The OPDC consortium has generated several lines of rats that express human *LRRK2* through a BAC transgene on a non-transgenic (*nTG*) background with wild-type endogenous *LRRK2*. One line of BAC transgenic rats expresses the G2019S mutant form of *LRRK2*, here named **G2019S**, and the other line expresses the R1441C mutant form of *LRRK2*, here named **R1441C**. The BAC transgenic lines were generated and maintained as described in Chapter 2. The G2019S and R1441C lines were created on the same genetic background, and are therefore more suitable for comparing results between the lines, which is an advantage over existing combinations of transgenic animals. There are furthermore, three potential advantages of using the rats created by the OPDC rather than following up on previous mouse models. Firstly, current BAC transgenic *LRRK2* models in

the literature are limited to mice, and using rats as a different rodent species expands the repertoire of transgenic animals available. This may help to distinguish species-specific findings from findings that are relevant to humans. Secondly, rats are larger than mice, which leaves more potential for future studies that use complex instrumentation, such as electric field recordings. Finally, the average life span of rats is longer than mice which creates more potential for studying an age-related and progressive disorder like PD.

To explore if either the G2019S or the R1441C rats have deficits in DA release, evoked striatal $[DA]_o$ was sampled using FCV at a CFM as done previously (Bull et al., 1990; Janezic et al., 2013). To study whether G2901S and R1441C rats develop changes in DA release with age, experiments were performed at 6 months, 12 months, and 18 months. Evoked $[DA]_o$ was sampled in four different striatal sites to explore sub-regional changes in DA release. Striatal DA content was also measured by HPLC with electrochemical detection to investigate whether G2019S or R1441C rats had any changes to DA content.

6.2. Methods

Unless otherwise specified, experimental procedures were as described in Chapter 2.

6.2.1 Electrical stimulation and FCV

Figure 6.1 shows the location of FCV sampling sites for this chapter. Each of the four sites sampled, shown in Figure 6.1, received both a single pulse electrical stimulation (1p) and a four-pulse train at 100Hz (4p). Evoked $[DA]_o$ in the striatum of nTG rats was found to be dependent on Ca^{2+} , sensitive to TTX, and influenced by changes to amplitude of the electrical stimulation current, blockade of nAChRs, and K^+ concentration (data not shown) (Bull et al., 1990; Robinson et al., 2003). The lifetime of $[DA]_o$ signals were also not affected by application of Desipramine, which inhibits the noradrenaline transporter, but was prolonged by application of GBR 12909, which inhibits DAT (data not shown) (Bull et al., 1990).

6.2.2 Experimental design and statistical analysis

Due to the reduced time of slice viability, experiments were shortened by only sampling 4 sites in each slice, as opposed to 8 sampled in the SNCA mice (Chapter 3). Due to their relative sizes, three sites were sampled in the CPu, and only one in the NAc. For some analyses, data was pooled from the two most dorsal sites to represent the dorsal striatum, and from the two most ventral sites to represent the ventral striatum. It is worth noting that sites pooled together for the ventral striatum are not all from the NAc, but that one of the sites is from the ventromedial CPu and the other from the NAc. However, the

ventromedial region of the CPu has been known to mediate some functions ascribed to the ventral striatum (Voorn et al., 2004), and receives substantial input from the VTA (Gerfen et al., 1987). Therefore, a site from the ventromedial CPu was pooled with a site from the NAc to represent the ventral striatum.

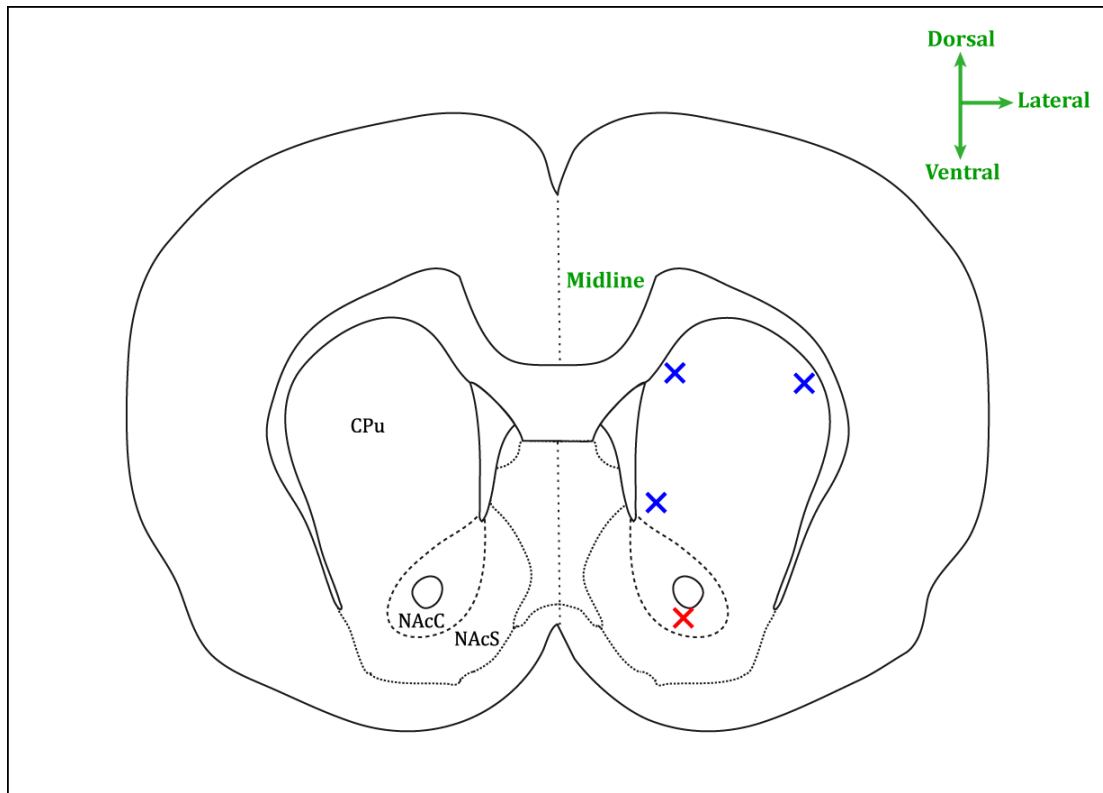


Figure 6.1: Coronal rat brain section sampled by FCV. Evoked DA release was sampled in acute coronal sections of rat striatum at bregma 1.8 mm. Due to the variation of DA release throughout the striatum, evoked $[DA]_o$ was sampled at 4 different sites in the CPu (3 blue sites) and NAc (red site). Individual sites were sampled in random order, with corresponding sites from human *LRRK2*-expressing and nTG rats sampled in immediate succession, and in the same bath. The different sites were approximately matched in all slices to control for regional variation, and to allow for sub regional analysis. Green arrows show dorsal-ventral, and lateral-medial axis. Diagram adapted from (Paxinos and Watson, 2007).

6.3. Results

6.3.1 G2019S rats at 18 months have a significant deficit in evoked [DA]_o in the dorsal striatum compared to nTG controls.

To investigate whether DA release is affected in G2019S rats, evoked striatal [DA]_o was sampled using FCV at a CFM. Due to regional variation of DA release, FCV was used to sample evoked [DA]_o at four distinct sites in the striatum. To explore the progressive nature of a possible phenotype, evoked DA was sampled in rats at 6, 12, and 18 months.

The monoamine detected fitted the electrochemical profile of DA (Figure 6.3 h). At age 6 months, electrically evoked [DA]_o was detected from each of the four sites sampled (Figure 6.2, a-d). Mean profiles of evoked [DA]_o vs. time show similar peak amplitudes of [DA]_o in G2019S and nTG rats. At age 12 months, evoked [DA]_o was also detected from each of the four sites sampled (Figure 6.2, e-h). Mean profiles of evoked [DA]_o vs. time show some regional variation with greater [DA]_o in dorsal than ventral sites. The peak amplitude of [DA]_o in G2019S and nTG rats are however similar at all sites. At age 18 months, evoked [DA]_o was detected from each of the four sites sampled (Figure 6.2, i-l). Unlike at ages 6- and 12 months however, at 18 months, evoked [DA]_o was reduced by up to 50% in the dorsal sites of G2019S vs. nTG rats (Figure 6.2, i,j). Evoked [DA]_o was similar in the ventral sites between G2019S and nTG rats at 18 months.

Data from individual sites was pooled into the dorsal region, which predominantly receives innervation from the nigrostriatal pathway, and the ventral region, which receives input from both the nigrostriatal and mesolimbic pathways (Gerfen et al., 1987; Prensa and Parent, 2001) (Figure 6.3). At age 6 months, evoked [DA]_o from both dorsal and ventral

regions is no different between G2019S and nTG rats (Figure 6.3 a,d). At age 12 months, evoked $[DA]_o$ from the dorsal and ventral regions is also no different between G2019S and nTG rats (Figure 6.3 b,e). However, at age 18 months, the average evoked $[DA]_o$ from the dorsal region is significantly reduced by about 30% in G2019S vs. nTG rats. Evoked $[DA]_o$ from the ventral region is no different between G2019S vs. nTG rats at 18 months. The peak values of evoked $[DA]_o$ were plotted in box and whiskers plots (Figure 6.3, g). The peak evoked $[DA]_o$ from G2019S and nTG rats are no different at 6- and 12 months. The peak evoked $[DA]_o$ from the dorsal region of G2019S and nTG rats at 18 months is significantly different at 0.36 μM and 0.55 μM respectively. The peak evoked $[DA]_o$ from the ventral region of G2019S and nTG rats at 18 months is no different (Figure 6.3 h). There appears to be a trend of total evoked $[DA]_o$ declining with age, however, adequate controls were only implemented to compare the two genotypes at each time point, and the data is not suitable for comparing DA release at different time points. Therefore, no comment can be made on changes to peak evoked $[DA]_o$ with age.

The data presented here reveal a deficit of evoked striatal $[DA]_o$ in G2019S rats vs. nTG controls. This deficit in DA release is most obvious in the dorsolateral striatum, and is also significant in the dorsal region of the striatum, which suggests that the nigrostriatal subpopulation of DA neurons is preferentially affected. Furthermore, this deficit is only observed at 18 months, and not at ages 6- or 12 months, which suggests an age dependent deficit of DA release.

