Impact of human alpha-synuclein overexpression on the nigrostriatal dopaminergic neurotransmission

Doctoral dissertation at the Faculty of Biology

Ludwig-Maximilians-Universität München

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Born in Málaga, Spain

München, 2021

Diese Dissertation wurde angefertigt
unter der Leitung von Prof. Dr. Jochen Herms
im Bereich der translationalen Hirnforschung
an der Ludwig-Maximilians-Universität München
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an der Fakultät für Biologie vertreten.

Erstgutachterin:	Prof. Dr. Hans Straka
Zweitgutachter:	Prof. Dr. Anja Horn-Bochtler
Tag der Abgabe:	21.10.2021
Tag der mündlichen Prüfung:	20.09.2022

ERKLÄRUNG

Ich versichere hiermit an Eides statt, dass meine Dissertation selbständig und ohne unerlaubte Hilfsmittel angefertigt worden ist. Die vorliegende Dissertation wurde weder ganz, noch teilweise bei einer anderen Prüfungskommission vorgelegt. Ich habe noch zu keinem früheren Zeitpunkt versucht, eine Dissertation einzureichen oder an einer Doktorprüfung teilzunehmen.

München, den 21.10.2021

Jose Medina Luque

It's always best to start at the beginning

and all you do is follow the Yellow Brick Road

L. Frank Baum

Contents

Summary

Alpha-synuclein is a small 140 amino acid presynaptic protein associated with several neurodegenerative disorders such as Parkinson's disease (PD). In idiopathic PD, abnormally misfolded wild-type proteins aggregate in the cytosol of certain neurons, forming what is the main component of Lewy bodies (LBs) - a major PD hallmark. Besides its role in regulating synaptic vesicle functions, alpha-synuclein appears to modulate dopamine (DA), a neurotransmitter particularly important in PD symptoms manifestation. DA can be found sparsely distributed in the brain and is predominantly expressed in midbrain neurons. From the midbrain, two main dopaminergic (DAergic) pathways densely innervate the dorsal portion of the striatum. Degeneration of such DAergic terminals is known to dysregulate DA homeostasis in the striatum. Despite several decades of PD research, the physiological influence of alpha-synuclein on DAergic neurotransmission in the two main areas of the dorsal striatum is still poorly understood. These areas are the dorsomedial striatum (DMS), mainly receiving innervation from the ventral tegmental area, and the dorsolateral striatum (DLS), which receives input from the substantia nigra pars compacta and is more susceptible to neurodegeneration. To clarify how alpha-synuclein may interfere with DAergic neurotransmission in either area, fast-scan cyclic voltammetry experiments were carried out in the DMS and DLS of transgenic mice overexpressing human alpha-synuclein (Tg) at twelve, six and three months of age. Additionally, pharmacological assays, behavioural tasks, and *ex vivo* immunofluorescence staining were performed to support the electrophysiological *ex vivo* results.

In this thesis, it is hypothesized that due to its intrinsic characteristics, alpha-synuclein preferentially interferes with DA neurotransmission dynamics in the dorsolateral region of the striatum. Data analysis confirmed that overexpression of human alpha-synuclein differentially interfered with normal DA release in the DLS in an age-dependent manner. At older ages (twelve-month-old Tg mice), decreased evoked DA release and slower DA uptake kinetics were observed. In addition, alterations in dopamine transporter (DAT) distribution, which appeared as increasing amounts of DAT-positive clumps, were found only in the DLS. Moreover, at pre-symptomatic stages (six-month-old) DA neurotransmission appeared to be stabilised before DA disruption. Surprisingly, at younger ages (three-month-old) increased electrically evoked DA release was also recorded in the DLS. Such changes were in line with the motor learning enhancement observed in their behavioural phenotype. In addition, DA uptake appeared to be impaired as evidenced by reduced extracellular

DA withdrawal. Further pharmacological experiments demonstrated that such alterations were mediated by DAT.

In summary, the present findings indicate that abnormal DAergic neurotransmission and function of DLS can be identified before the onset of structural pathologies in a model of transgenic expression of human alpha-synuclein. Depending on the progression of the pathology, human alpha-synuclein has different impacts on neurotransmission, initially enhancing it but impairing it at later stages. It is here proposed that assessment of DLS function by non-invasive brain imaging and neuropsychological techniques might be relevant in early PD diagnosis and help design appropriate therapeutic interventions.

Abbreviations

aCSF, Artificial cerebrospinal fluid

Aa, Amino acid

AAAD, Aromatic amino acid decarboxylase

ALDH, Aldehyde dehydrogenase

AD, Alzheimer disease

a.u., arbitrary unit

BAC, Bacterial artificial chromosome

BG, Basal ganglia

BCA, Bicinchoninic acid

CA, Catecholamine

CFD, Cumulative frequency distribution

CNS, Central nervous system

Coc, Cocaine

COMT, Catechol-O methyl transferase

CPu, Caudate putamen

D1R, Dopaminergic receptor 1

D2R, Dopaminergic receptor 2

DA, Dopamine

DAergic, Dopaminergic

DAT, Dopamine transporter

ddH2O, double-distilled water

DLB, Dementia with Lewis Bodies

DLS, Dorsolateral striatum

DMS, Dorsomedial striatum

DOPAC, 3,4-dihydroxyphenylacetic acid

DOPAL, 3,4-Dihydroxyphenylacetaldehyde

DPC, Dorsolateral prefrontal cortex

FSCV, Fast scan cyclic voltammetry

GP, Globus pallidus

hDAT, human dopamine transporter

HEK, human embryonic kidney

HVA, Homovanilic acid

I/O, Input/Output

LBs, Lewy bodies

L-DOPA, Levodopa

MAO, Monoamine oxidase

MSN, Medium spiny neurons

NAC, Non-amyloid component

NA, Nucleus accumbens

NM, Neuromelanin

NPY, Neuropeptide Y

O.D., Optical density

PB, Phosphate buffer

PBS, Phosphate-buffered saline

PD, Parkinson's disease

PDD, Parkinson disease dementia

PDGF, Platelet-derived growth factor

Phe, Phenylalanine

PVDF, Polyvinylidene fluoride

SEM, Standard error of the mean

ROS, Reactive oxygen species

Sth, Subthalamic nucleus

SNc, Substantia nigra pars compacta

SNr, Substantia nigra pars reticulata

Tg, PDGH-h-asyn

Tyr, Tyrosine

TH, Tyrosine hydroxylase

TVA, Tierversuchsantrag

VMAT-2, Vesicular monoamine transporter-2

VTA, Ventral tegmental area

WT, Wild-type

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Acknowledgements

First, I would like to thank Prof. Dr. Jochen Herms for his supervision and much support during these years of research and work aimed at completing my Doctoral Thesis in his laboratory.

I would also like to express my deepest gratitude to Carmelo Sgobio, who unawarely gave me what might have been the last chance to complete my Ph.D. Thank you very much for enriching and supporting conversations during these years.

I am also grateful to the members of my TAC meeting, whose attention and commitment during our talks brought about many fruitful conversations, as well as intense and constructive debate. Special thanks to Prof. Dr. Hans Straka for reviewing my thesis and supporting me during the submission process.

This journey would not have been possible without my amazing partners at the DZNE institute. Thanks to the wonderful people belonging to the AG Herms group, some already former members, others still working there: Laura (la Sueca), Elena, Fumy, Jeannine, Paul, Pri and Sophie. I would also like to thank the members of Los destructores de… (they know what follows), Florecilla, Gianmarquito, Maíra, Rachele and Katrin, for endless laughter and fun cheering my days, weeks, and years in the laboratory. Special thanks to Katrin, my partner in crime, my Austrian sibling. I thank, every single day, our ancestor, Charles II The Bewitched, for connecting us somehow. Your friendship, empathy, and the time we spent together meant the world to me and made everything easier. Our brotherhood will last forever (you know until when…). Mil gracias amiga.

I will not forget the members of the laboratory on the other side of the corridor. Thank you, Alessio, Ana, Katrin, Jasenka, Laura (la Rubia) and Lini (a.k.a. Tweety) for good vibes and all the support and advice. Special thanks to Laura for taking care of me; thank you for your patience, support, sympathy, nice chats and tireless encouragement throughout these years.

Querría aprovechar este momento para sinceramente dar las gracias a todas las personas que de algún modo u otro han contribuido a que llegue a este punto de mi carrera. Durante todo este camino, que empezó hace ya casi 8 años, he tenido la suerte de estar rodeado de mi familia, compañeros y amigos. Sin ellos, nada de esto habría sido posible. Es por eso por lo que merecéis que os dé las gracias. Gracias a mi Aleja, por todas esas llamadas, por escucharme, entenderme y, en definitiva, saber mejor que nadie lo que es estar en un país que no es el tuyo. Quien nos iba a decir que el chico

que no paraba de hacer bromas allí por el 2009, y era un poco distraído, y la chica que se quejó de él, se iban a convertir en los grandes amigos que son hoy. Agradezco tanto tu apoyo, y todo lo que has hecho durante todo este tiempo. Gracias también a Sara. Ya son muchos años de amistad. Juntos hemos vivido muchas cosas casi de forma paralela: la carrera, ese aprobado de Bioquímica, el “doctorado”, y ese momento de irnos al extranjero, pero sobre todo lo que más te agradezco es que estuvieras aquí el primer día que llegué, esperándome, dispuesta para ayudar, aunque tuvieras que venir desde Suiza. Me hiciste sentir como si estuviera en casa. Gracias por tu apoyo. Gracias a mi Familia de Múnich (Andrés, Carmen, Fran, Leti, Maria, Tito, Zosia) por todo ese tiempo juntos, y por disfrutar en la Rustikeria del mejor tiramisú del mundo. En especial, me gustaría dar las gracias a María, por ser ese apoyo que necesitaba. Por comprenderme tan bien. Eres una de las cosas bonitas que me llevo de mi tiempo en Múnich. También me gustaría agradecer a mis amistades de España, todos y cada una de ellas. Habéis sido de gran ayuda para terminar mi tesis doctoral, aunque muchos de vosotros no lo sepáis. Por último, me gustaría dar las gracias a mi familia. A mi madre, mi hermana, mis dos sobrinos (Martín y Moisés) y a Radek. Gracias, mamá. Gracias por dar todo lo que me has dado. Por el sacrificio, el esfuerzo y el amor que has intentado inculcarnos. Nunca podremos estar lo suficientemente agradecidos por todo lo que has hecho. Gracias a mi hermana. Puede que seas dos años mayor que yo, pero es como si fuéramos gemelos. Tenemos una conexión tan especial, que no se puede describir. Desde que éramos pequeños me has cuidado de una forma que solo una hermana puede hacer. Aunque ya lo sepas, eres mi apoyo, y te estoy tan agradecido por todo lo que has hecho. Siempre serás parte de mí. A Cuchufletito y mi Lori-Lori que, aun no siendo conscientes, me han alegrado mi vida con su existencia, amor y sonrisas. Aunque a veces gritéis un poquitín, me hacéis ser el tito más orgulloso del planeta. Gracias Radek, por ser mi mente fría, por ser uno de mis grandes apoyos, por creer que puedo hacer esto y mucho más, y por haberme ayudado de manera tan desinteresada a conseguir mi sueño. Dzięki Tysiąckrotne.

1 Introduction

The brain is a complex organ, where billions of reactions are constantly occurring to keep the fragile balance between neuronal connections and neurochemical reactions. Proper maintenance of such interactions is necessary for multiple cognitive functions, such as the operation of fine movement tasks or the creation of new memories. Those processes are mainly modulated by a number of chemical agents, many of which are released into the extracellular space, where they interact with surrounding receptors thus activating or inhibiting neurons. Although consensus has not been reached, some researchers classify these chemical agents according to either their molecular structure (small molecules, peptides, monoamines or nucleotides) or their effects (inhibitory or excitatory). Among them, neurotransmitters form an extensive group of messengers that carry information not only to neurons but also to muscle cells and glands. Neurotransmitters are defined as particles stored in presynaptic vesicles, which can be released into the synaptic cleft when an action potential occurs in the presynaptic neuron. Thus, any agent released at the synapse and producing an excitatory or inhibitory response in the postsynaptic or other neighbouring cell is considered a neurotransmitter. Among neurotransmitters, catecholamines (CAs) are one of the most complex molecule groups in the human body. They can be found both in the peripheral and central nervous systems (CNS), where they act either as neurotransmitters or hormones (Stanford & Heal, 2019). Quite versatile, CAs modulate neuronal activity and mediate a variety of functions, such as cognition, memory and motor skills, in the brain (Kobayashi, 2001; Goldstein, 2011). In the CNS, CAs are mainly represented by norepinephrine and dopamine (DA). DA is well known for its direct participation in motor behaviour, movement control, learning and executive functions. Deficiencies in DA biosynthesis or release are directly associated with multiple neurodegenerative diseases such as Parkinson's disease (PD) or dementia with Lewis bodies (DLB) (Franco et al., 2021).

1.1 Dopamine

DA was known as a compound many years before its neurotransmitter functions in the CNS were discovered. In 1910, George Barger and Arthur James Ewins synthesized DA based on previous studies undertaken by Henry Dale (Barger & Ewins, 1910). Until 1957, DA was considered only a precursor of epinephrine and norepinephrine; in 1957, Kathleen Montagu isolated several aromatic compounds from certain brain regions of different animals, while studying the occurrence of catechol compounds in the rat (Montagu, 1957). In 1958, studies conducted by Nils-Eke Hillarp and Avid Carlsson showed that CAs depletion

triggered dysfunctions similar to those observed in patients with PD (Carlsson et al., 1958). DA deficiency was then identified as a cause of PD-like symptoms and became an important research target. From then on, our knowledge of CAs has quickly increased with several studies describing their preferential expression in midbrain dopaminergic (DAergic) neurons (Andén et al., 1966; Dahlström & Fuxe, 1964), involvement in fine movement control and motor skill learning (Hikosaka et al., 2002) and association with certain neurodegenerative diseases (Ehringer & Hornykiewicz, 1960).

1.1.1 Molecular structure

First described in the early 60s, neurotransmitter DA – also known as 3-hydroxytyramine – can be found in the central and peripheral nervous systems (Arvid & Waldeck, 1958; Carlsson et al., 1958). DA is classified as a monoamine-like neurotransmitter and belongs to the CAs group (Gnegy, 2012). Structurally, DA contains a 3,4-dihydroxyphenyl (catechol) nucleus (Björklund & Dunnett, 2007) and an ethylamine side chain with a single amine group that may present additional substituents (Gnegy, 2012) (Figure 1).

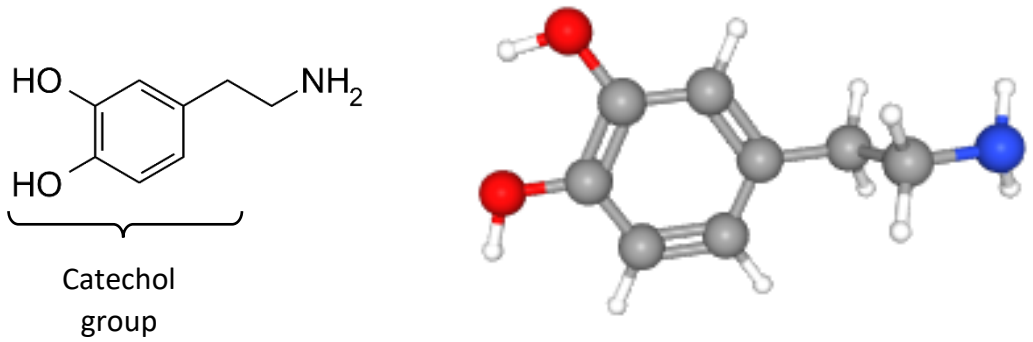

Figure 1. Detailed molecular structure of dopamine

The figure shows a two-dimensional representation of DA (left) and a non-experimentally determined three-dimensional one (right) (source: https://pubchem.ncbi.nlm.nih.gov/compound/Dopamine).

1.1.2 Dopamine biosynthesis

Although DA synthesis has been involved in several pathways, most studies report that this compound is synthesized from the non-essential amino acid tyrosine (Tyr) (Levitt et al., 1965). DAergic neurons use a different amino acid namely phenylalanine (Phe) as a source to synthesize DA in a cytosolic two-step pathway, both in the soma and axon terminals (Blascko, 1939; Best et al., 2009). In the first step, Phe is turned into Tyr by a hydroxylation reaction carried out by the enzyme Phe-hydroxylase. Tyr is then converted into 3,4-dihydroxy-l-phenylalanine (called Levodopa: L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) in a redox process regulated by cofactors oxygen (O_2), tetrahydrobiopterin (BH_4) and iron (Fe^{2+}) (Gnegy, 2012). Finally, L-DOPA is metabolised into DA by the enzyme aromatic amino acid decarboxylase (AAAD) (Figure 2). Due to its high sensitivity to pH and to prevent the formation of toxic compounds, DA is quickly sequestered from the cytoplasmic space and stored into presynaptic vesicles. The transport is mediated by vesicular monoamine transporter-2 (VMAT-2), which exchanges one H^+ ion and one DA molecule (Liu & Edwards, 1997; Liu & Kaeser, 2019). This process is facilitated by the ATPase proton pump, which not only promotes DA vesicular uptake but also provides a more acidic environment that prevents DA oxidation (Guillot & Miller, 2009; Figure 2).

1.1.3 Dopamine metabolism

Once released, DA may either directly act in the synaptic cleft or diffuse to neighbouring cells, where it may bind pre- or postsynaptic DAergic receptors (Fuxe et al., 2010, 2012). Extracellularly, DA may then be taken up into neurons or glial cells mainly through a transmembrane protein, the dopamine transporter (DAT), which regulates the duration of the postsynaptic signal. Besides the action of DAT, other DA-clearance processes such as diffusion, extracellular degradation, or non-neuronal uptake (Smiley et al., 1994) contribute to reducing extracellular DA levels, although to a lesser extent (Mulvihill, 2019).

Inside neuronal terminals, DA is cytosolically metabolized into 3,4-Dihydroxyphenylacetaldehyde (DOPAL) and hydrogen peroxide (H_2O_2) by the mitochondrial outer-membrane enzyme monoamine oxidase (MAO) (Schnaitman et al., 1967; Eisenhofer et al., 2004). Aldehyde DOPAL can be inactivated through oxidation to carboxylic acid or 3,4-dihydroxyphenylacetic acid (DOPAC) by enzymes alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH), respectively (Meiser et al., 2013). Although this is the

main DA metabolism pathway, synaptic DA may also be taken up into glial cells, where it is degraded by enzymes MAO or catechol-O methyl transferase (COMT) leading to the formation of homovanilic acid (HVA) (Tunbridge et al., 2006; Myöhänen et al., 2010) (Figure 2). The final product of both degradation pathways is released into the bloodstream, filtered in the kidneys and eventually excreted in the urine (Eisenhofer et al., 2004).

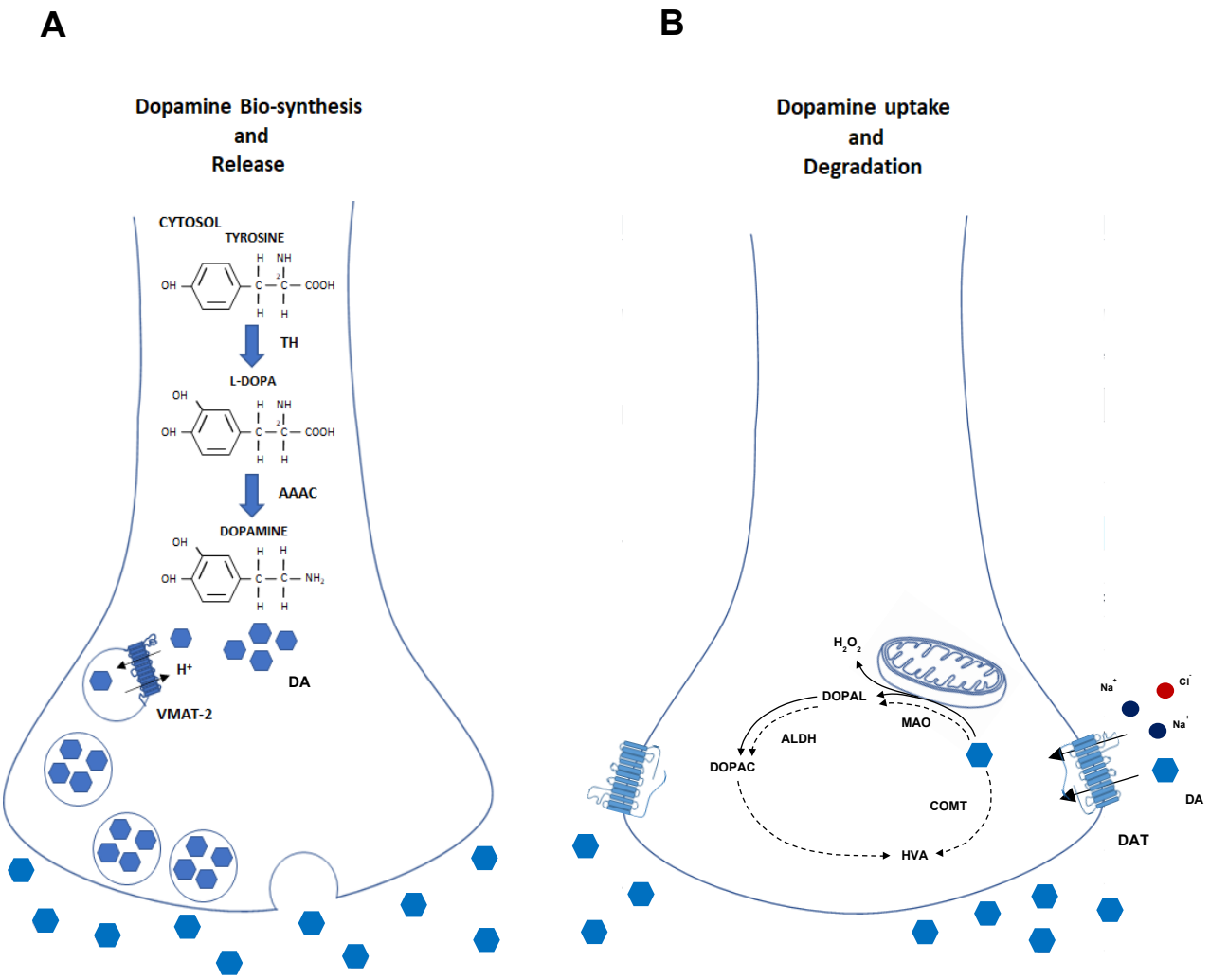

Figure 2. **Dopamine synthesis and metabolism pathways**

(**A**) The primary DA biosynthesis pathway takes place in two steps in the cytosol of catecholaminergic neurons. Tyrosine is quickly converted into 3,4-dihydroxy-l-phenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) and then metabolized to dopamine (DA), a highly pH-sensitive molecule, which is stored into presynaptic vesicles through the action of vesicular monoamine transporter 2 (VMAT-2). DA may be then released by phasic or tonic transmission. (**B**) Once released, DA diffuses in the extracellular space, where it can interact with DAergic receptors on neighboring cells. Simultaneously, DA is quickly re-taken into neurons or glial cells through dopamine transporter DAT, in order to prevent prolonged interaction or toxic compounds formation. DAT transports a DA molecule together with two Na^+ and one Cl^- ions into the cell. In the cytoplasm of neurons (dark line) or glial cells (dash line), DA is metabolized to DOPAC plus HVA as main degradation products.

1.1.4 Dopamine neurotransmission

1.1.4.1 Dopamine release

DA-containing synaptic vesicles are densely packed or docked in small clusters at certain compartments of the neuronal cell (presynaptic terminals, varicose axons or soma), ready to be released through exocytosis (Westerink, 2008; Liu & Kaeser, 2019). In general, DA release is mainly modulated by two processes: 1) fast synaptic transmission, which requires alignment of the presynaptic terminal and postsynaptic neuron, plus the accumulation of synaptic membrane proteins such as calcium channels and scaffolding proteins in the active zone (Südhof, 2012); and 2) volume neurotransmission, a process first described in the late 80s, by which a neurotransmitter diffuses into the extracellular medium and exerts its effect not only on postsynaptic receptors but also on receptors located on neighbouring neuronal or glial cells (Agnati et al., 1986, 2010; Grace, 1991; Tritsch & Sabatini, 2012). In the striatum, DAergic boutons are usually varicose instead of the typical synapse-to-synapse boutons. Intercellular communication would thus be mediated by a complex interaction between fast synaptic transmission and volume transmission. While volume neurotransmission may be the main communication mechanism (Agnati et al., 2010; Fuxe et al., 2010; Borroto-Escuela et al., 2018), DAergic boutons also present active zone-like hotspots that accumulate scaffolding proteins and facilitate quick fusion of presynaptic vesicles (Liu et al., 2018; C. Liu & Kaeser, 2019).

Besides their unique cell-to-cell communication features, DAergic neurons also exhibit two differential neurotransmission patterns that determine DA levels in the extracellular space: phasic and tonic transmission (Grace, 1991; Floresco et al., 2003; Sulzer et al., 2016). Phasic or burst-like activity is sporadic and has a short duration. It increases extracellular DA in a fast and transient way as a consequence of action potentials of DA neurons, and it involves calcium (Ca^{2+}) channels activity. Conversely, tonic DA transmission results in small extracellular DA increases and is not action potential-dependent but regulated through several factors, including DA terminal autoreceptors feedback system and the activity of prefrontal neurons (Rice et al., 2011; Klein et al., 2019). In both cases, DA release requires synaptic vesicles to fuse with cell membranes in an exocytosis process.

1.1.4.2 Dopamine uptake

Following exocytosis and DA release in the extracellular space, DA-clearance mechanisms are immediately activated. Inside the synaptic vesicle, DA stability is preserved by an acidic environment (pH ~ 5.6); as it enters into contact with the physiological pH in the cytosol or the extracellular space, DA may spontaneously oxidise to DA-o-quinone, a compound with potential neurotoxic effects such as oxidative damage to organelles or proteins (Cobley et al., 2018; Gainetdinov et al., 2008; Kristal et al., 2001). Quick DA sequestration into glial or neuronal cytosol via DAT transportation prevents such deleterious effects (Jones et al., 1998a, b). DA is then stored or metabolized.

1.1.4.3 Dopamine transporter

First identified in the early 60s, DAT is a 619 amino acid (Aa) protein composed of 12 transmembrane domains including N- and C- terminals, that contain most of the regulatory elements (Kilty et al., 1991; Giros et al., 1992; Shimada et al., 1992; Nirenberg et al., 1996; Brüss et al., 1999). Distributed in the periphery of synaptic boutons, DAT is found in brain regions connected by DAergic pathways, including the cerebral cortex, the striatum or midbrain and others (Hall et al., 1999). Being a member of the Na^+/Cl^- transporter-like protein family, DAT reduces DA neurotransmission by symport transportation of a DA molecule along with two Na^+ and one Cl^- ions into the cell (Shan et al., 2011). This process generates the necessary energy to pump DA molecules into the cell against the concentration gradient. Given that DAT is essential in determining the amplitude and duration of DA uptake into the synapse, and its consequent availability in the cytosol, this protein is dynamically regulated both at the transcriptional and post-transcriptional levels, mainly through mechanisms like protein-protein interaction, compartmentalization or post-translation modifications (Vaughan & Foster, 2013; German et al., 2015). The most important regulation mechanisms include DAT-alpha-synuclein C-terminal interaction (Lee et al., 2001; Butler et al., 2015), phosphorylation by PKC kinases (Foster et al., 2006) and D2-like family auto-receptor activation (Lee et al., 2007), which have been the object of much research.

Like in DA release, volume neurotransmission (both by phasic or tonic transmission) also modulates the involvement and activity of DAT. While phasic neurotransmission leads to high DA concentration in the extracellular space and modifies DAT-influx, tonic neurotransmission is not affected by this activity of DAT (Klein et al., 2019).

1.1.5 Dopaminergic pathways

In humans and mice, DA-expressing neurons are sparse both in the central and peripheral nervous systems; actually, they account for only 1% of all neurons in the brain. In the CNS, DA neurons are mainly grouped in the midbrain, forming one of the main DAergic brain nuclei (Chinta and Andersen, 2004). Midbrain DA neurons form a heterogeneous group of cells that can be classified by different genetic criteria (Roeper, 2013; Saunders et al., 2018). In addition to their varied gene-expression patterns (Tiklová et al., 2019), DAergic neurons can be topographically assorted on the basis of the striatal region their projections innervate (Beckstead, 1979; Fallon, 1981). Thus, according to their afferent innervation, DAergic neurons form four well-studied pathways (Arias-Carrián et al., 2010; Luo & Huang, 2016). Among them, two major pathways, the nigrostriatal and the mesolimbic pathways, project into the striatum (Dahlström & Fuxe, 1964). Additionally, the projections of many DAergic neurons in the substantia nigra pars compacta (SNc) innervate the dorsal-medial striatum or the caudate putamen (CPu) (Figure 3) (Fuxe, 1965 a,b). Furthermore, DAergic neurons in the ventral tegmental area (VTA) densely innervate the medial-ventral striatum or the nucleus accumbens (NA, Fuxe et al., 1985; Gerfen & Wilson, 1996; Horvitz, 2000; Farassat et al., 2019) (Figure 3). Both midbrain-striatal pathways together play a critical role in the neuromodulation of different functions directly substained by the striatum. While in the SNc, DA innervation primarily regulates procedural learning and controls for motor functions (Hikosaka et al., 2002; Joshua et al., 2009), DAergic neurons from the VTA are essential in reward, motivation and novelty-seeking behaviours (Ikemoto & Panksepp, 1999; Büchel et al., 2017). Since these pathways are involved in the modulation of vital functions, any neurotransmission imbalance affecting them may modify intrinsic functions in striatal areas. In humans, dysfunction of any of these pathways have been closely related to the development of several disorders such as addictions (Sulzer, 2011; Nutt et al., 2015; Wise & Robble, 2020), schizophrenia (McCutcheon et al., 2019), Huntington's disease (Schwab et al., 2015; Koch & Raymond, 2019), dementia with Lewis bodies (Piggott et al., 1999; Duda, 2004; Patterson et al., 2019;) and Parkinson's disease (Del Rey et al., 2018).

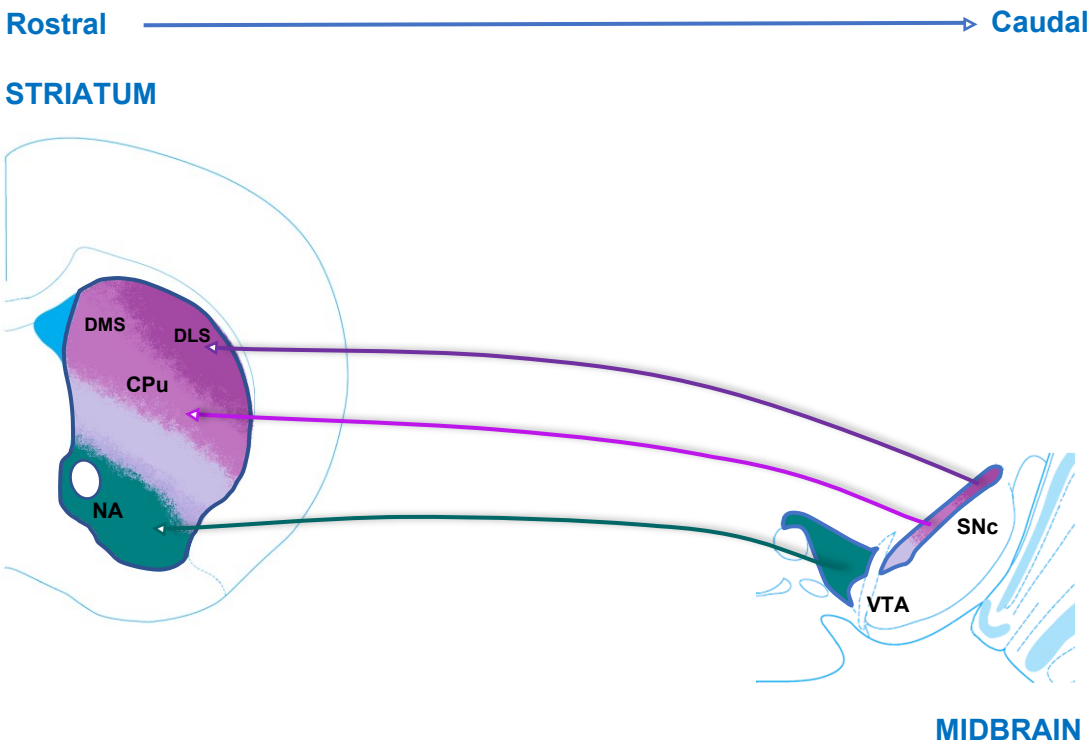

Figure 3. Distribution of midbrain striatal dopaminergic projections

Topographic organization of ascending dopaminergic innervation from different midbrain DAergic cell populations to the striatum. The VTA predominantly innervates the ventral striatum (green arrow), also called nucleus accumbens, while the lateral (pink arrow) and medial (purple arrow) regions of the Snc are connected to the DMS and DLS.

Abbreviations: CPu, caudate putamen; DMS, dorsomedial striatum; DLS, dorsolateral striatum; NA, nucleus accumbens; SNc, substantia nigra pars compacta; VTA, ventral tegmental area

1.2 Striatum

Basal ganglia (BG) are a well-characterized group of subcortical nuclei, which anatomically connect the striatum, the thalamus, the globus pallidus (GP), the cerebral cortex, and the midbrain (Fazl & Fleisher, 2018; Eisinger et al., 2019; Simonyan, 2019). Despite their diversity, these nuclei operate in close relationships to regulate and control motor learning, motor behaviour and emotions. Among the regions, the striatum is the largest nucleus and acts as the integrative gateway of the BG, processing most of the motor and limbic information (Lanciego et al., 2012; McCutcheon et al., 2019). Being the main afferent nucleus in the BG, the striatum receives most of its connections from three sources: pyramidal neurons in the cortical layer V, the thalamus and DAergic neurons in the midbrain (Shepherd, 2013; Haber, 2014).

1.2.1 Structure and cell populations

Based on its connectivity, the striatum is usually divided into two regions: the dorsal striatum or caudate-putamen (CPu) and the ventral striatum or nucleus accumbens (NA) (Gerfen & Surmeier, 2011). Such topographic parcellation is based on their different connections and abilities to discriminate between sensorimotor and limbic functions (Alexander & Crutcher, 1990; Howes et al., 2013). In rodents and humans, CPu can be subdivided into: the caudate nucleus or dorsomedial region (DMS) and the putamen nucleus or dorsolateral region (DLS) (Voorn et al., 2004; Graybiel & Grafton, 2015). At the cellular level, the neuron population of the striatum is composed of approximately 90-95% GABAergic neurons, also called medium spiny neurons (MSNs; Plenz & Wickens, 2016). MSNs receive glutamatergic and DAergic innervation and spread their projections into the striatum, as well as externally, with collateral axon projections to other BG nuclei (Loopuijt & Van der Kooy, 1985). Approximately half of the MSNs express the D1 DAergic receptor (D1R) and form a direct pathway projecting to the internal part of the GP and substantia nigra pars reticulata (SNr) (Gerfen et al., 1990; Surmeier et al., 1996). The other half of them, express the D2 DAergic receptor (D2R) and project to the external part of the GP and subthalamic nucleus (Sth), forming an indirect pathway. The remaining 5-10% neurons are interneurons, typically sub-classified into cholinergic, calretinin, neuropeptide Y (NPY) and parvalbumin GABA-expressing cells (Kawaguchi et al., 1995; Kubota & Kawaguchi, 1993).

1.2.2 Corticostriatal connectivity

Striatal functionality is inherently derived from the cortical connections that the dorsal and ventral striatal regions receive (Shepherd, 2013). Although cortical projections are not completely separated, they mostly innervate well-defined regions in the striatum, thus determining an association between those regions and certain functions. Generally, cortical inputs originate in the limbic area (related to motivation and reward) primarily reach the ventral striatum or NA (Figure 4) (Salgado & Kaplitt, 2015), while the associative cortex (dorsolateral prefrontal cortex) innervates the ventral-lateral and dorsomedial regions of the NA and the DMS in rodents (Figure 4) (Salgado & Kaplitt, 2015). Finally, premotor and motor cortex efferents mainly innervate the DLS (Figure 4) (McGeorge & Faull, 1989; Voorn et al., 2004). The complexity of the cortical-afferent distribution in the striatum entails

functional segregation of incoming information, where DMS and DLS are associated with behaviour/spatial learning and motor skill acquisition, respectively (Yin et al., 2009). The response strength and the relevant functions are directly controlled by DA release/uptake and DAergic pathways innervating the striatal regions (Gerfen & Surmeier, 2011; Zhai et al., 2018; Bogacz, 2020). Imbalances in DA release may disrupt the work of the striatal network thus underlying the symptoms of neurological disorders like schizophrenia or PD (Howes et al., 2013).

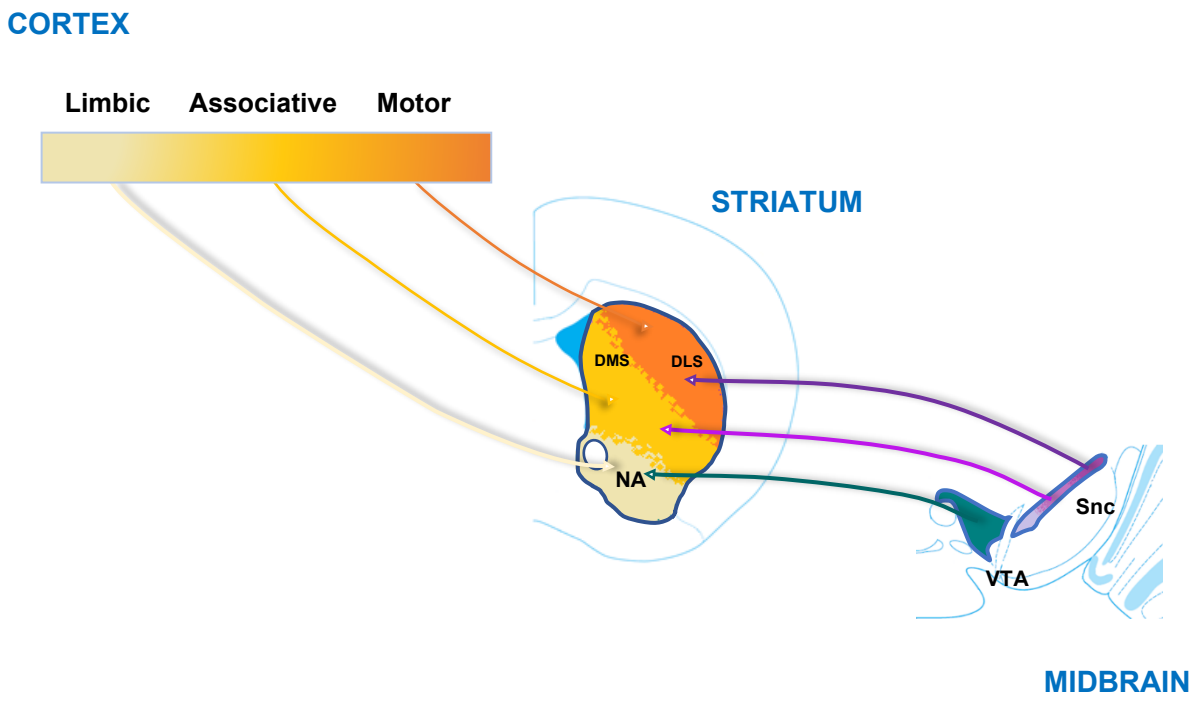

Figure 4. Topographic organization of cortical and midbrain afferents into rodent striatum

Striatum subdivision according to the topographic organization of corticostriatal innervation. Orange arrow: descending cortical afferents originated in the motor and premotor areas (somato-sensorimotor cortex, SMC) reach the putamen nucleus or DLS. Yellow arrow: corticostriatal connection of associative areas (dorsolateral prefrontal cortex, DPC) with ventrolateral and caudate nucleus or DMS regions. Beige arrow: association of the limbic cortex with the ventral region of the striatum. The purple, violet and green arrows represent the regional distribution of dopaminergic input into the dorsoventral and medial-lateral striatum (**see Figure 3**).

Abbreviations: DMS, dorsomedial striatum; DLS, dorsolateral striatum; DPC, dorsolateral prefrontal cortex; Snc, substantia nigra pars compacta; SMC, somatosensorimotor cortex; VTA, ventral tegmental area.

1.3 Parkinson's Disease

Parkinson's disease (PD) is a long-term neurodegenerative disorder that progressively impairs motor and non-motor functions and may result in injuries eventually leading to death (Stacy et al., 2010; Pfeiffer, 2016). Mainly affecting adult people around 50-60 years of age ,and 1.4 times more frequent in men than in women, PD is the second most common neurodegenerative disorder after Alzheimer's disease (Alzheimer's Association, https://www.alz.org/). Although its prevalence is hard to calculate, due to the high amount of undiagnosed cases (Van Den Eeden et al., 2003), it has been estimated in 150-200 cases per 100.000 people worldwide approximately and there is a prediction of nearly 13 million cases by 2040 (Lee & Gilbert, 2016; Nerius et al., 2017; The Lancet neurology, 2018). Nevertheless, such figures may be underestimated because of the lag time between the onset of the disease and the manifestation of motor symptoms (Savica et al., 2010). Despite our increasing knowledge about the progression of the disease, PD is still largely unknown and cannot be cured, with therapies only aiming to delay or relieve clinical symptoms.

1.3.1 Symptoms and neuropathology

James Parkinson described in 1817, a disease with an imperceptible onset but a severe impact on the quality of life, as symptoms later appeared. His notes included some of the most relevant clinical symptoms, still used in the diagnosis of Parkinson's disease (Goetz et al., 2011; Goedert et al., 2018). However, it was not until 1872 that Jean-Martin Charcot described the associated anatomopathological signs (Charcot, 1872).

Although not all patients present motor symptoms, the most important ones include limb tremor, difficulty initiating movement and muscle rigidity. Interestingly, as the disease progresses, patients develop additional non-motor symptoms such as depression or cognitive impairment (Dextera & Jenner, 2013; Williams-Gray & Worth, 2016). The manifestation of motor and non-motor symptoms is usually preceded by a decline period of years or even decades, during which no anomalies are observed. Studies have identified such a prodromal phase, in which symptoms are not yet apparent but degeneration has started. Non-motor signs include sleep disorders, depression, constipation and impaired olfaction (Postuma et al., 2012).

Histological atrophy of the brain is not marked in PD, since neurodegeneration only affects certain brain areas and neuronal populations rather than the whole brain (Poewe et al., 2017). Therefore, the onset of motor symptoms is generally attributed to a drop in DAergic content in the dorsal striatum due to the loss of DA-containing neurons (Andén et al., 1965; Grosch et al., 2016; Zhai et al., 2019). In healthy subjects, advanced age is associated with a linear decrease in the SNc DAergic neuron population of about 4.7% per decade of life (Fearnley & Lees, 1991). In PD patients, such decrease is exponential with a drastic loss of about 50%, leading to the onset of behavioural signs (Damier et al., 1999). DAergic neuron loss can be macroscopically observed because the SNc contains high levels of the intracellular pigment neuromelanin (NM). NM is only expressed in DA-containing neurons, which makes DAergic areas markedly dark (Haining & Achat-Mendes, 2017; Carballo-Carbajal et al., 2019). As the disease progresses, neuronal loss results in depigmentation of the midbrain, evidencing its deterioration.

Besides pigmentation loss, midbrain DAergic-neurons are heterogeneously affected during the progression of the disease. Neuronal degeneration tends to be more marked in the lateral ventral tier of the SNc, which reduces axonal projections into the dorsal striatum (Figure 5). In normal ageing, the SNc neuronal population decreases by approximately 2.1% per decade of life, with slightly higher rates in the medial ventral and dorsal tiers (5.4 and 6.9%, respectively). In advanced PD, the pattern appears to be the opposite, with the lateral tier exhibiting the largest neuronal loss (91%), while the cell-population of the medial and dorsal tier decreases from 71% to 56% (Fearnley & Lees, 1991; Damier et al., 1999; Kordower et al., 2013). Regarding the loss of DA terminals in the striatum, the integrity of DAergic fibres is generally more severely impaired in the putamen than in the caudate (corresponding to DLS and DMS, respectively) (Kish et al., 1988; Kordower et al., 2013). This heterogeneous susceptibility has also been described in pre-symptomatic stages since the loss of DA-producing neurons is restricted to the lateral ventral tier of the SNc, where the DAergic population decreases by about 52% (Huddleston et al., 2017; Surmeier et al., 2017).