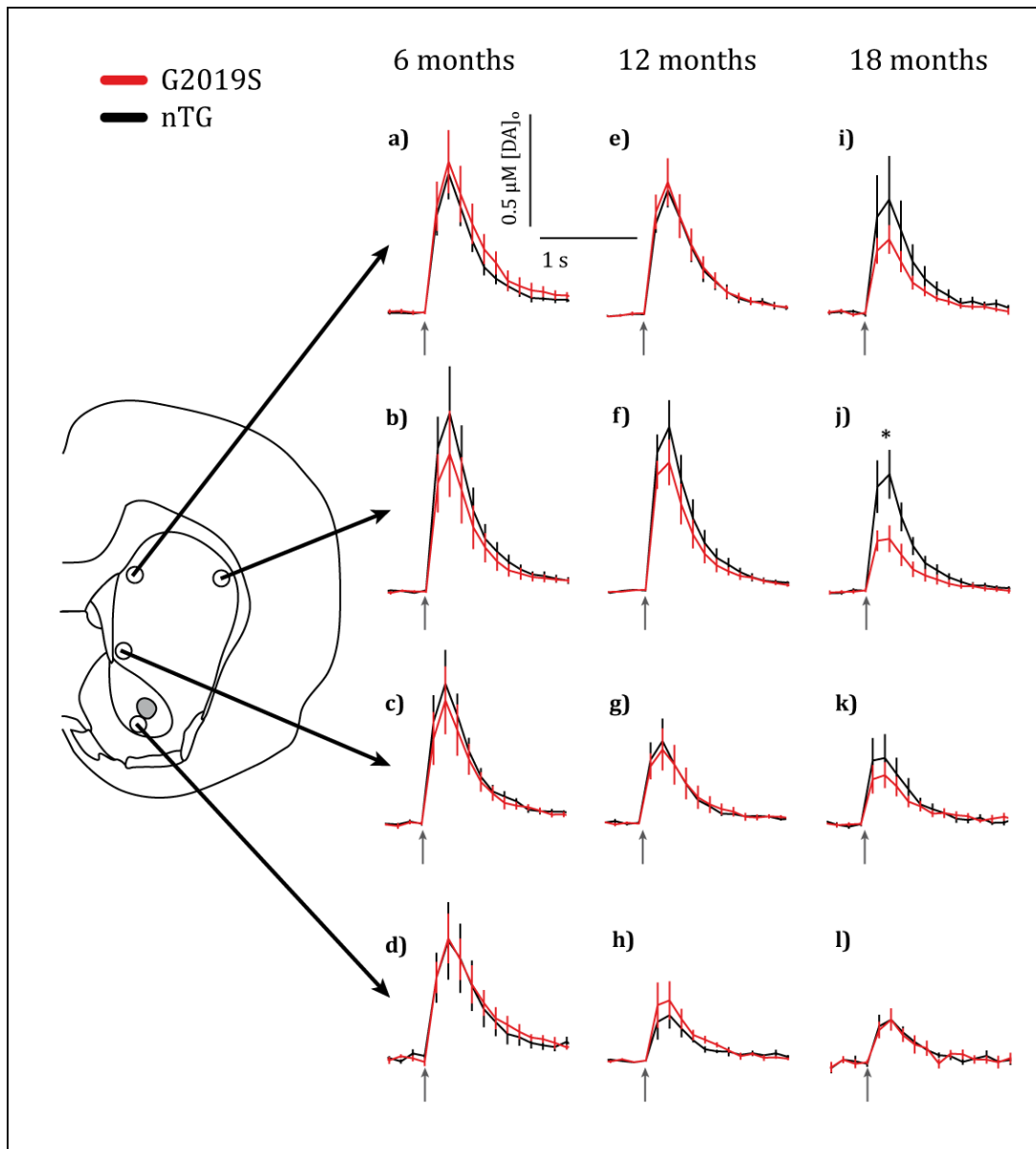


Figure 6.2: Evoked $[DA]_0$ from G2019S and nTG rats. Mean $[DA]_0$ profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in the dorsomedial CPu (a,e,i), dorsolateral CPu (b,f,j), ventromedial CPu (c,g,k), and NAc (d,h,l) of G2019S rats and nTG controls. Evoked $[DA]_0$ is reported at 6 months (a,b,c,d), 12 months (e,f,g,h), and 18 months (i,j,k,l). Experiments at different time points were Distribution of mean peak evoked $[DA]_0$ was not assumed to be normal after failing D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test, and not appearing normally distributed on Q-Q plot. Peak $[DA]_0$ is significantly reduced in G2019S vs. nTG controls in the dorsolateral site (j) at 18 months ($p < 0.05$, unpaired two-tailed Mann Whitney, $n = 19$). Peak $[DA]_0$ is however no different between G2019S vs. nTG controls in all other sites ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 5-17$). Data from the top two sites make up the dorsal region, and the bottom two sites make up the ventral region in Figure 6.3.

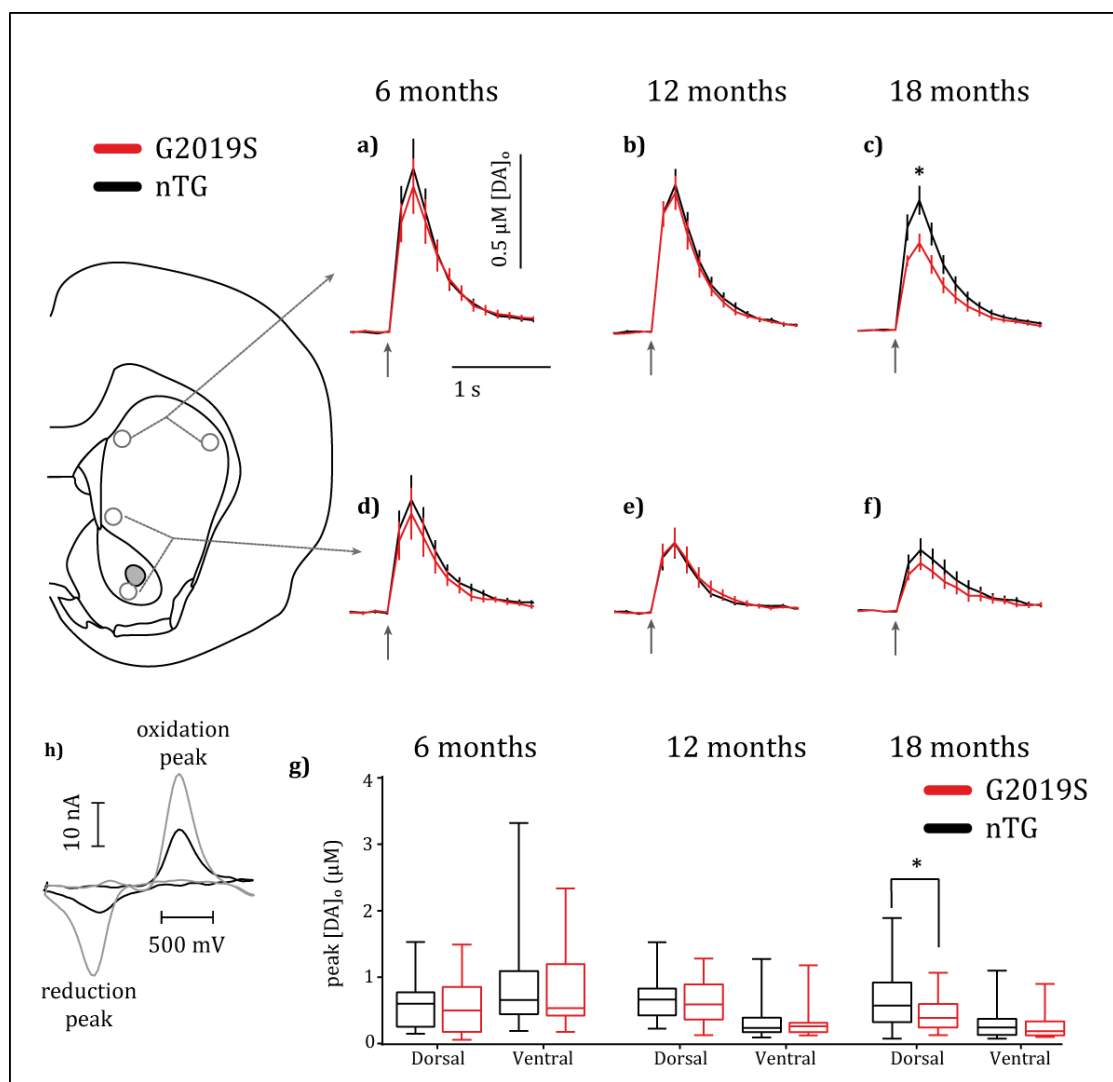


Figure 6.3. Pooled data of evoked $[DA]_0$ from G2019S and nTG rats. Mean $[DA]_0$ profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in the dorsal region (a,b,c) and ventral region (d,e,f) of G2019S rats and nTG controls. Evoked $[DA]_0$ is reported at 6 months (a,d), 12 months (b,e), and 18 months (c,f). Box and whiskers plots (g) of peak evoked $[DA]_0$ in dorsal and ventral regions at 6, 12, and 18 months, showing median, interquartile range, along with lowest- and highest observations. Cyclic voltammogram recorded in striatal tissue (h, black line) shows peaks at the same oxidation and reduction potential as a $2 \mu M$ DA calibration (h, grey line). Peak $[DA]_0$ is significantly reduced in G2019S vs. nTG controls in the dorsal region (c,g) at 18 months ($p < 0.05$, unpaired two-tailed Mann Whitney, $n = 36$). Peak $[DA]_0$ is however no different between G2019S vs. nTG controls in the dorsal region at different ages, or in the ventral region at any age ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 13-21$).

6.3.2 R1441C rats at 18 months have a significant deficit in evoked [DA]_o in the dorsal striatum compared to nTG controls.

To investigate whether DA release is affected in R1441C rats, evoked striatal [DA]_o was sampled using FCV at a CFM. Due to regional variation of DA release, FCV was used to sample evoked [DA]_o at four distinct sites in the striatum. To explore the progressive nature of a possible phenotype, evoked DA was sampled in rats at 6, 12, and 18 months.

At age 6 months, electrically evoked [DA]_o was detected from each of the sites sampled (Figure 6.4, a-d). Mean profiles of evoked [DA]_o vs. time show similar peak amplitudes of [DA]_o for R1441C and nTG rats. At age 12 months, evoked [DA]_o was also detected from each of the four sites sampled (Figure 6.4, e-h). Mean profiles of evoked [DA]_o vs. time show some regional variation with a greater [DA]_o in dorsal than ventral sites. The peak amplitude of [DA]_o in R1441C and nTG rats are however similar at all sites. At age 18 months, evoked [DA]_o was detected from each of the four sites sampled (Figure 6.4, i-l). Unlike at ages 6- and 12 months however, at 18 months, evoked [DA]_o was reduced by up to 40% in the dorsal sites of R1441C vs. nTG rats (Figure 6.4, i,j). Evoked [DA]_o was similar in the ventral sites between R1441C and nTG rats at 18 months.

Data was pooled into dorsal and ventral regions as in section 6.3.1, and statistical analyses were performed (Figure 6.5). At age 6 months, evoked [DA]_o from both dorsal and ventral regions is no different between R1441C and nTG rats (Figure 6.5 a,d). At age 12 months, evoked [DA]_o from the dorsal and ventral regions is also no different between R1441C and nTG rats (Figure 6.3 b,e). However, at age 18 months, the average evoked [DA]_o from the dorsal region is significantly reduced by about 30% in R1441C vs. nTG rats. Evoked [DA]_o from the ventral region is no different between R1441C vs. nTG rats at 18 months. The

peak values of evoked $[DA]_o$ were plotted in box and whiskers plots (Figure 6.5, g). The peak evoked $[DA]_o$ from R1441C and nTG rats are no different at 6- and 12 months. The peak evoked $[DA]_o$ from the dorsal region of R1441C and nTG rats at 18 months is significantly different at 0.23 μM and 0.47 μM respectively. The peak evoked $[DA]_o$ from the ventral region of R1441C and nTG rats at 18 months is no different. As with the G2019S rats, there appears to be a trend of total evoked $[DA]_o$ declining with age, however, because adequate controls were only implemented to directly compare the two genotypes, and no comment can be made on changes to peak evoked $[DA]_o$ with age.

The data presented here reveal a deficit of evoked striatal $[DA]_o$ in R1441C rats vs. nTG controls. This deficit in DA release is specific to the dorsal region of the striatum, which suggests that the nigrostriatal sub-population of DA neurons is preferentially affected. Furthermore, this deficit is only observed at 18 months, and not at ages 6- or 12 months, which suggests an age-dependent deficit.

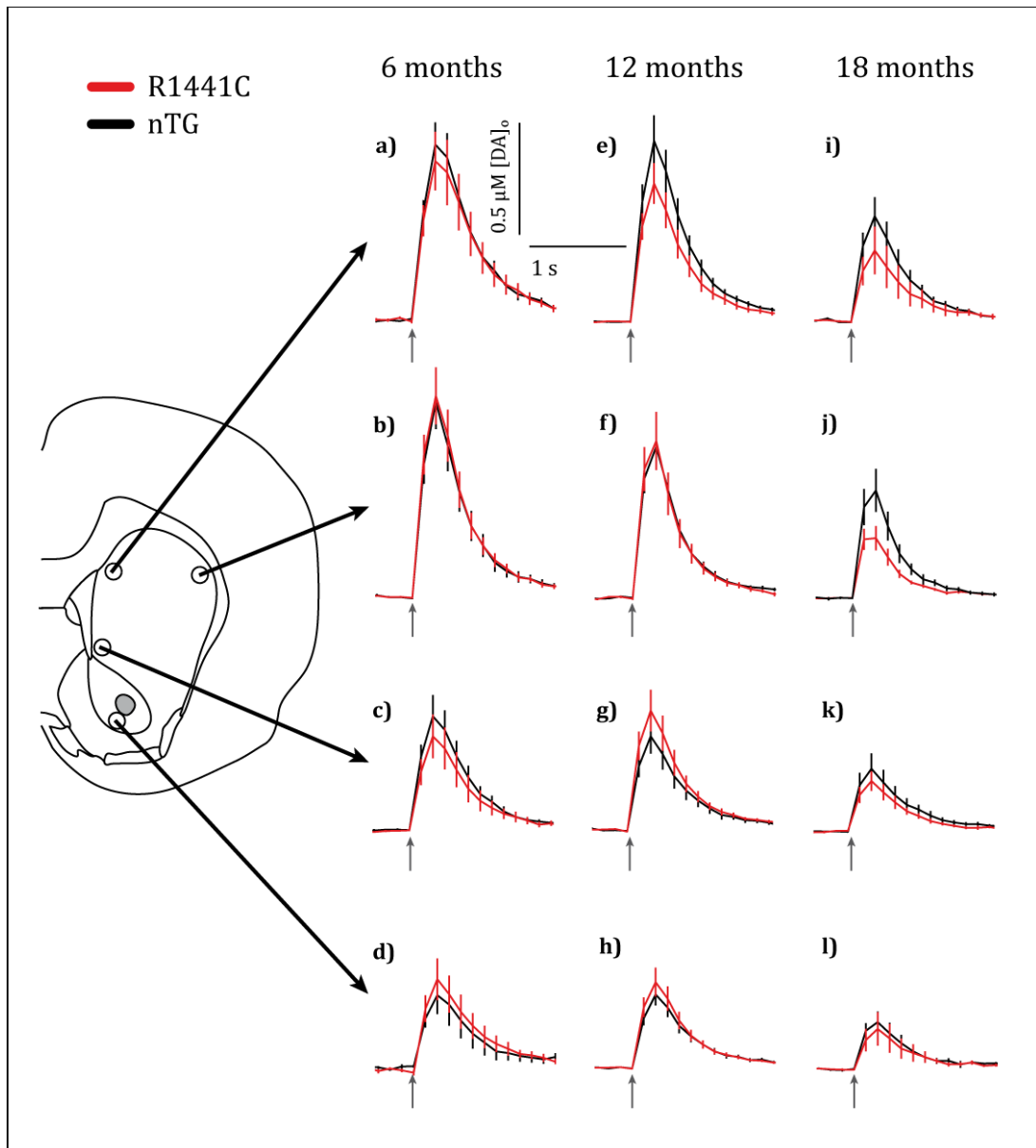


Figure 6.4: Evoked $[DA]_0$ from R1441C and nTG rats. Mean $[DA]_0$ profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in the dorsomedial CPu (a,e,i), dorsolateral CPu (b,f,j), ventromedial CPu (c,g,k), and NAc (d,h,l) of R1441C rats and nTG controls. Evoked $[DA]_0$ is reported at 6 months (a,b,c,d), 12 months (e,f,g,h), and 18 months (i,j,k,l). Distribution of mean peak evoked $[DA]_0$ was not assumed to be normal after failing D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test, and not appearing normally distributed on Q-Q plot. Peak $[DA]_0$ is however not significantly different between R1441C vs. nTG controls in any individual sites ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 5-15$). The top two sites make up the dorsal region, and the bottom two sites make up the ventral region in Figure 6.5.

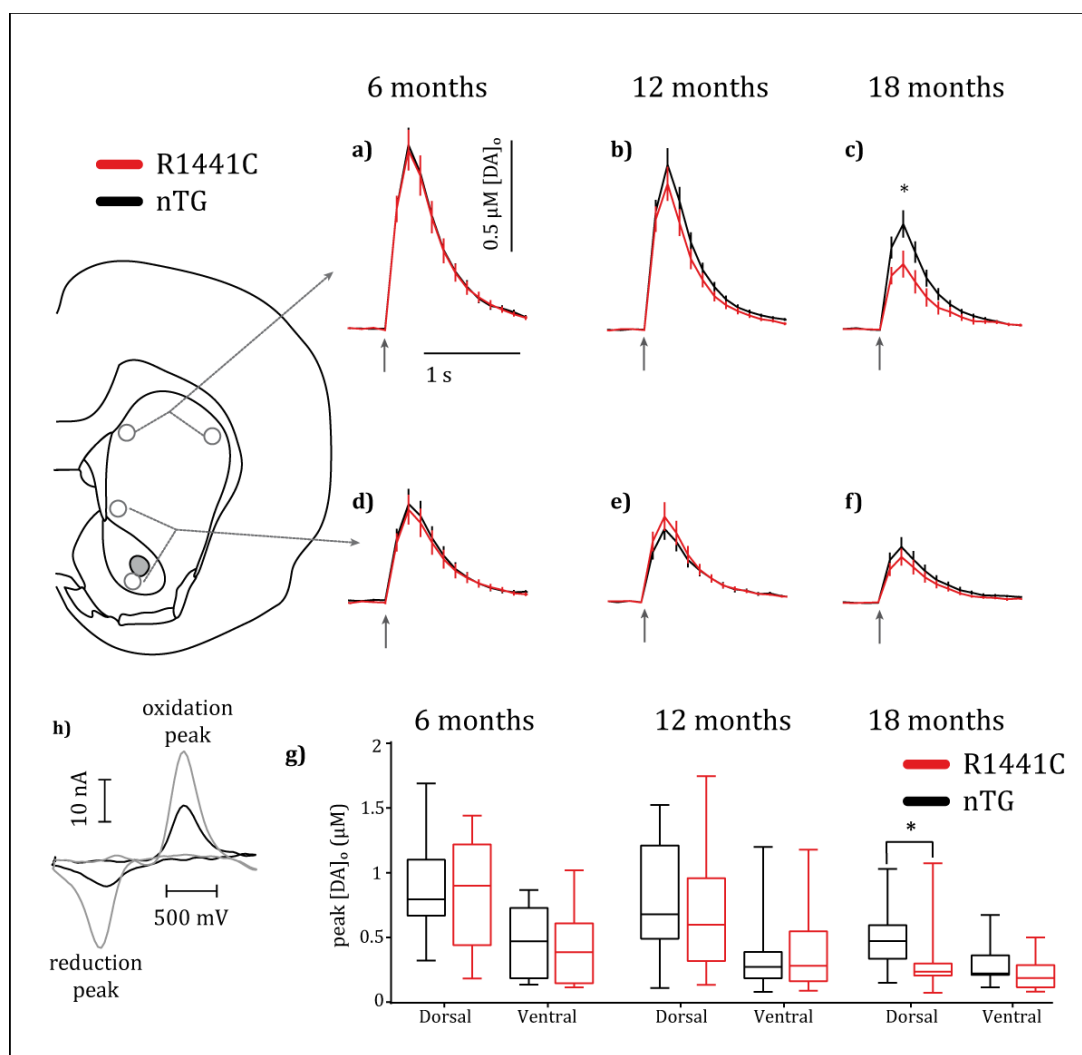


Figure 6.5: Pooled data of evoked $[DA]_0$ from R1441C and nTG rats. Mean $[DA]_0$ profiles vs. time (mean $\pm$ SEM) following single pulse stimulation ($\uparrow$) in the dorsal region (a,b,c) and ventral region (d,e,f) of R1441C rats and nTG controls. Evoked $[DA]_0$ is reported at 6 months (a,d), 12 months (b,e), and 18 months (c,f). Box and whiskers plots (g) of peak evoked $[DA]_0$ in dorsal and ventral regions at 6, 12, and 18 months, showing median, interquartile range, along with lowest- and highest observations. Cyclic voltammogram recorded in striatal tissue (h, black line) shows peaks at the same oxidation and reduction potential as a $2 \mu M$ DA calibration (h, grey line). Peak $[DA]_0$ is significantly reduced in R1441C vs. nTG controls in the dorsal region (c,g) at 18 months ($p < 0.05$, unpaired two-tailed Mann Whitney, $n = 36$). Peak $[DA]_0$ is however no different between R1441C vs. nTG controls in the dorsal region at different ages, or in the ventral region at any age ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 13-21$).

6.3.3 DA release deficits in G2019S and R1441C rats are both due to changes in DA releasability

Data from the previous sections show a progressive decline in evoked $[DA]_o$ from the dorsal striatum of R1441C and G2019S rats vs. nTG controls. There are a number of factors that affect the amount of evoked $[DA]_o$ including the amount of DA content, DA releasability from DA neurons, and the rate of DA uptake through DAT.

To investigate whether there was a reduction in the amount of striatal DA content, HPLC with electrochemical detection was used to quantify DA from striatal punches. For G2019S rats, DA was detected in both the CPu and NAc regions of the striatum at all ages (Figure 6.6, a). DA content in the dorsal striatum was about twice that of the ventral striatum. However, no significant reduction in DA content could be found in G2019S rats at any age compared to nTG controls. For R1441C rats, DA was also detected in both the CPu and NAc regions of the striatum at all ages (Figure 6.6, b). As with G2019S rats, no significant reduction in DA content of R1441C rats could be found at any age. These data reveal no changes to DA content in either G2019S or R1441C rats, suggesting that the deficit of evoked $[DA]_o$ seen in the previous two sections is not due to a loss of DA content.

As with the FCV data, there appears to be changes to DA content between mice with the same genotype at different ages, however, adequate controls were only implemented to compare the two genotypes within each age category, so no comment can be made about whether DA content changes with age in either genotype.

To investigate whether there were any changes to the rate of DA uptake in G2019S and R1441C rats, the decay phase of $[DA]_o$ profiles were compared, which depends on the rate of DA uptake (Anwar et al., 2011; Janezic et al., 2013). If the deficit of evoked $[DA]_o$ in 18-month old G2019S and R1441C rats is due to an increased rate of uptake, then the decay phase of $[DA]_o$ profiles can be expected to take a shorter amount of time, and therefore be at a steeper angle. The uptake of extracellular DA by DAT is concentration dependent, and the rate of uptake was therefore compared in the same DA concentration range. The decay slopes of $[DA]_o$ profiles in the dorsal striatum of 18-month old G2019S and R1441C rats are no different from nTG controls, (Figure 6.6 c,d). These data reveal that the deficit of evoked $[DA]_o$ in 18-month old G2019S and R1441C rats is not paired with a quicker decay of $[DA]_o$ profiles, and is therefore not due to an increase in the rate of DA uptake.

Taken together, the data presented here shows that the deficit of evoked $[DA]_o$ in 18-month old G2019S and R1441C rats is not due to changes in DA content, or the rate of DA uptake. The DA release deficits in G2019S and R1441C rats are therefore due to a reduction in DA releasability.