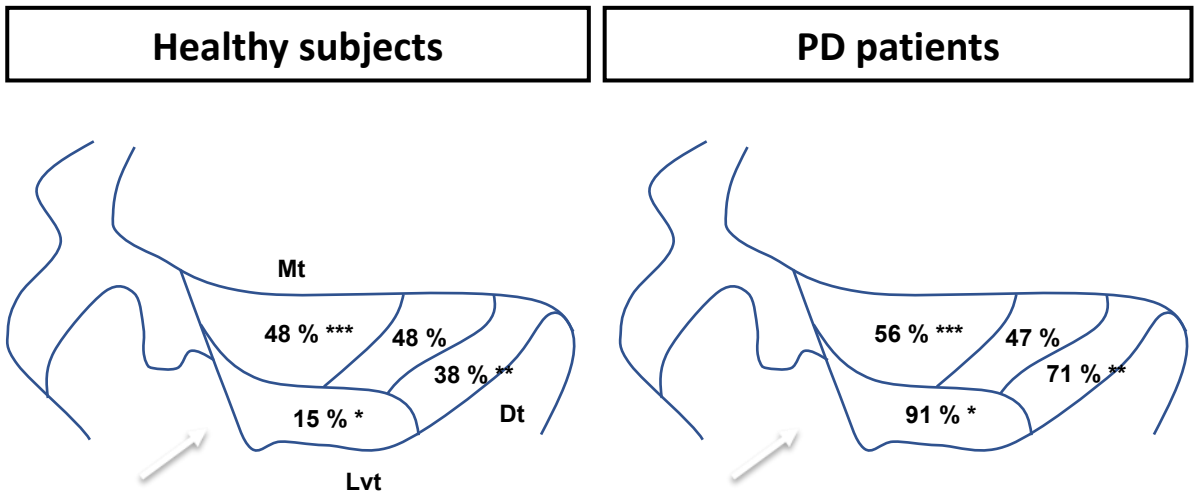

Figure 5. **Degeneration of dopaminergic neurons in human midbrain**

Neuronal loss distribution in the substantia nigra pars compacta in ageing healthy subjects or PD patients. The figure shows the consequences of aging on the dopaminergic cell population of the lateral ventral tier (*), medial tier (**) and dorsal tier (***) of healthy subjects (left) or Parkinson patients with several symptoms (right).

Modified from Fearnley & Lees, 1991 (License number: 5507041431882).

Abbreviations: Dt, Dorsal tier; Lvt, Lateral ventral tier; Mt, Medial tier

Besides SNc depigmentation, a further prominent pathological sign is the presence of abnormal intracellular depositions called Lewy bodies (LBs) (Schiller, 2000; Holdorff, 2002). Although LBs are regarded as a characteristic neuropathological sign of PD, they may also occur in different conditions like dementia with Lewy bodies (DLB) or other neurodegenerative disorders such as the Alzheimer's disease (AD) (Chung et al., 2015; Iacono et al., 2015). LBs have a rather heterogeneous protein composition, with aggregations of cytoskeleton proteins (neurofilaments, tubulin) or structural proteins (tau, parkin, ubiquitin) (Yacoubian & Standaert, 2014). However, the most abundant component of LBs is the presynaptic protein alpha-synuclein, which may be found as phosphorylated oligomers or fibrils (Goedert et al., 2012; Spillantini et al., 1997). These signs are easily detectable in different brain regions, e.g. the olfactory nucleus, the SNc or the cortex, and may be used to evaluate the stage and progression of the disease (Braak et al., 2003; Beach et al., 2009).

Up to date, the aetiology of PD remains unclear and most cases are classified as either idiopathic (sporadic) or familial (hereditary). Idiopathic cases account for about 90% of all diagnosed ones. Researches generally consider that they result from an interaction between environmental and genetic factors (Kalia et al., 2015; Wirdefeldt et al., 2011). Unlike idiopathic

cases, heritable forms of the disease only affect 5-10% of patients. Nonetheless, studies of familial cases seem to be essential to better understand the mechanisms of the disease. In pioneer studies of inherited patterns, gene SNCA – coding for protein alpha-synuclein – was the first gene to be identified as associated with PD (Golbe et al., 1990; Polymeropoulos et al., 1997). On this basis, alpha-synuclein has been consistently considered a factor in the pathogenesis of the disease.

1.3.2 Alpha-synuclein

Alpha-synuclein is a member of the synuclein-like protein superfamily, which also includes the gamma- and beta-synuclein forms (Lavedan, 1998; George, 2002). Highly conserved in vertebrates, alpha-synuclein is encoded in the SNCA gene. Its structure is composed of 140 amino acids (Ueda et al., 1993) and contains three major motifs: the acidic C-terminal domain, the non-amyloid component (NAC) and the N-terminal domain (Bendor et al., 2013; Meade et al., 2019). In the CNS, alpha-synuclein is most abundant in presynaptic terminals and neuronal nuclei (Iwai et al., 1995; Maroteaux et al., 1988), where it contributes to regulate many neuronal pathways (Benskey et al., 2018; Emanuele & Chieregatti, 2015). Although the exact function of this protein is still poorly understood, recent studies suggest that the above-mentioned motifs may actively participate in processes like oligomerization and aggregation of several protein units (Ulmer et al., 2005 a, b), protein-protein interaction (Benskey et al., 2016; Post et al., 2018) or modulation of distal synaptic vesicle pools, synaptic vesicle docking and vesicular trafficking (Bellani et al., 2010; Lautenschläger et al., 2018). Due to its presynaptic distribution and regulatory function of synaptic vesicle turnover, alpha-synuclein has been strongly associated with the maintenance of DAergic neurotransmission. Several studies have shown its modulatory effects on DA transportation and synaptic fusion (Butler et al., 2017; Ghiglieri et al., 2018). Additionally, studies on the interaction of alpha-synuclein with synaptic membranes in DAergic neurons showed that N-terminus-mediated binding allows rigid oligomeric cores to be inserted into membranes, thus disrupting them (Fusco et al., 2017). In the cytoplasm, alpha-synuclein can be found as dynamic soluble monomers or tetramers (Killinger et al., 2019; Wang et al., 2011). In pathological conditions, alpha-synuclein is phosphorylated and aggregated into oligomers, which alters its location and function (Bernal-Conde et al., 2020). Dysregulation of its normal distribution is a major pathogenic factor in synucleinopathies (Zunke, 2020). Phosphorylation and misfolding have been

observed in axon and presynaptic terminals of patients with neurodegenerative diseases such as PD, AD or DLB.

1.3.3 Animal models of human alpha-synuclein overexpression

Animal research is currently providing much insight into the physiological role of alpha-synuclein and its involvement in synucleinopathies. Thus, many genetically modified lines overexpressing or lacking this protein have been developed, by inserting or deleting the SNCA gene. Although a model including all PD features has not been achieved so far, these pivotal tools can help elucidate the role of this protein in PD-like symptoms (Magen & Chesselet, 2010). On the one hand, studies with alpha-synuclein knockout animals help assess its presynaptic functions in neurotransmission; on the other hand, overexpression of wild-type (WT) alpha-synuclein or expression of truncated human alpha-synuclein provide PD-like models that mimic several PD features. Animal models overexpressing the complete human alpha-synuclein gene under different promoters have been created since the early 2000s. As a result, several lines have been developed, which differ not only in the expression pattern of alpha-synuclein but also in the expressed phenotypes.

MOUSE LINE	PROMOTER	REGION EXPRESSION	LOCOMOTOR DEFICIT	DOPAMINERGIC ALTERATIONS	REFERENCE
Line D	PDGF-B	Neocortex, midbrain, olfactory bulb	X	X	Masliah et al., 2000
Line 61	Thy-αSyn	Cortex, brainstem, basal ganglia, Sn	X	X	Rockenstein et al., 2002
	BAC	Cortex, striatum, Sn, thalamus	X	X	Nuber et al., 2013

Table 1. **Mouse models overexpressing human alpha-synuclein**

Short overview of the most frequently used mice models overexpressing human wild-type alpha-synuclein. (X represents the development of deficiencies in locomotion tasks and dopaminergic innervation in the striatum).

Abbreviations: BAC: Bacterial artificial chromosome; Thy1-αSyn: murine Thy-1 promoter; PDGF-B: Platelet-derived growth factor; Sn: Substantia nigra.

1.3.4 Platelet-derived growth factor human alpha-synuclein (Tg) transgenic mouse

The Eliezer Masliah's research group was a pioneer in developing transgenic animal models using the PDGF or the Thy promoter, two of the most frequently used promoters for protein overexpression in neurons (Masliah et al., 2000; Rockenstein et al., 2002; Magen & Chesselet, 2010; Hall & Roberson, 2012; Grosch et al., 2016). In addition to increased protein expression, both mice lines express high levels of human alpha-synuclein RNA, although they differ in the expression level and distribution pattern. Despite the differences, both strains show marked dysregulation and alterations in DAergic neurotransmission (Table 1).

The PDGFβ-SNCA (Tg) line was one of the first transgenic mice lines carrying the complete sequence of the human alpha-synuclein gene. During its development, multiple transgenic parental lines were generated and selected according to their ability to overexpress the transgene. Line D was found to express the highest levels of human alpha-synuclein RNA and reproduce some of the primary PD features. Opposite to non-transgenic WT mice, Line D mice showed abnormally dense inclusions in midbrain neurons since young ages (two months old). However, although such aggregations were similar to human LBs, they lacked the fibrillary components typical of humans. Furthermore, in aged mice (twelve-month-old), continuous overexpression was observed to alter DAergic neurotransmission; DA contents were drastically affected and TH activity was lower in striatal DA terminals. Interestingly, such alterations were not reflected in the midbrain, as stereological studies failed to find significant neuronal loss. As a consequence of reduced DA contents, Tg mice exhibited marked alterations in motor learning and remarkable worsening of motor tasks. The characteristic of reproducing several PD-like neuropathologies has promoted the use of this mouse line in a large number of studies aimed at unraveling the mechanisms that trigger degeneration and malfunction of the DAergic system in synuclein-dependent pathologies.

Hypothesis and aims

From a physiological point of view, alpha-synuclein has multiple properties and modulates DA synthesis, release and uptake in DAergic midbrain neurons. Pathological accumulation of misfolded forms of this protein contributes to cellular dysfunction and triggers neurodegeneration. During PD progression, the selective vulnerability of certain neuron populations can be observed, with impaired striatal DA functions, especially in the DMS and DLS. Yet, the mechanisms through which alpha-synuclein overexpression preferentially affects definite areas in the dorsal striatum remain unknown. Understanding the reasons behind such preferential vulnerability may be important to understand the mechanisms of neurodegeneration onset. Considering its functions in DA neurotransmission and the preferential susceptibility of certain striatal neuron terminals in PD, it is hypothesized that alpha-synuclein may interfere with DA neurotransmission dynamics, preferentially in the DLS.

In pursue of new knowledge and a better understanding of the impact of alpha-synuclein on DA-system susceptibility in the dorsal striatum, the following objectives were formulated:

1. Studying the age-dependent impact of human alpha-synuclein overexpression on DA neurotransmission between the dorsomedial and dorsolateral striatum at the physiological and behavioural levels.

2. Analysing alpha-synuclein involvement in the modulation of DAT activity during DA uptake from the extracellular space.

3. Analysing the way alpha-synuclein affects TH and DAT distribution in the DMS and DLS.

2 Material and Methods

2.1. Genetically modified organisms and husbandry

2.1.1 Animal model

PDGFβ-SNCA

Tg(PDGFB-SNCA)4Ema (Tg) transgenic mice were developed on a combined genetic background involving C57BL/6 and DBA/2 strains (MG IDI:3528873) (Masliah et al., 2000). Cohorts of three-, six- and twelve-month-old animals overexpressing human alpha-synuclein protein under the PDGFβ promoter were used in this study.

2.1.2 Animal Husbandry

Laboratory mice welfare and experimental use followed the regulations of the Ludwig Maximilians University of Munich and the government of Upper Bavaria (Tierversuchsantrag, TVA: 55.2-1-54-2532-214-2016). Mice were bred and maintained in standard individual ventilated cages (IVC, 30 × 15 × 20 cm) with a 12/12 h dark/light cycle and *ad libitum* access to food and water. Nesting materials and plastic-made shelters were provided to enrich their environment. To prevent social deprivation effects, mice were lodged in groups of 5 individuals per cage, after weaning.

2.1.3 Genotyping

DNA extraction

Ear tissue biopsies were collected from four-week-old mice and kept at minus 20°C until required. For DNA isolation, ear samples were incubated in lysis buffer containing proteinase K and RNase in distilled water (ddH2O) at 52° C (400 µml per well, overnight). The resulting solution was loaded on a binding plate (PerfectBind) with 200 µl of Binding Buffer A to a final volume of 600 µml. The binding plate was then set onto a multiwell plaque, sealed and centrifuged for 5 minutes at 3700 rpm/ccf. The remaining pellet was washed and centrifuged in washing buffer (450 µml per well, 1 min 3700 rpm/ccf). Additionally, the plates were centrifuged at the same speed with no buffer. To finally get the isolate, samples were spun down with pre-heated elution buffer at 52° C and kept at 4° C.

DNA amplification (polymerase chain reaction, PCR)

After DNA extraction, the template DNA was amplified through the polymerase chain reaction (PCR, Thermocycler Eppendorf). To identify PCR results, samples were loaded on 2% agarose gel (diluted in TE buffer and SilverGold). ChemiDoc imaging system was used to detect the chemiluminescence reaction.

PDGFβ-SNCA

Primers

	Sequence 5´ to 3´	Length	
209F	CTG GAA GAT ATG CCT GTG GA	20	
50R	CAT CAA TGT ATC TTA TCA TGT CTG GAT TCT	29	

PCR solution

Volume	Compound	Concentration
12.5	OneTaq HotStart QuickLoad, NEB (x2mm)	
0.5	209F	1/10
0.5	50R	1/10
1	Template DNA	
10.5	H_20	

PCR programme

Step	Temperature (°C)	Time	Repeat
1	94	3 min	x1
2	94	30 sec	x27
3	60	1 min	
4	68	20 sec	
5	68	5 min	x1
6	10	unlimited	unlimited

2 % Agarose gel

	PDGF ca. 117 pp	Control
WT	-	+
Het	+	+

2.2 Drugs

Cocaine-hydrochloride (C5776, Coc) and dopamine-hydrochloride (H8502-10G, DA) were purchased from Sigma Aldrich. Following datasheet information, compounds were kept at room temperature or 2-8° C. Due to security requirements, the Coc container was stored in a safe box until its experimental use.

Coc pre-stock 1mM was diluted in ddH2O and stored at +4°C for no longer than four weeks. Right before the experiments, the stock was diluted in artificial cerebrospinal fluid (aCSF) to a 3 μM concentration.

A DA working solution was prepared in aCSF at room temperature and immediately protected from light due to its photosensitivity. For experiments, the stock was consecutively diluted to 5, 20 and 25 mM in aCSF.

2.3 Fast scan cyclic voltammetry (FSCV)

FSCV was used to detect temporal and spatial DA neurotransmission based on the voltage produced during reduction and oxidation processes. Briefly, the setup was composed of a stimulating electrode and a recording one. The stimulating electrode was a bipolar electrode connected to an isolated analogue device (Digitimer ltd, model S3D), which delivered electrical stimuli. The recording electrode was a glass capillary tube charged with continuous triangular waveforms (−0.4 to 1.3 V and back to −0.4 V vs Ag/AgCl with a scan rate of 400 V/s) designed to detect current fluctuations in the buffer.

After electrical stimulation of the region of interest, evoked DA release and uptake were recorded using the Demon voltammetry software. For determining functional changes in the DAergic innervation, DA parameters were subtracted from dorsomedial (DMS) and dorsolateral (DLS) striatum. *Ex vivo* studies were conducted with an animal model for synucleiophaties, namely twelve-, six- and three-month-old Tg mice.

2.3.1 Tissue preparation

Mice were deeply anaesthetised with inhaled isoflurane. As the absence of any reflex was confirmed, they were euthanized by cervical dislocation (as TVA reported). Brains were immediately harvested, taking extreme care not to damage them during removal. To facilitate brain extraction, four cuts (two perpendicular cuts, one on the sagittal suture and one between the eyes) were made. Once removed, brains were transferred into a semi-frozen cutting solution (Sucrose (194), NaCl (30), $NaHCO_3$ (26), Glucose (10), KCl (4.5), NaH_2PO_4 (1.2), $MgCl_2$ (1) mM) and placed on a vibratome tray (Leica VT1000S) pre-chilled at -4°C and 250-µm coronal striatal slices were obtained. Slices were put into a chamber, in a 150 ml beaker containing aCSF (NaCl (126), $NaHCO_3$ (25), KCl (2.5), HEPES (20), glucose (11), $CaCl_2$ (2.4), NaH_2PO_4 (1.2), $MgCl_2$ (1.2), L-ascorbic acid (0.4) mM, pH 7.4), where they were kept in a bath at 32°C for one hour and then at room temperature until required. To maintain adequate pH and oxygen levels, brains and slices were constantly oxygenated with carbogen (95% O_2/ 5% CO_2).

2.3.2 Recording electrodes

Recording electrodes were prepared by placing a single carbon fibre (6-7 µm diameter, Goodfellow) inside a glass capillary tube. The capillary was then pulled at 62.7°C (micropipette puller, Narishigue, Model PC-10) and trimmed at a length of 50-100 µm using a magnifying glass microscope (Leica MZ 10 F) (Figure 6). Before the experiments, the recording electrodes were filled with a 2M potassium chloride solution and cleaned with isopropanol saturated with carbon particles. Acclimatization in aCSF was needed to reduce background noise.

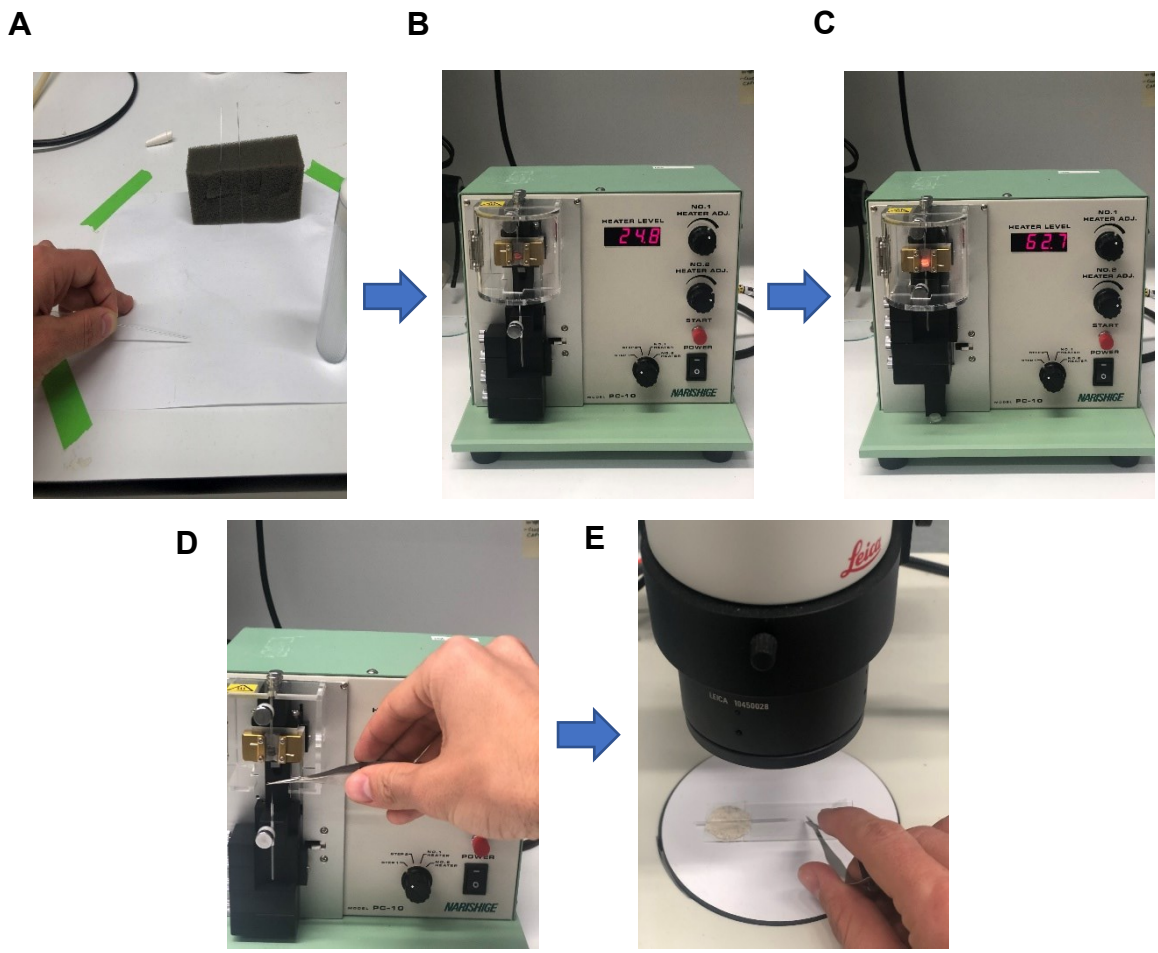

Figure 6. Step-to-step preparation of the recording electrode

A) A single carbon fibre 6-7 µm in diameter was isolated and inserted into a glass capillary tube by vacuum absorption. **B**) The capillary tube was loaded in the micropipette puller. **C**) The electrode was pulled at 62.7°C and the two resulting electrodes were cut. **D**) The electrodes were then removed and trimmed under light microscope, 8x magnification. **E**) A segment was cut, 50-100 µm long from the sealing point (easily recognisable because the point where the glass attaches to the fibre blinks when exposed to light).

2.3.3 Demon voltammetry software

Recordings from each slice were stored and analysed by using voltammetry software DEMON (Yorgason et al., 2011). The software interface consisted of three panels that provided the correct acquisition of DA stimulation (Figure 7). In addition, the Demon voltammetry software offers several tools useful to detect and analyse the level of evoked DA release and other DA parameters, such as DA uptake.

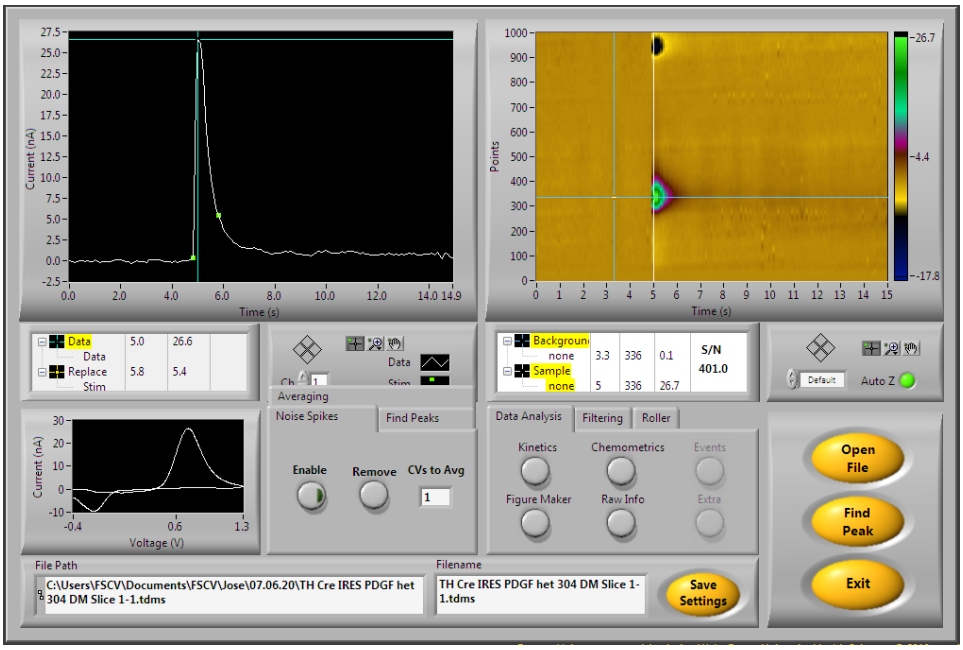

Figure 7. Demon Voltammetry interface

Upper left panel: recorded traces showing the subtracted dopamine current (nA) vs time (sec). Upper right panel: colour plot showing recording over time plus DA oxidation (green peak) and reduction peak (black circle). Lower left panel: specific voltammogram profile of DA current vs voltage curve.