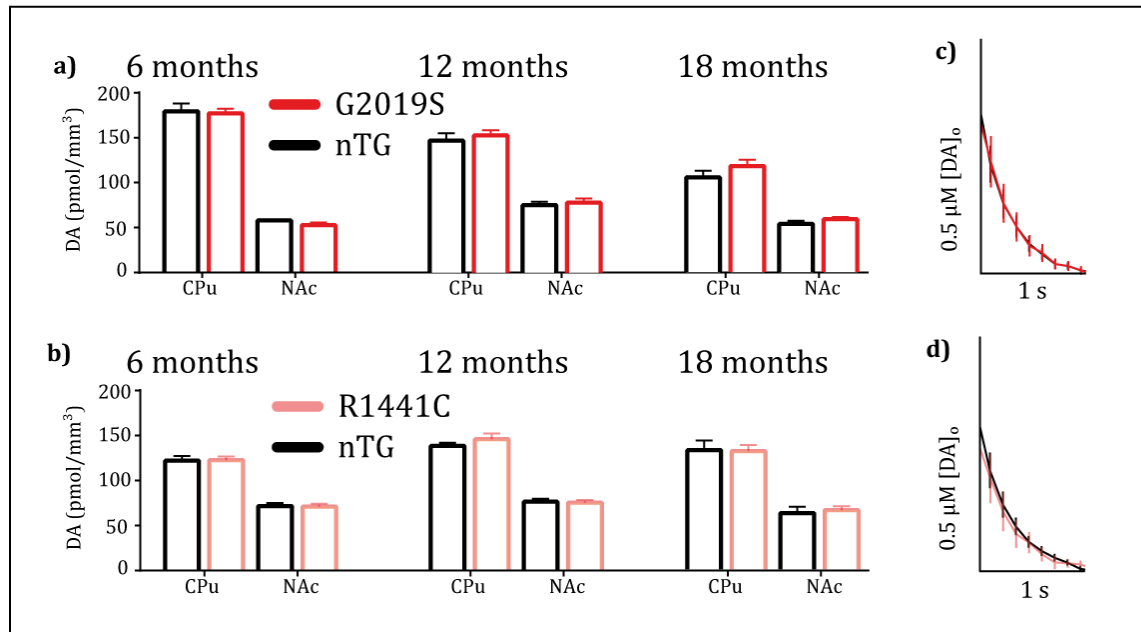


Figure 6.6: DA content, rate of DA uptake, and 4p:1p ratios a&b) Striatal DA content in G2019S (a) and R1441C (b) rats. Data was not assumed to be normally distributed after some data failed D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality tests, and didn't appear normally distributed on Q-Q plot. No significant difference in DA content was found between G2019S or R1441C rats and nTG controls in either dorsal or ventral regions at any age ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 6-10$). c&d) Decay phase of mean [DA]₀ profiles vs. time (mean $\pm$ SEM) from the dorsal striatum of G2019S (c) and R1441C (d) rats at 18 months. Profiles for G2019S and R1441C rats were taken from Figure 6.3, c and Figure 6.5, c respectively, and adjusted along the X-axis to align with nTG control.

6.3.4 Exploring changes in DA releasability of G2019S and R1441C rats

Data from the previous sections have shown that G2019S and R1441C rats have a DA release deficit in the dorsal striatum at 18 months, which is due to a reduction in DA releasability.

To further explore the reduced releasability of DA in the dorsal striatum of G2019S and R1441C rats, changes to the release probability was investigated. DA neurons in rats have been shown to fire action potentials at frequencies of up to 100 Hz (Hyland et al., 2002). Furthermore, when DA neurons are stimulated by more than one electrical pulse, they undergo short-term synaptic plasticity, with ratio of multi-pulse release to 1p being inversely proportional to the initial release probability (Cragg, 2003; Rice and Cragg, 2004). Therefore, if the DA release deficit in the dorsal striatum of G2019S and R1441C rats involves any changes to release probability, then a difference in the ratio of multi-pulse release to 1p release can be expected. To investigate whether there are any changes in the release probability of DA from G2019S and R1441C rats, $[DA]_o$ evoked from four pulse train stimulation (4p) at 100 Hz was sampled and normalised to the peak $[DA]_o$ evoked by 1p stimulations (Figure 6.7 a-d). For G2019S rats, normalised profiles of $[DA]_o$ evoked from 4p stimulations are very similar to nTG controls in both the dorsal- (Figure 6.7 a) and the ventral (Figure 6.7 c) striatum. Likewise, for R1441C rats, normalised profiles of $[DA]_o$ evoked from 4p stimulations are very similar to nTG controls in both the dorsal- (Figure 6.7 b) and the ventral (Figure 6.7 d) striatum. No significant difference was found in peak $[DA]_o$ evoked from 4p stimulations. These data reveal no changes to the 4p:1p ratio for G2019S and R1441C rats at 18 months, suggesting that the DA release deficit is not due to changes in release probability.

Some factors that control DA releasability in the striatum including, but not limited to, activation of presynaptic nAChRs and nitric oxide (NO) signalling, can affect the dependence of the magnitude of DA release on the frequency of electrical stimulation (Exley et al., 2007; Hartung et al., 2011). If any such changes are involved in the DA release deficit observed in G2019S and R1441C rats, then a corresponding difference in the frequency dependence of DA release can be expected. To investigate the possibility of factors that relate to frequency sensitivity, such as nAChRs activation or nitric oxide signalling, playing a role in the DA release deficit of G2019S and R1441C rats, evoked $[DA]_o$ was sampled at stimulation frequencies of 25 Hz and 100 Hz. DA was evoked using 4-pulse train stimulations at 25 Hz and 100 Hz in the dorsal striatum of 18-month old G2019S and R1441C rats, along with nTG controls. For the G2019S rats, peak $[DA]_o$ evoked from 4-pulse train stimulations at 25 Hz and 100 Hz was no different from nTG controls (Figure 6.7, e). Likewise for the R1441C rats, peak $[DA]_o$ evoked from 4-pulse train stimulations at 25 Hz and 100 Hz was no different from nTG controls (Figure 6.7, f). The data here shows that the DA release deficit in G2019S and R1441C rats does not involve changes to the stimulation frequency dependence of DA release, and suggests that there are no changes to nAChR activity or NO signalling.

A previous study using BAC transgenic mice with the G2019S *LRRK2* mutation revealed a greater decline in peak evoked $[DA]_o$ from consecutive stimulations at 2 min intervals. Such data suggest that striatal DA synapses with the G201S mutation have an impaired ability to maintain consistent DA release over a long time period. To investigate whether the G2019S and R1441C rats described here have an impaired ability to maintain DA release, electrically evoked $[DA]_o$ was sampled three times over a period of 5 min with a recovery

time of 2.5 min. Evoked $[DA]_o$ from the third sample (3rd 1p) was normalised to the peak $[DA]_o$ from the first sample (1st 1p), and plotted over time (Figure 6.8 a-d). For G2019S rats, normalised profiles of $[DA]_o$ evoked from the 3rd 1p are very similar to nTG controls in both the dorsal- (Figure 6.8 a) and the ventral (Figure 6.8 c) striatum. Likewise for R1441C rats, normalised profiles of $[DA]_o$ evoked from the 3rd 1p are very similar to nTG controls in both the dorsal- (Figure 6.8 a) and the ventral (Figure 6.8 c) striatum, with profiles overlapping. The ratio between the peak $[DA]_o$ from the 3rd stimulation vs. 1st stimulation (3rd 1p: 1st 1p ratio) in the dorsal striatum was also calculated (Figure 6.8 e). The median 3rd 1p: 1st 1p ratios of 18-month old G2019S rats and matching nTG controls are 0.83 and 0.81 respectively. The median 3rd 1p: 1st 1p ratios of 18-month old R1441C rats and matching nTG controls are 0.65 and 0.71 respectively. These data show no significant change to the normalised 3rd 1p: 1st 1p of either G2019S or R1441C rats at 18 months, which suggests that the DA release deficit in these lines is not accompanied by an impaired ability to maintain DA release over a period of five minutes.

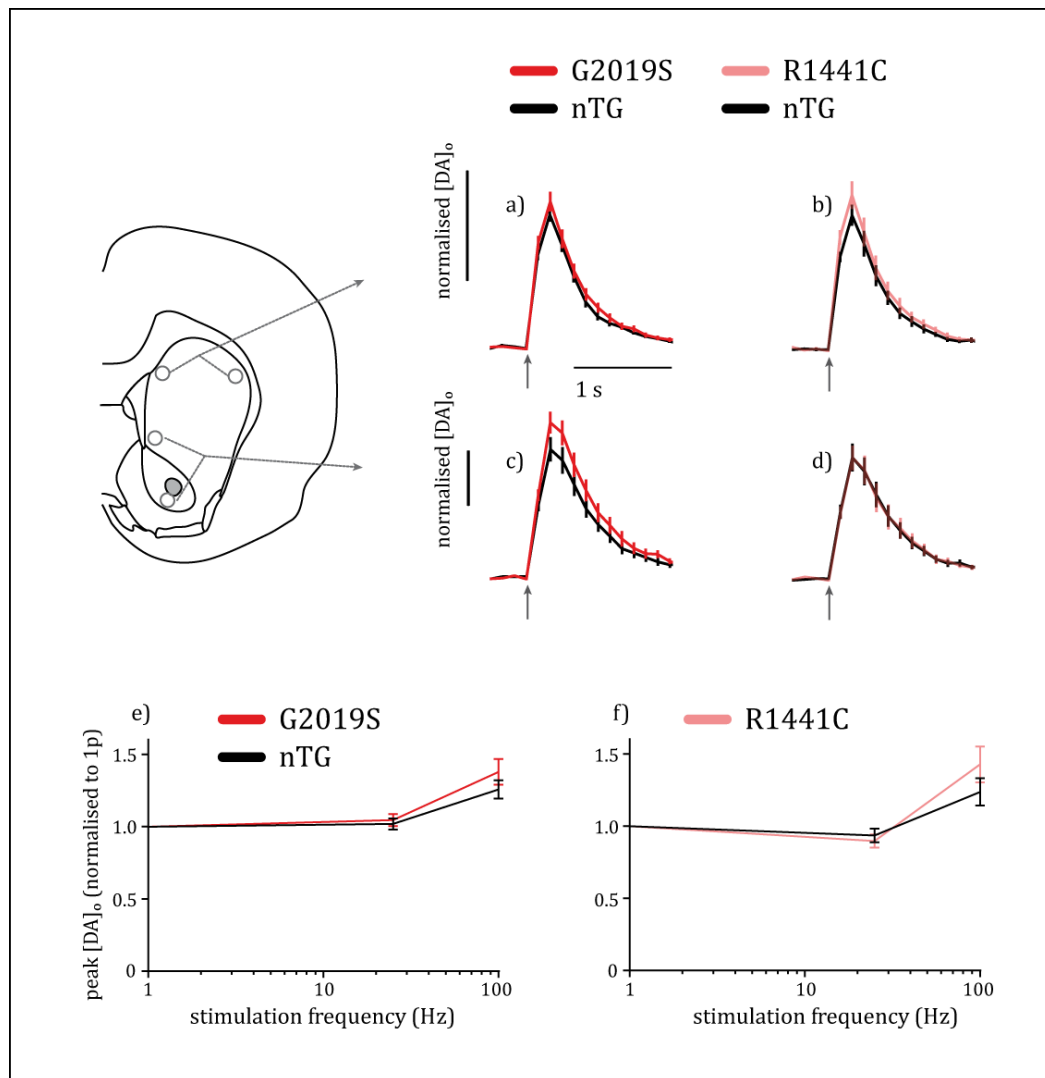


Figure 6.7: Effects of G2019S and R1441C mutations on [DA]_o evoked by different frequency stimulations. a-d) Mean profiles of evoked [DA]_o (mean $\pm$ SEM) from 4p stimulations at 100Hz (normalised to peak [DA]_o from 1p stimulation). Profiles are from G2019S (a,c) and R1441C (b,d) rats, recorded in dorsal (a,b) and ventral (c,d) striatum. No significant difference in peak [DA]_o was found between G2019S or R1441C rats and nTG controls ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 14-35$). e) Frequency response of [DA]_o evoked from 4-pulse train stimuli at 25 Hz and 100Hz in G2019S rats (mean peak [DA]_o $\pm$ SEM). f) Frequency response of [DA]_o evoked from 4-pulse train stimulations at 25 Hz and 100Hz in R1441C rats (mean peak [DA]_o $\pm$ SEM). No significant difference in the frequency response was found between G2019S or R1441C rats and nTG controls ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 14-35$). No data was assumed to be normally distributed after some data in each set failed D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality tests, and didn't appear normally distributed on Q-Q plot.

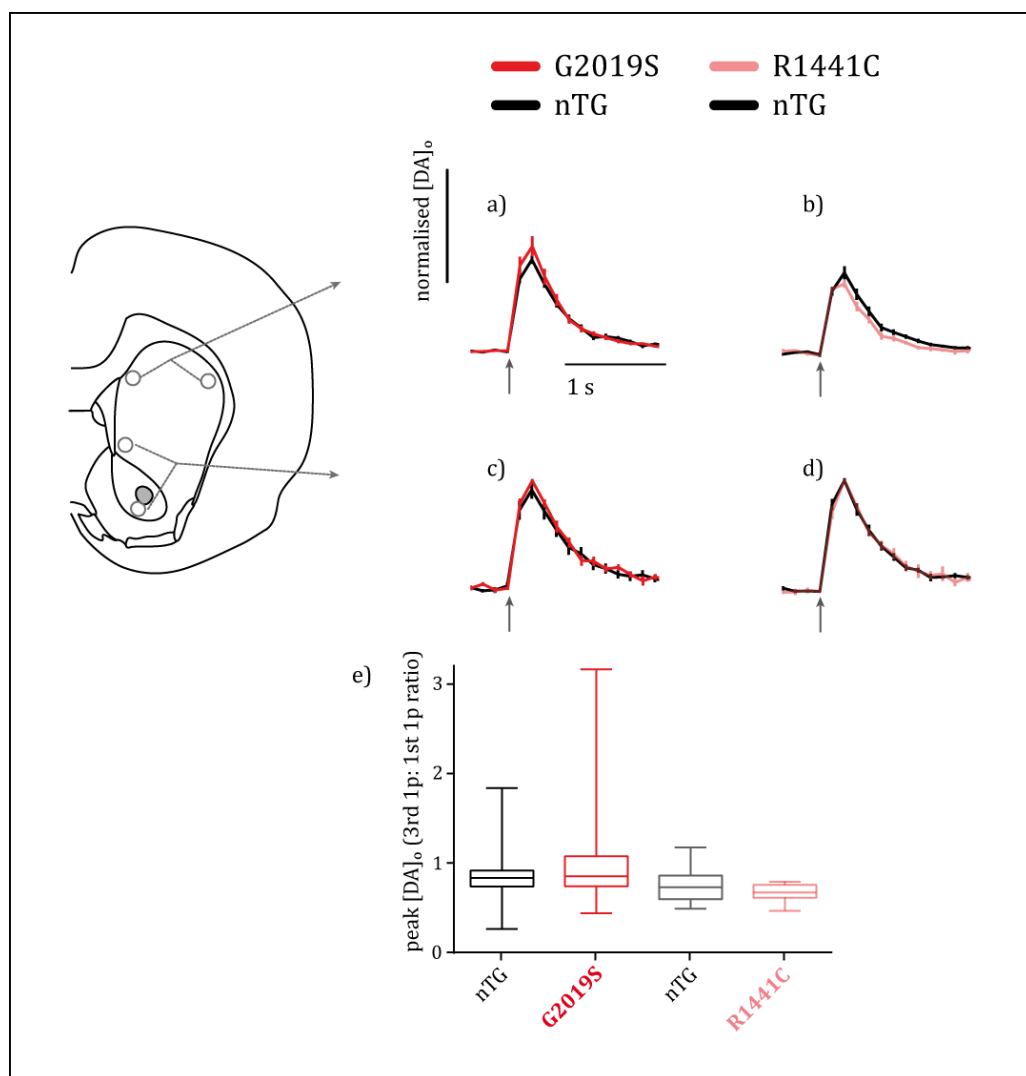


Figure 6.8: Ability of G2019S and R1441C rats to maintain evoked DA release. Mean profiles of evoked [DA]_o (mean $\pm$ SEM) from 3rd 1p stimulation delivered 5 min after naïve release (normalised to peak [DA]_o from 1st 1p stimulation). Profiles are from G2019S (a,c) and R1441C (b,d) rats, recorded in dorsal (a,b) and ventral (c,d) striatum. e) Box and whiskers graph showing the ratio of peak [DA]_o (median peak [DA]_o $\pm$ interquartile range) released in the dorsal striatum from the third stimulation (3rd 1p): peak [DA]_o released from the first stimulation (1st 1p), with each stimulation separated by 2.5 min. Second stimulation data is not shown. No significant difference in the 3rd 1p:1st 1p ratio was found between G2019S or R1441C rats and nTG controls ($p > 0.05$, unpaired two-tailed Mann Whitney and Kolmogorov-Smirnov, $n = 15-36$). No data was assumed to be normally distributed after some data in each set failed D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality tests, and didn't appear normally distributed on Q-Q plot.

6.4. Discussion

This chapter set out to explore whether the G2019S and R1441C rats created by the OPDC had any changes to evoked striatal $[DA]_o$ release. The data presented here shows a deficit in evoked $[DA]_o$ from the dorsal striatum of R1441C and G2019S rats vs. nTG controls (Section 6.3.1,2). This deficit is present only at 18 months, and not at 6- or 12 months, which suggests that the effects of mutant *LRRK2* on these rats are age-dependent. Observing a deficit of evoked $[DA]_o$ in the dorsal striatum, which is predominantly innervated by DA neurons from the SNc, and not in the ventral striatum, which is predominantly innervated by DA neurons from the VTA (Gerfen et al., 1987; Voorn et al., 2004), is also in line with previous findings that DA neurons in the SNc are more susceptible to being affected during PD (Gibb and Lees, 1991; Kordower et al., 2013).

The DA release deficit in G2019S and R1441C rats was not caused by a reduction in striatal DA content or an increased rate of DA uptake (Section 6.3.3), and is therefore due to a reduction in DA releasability. The releasability of DA can be influenced by many different factors including, intracellular $[Ca^{2+}]$ buffering, vesicle pool mobilisation, and availability of DA vesicles at the active zone (Cragg, 2003; Cragg et al., 1997b; Stamford et al., 1988). A few of these mechanisms have been probed indirectly in this chapter in an attempt to narrow down the possible mechanisms responsible for the DA release deficit. For example, the 4p:1p ratio was explored in G2019S and R1441C rats, to report on the release probability of DA, which is influenced by factors that include intracellular $[Ca^{2+}]$ buffering and availability of DA vesicles at the active zone. No changes were found to the 4p:1p ratio or frequency response of evoked $[DA]_o$ in G2019S or R1441C rats, which suggests that the

DA release deficit is not due to changes in release probability (Section 6.3.4). However, the 4p:1p ratio is not the most sensitive test for the factors mentioned here, and more tests may still be needed to investigate the role of intracellular $[Ca^{2+}]$ buffering, availability of readily releasable DA vesicles, and the SNARE machinery function in the DA release deficit of G2019S and R1441C rats. The frequency dependence of DA release was explored in G2019S and R1441C rats, to report on changes to nAChR receptor function or NO signalling. No changes were found to the frequency dependence of DA release in G2019S or R1441C rats, which suggests that the DA release deficit is not due to changes in nAChR receptor function or NO signalling (Section 6.3.4). However, sampling the frequency dependence of DA release is not the most sensitive test for ACh or NO signalling, and more sensitive measures, such as sampling DA in the presence of DH β E may be required.

A previous study using BAC transgenic mice revealed that the G2019S mutation reduces the capacity for maintaining consistent DA release (Li et al., 2010). This finding may report on the capacity of DA synapses to maintain the readily releasable pool of presynaptic vesicles, and a test was therefore performed to investigate the ability of G2019S and R1441C rats to maintain consistent DA release. No changes were found in the 3rd 1p: 1st 1p ratios of either G2019S or R1441C rats, revealing no changes in the capacity to sustain DA release over a period of 5 min (Section 6.3.4). Due to limitations in slice viability, the test reported here was however only carried out over five minutes, and the test by *Li et al.* was performed over 20 minutes, with a maximum deficit in DA release in G2019S transgenic mice appearing after around 14 minutes. It may therefore be possible that G2019S or R1441C rats have a greater decline in peak evoked $[DA]_o$ when tested over a longer time period.

The deficit of evoked DA seen here in R1441C and G2019S rats is in agreement with previous reports of *LRRK2* models. Previous investigations in mice that express the G2019S mutant form of *LRRK2* in a BAC transgene showed lower baseline [DA]_o detected by microdialysis, an age-dependent decrease in striatal DA content, along with changes to DA uptake, and evoked release detected by FCV (Li et al., 2010; Melrose et al., 2010). The deficit in DA release found by *Li et al.*, albeit progressive, was however accompanied by a comparable deficit in DA content (Li et al., 2010). The investigation by *Melrose et al.* did not find changes to DA content, which is in agreement with data presented here (Melrose et al., 2010). The study by *Melrose et al.* did however use microdialysis in the presence of amphetamine, which does not report on DA releasability. The data in this chapter therefore presents the novel finding that the G2019S mutant form of *LRRK2* has a progressive effect on DA releasability in the dorsal striatum. Other models that express the G2019S mutant form of *LRRK2* under BAC, doxycycline inducible, or PDGF promoters found impaired DAT activity, which is not observed from the G2019S rats studied here (Li et al., 2010; Zhou et al., 2011; Chen et al., 2012). It might be possible that impaired DAT activity is a feature of more severe effects of synapse dysfunction, and might be present in G2019S rats that are older than 18 months.

One study that expressed the R1441C mutant form of *LRRK2* in BAC transgenic mice found reduced striatal [DA]_o detected from microdialysis *in vivo* (Li et al., 2009). This study however, like *Melrose et al.*, used microdialysis, which does not report on DA releasability. Another study on chromaffin cells with the R1441C mutation also found reduced catecholamine release (Tong et al., 2009), which is in agreement with the results presented here. Chromaffin cells, however, do not release DA *in vivo*, which means the data in this

chapter presents the novel finding that the R1441C mutant form of LRRK2 has a progressive effect on DA releasability in the dorsal striatum.

The mechanism by which mutant LRRK2 affects DA releasability in the animals tested here may play a role in early stages LRRK2-related pathology, and is therefore relevant to understanding the underlying mechanisms of PD. LRRK2 is associated with presynaptic vesicles where it has been shown to affect neurotransmission (Shin et al., 2008; Piccoli et al., 2011). Previously suggested roles for LRRK2 however mainly relate to endocytosis (Belluzzi et al., 2012), which is unlikely to be a direct contributor to the data presented here since no changes to the rate of DA uptake were observed. Furthermore, the progressive effects of mutant LRRK2 seen here might not be due to LRRK2 function in a healthy state, and will be discussed further in chapter 7. Data presented here suggest that there are no changes to short-term DA release probability or frequency dependence. Future work beyond the scope of this project should explore whether DA release in G2019S or R1441C rats at 18 months have changes in dependence on D₂ autoreceptor activation, Ca²⁺ regulation, nAChR activation, changes to presynaptic SNARE machinery, and presynaptic vesicle mobilisation.

The findings presented in this chapter reveal that the G2019S and R1441C mutations of human LRRK2 can cause neurotransmission deficits in nigrostriatal DA neurons, which is due to a reduction in DA releasability. These data contribute to a broader understanding of presynaptic dysfunction caused by LRRK2 mutations, and also provide a basis for further investigations into G2019S and R1441C rats as a novel model of PD.

CHAPTER 7. Discussion

This thesis focuses on how monoamine synapse function is affected in several transgenic models relating to PD. Monoamine content and evoked monoamine release was investigated in mice that express different levels of *SNCA*, and rats overexpressing different mutations of *Leucine Rich Repeat Kinase 2 (LRRK2)*.

The following sections will firstly discuss the findings in mice that express human α -synuclein, and a potential mechanism by which α -synuclein affects neurotransmitter release will be suggested. Secondly, the findings in rats that express human LRRK2 will be discussed, and suggestions will be made for future directions of investigations. Finally, all the findings reported here will be drawn together to discuss the implications for PD.