2.3.4 FSCV acquisition

Slices were transferred to a chamber under continuous perfusion with freshly prepared aCSF at 32°C (perfusion rate 2 ml/min). Once acclimated to the medium (ten minutes), a stimulating electrode was placed into the corpus callosum, close to the region of interest, i.e. the DMS or the DLS. To ensure proper reading, the recording electrode was poked into several points between the two ends of the bipolar stimulating electrode until two consecutive DA peaks reached similar values. Once stable peaks were obtained, DA release was evoked every three minutes following different experimental protocols. Traces were recorded for 15 seconds and a stimulus was generated after five seconds. All the experiments were performed both in the DMS and the DLS.

Since FSCV might evidence other catecholamine neurotransmitters, the current was monitored to verify that the oxidation potential was around 500 – 700 mV and reduction around -200 mV, which are typical for DA.

2.3.4.1 Input/Out stimuli

In order to evaluate the effects on phasic evoked DA dynamics, stimulation intensities of 100, 200, 400 and 600 µA were consecutively applied (Figure 8). Input/output (I/O) stimulation was directly generated in the corpus callosum directly above the DMS and the DLS areas following the above-described protocol.

2.3.4.2 Burst-firing stimuli

Besides isolated electrical stimuli, 5p 50Hz burst-like stimuli were applied to the DMS and DLS areas after the I/O experiments.

2.3.4.3 Dopamine blockade

To evaluate the impact of DAT-mediated DA uptake, fresh 3 µM Coc buffer diluted in aCSF was pumped into the perfusion system. Slices were stimulated every three minutes and tracked for 15 minutes. DA was electrically evoked at 400 µA. Between one slice and the next one, perfusion tubes were rinsed with ddH20 for 30 minutes to ensure that no rests of Coc remained in the recording setup.

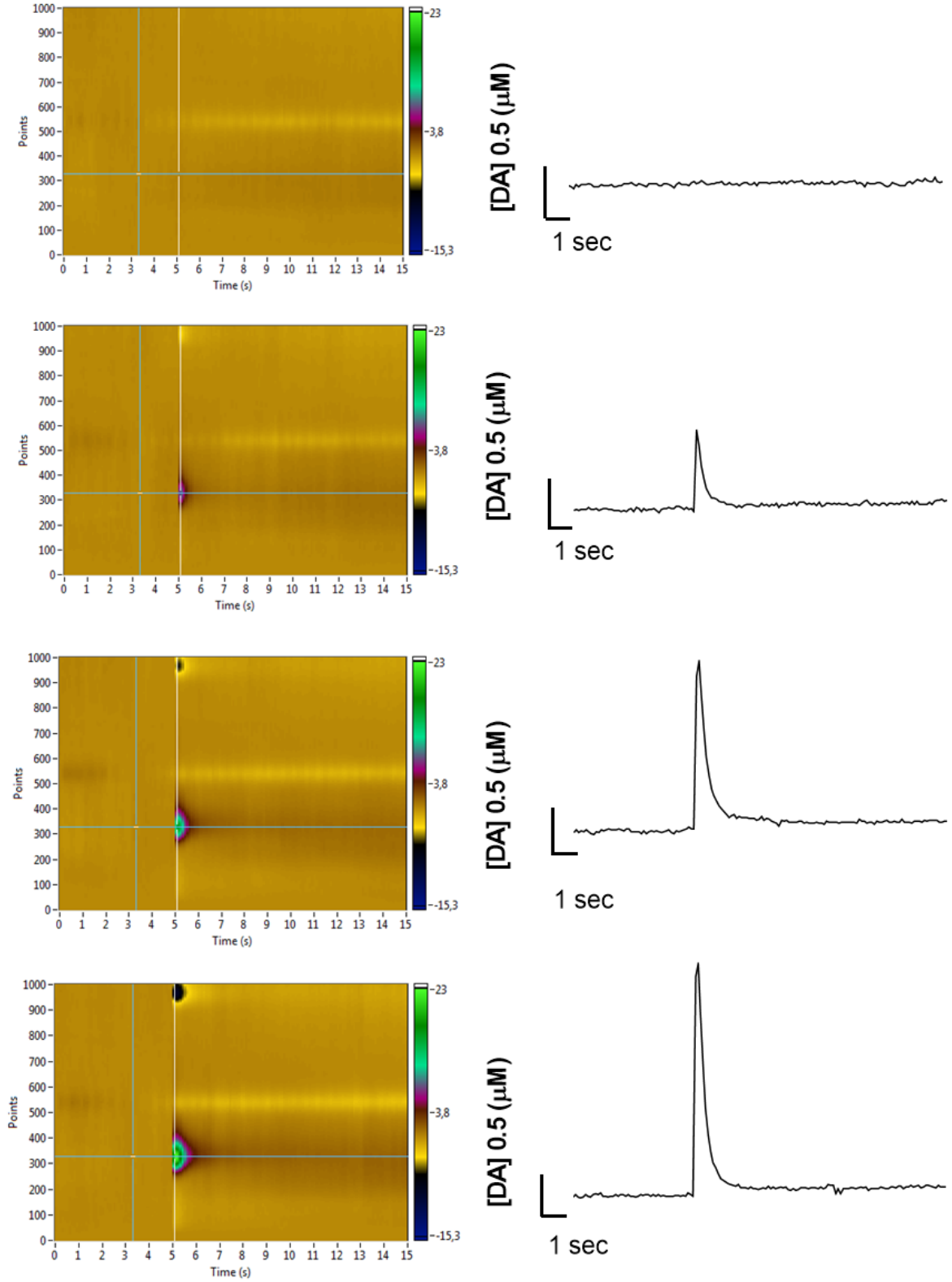

Figure 8. Representative pictures and subtracted traces of dopamine-release recordings in the I/O experiments

Left panel: colour plot of the voltammetric current (colour in z-axis) vs applied potential (y-axis) and acquisition time (x-axis). Colour plots are magnified to better show DA spikes. Right panel: normalized recording traces after electrical stimulation.

2.3.5 Data analysis

DA-release data from DAergic terminals in the DMS and the DLS were analysed by using the Demon voltammetry software. In these studies, DA peaks and DA uptake were evaluated in at least four striatal slices.

2.3.5.1 Dopamine release

To convert digital current peaks to actual DA concentrations values, standardized solutions of DA (H8502, 10G) 5, 20 and 25 M were consecutively injected into a petri dish containing 5 ml of aCSF. Subsequently, the current corresponding to each concentration was measured for 120 seconds with the recording electrode. The correlation between readings and concentrations gave a linear equation, where DA peak levels could be extrapolated and DA concentrations calculated for the different I/O intensities and burst-firing stimuli (Figure 8).

2.3.5.2 Dopamine uptake

The software also provided tools to evaluate DA uptake based on the Michaelis-Menten model. After reaching a peak value, DA declined to basal levels. The decay portion, which indicates DA removal from the extracellular space, could be calculated by extracting the time constant between the 10th and 40th percentiles of the slope (τ) (Figure 9). The τ value illustrated the speed of DA levels decline in the extracellular medium. DLS τ values were normalized with the DMS ones collected from the same slice, and data were thus shown as percentages of τ DLS/ τ DMS.

2.3.5.3 Dopamine uptake blockade

DAT activity was studied by comparing how the decay portion changed over time due to continuous DAT blockade. As described above, the τ parameter was used to identify the functionality of the DAT protein. Since exposure to Coc lead to depletion of the DA pool in the presynaptic DA terminals, every slice was used only once. Therefore, DLS τ values were normalized with the DMS τ value from another slice at the same Bregma level. As above, data are shown as percentages of τ DLS/ τ DMS.

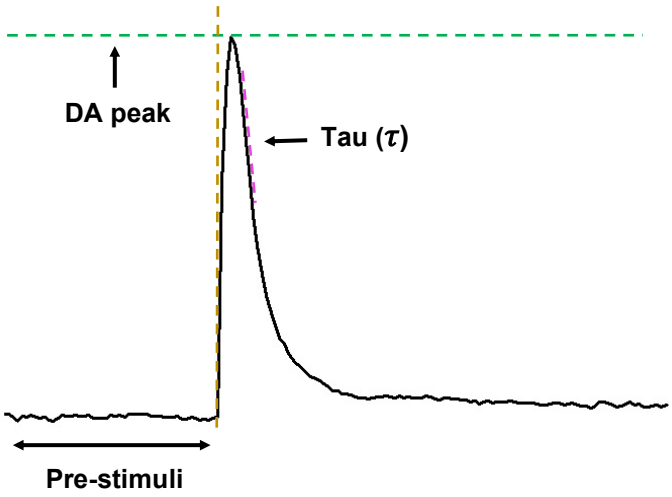

Figure 9. **Representation of dopamine neurotransmission parameters**

The trace represents the DA peak (green dash line) and decay portion (pink dash line), where DA levels and time constant were subtracted (Tau value τ, pink dash line).

2.4 Immunofluorescence (IF)

2.4.1 Sample fixation and slicing

Mice were deeply anesthetized with ketamine/xylamine (60 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Brains were rapidly removed and post-fixed in the same fixative medium overnight. Coronal sections of 50 µm were obtained from the whole brain using a vibratome (VT1000, Leica) and stored in PBS containing 0.02% sodium azide. Free-floating sections were taken from the CPu and processed for standard immunofluorescence assay.

2.4.2 Immunofluorescence

Sections were washed in PBS 0.1 M three times, for ten minutes. Subsequently, tissues were pre-blocked overnight with either donkey or goat serum 5% in PBS with 0.2% Triton X-100 (PBS-TX), in order to reduce unspecific binding. Sections were then incubated with the corresponding primary antibody (Table 2) diluted in PBS with 0.2% Triton X-100

(PBS-TX) and 3% of the serum used in the pre-blocking solution. Incubation was carried out for 24 or 48 hours in cold room.

	CATALOGUE NUMBER	SOURCE	HOST	DILUTION	INCUBATION TIME
TH	MAB318	Sigma-Aldrich	Mouse	1:500	o/n / 4°C
DAT	AB1591P	Sigma-Aldrich	Rabbit	1:500	48 h / 4°C
H-A-SYN	15G7	(Neumann et al., 2002)	Rat	1:2000	o/n / 4°C

Table 2. Primary antibodies

After repeated rinse in PBS, sections were incubated in Alexa fluor 488 anti-mouse, Alexa fluor 647 anti-rat or Alexa fluor 647 anti-rabbit (1:1000), for 1-2 hours. Finally, sections were mounted on coated slides with mounting medium (DAKO fluorescence mounting medium).

2.4.3 Quantitative and qualitative analysis

Confocal microphotographies were used to study the DAergic innervation in the DMS and DLS of WT and Tg mice. Images were obtained with a laser-scanning confocal microscope (LSM 780, Carl ZEISS, Germany) (objective 20x or 40x) and analysed with the free software ImageJ (National Institutes of Health, NIH). Three sections of the rostrocaudal striatal series were analysed and two pictures were taken of each area and brain hemisphere (15 µm of optical section thickness).

2.4.3.1 Optical density and area

For semi-quantitative and qualitative studies of the protein level, optical density (O.D.) and area cover were measured in TH, DAT, and human alpha-synuclein staining. First, z-stack images were projected at maximum intensity and transformed into 8-bit images. Then, the threshold was adjusted to the desired signal intensity. O.D. raw data were corrected with the values of immunonegative areas and represented as the mean of grey values.

The area was calculated as the percentage of the signal considered specific of a marker and represented in μm^2.

2.4.3.2 Dopamine transporter distribution

To evaluate the size of DAT puncta, a particle analysis was carried out in the DMS and the DML (size parameter was set from 1.5 μm^2 to infinity). Results were then classified into size intervals by using the cumulative frequency distribution tool (CFD), which also generated a frequency table with the number of observations in each category. Bin width was set at 0.1.

2.5 Rotarod task

To examine the effect of DA release in a motor learning process, three-month-old WT (N=6) and Tg (N=6) mice were studied while performing a rota rod (or rotarod) task. In this task, animals were placed on a rotating rod (a 10 cm cylinder), which was hanging high enough to discourage them from jumping. The rod was connected to a working device that controlled the rotating speed and stopped at the moment the mouse fell from it. The apparatus measured the time mice were able to stay on the rod, which was later considered as the latency to fall. The rotation speed was set to accelerate from 0 to 40 rpm in 60 sec and keep constant afterward (at 40 rpm). Before the experiment, animals were weighed to analyse any possible effect of body weight on the performance of the task.

2.5.1 Procedure

The rotarod experiment consisted of two phases: a first familiarization phase, where animals got used to the apparatus, and a subsequent four-day testing phase, where their performance was measured. On the first day, animals were taken to the room where the experimental apparatus was located and left there for at least 30 minutes. Once acclimated to the environment, they were gently placed on the rod (not rotating) and returned to their enclosures after five minutes approximately. If a mouse fell before that time, the process was repeated until the animal was able to stay on the rod for several minutes. On the subsequent testing phase, animals were left on the rotating rod (first accelerating and then rotating at a constant speed as described above) until they fell and this time was considered the latency to fall. This latency was measured ten times per day, in sessions separated by five minute periods, for four testing days

2.5.2 Data analysis

Motor learning was evaluated by comparing WT versus Tg mice in terms of mean latency per day and possible latency changes during the four testing days.

2.6 Statistical analysis

The statistical analysis was carried out with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA USA). Parametric tests were used for data with a normal distribution (Student´s t-test and two-way ANOVA) and non-parametric tests for data with non-normal distribution (unpaired two-tailed Student's t-test and two-way ANOVA followed by Bonferroni post hoc test); $p < 0.05$ was considered significant.

3 Results

3.1. Impaired dopaminergic neurotransmission in the striatum of mice overexpressing human alpha-synuclein

The Tg(PDGFB-SNCA)4Ema (Tg) mouse strain is a genetically modified animal model developed at the beginning of the 2000s (Masliah et al., 2000), suitable to study the diseases classified as synucleopathies. According to the relevant literature and due to their phenotype – mimicking several of the features observed in human patients – these animals are the best choice to study the effects of human alpha-synuclein overexpression in the brain. Studies previously conducted with this model revealed that overexpression of human alpha-synuclein interferes with the DA system in the striatum (Rockenstein et al., 2002; Haggerty et al., 2011). Yet, little is known about its effects on other projecting areas of the DAergic pathways, or the way the vulnerability of certain DAergic innervations may contribute to the progression of the disease. To better understand the impact of human alpha-synuclein overexpression on DA release and uptake in the DMS and DLS, experiments with twelve-, six- and three-month-old Tg mice were carried out.

3.1.1 Dopamine dynamics is preferentially impaired in the dorsal striatum of twelve-month-old Tg mice

FSCV is a versatile technique useful to identify many analytes. To ensure proper DA readings, the analyte (in this case, neurotransmitter DA) must be redox-active within the potential window to be scanned. In our experimental setting, to validate DA recordings, the oxidation (~ 600 V) and reduction (- 200 V) point values of this neurotransmitter were used as a reference for correct acquisition, after calibrating the electrodes with a DA standard solution. In the below colour plots, such values are represented as an oxidation peak around 400 and a reduction peak at 950 (Figure 10A). As observed in Figure 10, graphs, traces and voltammogramms confirmed the recording of DA (Figure 10A and B). As a convention, the oxidation peak has been used as a variable to monitor the extracellular level in the biological substrate (striatum slices).

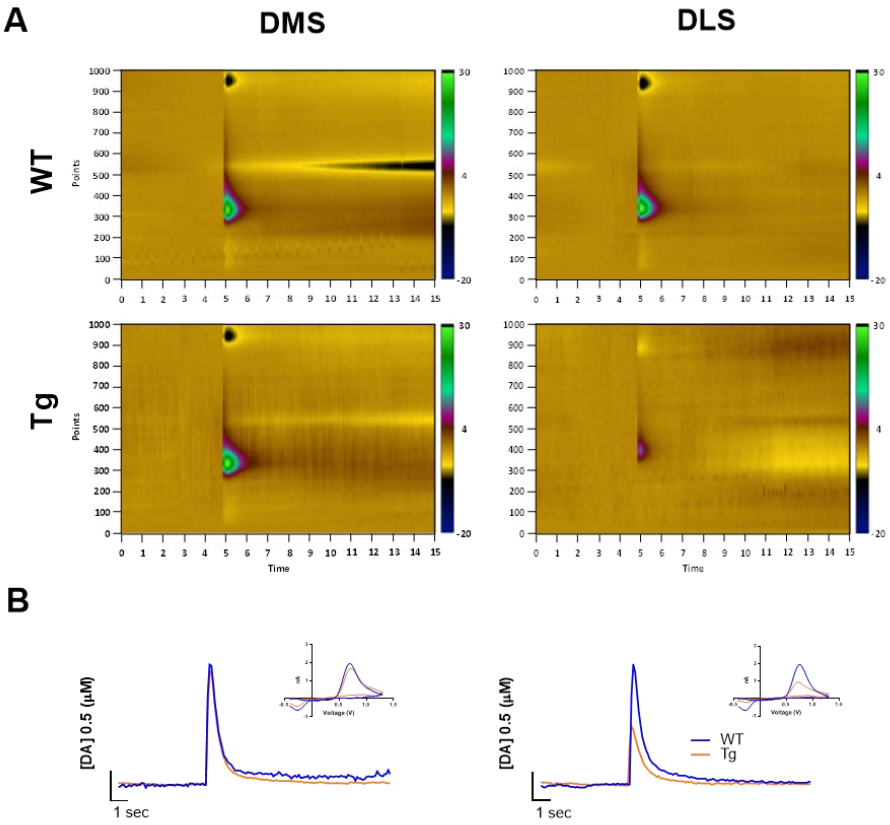

Figure 10. Representative recordings with 400 µA stimulation in WT and Tg mice

(**A**) Colour plots of 400- µA stimulation in the DMS and DLS of WT and Tg mice, (**B**) Recording traces and voltammograms represent DA release evoked with 400 µA stimulation in twelve-month-old WT and Tg mice.

3.1.1.1 Dopamine release and uptake are impaired in the dorsolateral striatum but not in the dorsomedial striatum

Upon electrical stimulation of DAergic terminals with single stimuli of increasing intensity, i.e. 100, 200, 400 and 600 milliampere (µA), a corresponding elevation in the DA release response was observed in the DMS region (Figure 11A; DMS: two-way ANOVA, Stimulation Main Factor, $F_{(3,32)} = 20.43$, $P < 0.0001$). However, no significant differences were found between genotypes at any stimulus intensity (Figure 11A; DMS: Stimulation x Genotype interaction $F_{(3, 32)} = 0.01336$, $P = 0.9978$). Interestingly, although evoked DA release in the DLS was also increased with the different stimulation intensities (Figure 11A; DLS: two-way ANOVA Stimulation main factor $F_{(3, 36)} = 101.7$, $P < 0.0001$), DA levels were substantially lower in Tg than in WT mice (Figure 11A; DLS: Stimulation x Genotype interaction $F_{(3, 36)} = 6.006$, $P = 0.0020$). Such differences were observed with 200, 400 and 600 µA stimuli and reached about 75%, 50% and 55%, respectively (**, 0.1303 to 1.073, P = 0.0075; ****, 0.4799 to 1.423,

P < 0.0001; ****, 0.7381 to 1.681, P < 0.0001). Burst firing-like stimulation (which resembles the physiological activity of DAergic populations) produced similar results (Figure 11B). While the DA level evoked by burst-firing stimuli in the DMS did not differ between genotypes (Figure 11B. DMS: WT = 2.037 ± 0.5704; Tg = 1.721 ± 0.2732), it was significantly lower in the DLS of Tg mice than in that of WT (Figure 11B. DLS: WT = 1.820 ± 1.036; Tg = 11.036 ± 9.116; * P < 0.05).

To assess whether DA level alterations could be accompanied by DA uptake modification, time constant (τ) was calculated from the slope of DA traces at 400 μA in both the DMS and the DLS (Figure 11C). Since DA levels in the DMS were statistically comparable for both genotypes and the slope seemed to return to baseline levels in the same way (Figure 11A and C), the τDMS value was considered an internal reference for τDLS, to prevent inter-individual or electrode/session-related biases. Additionally, to reduce experiment-associated variability (e.g. from recording in different sessions), the τDLS/ τDMS ratio was calculated using data acquired on the same recording day and from the same striatal slice. Kinetics studies revealed that the τDLS/τDMS ratio of Tg mice was significantly higher than that of WT mice by approximately 28% (Figure 11D. WT = 80.19 ± 3.640; Tg = 108.0 ± 9.116; * P < 0.05). Higher values of decay time ratio were associated with slower DA uptake and consequently, longer DA persistence in the extracellular space before returning to pre-stimulation baseline levels. The faster DA-clearance kinetics observed in the DLS of WT mice was not evident in Tg animals, which posed questions on the possible pathophysiological role of DAT in our model.