7.1.1 Neurotransmission deficits in transgenic models of PD

The two genes investigated in this thesis, *SNCA* and *LRRK2*, both cause neurotransmission deficits in their pathogenic forms. Expressing high levels of WT α -synuclein, which is coded for by *SNCA*, causes deficits in evoked striatal DA release. Likewise, expressing the G2019S or R1441C mutant forms of *LRRK2* also causes deficits in evoked striatal DA release. For both of these models, neurotransmission deficits are among the first phenotypes displayed (Janezic et al. 2013, personal communication). When taking these data together, there appears to be a common theme that PD-related genotypes cause neurotransmission deficits as one of the earliest stages of pathophysiology. This idea is supported by previous observations of α -synuclein overexpression (Lundblad et al., 2012; Nemani et al., 2010), and mutant forms of *LRRK2* (Li et al., 2010, 2009). Furthermore, neurotransmission deficits that precede other major phenotypes have been observed in other rodent models with PD-related genes. For example, mice with a deletion of *DJ-1*, which is associated with early onset Parkinsonism, have a reduction in evoked striatal $[DA]_o$ in the absence of nigrostriatal cell loss (Goldberg et al., 2005). Likewise, mice with a deletion of *Parkin*, which is also associated with early onset Parkinsonism, have an increase in basal striatal $[DA]_o$ in the absence of nigrostriatal cell loss (Goldberg et al., 2003). Furthermore, mice with a deletion of *PINK1*, yet another condition associated with PD, have a decrease in evoked striatal $[DA]_o$, without any cell loss or changes to DA content. Taken together, the findings presented here add to a growing body of literature highlighting neurotransmission deficits as one of the earliest stages of PD pathophysiology.

The deficits in DA release caused by expressing high levels of WT α -synuclein or mutant *LRRK2* have several factors in common, which may help elucidate the underlying

mechanisms by which these proteins affect neurotransmission. Firstly, the deficit in DA release is restricted to the dorsal part of the striatum (chapter 3.3.1, chapter 6.3.1-2), and not observed in the ventral striatum. This highlights a common regional selectivity for the effects of α -synuclein and LRRK2, which suggests that these two proteins may utilise a converging downstream mechanism. Secondly, the DA release deficit caused by expressing high levels of WT α -synuclein, or mutant LRRK2, did not depend on cell loss in the SNc or changes to DA content, suggesting that the protein produced from these genes did not make use of mechanisms involving increased apoptosis, significant changes to DA production, or any other gross intracellular pathology. Thirdly, the deficit of DA release caused by both genes was mediated through changes in DA releasability. The observation that DA releasability is affected, rather than DA production or cell death, suggests that the effect of α -synuclein and LRRK2 on DA release is primarily at the axon terminals.

Moreover, observing changes to neurotransmitter releasability as one of the earliest signs of cell dysfunction in transgenic PD models, suggests that dysfunction at the axon terminal may be involved in one of the earliest stages of PD pathogenesis. The exact mechanism by which DA releasability is affected remains unknown, and certainly deserves further investigation beyond the scope of this project. The exact mechanism by which α -synuclein- or LRRK2-related pathophysiology contribute towards other cell pathology, such as protein aggregation and mitochondrial dysfunction, remains unclear, but will be discussed more in section 7.2.3

In contrast to the factors that expressing high levels of WT α -synuclein or mutant LRRK2 have in common in the investigations mentioned here, at least one factor is worth

mentioning that these two models do not have in common. The SNCA-C mice show an early and sustained deficit in DA release starting at 3 months and observable to a similar extent at 18 months (chapter 3.3.1, Janezic et al., 2013). Furthermore a lower dose of α -synuclein expressed in SNCA-B mice, has no effect on DA release, which suggests that the effect of α -synuclein overexpression on DA release has is more dependent on the dose of α -synuclein, than the age of the animal. In contrast to this, the G2019S and R1441C rats have no deficit in DA release at 6- or 12 months, but develop a deficit at 18 months (chapter 6.3.1-2). This suggests that the effects of mutant LRRK2 strongly depend on the age of the animal. However, the observation that SNCA-C mice develop a DA release deficit at a much younger age than G2019S or R1441C rats, does not rule out the possibility that the effects of α -synuclein and LRRK2 are mediated through a converging downstream mechanism. Therefore, the data discussed here does not allow us to directly comment on whether there is any overlap in the mechanisms by which α -synuclein and LRRK2 affect evoked striatal DA release.

7.2. Overexpressing human α -synuclein affects DA releasability in nigrostriatal DA neurons.

Chapters 3, 4, and 5 of this thesis investigated changes to evoked monoamine (DA and 5-HT) release in mice that express varying levels of human α -synuclein. In chapter 3, earlier findings were reproduced that SNCA-C mice, which express human α -synuclein at levels 5-6 fold of human brain tissue, have a $\sim 30\%$ deficit in electrically evoked $[DA]_o$ in the CPu, but not NAc (section 3.3.1). It was subsequently shown that SNCA-A mice, which express human α -synuclein at a comparable level to human brain tissue, do not develop changes to evoked CPu $[DA]_o$ (section 3.3.2). Furthermore, it was then shown that SNCA-B mice, which express α -synuclein at levels 2-3 fold of human brain, also develop no changes in evoked striatal $[DA]_o$ (section 3.3.3). Taken together, these data suggest that the overexpression of human α -synuclein in mice causes a deficit of evoked $[DA]_o$, which is selective to nigrostriatal neurons projecting to the CPu, and dependent on the relatively high level of α -synuclein expression. The effect of α -synuclein overexpression on evoked $[DA]_o$ is not accompanied by cell death, changes to DA content, or changes to the rate of DA uptake (Janezic et al., 2013), which suggests that the deficit of DA release is due to changes in releasability of DA at a presynaptic level.

Chapter 4 of this thesis expanded on previous reports that the deficit of DA release in SNCA-C mice is due to changes in DA releasability (Janezic et al., 2013), and explored two potential mechanisms to mediate the DA release deficit in these mice. Firstly, findings in chapter 4 revealed no changes in the response of D_2 activation in SNCA-C mice (section 4.3.1), suggesting that the deficit in DA release is not mediated by changes to D_2

autoreceptor function. The second part of chapter 4 expanded on previous observations that striatal presynaptic vesicles in the SNCA-C mice have increased clustering, which suggests a decrease in vesicle mobility (Janezic et al., 2013). Investigations in this section revealed that DA releasability in the CPu of SNCA-C mice is more sensitive to the vesicle mobilisation properties of cocaine (section 4.3.2). This data provides functional support for the idea that SNCA-C mice have a reduction of presynaptic vesicle mobility, selectively found in nigrostriatal DA neurons. This finding is of particular interest, because a reduction in presynaptic vesicle mobility may contribute towards the DA release deficit seen in SNCA-C mice.

Chapter 5 investigated whether SNCA-C mice had any changes to 5-HT signalling. It was found that SNCA-C mice have no changes to evoked $[5\text{-HT}]_o$ in the SNr (section 5.3.2-3), which suggests that α -synuclein overexpression does not affect 5-HT release to the same extent as nigrostriatal DA release. This finding is in agreement with other observations that noradrenaline release in the BNST is also unaffected in SNCA-C mice (Janezic et al., 2013). Taken together with observations from chapter 3 and 4, these data suggests that the neurotransmission deficit in SNCA-C mice is specific to DA neurons of the nigrostriatal pathway, which are particularly susceptible to degeneration in PD (Fearnley and Lees, 1991).

Understanding how α -synuclein affects DA releasability in nigrostriatal neurons may contribute to elucidating the pathophysiological mechanisms that lead to the death of these neurons in PD. The following section will discuss what can be learnt from the findings

presented here, and will also suggest how these findings may contribute to developing a greater understanding of the underlying pathophysiology in PD.

7.2.1 The preferential effect of SNCA overexpression in nigrostriatal DA neurons depends on the dose of α -synuclein.

The effects of WT α -synuclein on DA neurotransmission depends on the amount of α -synuclein present. In human brains, α -synuclein has been observed in the dorsal and median raphe nuclei, which innervate the SNr with 5-HT, and also the VTA and SNc, which innervate the NAc and CPu respectively with axons from DA neurons (Braak et al., 2003; Chu and Kordower, 2007). Furthermore, immunohistochemical analyses of SNCA-C mice, reveal a homogeneous distribution of α -synuclein across the SNc and raphe, with an equally homogeneous distribution across the striatum and SNr (Janezic et al., 2013). These data suggest that the selective effects of α -synuclein on nigrostriatal DA neurons are not due to α -synuclein being exclusively expressed in such neurons. However, the preferential effects of α -synuclein in nigrostriatal DA neurons may be due to higher levels of sub-cellular α -synuclein expression in those cells. The abundance of α -synuclein in healthy people is lower in the VTA than SNc (Chu and Kordower, 2007), and immunofluorescence labelling in SNCA-C mice reveal a more pronounced expression of α -synuclein in the SNc than VTA (Janezic et al., 2013). These data suggest that more α -synuclein protein is present in nigrostriatal DA neurons than VTA DA neurons. Furthermore findings in chapter 3, and in human patients (Eriksen et al., 2005), suggest that the effects of α -synuclein depends on the amount of protein present. Therefore, the fact that α -synuclein overexpression affects DA releasability from SNc neurons, but not VTA neurons, may be explained, at least in part, by the regional distribution of α -synuclein expression.

7.2.2 α -synuclein reduces vesicle pool mobility in SNc DA neurons, possibly through a converging mechanism with synapsin III

Data in chapter 4 revealed that α -synuclein overexpression in SNCA-C mice causes increased sensitivity of DA release in the CPu, but not NAc, to the vesicle-mobilising properties of cocaine. This finding suggests that α -synuclein may cause impaired presynaptic vesicle mobility through unique interactions in SNc DA neurons.

It has already been mentioned in the introduction that SNc DA neurons have several unique characteristics, such as a high level of axonal branching and a high flux of Ca^{2+} , that distinguishes them from VTA DA neurons. One distinguishing feature that might be related to the SNc-selective DA release deficit, is the function of synapsins in these neurons. In a similar fashion to α -synuclein, the loss of synapsins affect DA release in the CPu, but not in the NAc (Nicola Platt, 2012), and not 5-HT release in the SNr (Kile et al., 2010). Among the sub-populations of neurons tested, the effect of synapsin loss on DA release has the same preferential selectivity towards SNc DA neurons, as the overexpression of α -synuclein. Furthermore, loss of the synapsin family, and in particular synapsin III have also been found to cause an increase DA releasability, without changes to DA tissue content or the rate of uptake (Kile et al., 2010). This finding, in conjunction with the data presented in Chapter 4, suggests that synapsins can suppress DA releasability with a similar regional selectivity to α -synuclein, which provides support for speculating that α -synuclein and synapsins affect DA releasability through a converging mechanism.

In further support of the idea that synapsins and α -synuclein utilise a converging mechanism, both proteins have been shown to regulate mobilisation of the reserve pool of

presynaptic vesicles (Gitler et al., 2004; Nemani et al., 2010). Furthermore, the vesicle mobilising effects of cocaine, which can partially rescue a DA release deficit due to α -synuclein overexpression, has been shown in a separate study to depend on the presence of synapsins I, II, and III (Venton et al., 2006).