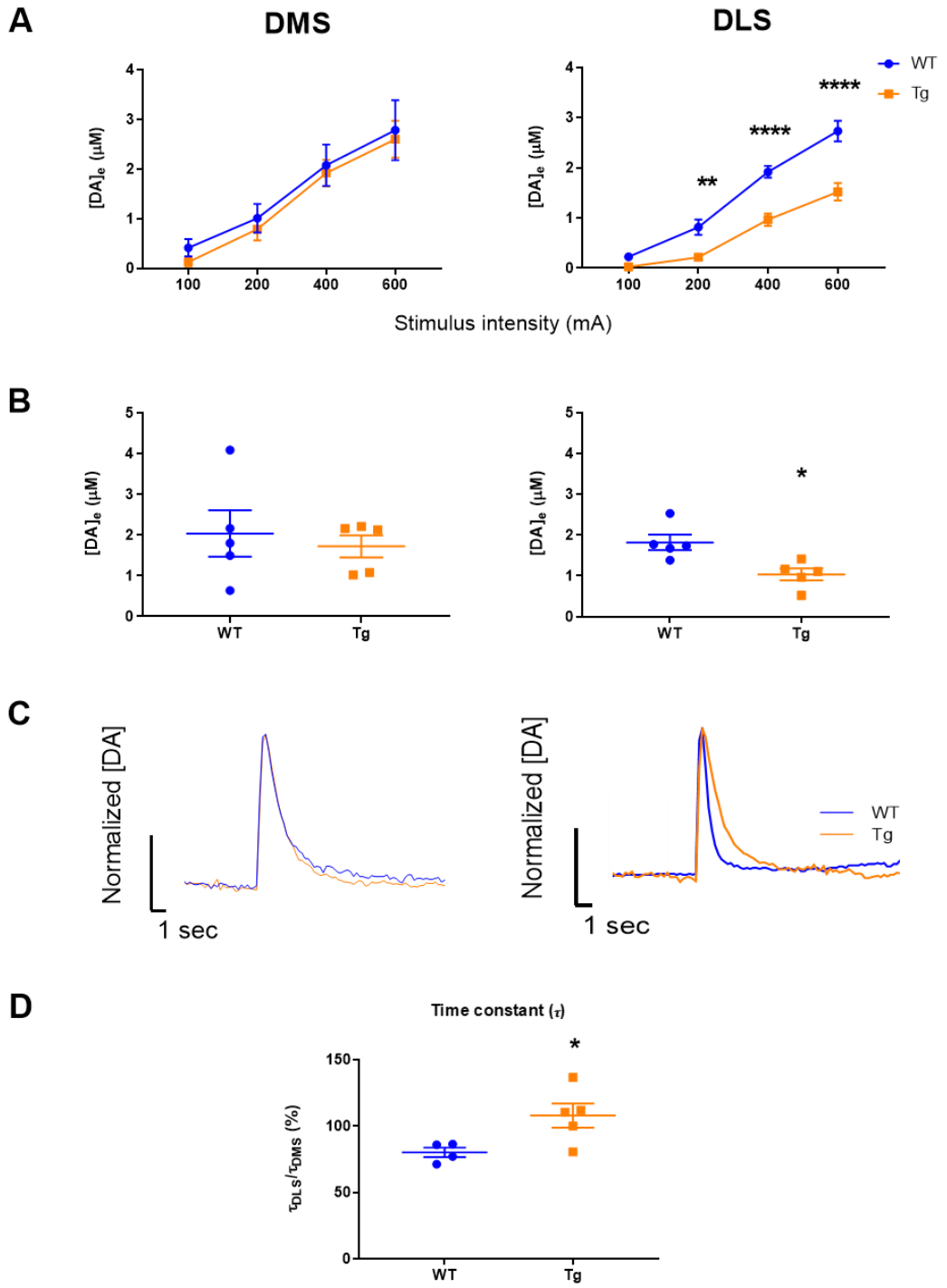

Figure 11. Dopamine release evoked by electrical stimulation of the dorsomedial (DMS) and dorsolateral striatum (DLS) of twelve-month-old WT and Tg mice

(**A**) Input/output stimulation to the DMS and DLS of WT and Tg mice with increasing intensities: 100, 200, 400 and 600 µA. (**B**) Burst-firing stimulation with 400 µA (5p 50Hz) to the DMS and DLS of WT and Tg mice. (**C**) Normalized traces for evoked DA release in the DMS and DLS of WT and Tg animals. (**D**) Percentages of the τDLS/τDMS ratio in WT and Tg mice. Data were expressed as mean ± SEM. The statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests. Asterisks indicate significant interaction between mouse strain and stimulus intensity; $F_{3,36}$ = 6.006, P = 0.0020. Unpaired two-tailed t Student´s test for burst-firing results (DMS, t = 0.4999, df = 8; DLS, t = 3.259, df = 8, P < 0.05) and time constant ratio (t = 2.567, df = 7, P < 0.05) in WT and Tg mice.

3.1.1.2 Dopamine transporter blockade indicates that dopamine uptake alterations may result from modifications in its function

During DA uptake, DAT plays a crucial role in the DA clearance and recovery into presynaptic terminals (Jones, 1998a,b). Since traces evidenced alterations of τ in Tg mice, the possible involvement of the DA transporter was assessed by incubating brain slices with cocaine (Coc, 3 µM). Coc directly binds DAT thus reducing the rate of DA reuptake into the DAergic terminal. The use of Coc allowed the analysis of possible variations in DA uptake system functionality. In line with previous results, colour plots (Figure 12A) and traces (Figure 12B and C) showed reduced DA released baseline levels in Tg and slower kinetics compared with WT littermates (Figure 11A and D). Moreover, results also confirmed that

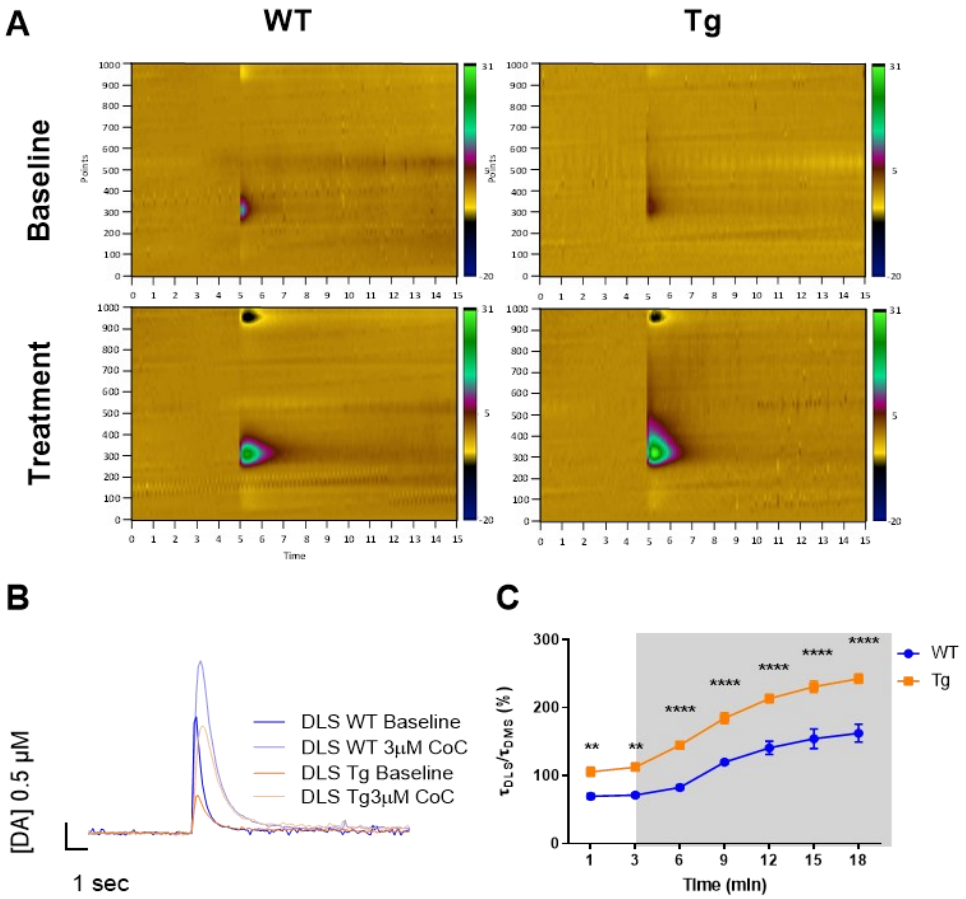

Figure 12. Blockade of dopamine transporter uptake results in decreased dopamine-clearance in the DLS of twelve-month-old Tg mice

(**A**) Colour plots of pre- and post-treatment peaks in the DLS of WT and Tg mice. (**B**) Representative traces corresponding to plots in the upper panel. (**C**) Time constant ratio between DLS and DMS in WT and Tg animals. Data were expressed as mean ± SEM. The statistical analysis consisted in a two-way ANOVA followed by Bonferroni's post hoc tests. Data indicate significant Genotype x Stimulation interaction $F_{(6,49)} = 2.599$, $P = 0.0288$. ** $P < 0.01$; **** $P < 0.0001$.

prolonged exposure to Coc modified the decay portion of the slope (Figure 12C, grey block, WT = 162.7 ± 13.13; Tg = 243.0 ± 6.545; **** P < 0.0001), increasing the time constant ratio. Altogether, these results suggest that DAT may be involved in mediating DA persistence in the extracellular space right after the initiation of the release.

3.1.2 Overexpression of human alpha-synuclein does not impair dopamine release or uptake in the dorsal striatum of six-month-old Tg mice

Given that human alpha-synuclein overexpression at advanced ages had evident region-specific disruptive effects in mice DAergic neurotransmission, we investigated whether such disruptions might occur in six-month-old Tg animals, which are at a time point without observed PD-like features and, are therefore, considered as pre-symptomatic.

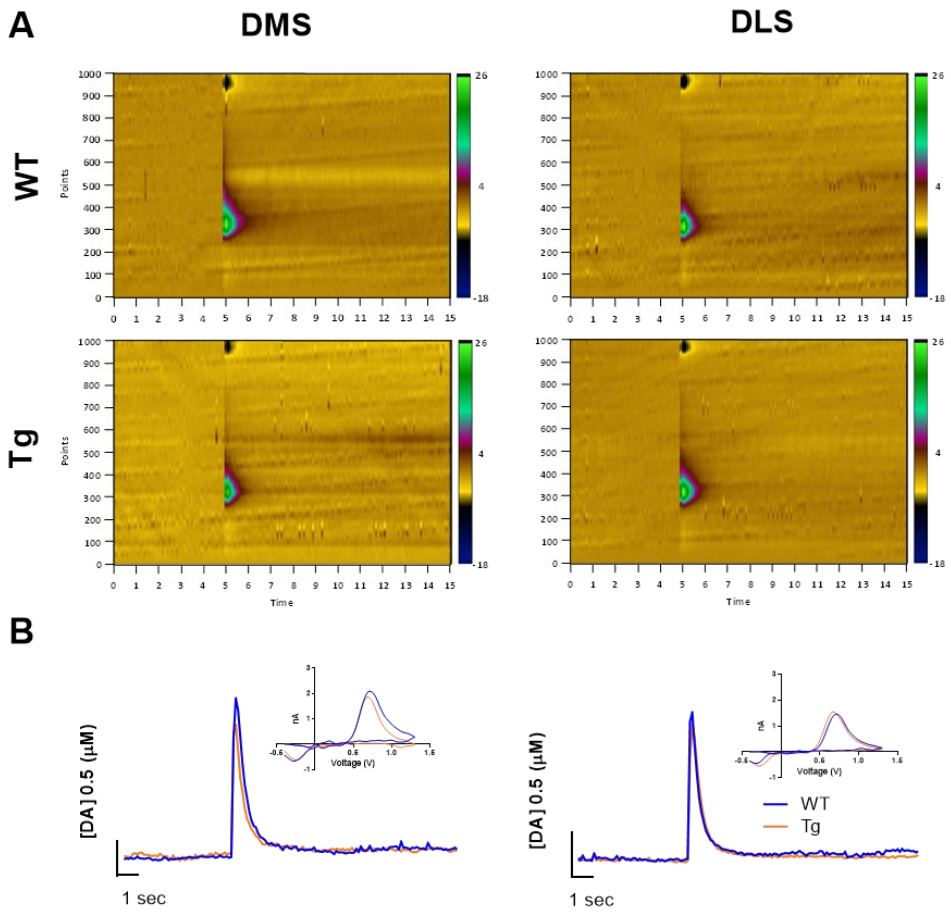

Figure 13. Electrically elicited dopamine release in WT and Tg mice at six months of age

(**A**) Colour plots and (**B**) recording traces and voltammograms for 400-µA stimulation in the DMS and DLS of WT and Tg mice.

Colour plots (Figure 13A) and traces (Figure 13B) showed the same oxidation voltage peak as DA standard solutions used for calibrating the electrode, which demonstrated that our recording carbon-fibre electrode specifically recognized the release of DA after electrical stimulation.

Also with I/O stimulation trains, increasing the intensity of the electrical stimuli generated larger evoked DA release, both in the DMS (Figure 14A. DMS: Stimulation main factor $F_{(3, 24)} = 17.36$, $P < 0.0001$) and the DLS (Figure 14A. DLS: Stimulation main Factor $F_{(3,24)} = 77.72$, $P < 0.0001$). Unlike the results observed in twelve-month-old animals, no significant differences were found between genotypes in either region of these mice (Figure 14A. DMS: Stimulation x Genotype interaction $F_{(3, 20)} = 0.01363$, $P = 0.9978$; DLS: Stimulation x Genotype interaction $F_{(3,24)} = 0.1964$, $P = 0.8978$). Similarly, no significant differences were observed with burst-firing stimulation (Figure 14B. DMS: WT = 1.624 ± 0.382, Tg = 1.345 ± 0.1998; DLS: WT = 1.370 ± 0.1430, Tg = 1.147 ± 0.1310). Opposite to the results in twelve-month-old mice, no significant change was observed in the τDLS/τDMS ratio of these animals (Figure 14D. WT = 88.06 ± 6.218; Tg = 85.84 ± 5.048). These findings suggest that overexpression of human alpha-synuclein does not alter DA release or uptake in the dorsal striatum at the pre-symptomatic age of six months.

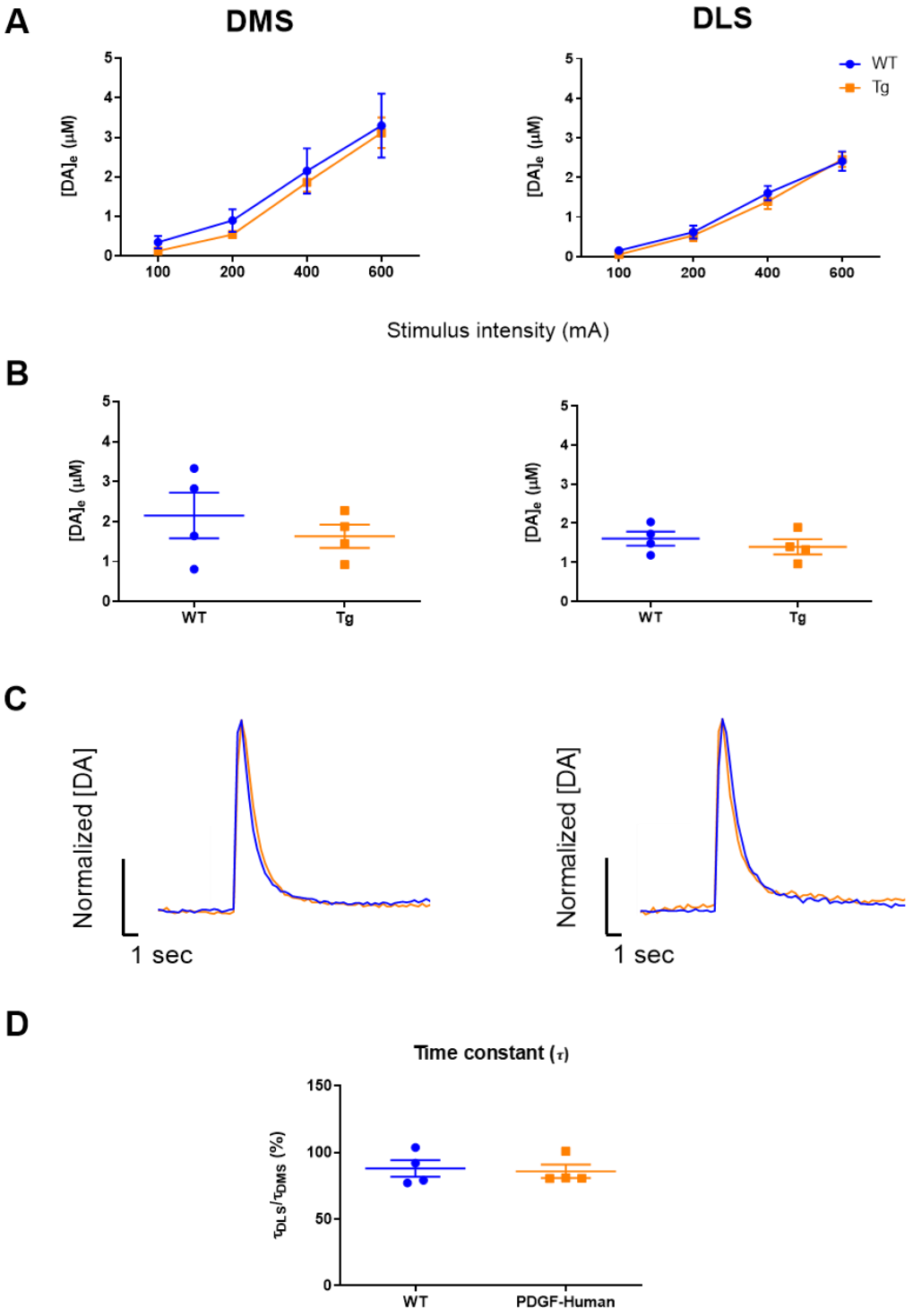

Figure 14. DMS and DLS evoked dopamine release and uptake remain unchanged in six-month-old Tg animals

(**A**) Input/output stimulation to the DMS and DLS at different intensities: 100, 200, 400 and 600 µA. (**B**) Burst-firing stimulation with 400 µA (5p 50Hz) to the DMS and DLS of WT and Tg mice. (**C**) Representative normalized traces from the DMS and DLS of WT and Tg mice. (**D**) Time constant ratio of the decay portion of WT and Tg animals. Data were expressed as mean ± SEM. Statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests (Stimulation x Genotype Interaction, $F_{(3,24)} = 0.1964$, $P = 0.8978$; Stimulation Main Factor, $F_{(3,24)} = 77.72$, $P < 0.0001$; Genotype Factor, $F_{(1,24)} = 0.5608$, $P = 0.4612$). Two-tailed Student´s test results of the time constant ratio comparison between WT and Tg ($t = 0.2770$, $df = 6$, $P > 0.05$).

3.1.3 Human alpha-synuclein overexpression at early age increases dopamine release and affects dopamine uptake

As reported above, human alpha-synuclein impairs DA neurotransmission in twelve-month-old (Figure 11) but not in six-month-old (Figure 14) mice. Since understanding the way human alpha-synuclein may affect DAergic neurotransmission is essential, FSCV experiments were also conducted with three-month-old Tg mice because this age marks the onset of human alpha-synuclein accumulation. Colour plots (Figure 15A), traces and voltammograms (Figure 15B) not only evidenced that DA release was being properly detected but also showed differences in the DA neurotransmission dynamics between both genotypes. As in the previous experiments, FSCV showed comparable DA release in the DMS of Tg and WT mice (Figure 16A. DMS: two-way ANOVA, Stimulation Main Factor $F_{(3, 24)} = 41.53$, $P < 0.0001$) but surprisingly, the DA peak amplitude was markedly increased in the DLS of Tg mice by approximately 75% and 44% with 400 and 600 µA, respectively (Figure 16A. DLS: Genotype x Stimulation interaction $F_{(3, 24)} = 3.680$, $P = 0.0260$). The same effect was observed for DA release elicited by burst-like stimulation (Figure 16B. DMS: WT = 0.9132 ± 0.1074. Tg = 1.104 ± 0.1174; DLS: WT = 0.6330 ± 0.1400, Tg = 1.294 ± 0.06842, ** $P < 0.005$). Interestingly, like in twelve-month-old mice, the decay time ratio was higher in Tg than in WT (Figure 16D. WT = 87.91 ± 4.475; Tg = 108.1 ± 2.743; ** $P < 0.05$). Altogether, these findings indicate that the onset of human alpha-synuclein accumulation at early ages is associated with increased DA release and altered DA uptake only in the DLS.

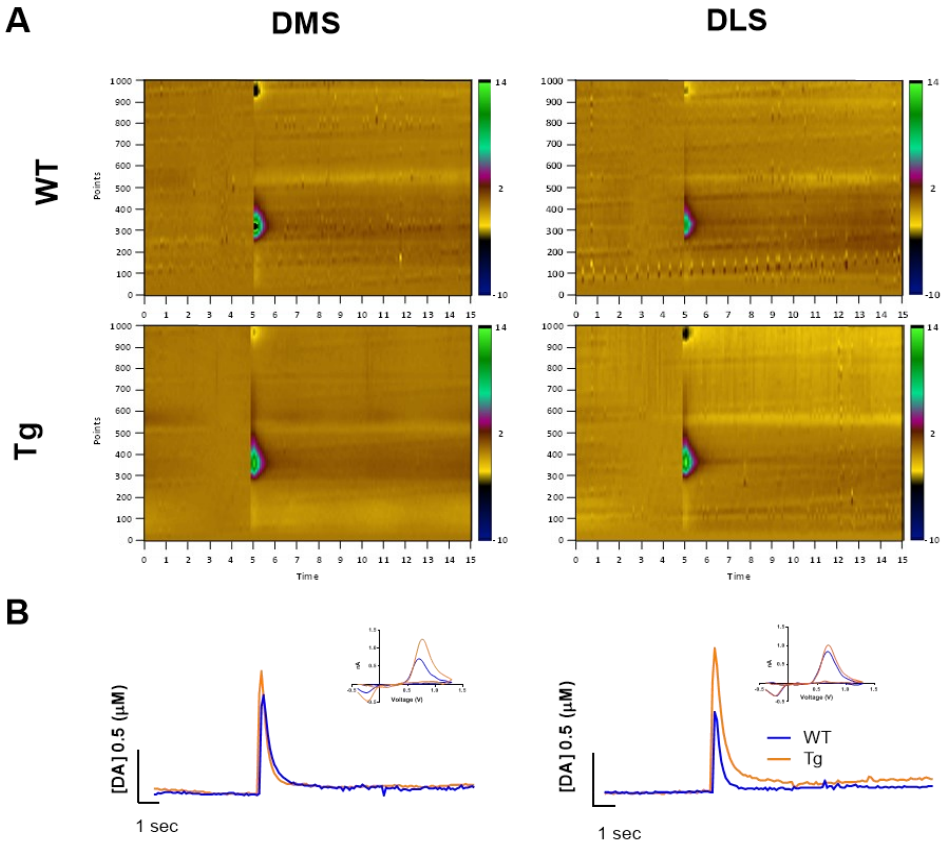

Figure 15. Electrically induced dopamine release in WT and Tg mice at three months of age

(**A**) Colour plots, (**B**) recording traces and voltammograms representing 400 µA evoked DA release in the DMS and DLS of three-month-old WT and Tg mice.