If synapsins and α -synuclein affect DA release through converging mechanisms, then the two proteins can be expected to act at the same target. To date, no literature has described direct interactions between α -synuclein and synapsins (Fdez and Hilfiker, 2006; Feng et al., 2002; Hosaka and Südhof, 1998; Kile et al., 2010), but indirect observations suggest at least one converging point of interaction exists. Both synapsins and α -synuclein have been shown to bind to presynaptic vesicles through direct interactions with GTP-Rab3 (Chen et al., 2013; Giovedì et al., 2004). Synapsin I has been shown to stimulate GTP binding of endogenous synaptic vesicle associated Rab3a, (Giovedì et al., 2004), and GTP bound Rab3a has in turn been shown to stabilise α -synuclein interactions with presynaptic vesicles. Furthermore, knocking down α -synuclein expression, with antisense oligonucleotides, in primary hippocampal cultures leads to a significant reduction in the levels of synapsin I (Murphy et al., 2000). These data suggest not only that α -synuclein and synapsin interactions converge in presynaptic vesicles, but also that the functions of synapsins can depend on the availability of α -synuclein.

In spite of the strong links between α -synuclein and synapsins, these two proteins differ in their dependence on Ca^{2+} . Knocking out synapsins affects evoked $[\text{DA}]_o$ in a manner that is sensitive to Ca^{2+} concentration (Kile et al., 2010), whereas either knocking out synucleins or overexpressing α -synuclein, affects evoked $[\text{DA}]_o$ in a manner that is not sensitive to

Ca²⁺ concentration (Anwar et al., 2011; Janezic et al., 2013; Nemani et al., 2010). However, the Ca²⁺ dependence for synapsins observed by *Kile et al.* can possibly be explained by the inhibitory role that Ca²⁺ plays on the ATP binding of synapsin III (Hosaka and Südhof, 1998), which may act to suppress DA release. Therefore, the idea that α -synuclein and synapsins may affect nigrostriatal DA release through a converging mechanism is not incompatible with differences in the Ca²⁺ dependence of synapsin and α -synuclein function.

Taken together, the findings to date suggest that α -synuclein overexpression reduces DA releasability in nigrostriatal neurons by reducing the mobility of the presynaptic pool of vesicles. Furthermore, it may be possible that α -synuclein causes this reduction in vesicle mobility by binding to the highly curved membranes of presynaptic vesicles (Westphal and Chandra, 2013), and then stabilising interactions with Rab3a, actin, and synapsins (Hilfiker et al., 1999; Pechstein and Shupliakov, 2010; Sousa et al., 2009).

7.2.3 Linking DA release deficits to cell death: Implications for PD

It has been shown that the DA release deficit in SNCA-C mice is followed by a significant loss in SNc DA neurons at 18 months (Janezic et al., 2013). There is currently no consensus on how presynaptic dysfunction due to α -synuclein overexpression leads to cell death, but a growing body of research suggests that cell death occurs from the cumulative effects of pathogenic mechanisms that may vary between cells (Collier et al., 2011; Obeso et al., 2010; Sulzer, 2007). It may therefore be possible that synaptic dysfunction makes a contribution to other pathogenic mechanisms, such as impaired protein degradation, oxidative stress, and mitochondrial dysfunction, against a backdrop of risk factors in SNc DA neurons, such as a high flux of Ca²⁺, a high level of arborisation, and a lack of calbindin. The following

section will provide a brief overview of some risk factors that make SNc neurons vulnerable to degeneration, followed with a proposed mechanism by which the presynaptic dysfunction caused by α -synuclein can contribute to cell death.

The first risk factor that confers selective vulnerability of SNc neurons, is the significant use of Ca^{2+} for pacemaking activity (Saxena and Caroni, 2011). The intrinsic pacemaking activity of SNc DA neurons is driven by L-type Cav1.3 low-voltage-dependent Ca^{2+} channels, which produce high Ca^{2+} flux loads (Chan et al., 2007). It has been shown that pacemaking in SNc DA neurons contributes to oxidative stress (Guzman et al., 2010), which may be due to the high Ca^{2+} flux loads. It has also been shown that the use of Ca^{2+} channel antagonists stopped Cav1.3 pacemaking, and provided protection against 6-OHDA toxicity in mice (Chan et al., 2007). Taken together, these findings suggest that the high Ca^{2+} flux caused by pacemaking activity increases the susceptibility of SNc DA neurons to degeneration in PD.

Another risk factor that confers selective vulnerability of SNc neurons is the relatively large axonal arborisation. A study that imaged GFP in rat SNc neurons, expressed through a viral promoter, observed that SNc DA neurons innervate a substantially larger area than VTA DA neurons (Matsuda, 2009). It has also been estimated that a single SNc DA neuron in the rat brain form up to 245,000 synapses, which is considerably higher than the estimate of 30,000 synapses formed by a VTA DA neuron (Bolam and Pissadaki, 2012). Furthermore, a recent biology-based computational study revealed that the energy demand on the cell increases exponentially with the level of axonal branching (Pissadaki and Bolam, 2013). The magnitude of axonal arborisation in SNc DA neurons will place a correspondingly high

energy demand on the cells, which can lead to oxidative stress and mitochondrial dysfunction (Collier et al., 2011; Saxena and Caroni, 2011).

In addition to a high Ca^{2+} flux and large axonal arbour, SNc DA neurons are relatively devoid of the calcium binding protein, calbindin-D28k, which is more widely found in the VTA (Chung et al., 2005; Pan and Ryan, 2012). Parkinsonian monkeys from MPTP injections show lower rates of loss of calbindin-positive neurons, which suggests that these neurons have some protective factor against degeneration (Lavoie and Parent, 1991). Calbindin has also been shown to regulate DA release in dissociated neuronal cultures from the rat VTA, by buffering intracellular levels of Ca^{2+} (Pan and Ryan, 2012). Therefore, the presence of calbindin-D28k may provide resistance against cell death for VTA DA neurons, which are less susceptible to degeneration in PD (Lavoie and Parent, 1991).

The data presented here suggest that α -synuclein overexpression in SNCA-C mice causes a deficit in evoked DA release by decreasing mobility of the reserve pool of synaptic vesicles. Taken together with the risk factors that confer a vulnerability of SNc neurons, it may be possible that overexpression of α -synuclein can lead to an increased energy demand, mitochondrial dysfunction, and eventually cell death. In support of this idea, it has been shown that α -synuclein can cause oxidative stress and mitochondrial dysfunction, in GT1-7 hypothalamic neuron cultures (Hsu et al., 2000). Interestingly, α -synuclein overexpression in this model caused a decrease in the secretion of gonadotropin-releasing hormone (Hsu et al., 2000), which is also released from action potentials generated by L-type calcium channels (Vazquez-Martinez et al., 2001). More recently, the A53T and A30P mutant forms of α -synuclein cause mitochondrial degeneration and cell death in mice (Martin et al.,

2006). Furthermore, a study in mouse primary DA neuron culture revealed that intracellular Ca^{2+} entry through L-type channels, together with α -synuclein overexpression, increased mitochondrial oxidative stress (Dryanovski et al., 2013). This finding suggests that the effects of α -synuclein overexpression can act in conjunction with elevated levels of Ca^{2+} to cause mitochondrial dysfunction, which may explain why SNc DA neurons are particularly susceptible to the effects of α -synuclein overexpression.

The exact mechanism by which α -synuclein contributes to mitochondrial dysfunction is incompletely understood. It has been suggested that α -synuclein acts directly inside mitochondria to impair complex one function (Chinta et al., 2010). However, the observation was made from mice under chronic administration of a proteasome inhibitor, and whether α -synuclein can directly affect mitochondrial function without proteasome inhibition *in vivo* remains unknown. Furthermore, a conflicting report from an *in vitro* study suggest that α -synuclein affects mitochondrial oxidative phosphorylation without inhibiting any of the respiratory chain complexes (Banerjee et al., 2010).

In light of the findings presented here that α -synuclein overexpression may reduce vesicle mobilisation, and against the background of SNc DA neuron susceptibility to increased oxidative stress, an alternative pathway to mitochondrial dysfunction will be suggested through speculation by bringing together three points (illustrated in Figure 7.1). Firstly, synapsin levels can be dynamically changed by altering the expression level of α -synuclein (Murphy et al., 2000), and the level of Rab3a decreases in synapsin knockout mice (Giovedì et al., 2004). These reports suggest that α -synuclein activity influences other energy-dependent mechanisms. Secondly, the results reported in this thesis reveal that

overexpressing α -synuclein has significantly attenuating effects on DA release and synaptic vesicle mobilisation. Therefore, α -synuclein overexpression will likely affect energy-dependent compensatory mechanisms. It is worth noting here that both Rab3a and synapsins require ATP (Giovedì et al., 2004; Hosaka and Südhof, 1998). Thirdly, energy-dependent compensatory mechanisms will contribute towards the energy burden on SNc DA neurons, which is already substantially high in comparison to VTA neurons (Bolam and Pissadaki, 2012). Taken together, these points can be used to make a speculative suggestion that homeostatic mechanisms responding to α -synuclein overexpression could create mitochondrial dysfunction by increasing the energy burden on the cell.

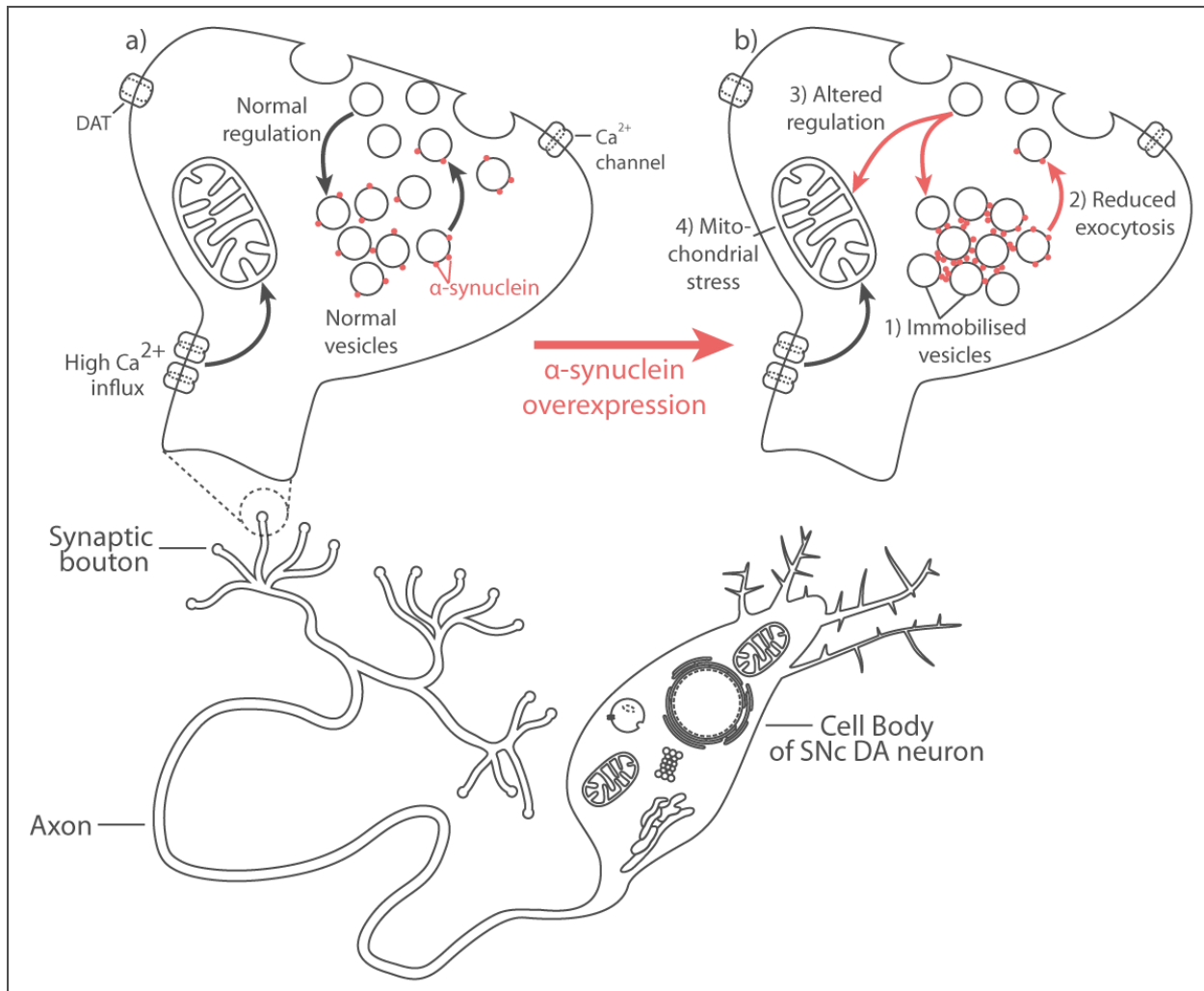


Diagram 7.1: Schematic diagram illustrating how α -synuclein overexpression leads to neuronal dysfunction. The two hypothetical boutons of an SNc DA neuron have normal levels of α -synuclein (a), and abnormally high levels of α -synuclein (b) 1) Where levels, of α -synuclein are too high, the reserve pool of vesicles have a reduced mobility, or are 'immobilised'. 2) Reduced vesicle mobility causes a reduction in DA exocytosis. 3) Energy-dependent homeostatic mechanisms that regulate vesicle distribution respond to the reduced vesicle mobility and reduction in DA release. 4) The altered regulation increases the energy burden on the cells and contributes towards oxidative and mitochondrial stress, which is exacerbated by the increased Ca^{2+} flux and the high level of axonal branching of SNc DA neurons.