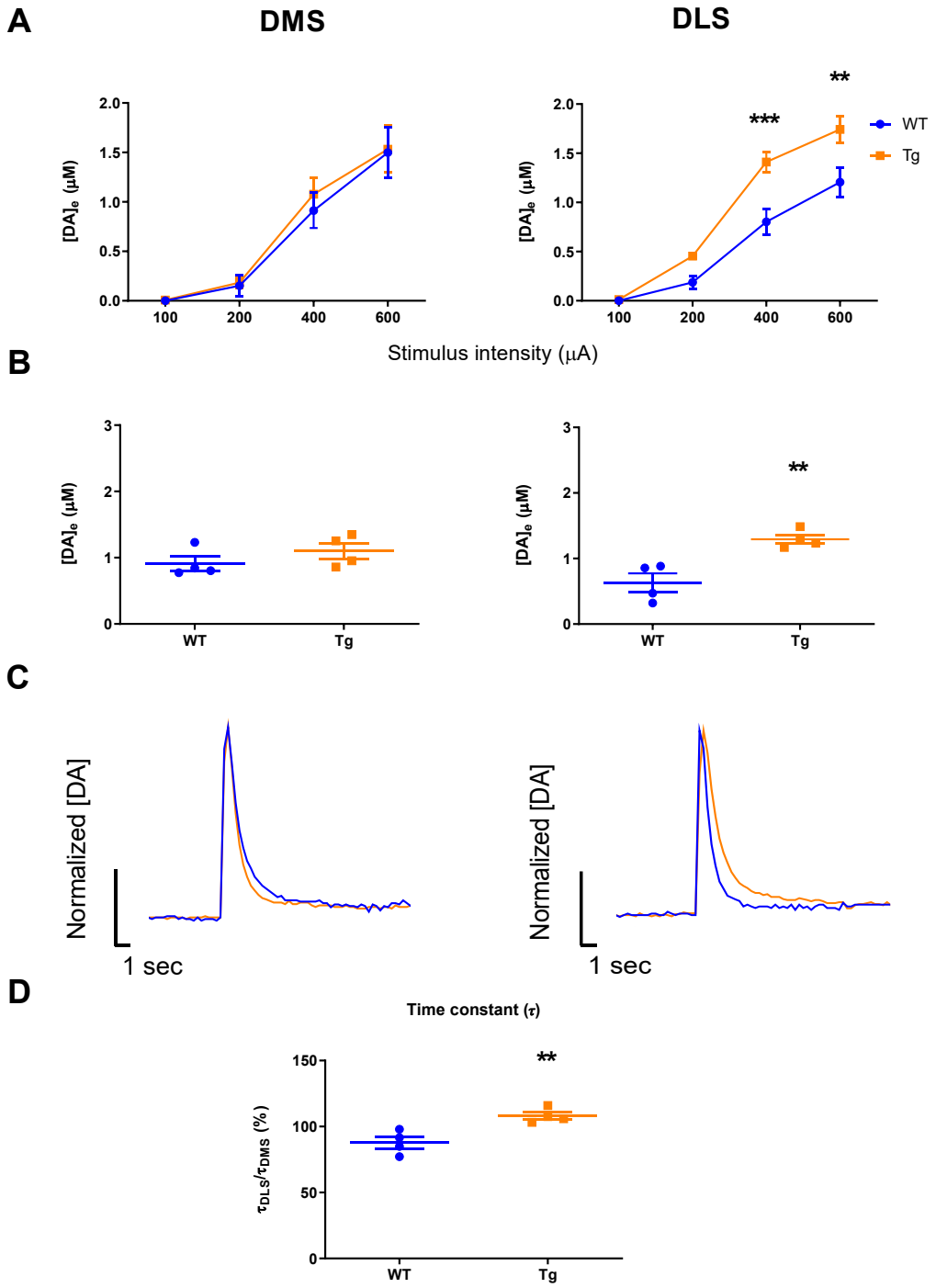

Figure 16. Human alpha-synuclein overexpression increases dopamine release but slowed dopamine uptake in three-month-old Tg mice

(**A**) Input/output stimulation to the DMS and DLS of three-month-old WT and Tg mice. Graphs represent electrical stimulation at different intensities: 100, 200, 400 and 600 µA. (**B)** Plot of current vs time with 400- µA stimulation to the DMS and DLS. (**C**) Representative traces and voltammograms of evoked DA release in the DMS and DLS of WT and Tg mice. (**D**) Time constant ratio in the DLS and DMS of WT and Tg animals. Data were expressed as mean ± SEM. Statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests (asterisk indicates significant interaction between genotype and stimulation intensity $F_{(3,24)} = 3.680$, $P = 0.0260$). Unpaired two-tailed t Student´s test was carried out for comparison of the time constant ratio of WT and Tg ($t = 3.853$, $df = 6$, ** $P < 0.0084$).

3.1.3.1 Early overexpression of human alpha-synuclein also reduces the magnitude of dopamine uptake without influencing dopamine transporter function

Since the decay time was found to be different in young mice, the above-described pharmacological experiments carried out with twelve-month-old mice (blocking DAT activity) were repeated with three-month-old WT and Tg animals. In line with previous results (Figure 16D), baseline τDLS/ τDMS ratio between Tg and WT animals changed equivalently to that of I/O stimulation experiments (Figure 17A and B). However, DAT blockade in young littermates only showed differences between genotypes (Figure 17C), unlike what we observed in adult mice (Figure 12C). Such results suggest that younger animals may have alterations in DAT availability, although not in DAT function.

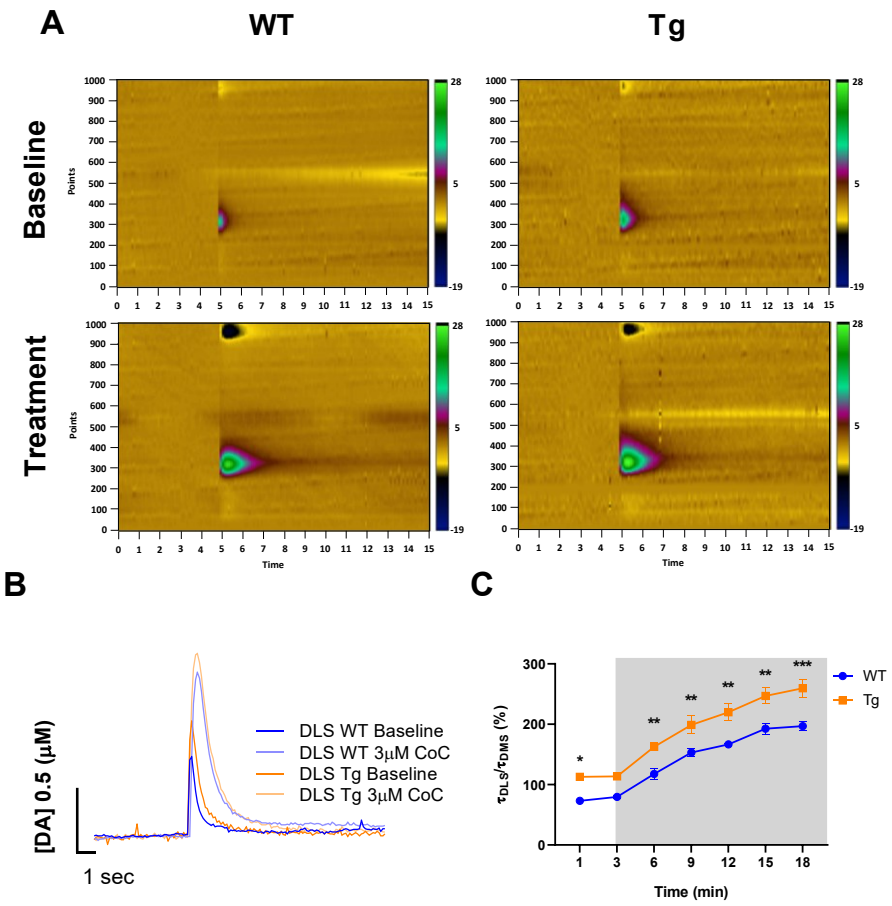

Figure 17. Dopamine transporter uptake blockade proved alterations in dopamine dynamic at three-month-old

(**A**) Pre- and post-treatment colour plots of WT and Tg mice with 400 µA electrical stimulation. (**B**) Representative traces corresponding to plots in the upper panel. (**C**) Time constant ratio between DLS and DMS in WT and Tg animals. Data were expressed as mean ± SEM. Statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests (asterisk indicates significant Genotype Main Factor $F_{(6,42)} = 71.76$, $P < 0.0001$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

These results prove that steady overexpression of human alpha-synuclein has a specific impact on the physiology of DAergic neurotransmission in the DLS but not in the DMS. Interestingly, results also show that in the early human alpha-synuclein accumulation (three-month-old Tg mice), DA release was increased and its kinetics in the DLS was slower than in WT. Thus, this finding might indicate a possible role of DAT in the alpha-synuclein-mediated alterations.

3.2 Effect of human alpha-synuclein overexpression on motor learning performance

3.2.1 Increased dopamine release in young mice leads to enhanced motor learning

DAergic signaling plays an essential role in the acquisition and consolidation of new motor learning skills, supported by the striatum (Marinelli et al., 2017; Wood et al., 2021). Previous studies reported a decline in motor performance in twelve-month-old Tg mice (Masliah et al., 2000), which correlates with the defective DA release observed in our experiments (Figure 11A and B). As young animals revealed a remarkable increase in DA release (Figure 16A and B), we performed the same procedural learning paradigm used in the previous study to elucidate whether such increase may also induce changes in a procedural learning task (Masliah et al., 2000). This test, namely Rotarod, provides a tool to evaluate motor learning activity in the dorsal striatum and consequently, to evaluate DA release since DA deficiencies impair motor performance (Yin et al., 2009; Beeler et al., 2010). Although both genotypes similarly improved in motor learning performance during the first two testing days (Figure 18A. Day 1: WT = 17.57 ± 0.7954, Tg = 19.22 ± 0.6591; Day 2: WT = 20.02 ± 0.9310, Tg = 20.66 ± 0.3454), the latency to fall was notably higher in Tg than in WT mice in the two last testing days. Compared to WT, Tg animals stayed on the rod approximately 21% and 26% longer, in the third and fourth testing day, respectively (Figure18A. Two-way ANOVA, Genotype main factor $F_{(1,40)} = 12.74$, * P = 0.0009). To rule out body weight-associated bias, mice were weighed on the first testing day. Although statistical comparison showed no weight differences between both groups (Figure18B. WT = 28.33 ± 1.682, Tg = 25.23 ± 1.6108), a Pearson correlation test was additionally conducted between body weight and latency to fall. As shown in Figure 18C, the correlations in both genotypes were not significantly different (WT, p = 0.151, r = - 0.2627; Tg, P = 0.0.5793, r = 0.2885) and body weight influence on the results was thus disregarded.

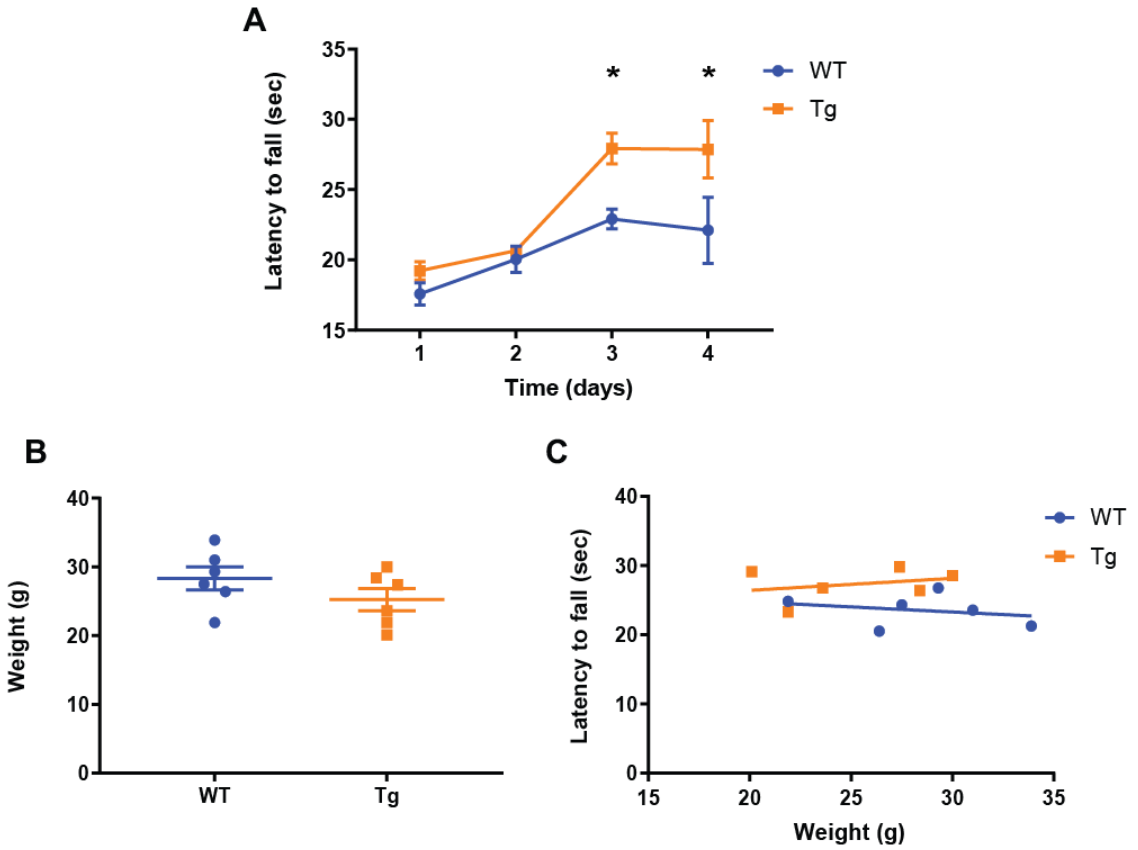

Figure 18. Procedural learning paradigm in rotarod test shows improved performances of three-month-old Tg mice as compared to WT animals

(**A**) The latency to fall (in seconds) during the four testing days, when the time on the rod was measured. Every timepoint corresponds to the mean of 10 consecutive measurements (statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests; * $P < 0.05$). (**B**) Body weight on the first testing day (unpaired two-tailed t Student´s $t_{(10)} = 1.332$, df = 10). (**C**) Pearson's correlation between the total time on the rotor by the end of the fourth testing day vs body weight. Data were expressed as mean ± SEM. Pearson's correlation was used to compare WT and Tg mice's body weight ($n = 6$, $P > 0.05$).

3.3 Impact of human alpha-synuclein overexpression on dopaminergic system markers

As mentioned, FSCV experiments revealed DAergic neurotransmission alterations in the DLS of Tg mice. In young individuals (three-month-old), human alpha-synuclein overexpression seemed to disrupt DA dynamics by modifying DA release and uptake. However, such changes were not persistent over time and the results of Tg mice matched those of WT in older animals (six-month-old mice). Interestingly, DA drastically decreased in advanced ages (twelve-month-old mice) and DA uptake capability was modified in the same way as in young mice. Although our readings showed opposite effects on DA release, in twelve- versus three-month-old animals, both showed an intriguing similar delay in τDLS/τDMS

ratio. Because these changes might be related to either impaired DA availability or impaired neurotransmitter content regulation, the distributions of tyrosine hydroxylase (TH) and dopamine transporter (DAT), which are key proteins in DA release and uptake respectively, were investigated.

3.3.1 Human alpha-synuclein overexpression does not induce changes in tyrosine hydroxylase-positive axon distribution

In DAergic neurotransmission, the rate-limiting enzyme TH produces most of the DA that is then stored into synaptic vesicles. Due to the scarce noradrenergic or catecholaminergic innervation other than DAergic in the striatum (Fuxe et al., 1985; Gerfen and Wilson, 1996), TH can be used to identify DAergic terminals in this region. Given its importance in regulating DA availability and because of the mentioned DA release and uptake alterations, DAergic innervation and relative TH expression in the dorsal striatum were compared between both genotypes.

As Figure 19 illustrates, the dorsal striatum showed the typical TH distribution, with the characteristic staining density of DA-positive striatal terminals. Different from DAT, which is mainly found in neuronal terminals, TH immunolabeling was observed along the whole axons. Initial observations failed to reveal clear differences between regions or genotypes, in mice of different ages (Figure 19).

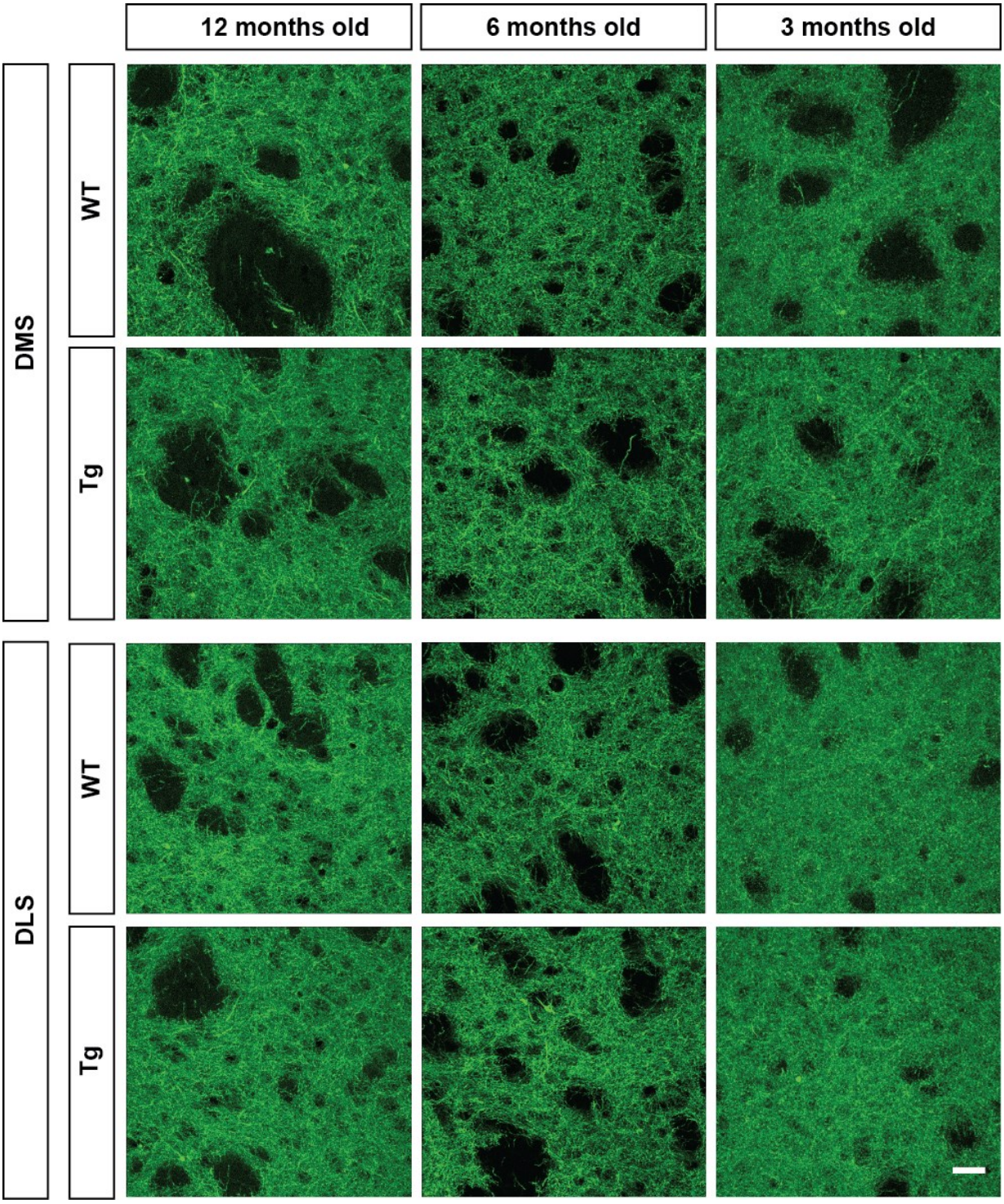

Figure 19. Tyrosine hydroxylase distribution in the dorsal striatum

Representative confocal microphotographies of TH immunostaining in the DMS and DLS showing DAergic terminals of twelve-, six- and three-month-old WT and Tg mice. Images were acquired with a x40 oil immersion objective. Bars scale: 20 µm.

Subsequent semiquantitative analysis of O.D. values did not reveal alterations in the TH-positive signal and terminal innervation area at any age (Figure 20A, B and C). It is worth noting that the TH signal in the DLS was slightly lower in twelve-month-old Tg mice than in WT animals of the same age (Figure 20A), although differences were not significant. Further quantitative analysis of the TH-positive area in the striatum did not reveal changes at any age (Figure 20D, E and F).

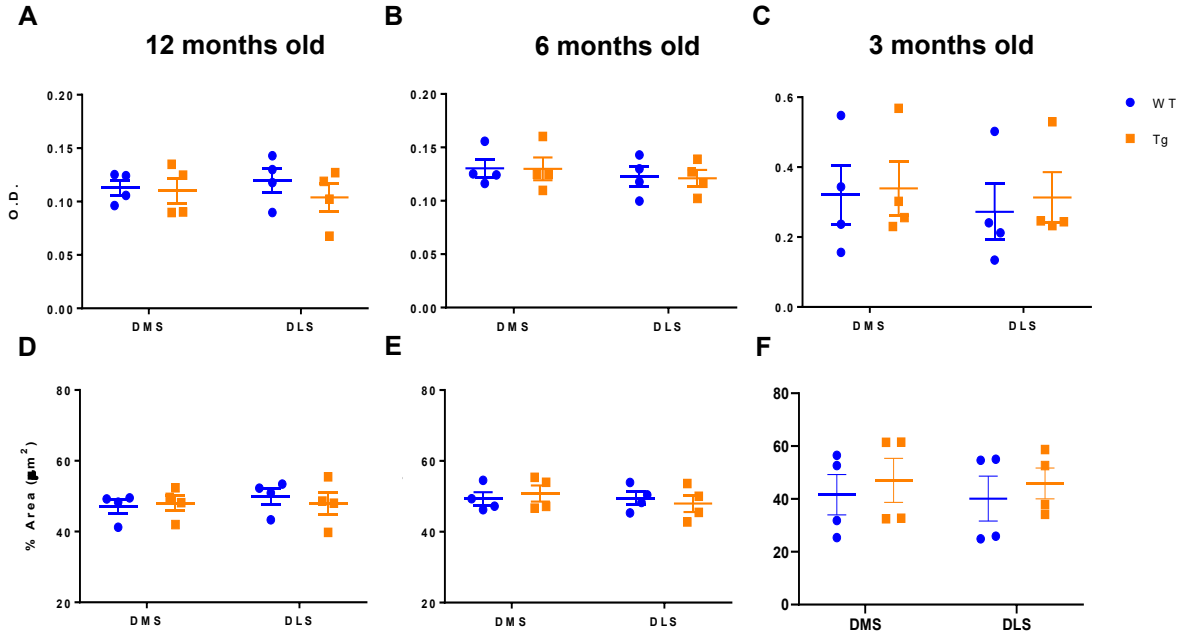

Figure 20. Tyrosine hydroxylase staining shows equivalent relative protein levels and innervation in the dorsal striatum of both genotypes

Graphs show the means ± SEM of O.D values, as well as the percentage of the area covered in the DMS and DLS of WT (blue) and Tg (orange) mice of twelve (**A, D**), six (**B, E**) and three (**C, F**) months of age. Statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests ($P > 0.05$).