7.3. G2019S and R1441C mutant human *LRRK2* affects DA releasability in nigrostriatal DA neurons.

Chapters 6 of this thesis investigated changes to evoked DA release in rats that express the G2019S and R1441C mutant forms of human *LRRK2*. For G2019S rats, a DA release deficit was observed at 18 months, but not at 6-months or 12 months (section 6.3.1). This suggests that the G2019S mutant form of *LRRK2* causes a progressive deficit in evoked DA release. Likewise, R1441C rats also exhibited a DA release deficit at 18 months, but not 6 months or 12 months (Section 6.3.2), suggesting a similar progressive deficit of DA release.

For both G2019S and R1441C rats, the deficit in DA release was primarily in areas innervated by the SNc (section 6.3.1-2), suggesting that the effects of mutant *LRRK2* in these rats has the same region selectivity as PD pathophysiology (Fearnley and Lees, 1991).

The deficit of DA release in G2019S and R1441C rats was not dependent on changes to DA content or the rate of DA uptake (section 6.3.3), which suggests that it is due to changes in DA releasability. It was also observed that the DA release deficit in both G2019S and R1441C rats is not accompanied by changes in release probability, stimulation frequency dependence, or ability to maintain DA release (section 3.3.4).

The use of both a G2019S and an R1441C mutation in this study allows for comparisons to be made between the effects of different genotypes on *LRRK2*, which may contribute to developing a deeper understanding of *LRRK2* function in PD pathogenesis. There is at least one notable difference between the G2019S and R1441C rats. In the methods, it was mentioned that G2019S rats express human *LRRK2* at about two fold the level of R1441C rats (section 2.2.2). Furthermore, the R1441C rats revealed a deficit in DA release from just

three pairs of animals, whereas seven pairs were used for the G2019S rats. The R1441C rats therefore have a lower expression of human LRRK2 and revealed a deficit with fewer pairs of animals tested, which may suggest a more robust phenotype for R1441C rats than G2019S rats. Conversely however, findings in the literature reports a reduction in striatal [DA]_o in BAC transgenic mice that express the R1441C mutant at 5 – 10 fold the level of endogenous mouse LRRK2 (Li et al., 2009), and also from BAC transgenic mice that express the G2019S mutant at 2.5-3.5 fold the level of endogenous mouse LRRK2 (Melrose et al., 2010). Both investigations sampled [DA]_o through *in vivo* microdialysis and found a ~35% deficit in [DA]_o. Such reports, in conjunction with the findings presented here, suggest that the level of mutant LRRK2 expression may not play as significant a role as the mutation itself. Furthermore, changes of catecholamine release from chromaffin cells have been reported from R1441C mutant mice that have endogenous levels of expression (Tong et al., 2009), which suggests that overexpression of a mutant gene may not be necessary to see changes in neurotransmission. Therefore, the observation that G2019S and R1441C rats, which express different levels of LRRK2 both have a DA deficit of about ~30% (sections 6.3.1-2), does not mean that the R1441C mutation has more pronounced effects than the G2019S mutation. Exploring to what extent the effect of G2019S and R1441C LRRK2 mutations depend on the level of protein, may deserve further investigation.

As well as differences between the G2019S and R1441C rats, there are very notable similarities. Both G2019S and R1441C rats have a progressive deficit in evoked DA release of ~30%, which appears at 18 months. This suggests that both mutations may affect DA release through the same mechanism. As mentioned in the introduction, the R1441C mutation is on the GTPase domain (Liu et al., 2011), and the G2019S mutation is on the

kinase domain of LRRK2 (Chen et al., 2012). It has been shown that the kinase activity of LRRK2 can regulate GTPase function (Greggio et al., 2009), and also that GTPase activity can regulate kinase function (Dachsel and Farrer, 2010; Ito et al., 2007). Therefore it may be possible that a mutation in either the kinase or the GTPase domain will affect LRRK2 function to the same extent.

It has been shown that LRRK2 affects neurotransmission (Piccoli et al., 2011; Shin et al., 2008), although mechanism by which this happens remains elusive (Dihanich and Manzoni, 2011). LRRK2 has been shown to interact with proteins such as Rab5b and synapsin I *in vitro* (Shin et al., 2008), which suggests that LRRK2 may act indirectly through downstream effector proteins. Interactions with Rab5 and synapsin I also suggest that LRRK2 might interact with similar vesicle associated proteins, such as Rab3 or synapsin III, in nigrostriatal DA neurons. It has also been reported that LRRK2 silencing alters the presynaptic vesicle distribution, possibly by relieving an attenuating effect on synaptic vesicle endocytosis (Dihanich and Manzoni, 2011; Piccoli et al., 2011).

7.4. Exploring a converging mechanism for α -synuclein and LRRK2 on nigrostriatal DA release

Both α -synuclein and LRRK2 have been implicated in the pathophysiology of PD (Eriksen et al., 2005; Funayama et al., 2002). The findings presented here suggest that both mutant LRRK2 and unmutated α -synuclein can cause a reduction in nigrostriatal DA release.

Exploring the similarities and differences in the effects of LRRK2 or α -synuclein may contribute to a better understanding of the underlying mechanisms by which they change DA release.

The G2019S and R1441C rats, as well as the SNCA-C mice described here, all showed a regional selectivity in the deficit of DA release, which suggests that nigrostriatal DA neurons have a predisposition to be affected by either protein. Furthermore, the G2019S and R1441C rats, as well as the SNCA-C mice described here, all caused a deficit in DA releasability, which was independent of changes to DA content or the rate of DA uptake. This highlights further similarities between the effects of α -synuclein and LRRK2 on DA release, and suggests a converging mechanism by which these two proteins act.

On the other hand, there are some notable differences in the effects of α -synuclein and LRRK2 on DA release. α -Synuclein causes an early and sustained DA release deficit in SNCA-C mice at 3 months, which remains steady with age until 18 months. This suggests that the deficit in DA release is not a progressive phenotype. The deficit in DA release at 3 months is observable in the absence of any other phenotype, which suggests that it is one of the first effects of α -synuclein overexpression (Janezic et al., 2013). In contrast to the SNCA-C mice, the DA release deficit in G2019S and R1441C rats is not present at 6 months,

but develops with age to be present at 18 months. This suggests that the G2019S and R1441C mutations of LRRK2 do not affect nigrostriatal DA release through the same mechanism as the overexpression of α -synuclein. However, it may be likely that a converging mechanism exists, perhaps through alteration of the presynaptic vesicle distribution as suggested before (Dihanich and Manzoni, 2011; Murphy et al., 2000; Nemani et al., 2010).

7.5. Future Investigations

The findings presented in this thesis reveal that high levels of human α -synuclein in BAC transgenic mice leads to a region-selective deficit of DA release in the CPu, and a concomitant increased sensitivity to presynaptic vesicle mobilisation in the same region. Future investigations into the mechanism by which α -synuclein reduces DA release should focus on two areas in particular. Firstly, it is important to explore to what extent the increased sensitivity of SNCA-C mice to cocaine can rescue the deficit in DA releasability. Findings in chapter 4.3.2 revealed that DA releasability in SNCA-C mice is increased to a greater extent, in comparison to *Snca* $-/-$ controls, by the vesicle mobilising properties of cocaine. The effect of cocaine therefore rescues the DA release deficit, at least in part. It is important to investigate the extent to which cocaine can rescue the DA release deficit in SNCA-C mice, because this will report on the extent to which impaired vesicle mobilisation is responsible for the release deficit. The extent to which cocaine can rescue the DA release deficit in SNCA-C mice can be investigated by sampling DA release in SNCA-C and *Snca* $-/-$ mice as performed in chapter 3.3.1, and then sampling the same sites in the presence of 5 μ M cocaine. If a reduction in vesicle mobilisation is entirely responsible for the DA release deficit in SNCA-C mice, then the deficit will be fully rescued by the vesicle mobilising properties of cocaine. A second area for future investigations on SNCA-C mice to focus on, is whether the mechanism by which α -synuclein overexpression affects DA release depends on synapsins. This question can be addressed by crossing synapsin I,II,III triple knockout mice with SNCA-C mice to create a strain of mice that lack synapsins, but overexpress α -

synuclein. If α -synuclein acts on DA release through a synapsin-dependent mechanism, then the new strain of mice will have no deficit in DA release.

The findings in this thesis also reveal that the G2019S and R1441C mutant forms of LRRK2 cause an age-dependent deficit in evoked DA release in the CPu. The OPDC is currently performing several ongoing investigations on the LRRK2 rats, including rats with other forms and expression levels of human LRRK2. Ongoing investigations on LRRK2 rats include behaviour, stereology, and FCV experiments. Behavioural studies are being conducted, and have to some extent found motor deficits in G2019S and R1441C rats at 18 months (personal communication). FCV studies are being conducted on BAC transgenic rats that express WT human LRRK2 to investigate whether the DA release deficits seen in G2019S and R1441C rats are due to the genetic mutations or just the presence of the transgene. Further FCV studies will also be conducted on rats that express the G2019S or R1441C mutation at lower levels than the animals used for this thesis, in order to explore whether the effects of the G2019S and R1441C mutations depend on high levels of protein expression.

Future studies on the G2019S and R1441C rats used for this thesis should aim to explore the mechanisms by which mutant LRRK2 reduces DA releasability. Given that DA release in SNCA-C mice is more sensitive to the vesicle mobilising properties of cocaine, it should be investigated whether DA release in G2019S and R1441C rats is also more sensitive to cocaine. Such a question can be addressed by sampling the effects of cocaine on evoked $[DA]_o$ in G2019S and R1441C rats, in comparison to nTG controls, using the experimental protocol for cocaine described in Chapter 4. Furthermore, the electron microscopy imaging

study that showed increased presynaptic vesicle clustering in SNCA-C mice (Janezic et al., 2013) can be used to investigate vesicle clustering in G2019S and R1441C LRRK2 rats. In addition to testing the response to cocaine, other potential mechanisms by which mutant LRRK2 can affect DA release should also be tested. These include testing the dependence of DA release in G2019S and R1441C rats to nAChR activation, DA D₂ autoreceptor activation, and extracellular [Ca²⁺].

7.6. Concluding remarks

This thesis explored presynaptic dysfunction in rodent models of PD. It was observed that PD-related forms of *SNCA* and *LRRK2* lead to a selective deficit of DA releasability in nigrostriatal neurons. The PD-related forms of α -synuclein and LRRK2 may affect DA releasability through a converging mechanism. Further investigations into the exact mechanism by which PD-related proteins, such as α -synuclein and LRRK2, cause PD dysfunction may contribute to understanding the earliest stages of PD pathogenesis.

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