3.3.2 Dorsomedial and dorsolateral striatum express similar levels of human alpha-synuclein in three-month-old Tg mice

To study whether the physiological alterations previously described may result from differences in the expression of human alpha-synuclein between the DMS and the DLS of three-month-old Tg mice, O.D. and area values were calculated for human alpha-synuclein protein. Immunohistochemical analysis (Figure 21A and B) failed to reveal changes in the expression of this protein (Figure 21B. DMS = 0.2104 ± 0.0119; DLS = 0.2005 ± 0.0095, $P < 0.05$). Similarly, in a quantitative analysis of the area positive for the protein, no variations in the distribution pattern were found (Figure 21C. DMS = 15.84 ± 2.879; DLS = 16.39 ± 2.556,

p < 0.05). These results suggest that human alpha-synuclein overexpression in the DMS is not different from that in the DLS. Thus, the findings described up to now do not seem to account for the observed differences in the expression level between both regions.

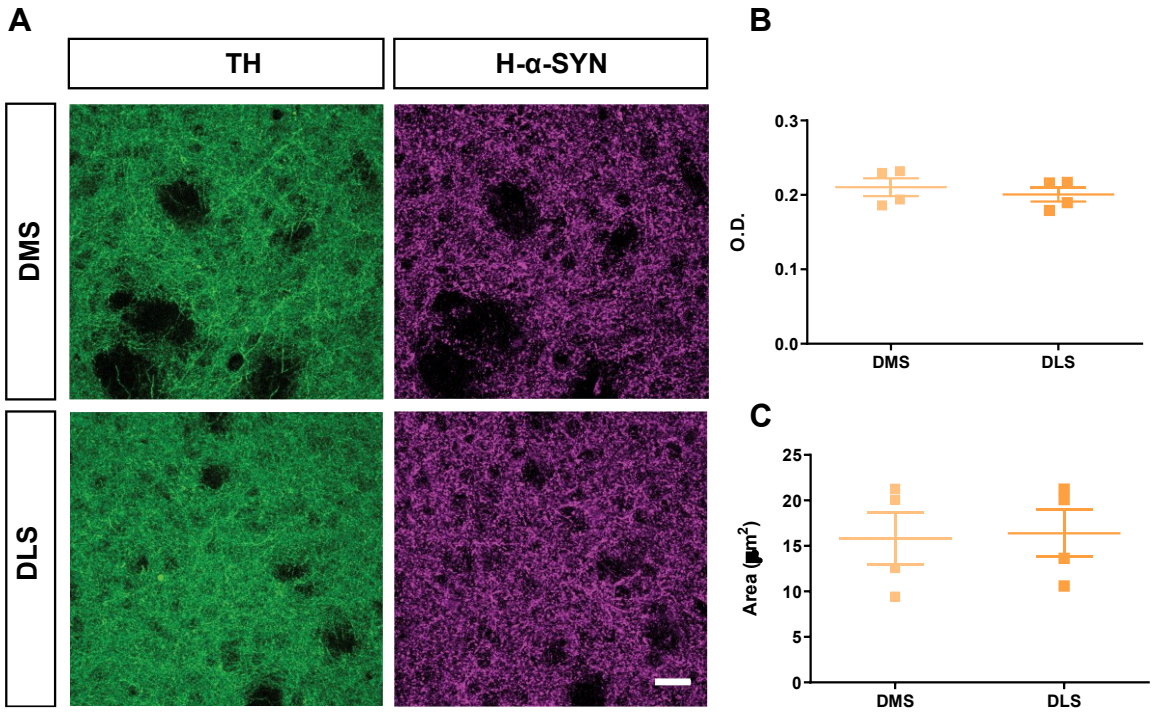

Figure 21. Human alpha-synuclein is equally overexpressed in the DMS and the DLS of three-month-old Tg mice

(**A**) Representative images of TH (green) and H-α-Syn (magenta) in the DMS and DLS**.** (**B**) O.D. values for DMS (light orange) and DLS (bright orange). (**C**) Analysis of the area covered by H-α-SYN. Represented values correspond to means ± SEM. Unpaired two-tailed Student´s test results O.D. (t = 0.6412, df = 6; DLS, P > 0.05) and % area (t = 0.1437, df = 6, P > 0.05) in WT and Tg mice.

3.3.3 Dopamine transporter structure changes in the dorsolateral striatum

DAT is a transmembrane protein selectively expressed in DAergic neurons (Ciliax et al., 1995; Freed et al., 1995; Nirenberg et al., 1996). As any other protein, DAT locates heterogeneously within those neurones, expressed in both soma and dendrites (somatodendritic region) but also, in axons and terminals. Following electrical stimulation, DA clearance processes are rapidly activated. Although during DA withdrawal there are several processes involved in reducing DA tone, the main mechanism seems to imply the activation of DAT (Giros et al., 1996; Jones et al., 1998; Benoit-Marand et al., 2000;

Gonon et al., 2000). Therefore, according to our decay time results, I decided to investigate the immunoreactivity as well as the distribution of DAT in the DMS and the DLS of WT and Tg animals at the aforementioned three time points.

Preliminary qualitative assessment clearly revealed abnormalities in the staining distribution. While the DMS appeared to display the characteristic DAT-like puncta distribution (Figure 22) at all the time points, the DLS did not do so. As shown in Figure 22, DAT staining was most concentrated at certain spots in the DLS of twelve-month-old Tg mice (white arrows, DLS twelve-month-old). Furthermore, three-month-old mice showed similar abnormalities (Figure 22, DLS, three-month-old), while those in the pre-symptomatic stage (six-month-old) did not exhibit such aggregation (Figure 22, DLS, six-month-old).

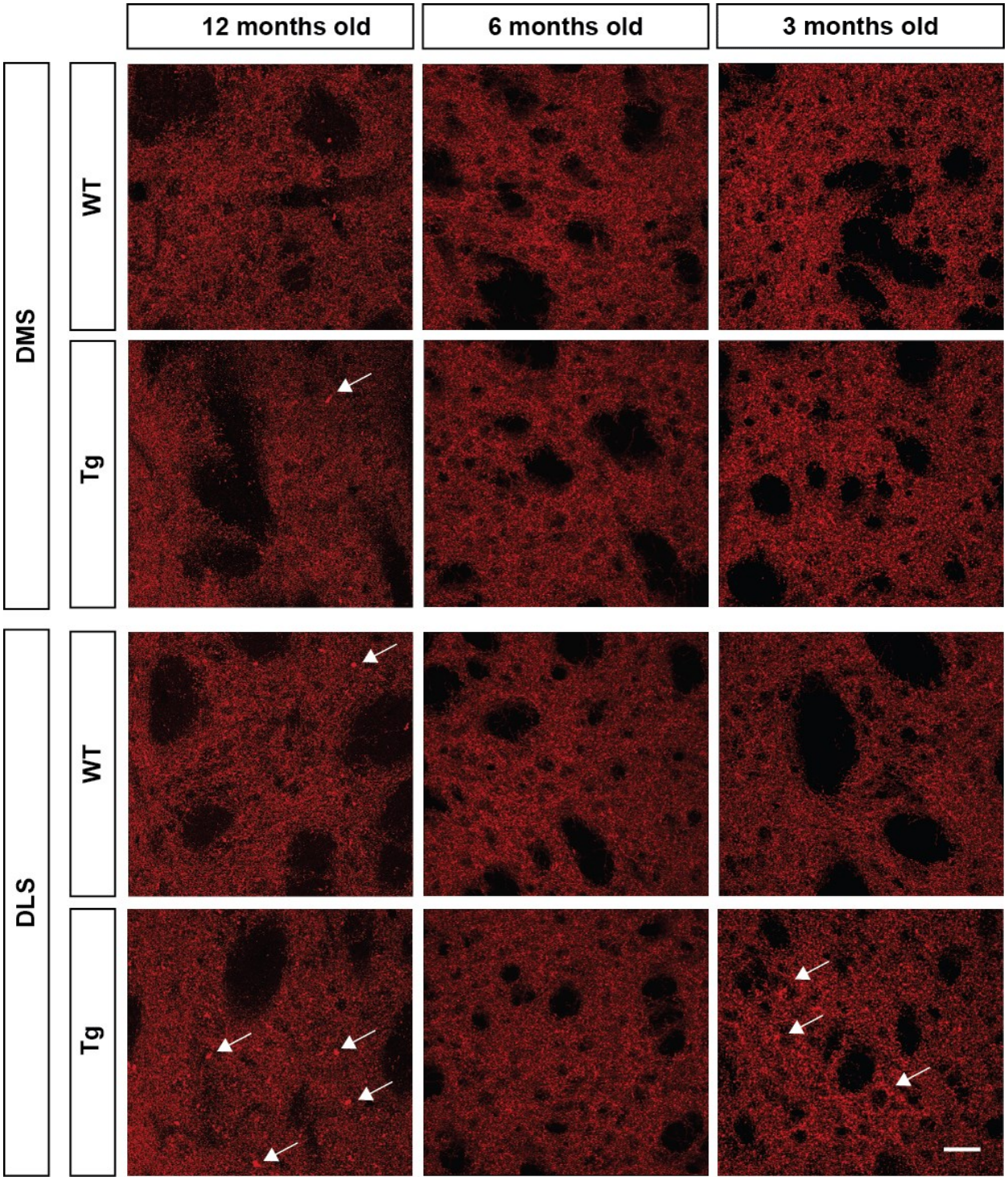

Figure 22. **Dopamine transporter distribution in the dorsal striatum**

Selected confocal microphotographies representing DAT staining in DMS and DLS DAergic afferents of twelve-, six- and three-month-old WT and Tg mice. Images were acquired with a x40 oil immersion objective. Arrows highlight abnormal DAT clumps. Bars scale: 20 µm.

3.3.4 Relative dopamine transporter levels are equivalent in both regions of the dorsal striatum

In order to assess whether the described morphological alterations corresponded to actual changes in DAT expression, semiquantitative O.D. analysis was performed in the DMS and the DLS. As shown in Figure 23, O.D. measurements did not change at any of the studied ages (Figure 23A, B and C).

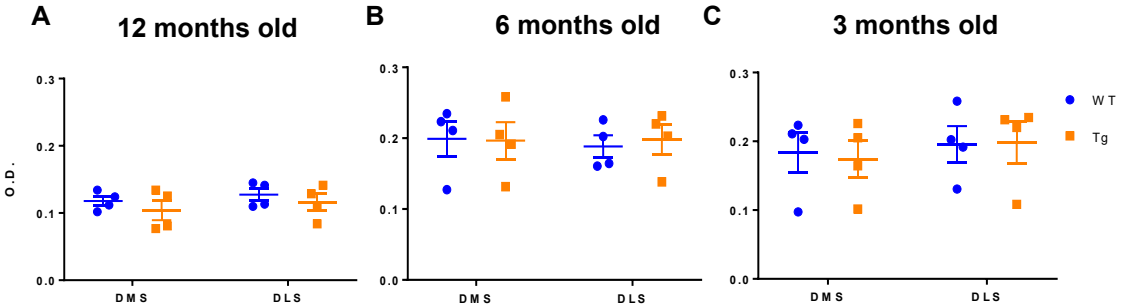

Figure 23. Human alpha-synuclein does not induce changes in the dopamine transporter O.D. at any of the studied ages

Graph values expressed as mean ± SEM correspond to the DMS and DLS of (**A**) twelve-, (**B**) six- and (**C**) three-month-old WT (blue) and Tg (orange) mice. The statistical analysis consisted of a two-way ANOVA followed by Bonferroni's post hoc tests (P > 0.05).

3.3.5 Dopamine transporter mainly clumps in the dorsolateral striatum of Tg animals

Since O.D. measurements did not reveal any change, a deeper analysis was carried out by applying a threshold-based mask to confocal DAT staining images and displaying the frequency of different particle sizes through a cumulative frequency distribution (CFD) study. In a comparison between both genotypes, twelve-month-old mice exhibited the same DAT-positive puncta size distribution in the DMS (Figure 24A). However, the size distribution was shifted to the right in the DLS of Tg as compared to WT mice (Figure 24A. Two-way ANOVA, DAT puncta size Factor, $F_{(85,860)} = 1173$, * $P < 0.05$). This finding indicated that the proportion of larger DAT-positive clumps was higher in Tg than in WT animals and supported the above described qualitative observations. In an equivalent comparison of six-month-old mice, no differences were found (Figure 24B. Two-way ANOVA, DAT puncta size x Region interaction, $F_{(255,688)} = 0.4813$, $P > 0.9999$). However, the comparison of three-month-old mice yielded clear differences resembling those of twelve-month-old animals (Figure 22 and

Figure 24) namely, equivalent size distributions in the DMS but higher proportion or larger DAT puncta in the DLS of Tg versus WT mice (Figure 24C. Two-way ANOVA, DAT puncta size $F_{(86,1044)} = 1390$, * $P < 0.05$). Thus, these findings indicate modifications in the distribution of DAT puncta only in the DLS.

Such remarkable DAT distribution differences in the DLS are in line with the previous observations of altered DA uptake in Tg mice at both twelve and three months of age. These findings suggest that human alpha-synuclein overexpression might trigger a reorganization of DAT distribution in an age-dependent manner, something that may have striking effects not only on DA release but also on DA uptake.

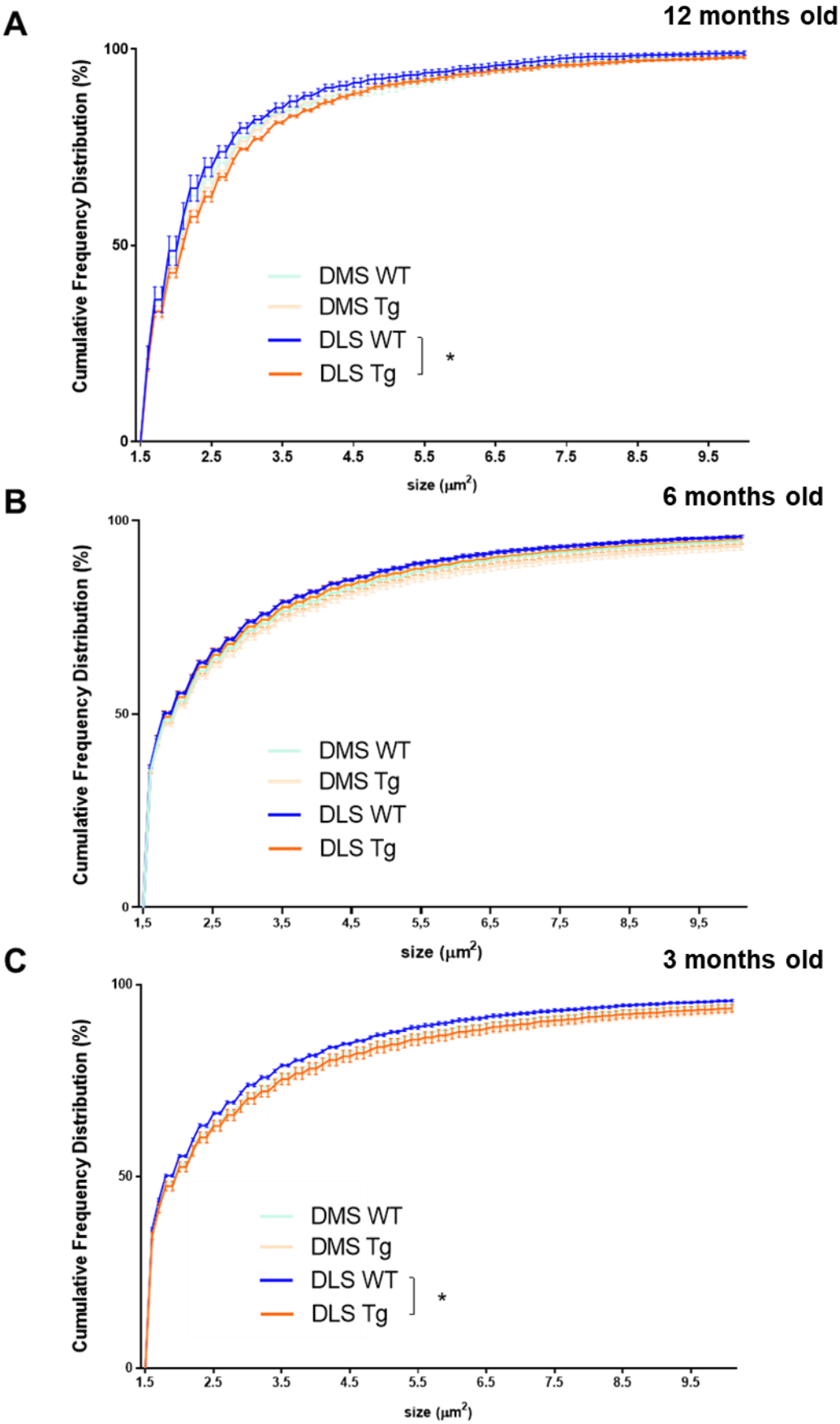
A
12 months old
Cumulative Frequency Distribution (%)
100
50
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
size (µm²)
DMS WT
DMS Tg
DLS WT
DLS Tg
*
B
6 months old
Cumulative Frequency Distribution (%)
100
50
0
1,5
2,5
3,5
4,5
5,5
6,5
7,5
8,5
9,5
size (µm²)
DMS WT
DMS Tg
DLS WT
DLS Tg
C
3 months old
Cumulative Frequency Distribution (%)
100
50
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
size (µm²)
DMS WT
DMS Tg
DLS WT
DLS Tg
*

Figure 24. **Cumulative frequency distribution of dopamine transporter-positive puncta revealed an increased proportion of larger clumps in the DLS of twelve- and three-month-old Tg mice**

Graphs of percent accumulative range values in the DMS (WT, bright blue; Tg, bright orange) and DLS (WT, dark blue; Tg dark orange) of mice at (**A**) twelve, (**B**) six and (**C**) three months of age. Data express the sum of the percentage of the range at bin 0.1. Statistical analysis consisted of two-way ANOVA followed by Bonferroni's post hoc tests. Asterisk indicates significant $P < 0.05$ ($n = 4$).

4 Discussion

4.1 Role of alpha-synuclein in nigrostriatal dopaminergic neurotransmission

Our knowledge of the function of alpha-synuclein has grown exponentially in the last few decades. Its modulatory effects on DA synthesis (Mori et al., 2006; Perez et al., 2002), synaptic vesicle docking (Burré et al., 2010; Diao et al., 2013; Fusco et al., 2016) and DA release and uptake (Butler et al., 2015; Lautenschläger et al., 2017) have directly led to propose a causal role for alpha-synuclein in PD (Moussaud et al., 2014; Visanji et al., 2019). Although the aetiology of idiopathic PD remains uncertain, its progression has been closely related to the aggregation and accumulation of misfolded forms of this protein. Major pathological features of PD include initial degeneration of DAergic terminals, followed by DAergic neuron loss at later stages (Riederer & Wuketich, 1976; Fearnley & Lees, 1991; Nandhagopal et al., 2008). Interestingly, different DA midbrain afferents preferentially innervate defined regions in the striatum. Throughout the progression of the disease, differential susceptibility has been observed for a fraction but not all DAergic axons. While DA axons in the DMS seem to be more resilient to PD-associated deterioration, DAergic terminals in the DLS appear to be more prone to degenerate (Shepherd, 2013; Vitrac & Benoit-Marandmarianne, 2017). Alterations or dysregulations in DA neurotransmission in either of these DAergic terminals have detrimental consequences on intrinsic dorsostriatal functions and consequently, on movement control and motor abilities (Kish et al., 1988; Surmeier & Kitai, 1994; Kordower et al., 2013). Hence, studying the equilibrium between DA release and uptake in the dorsal striatum is crucial to understand the aetiology and progression of PD. In this thesis, the age-dependent effects of steady human alpha-synuclein overexpression were studied in the DMS and DLS and the related DAergic neurotransmission was characterized in terms of DA release and uptake.

Results indicate that steady overexpression of human alpha-synuclein impairs striatal DA neurotransmission by disrupting DA release and uptake in an age-dependent manner, specifically in the DLS. Interestingly, the progressive decline in DA release capability did not follow the same changes as in the uptake, thus suggesting that the causes of the DA kinetics slowdown may have different explanations at early than at late stages of alpha-synuclein pathological progression.

4.1.1 Continuous human alpha-synuclein overexpression has age-dependent effects on dopamine release in the dorsolateral striatum

One of the most robust results of this study was the observation of changes in DA release in the DLS across different ages of the mouse model, while DA neurotransmission in the DMS remained unaffected. FSCV *ex vivo* studies revealed decreased electrically evoked DA release, both with single and burst-like stimuli, in twelve-month-old mice. Interestingly, no changes were observed in DA neurotransmission in six-month-old animals, an age that has been here considered as a pre-symptomatic stage. This latter finding suggests that there is a phase of unaltered DA release before neurotransmission is disrupted by alpha-synuclein disbalances. However, experiments conducted close to the onset of transgenic alpha-synuclein accumulation (three-month-old mice) revealed a marked increase in evoked DA release, i.e. 1.6-fold higher in Tg than in WT mice. Understanding the consequences of alpha-synuclein accumulation for DAergic neurotransmission would help unravel the progression of PD. Therefore, several studies have extensively assessed its role using *in vivo* and *in vitro* models. In line with our results, Lam et al. (2011) described a similar age-dependent effect in extracellular DA contents measured with microdialysis and high-performance liquid chromatography (HPLC) in Thy-αSyn transgenic mice (Line 61). They observed a marked decrease of striatal DA extracellular contents in the dorsal striatum of aged mice (14 months old) but also a remarkable increase in extracellular DA contents at earlier ages (six months old). However, these authors measured DA by using *in vivo* microdialysis, which has a temporal resolution of 15-30 minutes while the neurotransmission activity of DAergic terminals takes place in a sub-second time window. Furthermore, microdialysis is rather invasive and less accurate than FSCV in terms of discriminating between DMS and DLS. Thus, although providing relevant information on the effect of accumulated human alpha-synuclein on striatal DA extracellular concentration, Lam et al. could not address the neurotransmission fast kinetics in their study. Even so, their findings confirm that striatal extracellular DA contents increase in the early phase of alpha-synuclein overexpression and decrease at later stages. Additionally, it is important to note that neurotransmission in Thy-αSyn and PDGF-β transgenic mice might be also affected by differences in the pattern of human alpha-synuclein expression. Studies using the recombinase polymerase technique, western blot and *in situ* hybridization showed that three-month-old Tg mice expressed moderate levels of the protein (1.5-fold higher than controls), while Thy-αSyn mice expressed much higher ones, equivalent to ten-fold the human level (Rockenstein et al., 2002). Moreover, the onset of human alpha-synuclein accumulation and protein detection did not follow identical expression patterns in the brains of those lines.

Given that the PDGF-β promoter provides a steady transgenic expression of human alpha-synuclein, the Tg transgenic model is particularly reliable to mimic PD symptoms as observed in human patients. The analysis of striatal human alpha-synuclein levels evidenced a progressive accumulation of the protein with increasing age in Tg mice (Amschl et al., 2013). Quantitative immunofluorescence protein measurements showed a pronounced increase in protein levels at twelve months of age, as compared with the results of six- or three-month-old Tg mice (Amschl et al., 2013). Interestingly, although protein-related intensity values were similar between three- and six-month-old mice, the latter ones appeared to have lower total alpha-synuclein levels.

It is important to notice that, increased protein levels, TH activity and protein expression in twelve-month-old mice tend to decrease in the striatum of Tg mice (Masliah et al., 2000; Amschl et al., 2013), especially in the DLS (preliminary data. TH protein levels by western blot. DLS TH; WT: 100 ± 9.610 a.u.; Tg: 78.07 ± 11.57 a.u.; DMS TH; WT: 100 ± 7.777a.u.; Tg: 90.83 ± 16.29 a.u.). Accordingly, Tg mice showed motor dysfunction in the rotarod task at that age (Masliah et al., 2000), which suggests underlying impairment of DA release in the DLS, since that region is critical in motor learning performance (Thorn et al., 2010). Thus, the impaired motor-related performance of aged Tg mice might be a consequence of a broad range of alpha-synuclein-dependent synaptic dysfunctions, presumably involving the DLS in particular. It can be postulated that the observed stable DA neurotransmission during the so-called pre-symptomatic stage (six-month-old mice) may result from compensatory mechanisms that counteract the increasing – though not yet excessive – alpha-synuclein accumulation. PET studies also reported that PD patients with early onset of the disease show more efficient compensation and better endurance to TH-terminal degeneration and neuronal loss in the SNc (Ming et al., 2007; De La Fuente-Fernández et al., 2011). Furthermore, clearance deficits in the autophagy pathways have been described in nine-month-old Tg mice (Crews et al., 2010; Klucken et al., 2012). Analysis of brain lysates revealed consistently increased levels of mTor and decreased levels of Atg7, which are proteins promoting the initiation of the autophagy process. Hence, it can be postulated that autophagy mechanisms are undamaged and functional in the DLS of six-month-old mice but get disrupted by reaching a critical level of alpha-synuclein accumulation at older ages. Moreover, other experiments demonstrated that reducing alpha-synuclein accumulation with rapamycin reverted the deficiencies in the autophagy pathway (Spencer et al., 2009).

Regarding the increased DA release at early stages of human alpha-synuclein overexpression (three-month-old mice), although surprising, data were quite robust and supported by a distinctive motor behaviour phenotype. Tg animals showed longer latencies to fall than WT mice in the rotarod task, which means that young Tg mice performed significantly better than WT. DA participation in the reinforcement of motor learning has its biological substrate in the DLS, in the cortico-striatal loop (since it is densely innervated by motor and associative cortical afferents) (Obeso et al., 2008; Graybiel & Grafton, 2015; Florio et al., 2018). In humans, PD patients with predominant tremor-related symptoms under L-DOPA medication reflected enhanced striatal DA-dependent learning in a motivational Go/NoGo learning paradigm, with a task designed to dissociate the effects on the learning rate from the effects on motivational choice (Steinberg et al., 2013; Van Nuland et al., 2020). In another study, transgenic M20 mice were administered a battery of motor behaviour tests. The authors concluded that human alpha-synuclein overexpression improved their performance in several motor tasks (Giraldo et al., 2018).

Additionally, it has been well-established that alpha-synuclein actively participates in key processes of neurotransmitter release. Recent publications reported that alpha-synuclein promotes the dilation of fusion pore (Logan et al., 2017), facilitates DA release in burst-firing stimulation in substantia nigra neurons (Somayaji et al., 2020) and interferes with intracellular $^{2+}$Ca dynamics (Angelova et al., 2016; Zaichick et al., 2017). Thus, a possible mechanism for DA release facilitation in early alpha-synuclein accumulation stages might be related to its functions in synaptic vesicle traffic and calcium efflux. In particular, non-aggregated alpha-synuclein has been found to enhance mobilization of active-zone proteins, such as the SNARE (Burré et al., 2014; Hawk et al., 2019) or Rab proteins (Cooper et al., 2006), as well as vesicle docking (Hawk et al., 2019). Notably, midbrain neurons and their terminals express different $^{2+}$Ca channels (Liss & Striessnig, 2019; Sgobio et al., 2019), which have been shown to confer resiliency to the DAergic neuron population (Minakaki et al., 2020).

Despite the increasing amount of related literature, the effects of alpha-synuclein on DA release in different striatal areas and DAergic neuron populations remain unclear. The results of this work provide a further layer of complexity to our knowledge of striatal neurotransmission, but also increase our insight into the way alpha-synuclein modulates different DAergic afferents in the striatum. Altogether, these data support the hypothesis that certain DA-positive afferents are more sensitive than others to progressive human alpha-synuclein overexpression, even at very early accumulation stages.

The next step in this work was focused on the structural components of DA neurotransmission and was aimed at exploring the potential mechanism underlying the impact of alpha-synuclein overexpression on presynaptic functions.

4.1.2 Impact of alpha-synuclein on tyrosine hydroxylase distribution and expression in striatal dopaminergic terminals

Given that TH is the rate-limiting enzyme in DA synthesis, its expression pattern has traditionally been used as a marker for DA-positive fibres in the striatum (Fallon & Moore, 1978 Fallon, 1981). In PD patients, deterioration of DAergic fibres is more marked in the putamen than in the caudate area, already before the onset of motor symptoms (Kish et al., 1988; Kordower et al., 2013). Moreover, alpha-synuclein has been postulated to directly interact with TH (Khan et al., 2012; Post et al., 2018). Since denervation and changes in TH expression levels could account for the DA release alterations recorded in the here-described FSCV experiments, its density was measured in the DMS and DLS of Tg mice in terms of O.D. values and percentage of covered area.

No significant differences were found in the DMS or the DLS of Tg and WT mice, neither in O.D. nor in TH fibre density, at any age. However, O.D. only provides a semiquantitative measure of protein distribution. Thus, based on the observation that TH levels tended to decrease in twelve-month-old animals (Figure 20), a western blot study was carried out, which confirmed the decrease in protein load (western blot preliminary data: DLS TH; WT: 100 ± 9.610 a.u.; Tg: 78.07 ± 11.57 a.u.; DMS TH; WT: 100 ± 7.777a.u.; Tg: 90.83 ± 16.29 a.u.). Consistent with previous results in the same mouse model, human alpha-synuclein overexpression was associated with reduced TH activity and levels in the striatum of twelve-month-old mice (Masliah et al., 2000). These results were similar to those reported in Thy-αSyn and BAC transgenic rodents (Lam et al., 2011; Nuber et al., 2013). Similarly, rAAV-alpha-synuclein models overexpressing WT human alpha-synuclein in the nigrostriatal pathway showed reduced TH signal and moderate terminal degeneration (Oliveras-Salvá et al., 2013).

It appears to be unquestionable that alpha-synuclein regulates TH activity and several studies have postulated that TH/alpha-synuclein interaction can modulate DA synthesis (Mori et al., 2006; Perez et al., 2002). Expectedly, changes in alpha-synuclein due to disease or normal aging would be associated with abnormal DA content in neurons and terminals, thus impairing TH-mediated regulation and leading to degeneration caused by the production of DA

toxic forms and reactive oxygen species (ROS). In this work, TH distribution and expression were suggested to vary in the different areas of the dorsal striatum. Differences in DA synthesis derived from differences in TH activity could partially account for the selective vulnerability of certain DA axon subtypes in the dorsal striatum. However, further experiments are needed to clarify this issue in the context of alpha-synuclein-related insults.

4.2 Human alpha-synuclein overexpression regulates dopamine uptake by interfering with the dopamine transporter

The amplitude and strength of DA neurotransmission are also markedly regulated by the activation of DA uptake mechanisms. Following DA release, DA removal processes are immediately activated (Vaughan & Foster, 2013) aimed at removing DA from the extracellular space into presynaptic terminals. Eventually, DA is metabolized or re-stored into synaptic vesicles. Among such regulatory processes (including extracellular DA diffusion and non-neuronal degradation), DAT is primarily involved in maintaining optimal extracellular DA levels (Jones et al., 1998). Due to the physiological importance of DAT and its direct relationship with DA uptake, it is here hypothesised that human alpha-synuclein overexpression could potentially influence DA uptake by interfering with DAT activity and distribution, especially in the DLS.

4.2.1 Human alpha-synuclein modulates dopamine transporter functionality

In addition to DA release, FSCV experiments provided relevant information about the mechanisms of extracellular DA removal during release. In particular, our data analysis indicated that DA clearance kinetics was slower in Tg than in WT animals at younger and older ages, but not in those at pre-symptomatic age (six-month-old). Moreover, further pharmacological experiments blocking DAT activity confirmed that the slow kinetics was driven by changes in DAT functionality at very early or late ages. Similarly, Lundblad et al. (2012) described an early reduction of the striatal DA reuptake rate in rats stereologically injected with a viral vector designed to induce human alpha-synuclein overexpression in the SNc. This result is in line with our alpha-synuclein-dependent slow kinetics in the DLS of Tg mice at early ages. In cell cultures, overexpression of alpha-synuclein and human-DAT (hDAT) was found to attenuate DAT activity as DA was infused into the medium, thus suggesting possible changes in either function or availability of

the protein at the membrane surface (Swant et al., 2011; Wersinger et al., 2003; Wersinger & Sidhu, 2003). Comparable to our results, Swant et al. showed that initial high levels of alpha-synuclein resulted in decreased extracellular DA clearance rate (Swant et al., 2011). Although these experiments were conducted in a different *in vitro* model, results showed a dynamics similar to what we observed in our experiments, with the difference that our results were specifically related to neurotransmission in the DLS. Assays with a mutated alpha-synuclein form also showed reductions in DAT functionality (Sidhu et al., 2004). However, experiments with either human embryonic kidney cells (HEKs) expressing hDAT or mice overexpressing human alpha-synuclein (Thy-αSyn) yielded opposite results, showing increased or unchanged DAT-mediated uptake, respectively (Lee et al., 2001; Lam et al., 2011). Such discrepancy between conclusions is probably the result of using experimental models with different expression levels of human alpha-synuclein, and/or different sampling methods, since these authors did not discriminate between DMS and DLS.

4.2.2 Alterations in dopamine transporter distribution in the dorsal striatum

The integrity of striatal DAergic innervation is certainly compromised in PD patients (Scherman et al., 1989; Porritt et al., 2005; Bédard et al., 2011). Neuropathological studies evidence faster and larger loss of DA fiber density in the putamen than in the caudate (DLS and DMS in rodents, respectively), even at the onset of motor symptoms (Schwartz et al., 2004; Tissingh et al., 1998). Since the development of clinical symptoms is closely related to the deterioration of DA innervation and given that Tg mice showed increased DA release amplitude and strength, experiments based on DAT distribution were designed to detect possible disturbances in DAT distribution at twelve, six or three months of age.

DAT staining in the DMS and DLS revealed a singular reorganisation of physiological DAT puncta distribution specifically in the DLS. Analysis of microphotography images showed regular DAT distribution in the DMS of Tg mice of all ages, as compared to littermate controls. But in the DLS of twelve- and three-month-old mice (though not in six-month-old ones), DAT-positive immunohistochemical signals appeared to be distributed into larger spots. Further CFD analysis confirmed that the widespread DAT pattern had been modified and that the number of larger spots in the DLS was higher in Tg than in WT mice.

It is widely accepted, that alpha-synuclein interacts with DAT through its C-terminal end (Lee et al., 2001; Wersinger et al., 2003). Up to date, several studies support that alpha-synuclein regulates not only DAT function but also its distribution (Wersinger & Sidhu, 2005; Eriksen et al., 2010; Kisos et al., 2014; Butler et al., 2015). Studies using traditional immunohistochemical methods in human striatal tissue reported co-occurrence of alpha-synuclein and DAT in the same spots in the caudate-putamen of PD patients. More important, redistribution of DAT alone or DAT/alpha-synuclein complexes was observed, describing the formation of so-called “DAT/alpha-synuclein clumps” (Longhena et al., 2018). Other researchers also reported a similar increase in the size of DAT/alpha-synuclein complexes in other animal models of PD (Garcia-Reitböck et al., 2010; Longhena et al., 2018). Using Proximity Ligation Assay (PLA), Belluci et al. revealed DAT/alpha-synuclein interactions and alterations in the distribution of these proteins (Bellucci et al., 2011).

The regulation of DAT distribution and activity is essential for the balance of cytoplasmic and extracellular DA concentrations, both in striatum and midbrain. DAT redistribution into larger clumps may be due to augmented DAT internalization from the cellular membrane. Disproportionate internalization would directly impact DAT availability at the membrane level and it might probably play a role in early disease onset. Promising research has been conducted with several proteins, such as Synaptogyrin-III, synapsin-III or Parkin, which have been recently reported to interact with both alpha-synuclein and DAT (Moszczynska et al., 2007; Egaña et al., 2009; Zaltieri et al., 2015; Blumenstock et al., 2019). However, the exact molecular mechanism underlying the observed preferential impact on the DLS has not been completely elucidated yet. Although the present work offers some hints of a wider interpretation of such mechanism, several aspects of the alpha-synuclein/DAT relationship and its effects on the differential vulnerability within striatal DA innervation will require further molecular studies.

In summary, the present work expands our knowledge on how changes in alpha-synuclein expression may interfere with striatal DAergic neurotransmission. The described results suggest that DA innervation probably implements intrinsic mechanisms to counteract the damaging impact triggered by alpha-synuclein overexpression at early stages. With advancing age, the brain seems to establish more compensatory mechanisms aimed at keeping the effects of alpha-synuclein accumulation under control. However, early compensatory mechanisms may not be powerful enough to compensate for long-term effects of ongoing alpha-synuclein overexpression and consequent accumulation. Ageing and prolonged exposure to high human alpha-synuclein levels may lead to other compensatory

modifications in the DAergic terminals e.g., alterations in TH activity or within the DA neurotransmission machinery. Low extracellular DA levels in aged mice may trigger adaptive DAT internalization as a physiological response to the DA deficit caused by the loss of terminals or dysregulation of TH. Such adaptations are common in aged individuals (and exacerbated in PD), in order to maintain optimal neurotransmitter concentrations for proper regulation and functionality of postsynaptic DA receptors (Paillé et al., 2010). Interpretations of the slow kinetics observed for DA uptake at early and late stages of alpha-synuclein overexpression may differ because of different causes. At early stages, acute and sudden increase of alpha-synuclein levels might directly impact DAT mobilization at the membrane level, where less functional transporters would directly result in reduced DA clearance and consequent increased overall release. Although not physiological, this augmented effect of enhanced DA neurotransmission would not impact negatively on the survival of the individual. At later stages, the burden of prolonged alpha-synuclein overexpression might reduce DA release by impairing TH activity or vesicle release; however, now the reduced DAT activity would be upheld in an attempt of maintaining ideal levels of extracellular DA in the aged, damaged brain.

5 Conclusions

1. Dopamine neurotransmission is altered in an age-dependent manner in transgenic mice that overexpress human alpha-synuclein, augmented at three months and decreased at twelve months.

2. Human alpha-synuclein overexpression preferentially affects dopaminergic neurotransmission in the dorsolateral striatum.

3. Increased DA release in the dorsolateral striata of three-month-old of transgenic mice is also associated with enhanced performance in a motor learning task.

4. Dopamine clearance after release in transgenic mice is slower at three and twelve months of age, resulting in a prolonged permanence of dopamine in the extracellular space.

5. Dopamine transporter-positive clumps are detected in the dorsolateral striatum simultaneously with DA uptake alterations, in three and twelve-month-old mice.

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7 List of publications

Peer-reviewed publications

1. Sun F., Salinas G. A., Filser S., Blumenstock S., **Medina-Luque J**., Herms J., and Sgobio C. (2021). Impact of alpha-synuclein spreading on the nigrostriatal dopaminergic pathway depends on the onset of the pathology. Brain pathology. Volume 32, Issue 2.

2. Biechele G., Franzmeier N., Blume T., Ewers, **Medina-Luque** J., Eckenweber F., Sacher C., Beyer L., Ruch-Rubinstein F., Lindner S., Gildehaus F. J., von Ungern-Sternberg B., Cumming P., Bartenstein P., Rominger A., Höglinger G. U., Herms J., Brendel M. (2020). Glial activation is moderated by sex in response to amyloidosis but not to tau pathology in mouse models of neurodegenerative diseases. Journal of Neuroinflammation.

3. Eckenweber F., **Medina-Luque J**., Blume T., Sacher C., Biechele G., Wind K., Deussing M., Briel N., Lindner S., Boening G., von Ungern-Sternberg B., Unterrainer M., Alber N. L., Zwergal A., Levin J., Bartenstein P., Cumming P., Rominger A., Höglinger G. U., Herms J., Brendel M. Longitudinal TSPO expression in tau transgenic P301S mice predicts increased tau accumulation and deteriorated spatial learning. Journal of Neuroinflammation.

4. Negrete-Díaz J. V., Shumilov K., Real M. A., **Medina-Luque J**., Valderrama-Carvajal A., Flores G., Rodriguez-Moreno A., and Rivera A. (2019). Pharmacological activation of dopamine D4 receptor modulates morphine-induced changes in the expression of Gad65/67 and GABAB receptors in the basal ganglia. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2019.01.024

5. Rivera A., Gago B., Suárez-Boomgaard D., Kehr J., Roales-Buján R., ValderramaCarvajal A., Bilbao A., **Medina-Luque J.**, Díaz-Cabiale Z., van Craenenbroeck K., Borroto-Escuela D.O., Rodríguez de Fonseca F., Santín L., de la Calle A., Fuxe K. (2016). Dopamine D4 receptor stimulation prevents nigrostriatal dopamine pathway activation by morphine: relevance for drug addiction. Addiction Biology. https://doi.org/10.1111/adb.12407

6. Suárez-Boomgaard D., Gago B., Valderrama-Carvajal A., Roales-Buján R., Van Craenenbroeck K., Duchou J., Borroto-Escuela D.O., **Medina-Luque J.**, de la Calle A., Fuxe K. and Rivera A. (2014). Dopamine D4 receptor counteracts morphine-induced changes in μ opioid receptor signaling in the striosomes of the rat caudate putamen. International Journal of Molecular Sciences. https://doi.org/10.3390/ijms15011481

Manuscripts in preparation

Medina-Luque J., Sgobio C. and Herms J. Dopaminergic neurotransmission is preferentially disrupted in the dorsolateral striatum of a transgenic mice modelling Parkinson's disease. *In preparation*.

Contribution to congresses

1. **Jose Medina-Luque**., Sgobio C. and Herms J. Role of alpha synuclein accumulation and lack of tau in nigro-striatal dopaminergic neurotransmission. International Conference on Alzheimer´s and Parkinson´s diseases (AD/PD). Lisbon, Portugal 2019. Poster presentation.

2. Maximilian Deussing., Eckenweber F., Blume T., **Medina-Luque J**., Sacher C., Lindner S., Unterrainer M., Alber N. L., Zwergal A., Bartenstein P., Rominger A., Höglinger G. U., Herms J., Brendel M. Longitudinal PET Monitoring of Microglial Activation in Tau Transgenic P301S Mice Predicts Cognitive Deterioration and Metabolic Decline. Nuklearmedizin. Germany, March 2019. Conference paper.

3. Kirill Shumilov, **J. Medina-Luque**, A. Valderrama-Carvajal, R. Roales-Buján, A. de la Calle y A. Rivera. Effect of selective ablation of striosomes on dopaminergic nigroestriatal inervation. XVI Congreso de la Sociedad Española de Biología Celular (SEBC). Spain, July 2015. Poster presentation.

4. H. Jimenez, B. Gago, D. Suárez, B. Gago, A. Valderrama-Carvajal, R. Roales-Buján, K. Van Craenenbroeck, J. Duchou, D O. Borroto-Escuela, **J. Medina-Luque**, A. de la Calle, K. Fuxe, M C. Rodríguez-Oroz. Dopamine D4 receptor counteracts morphineinduced changes in μ opioid receptor signaling in the striosomes of the rat Caudate Putamen. II Reunión vasca de neurociencias (Neurogune): Donostia-San Sebastián (Spain), 9 July 2014. Poster presentation

5. A. Rivera, D. Suárez, B. Gago, A. Valderrama-Carvajal, **J. Medina-Luque**, R. Roales-Buján, K. Fuxe, A. de la Calle. Dopamine D4 receptor activation counteracts nigrostriatal pathway activation by morphine: relevance in drug addiction. XV Congreso de la Sociedad Española de Neurociencia (SENC): Oviedo (Spain), 25-27 September 2013. Poster presentation.

8 Appendix

3.1. Fast scan cyclic voltammetry

Cutting solution

	[CONC.] mM	g/2L 1X
Sucrose	194	132.81
NaCl	30	3.51
KCl	4.5	0.671
$MgCl_2$	1	0.407
$NaHCO_3$	26	4.370
NaH_2PO_4	1.2	0.330
Glucose	10	3.6

aCSF 10 x Stock

	[CONC.] mM	1 L	500 ml
NaCl	126	73.6	36.8
KCl	2.5	1.86	0.93
NaH_2PO_4	1.2	1.66	0.83
CaCl2	1.4	3.52	1.765
$MgCl_2$	1.2	2.44	1.22

aCSF 1x solution, pH 7.3

	2L	1L	[nM]
Ascorbic Acid	0,14	0.07	0.4
HEPES	9.6	4.8	20
Glucose	3.96	1.98	11
$NaHCO_3$	4.2	2.1	25
NaOH (pellet)	4	2	
Voltammetry stock 10x	200 ml	100 ml	50 ml

Coc stock (1mM)

	mg	Final volume (ml)
Coc-Hydrochloride	0.0127	
ddH_2O		36.785

Dopamine solution (Calibration line)

	mg	Final volume (ml)
DA-Hydrochloride	9.482	
aCSF 1x		50

3.1. Immunohistofluorescence

Phosphate buffer (PB) 0.4 M pH 7.4

	g/2L
$NaH_2PO_4 – 2\ H_2O$	27.6
$Na_2HPO_4 – 2\ H_2O$	106.8

Phosphate buffered saline (PBS) 0.1M, pH 7.4

	g/1L	ml
NaCl	9	
$Na_2HPO_4 – 2\ H_2O$		250

Tissue preservation buffer

	g	ml
Sodium azide	0.02	
PBS 0.1 M pH 7.3		100

Phosphate buffered saline-Triton buffer (PBS Tx - 0.2 %)

	g	ml
Triton tx (reference)		0.2
PBS 0.1 M pH 7.3		10
Sodium azide	0.1	

Sudan black B

	g	ml
Ethanol 70 %		100
Sudan Black B	0.2	