IMPULSE-CONTROL DISORDERS IN PARKINSON'S DISEASE: DEVELOPMENT OF AN ANIMAL MODEL

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Impulse-control disorders in Parkinson's disease: Development of an animal model

TESIS DOCTORAL

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Abbreviations

[^{11}C]-PHNO	[^{11}C]-(1)-propyl-hexahydro-naphthooxazin
[^{18}F]FDG	[^{18}F]fluorodeoxy-D-glucose
[^{18}F]Fluorodopa	[^{18}F]-2-fluoro-5-hydroxy-l-tyrosine
6-OHDA	6-hydroxydopamine
α-syn	α-synuclein
ac	Anterior comissure
ACA	Anterior cingulate area
ACC	Anterior cingulate cortex
AMY	Amygdala
aPFC	Anterior Prefrontal cortex
AVV	Adenoassociated viral vectors
BAC	Bacterial artificial chromosome
BLA	Basolateral nuclei of the amygdala
DL/dl-	Dorsolateral
DM	Dorsomedial
cc	corpus callosum
Cd	Caudate nucleus
cdm-	Caudal dorsomedial
cl-	Caudolateral
cm-	caudomedial
COMT	Cathecol-O-methyltransferase
CPA	Conditiones place aversion
CPP	Conditioned place preference
D_1R/D_2R/D_3R/D_4R/D_5R	D_1, D_2, D_3, D_4 and D_5 dopamine receptors
DAT	Dopamine transporter
DAWS	Dopamine withdrawal syndrome
DDS	Dopamine dysregulation syndrome
DLPFC	Dorsolateral prefrontal cortex
DRL	Differential reinforcement of low rate of responding
FCN	Fixed consecutive number
FEF	Frontal eye fields
fMRI	Functional magnetic resonance imaging
GABA	γ-amminobutiric acid
GBA	Glucocerebrosidase
GP	Globus pallidus
GPe	External globus pallidus
GPi	Internal globus pallidus
hα-syn	Human α-synuclein
HPC	Hippocampus
HThal	Hypothalamus
ICB	Impulsive-compulsive behaviours
ICD	Impulse control disorders
ICSS	Intracranial self-stimulation
Ic	internal capsule
icv	Intracerebroventricular
ip	Intraperitoneal
ITI	Inter-trial interval
iv	Intravenous
KO	Knock-out

LB	Lewy body
l-	lateral
ldm-	lateral dorsomedial
LH	Limited hold
LRRK2	Leucin-rich repeat kinase 2
MAO-B	Monoamino-oxidase-B
m-	Medial
MD	Mediodorsal nucleus of the thalamus
mdm-	Medial dorsomedial
MDmc	Mediodorsal nucleus *pars magnacelullaris* of thalamus
MDpc	Mediodorsal nucleus *pars parvocellularis* of thalamus
MDpl	Mediodorsal nucleus *pars paralamellaris* of thalamus
MFN	Medial forebrain bundle
mPFC	Medial prefrontal cortex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
OFC	Orbitofrontal cortex
OR	Odds ratio
PCC	Posterior cingulate cortex
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
pm-	Posteromedial
PPN	Pedunculopontine nucleus
PPX	Pramipexole
PrP	Prion protein
PTSA	Post-training signal attenuation task
Put	Putamen nucleus
rCBF	Regional cerebral blood flow
rd-	Rostrodorsal
rl-	Rostrolateral
rm-	Rostromedial
ROS	Radical oxygen species
rt	Reticular nucleus of thalamus
sc	Subcutaneous
SD	Stimulus duration
SMA	Supplementary motor area
SN	Substantia nigra
SNc	Substantia nigra *pars compacta*
SNr	Substantia nigra *pars reticulata*
SPECT	Single photon emission computed tomography
STN	Subthalamic nucleus
Thal	Thalamus
UPS	Ubiquitine-proteasome system
VAmc	ventral anterior nucleus *pars magnocellularis* of thalamus
VApc	ventral anterior nucleus *pars parvollularis* of thalamus
VTA	Ventral tegmental area

VL/vl-	Ventrolateral
VM	Ventromedial
VLo	Ventral lateral nucleus *pars oralis* of thalamus
VLPFC	Ventrolateral prefrontal cortex
Vlm	Ventral lateral nucleus *pars medialis* of thalamus
VP	Ventral pallidum
VS	Ventral striatum
vSub	Ventral subiculum of the hippocampus

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Summary in Spanish

La enfermedad de Párkinson (EP) es una enfermedad neurodegenerativa caracterizada por el desarrollo de alteraciones motoras (temblor, rigidez y bradicinesia) que están asociadas a una progresiva muerte de neuronas dopaminérgicas de la sustancia negra compacta (SNc), la consecuente depleción dopaminérgica en el estriado y la aparición de agregados proteicos intracelulares (principalmente de α-sinucleína). La fisiopatología de la muerte neuronal es aún desconocida en gran medida, por lo que en la actualidad no existe ningún fármaco que ralentice o revierta la muerte neuronal. Así, los tratamientos disponibles están enfocados a mitigar los signos motores de la enfermedad mediante la reposición exógena del déficit dopaminérgico, principalmente mediante la administración de levodopa (precursor de la dopamina) y/o agonistas dopaminérgicos. Sin embargo, el uso crónico de estos fármacos induce el desarrollo de efectos secundarios motores y no motores, de los que las disquinesias (movimientos involuntarios de tipo coréico) y las complicaciones psiquiátricas como el trastorno de control de impulsos (TCI), que incluye el juego patológico, las compras compulsivas, la hipersexualidad y la ingesta compulsiva de comida, y otros trastornos impulsivos-compulsivos, como el síndrome de disregulación dopaminérgica (SDD), son los más frecuentes e incapacitantes (Fabbrini et al., 2007; Voon and Fox, 2007; Weintraub et al., 2015). Salvo el SDD, estos comportamientos se clasifican como adicciones conductuales (Potenza, 2006) se relacionan con el uso crónico de agonistas dopaminérgicos que actúan sobre los receptores dopaminérgicos D_2/D_3, aunque también pueden ser promovidas por la levodopa (Weintraub et al., 2015). Otros factores de riesgo para el desarrollo del TCI son edad joven al inicio de la EP, sexo (hombre) y tener una personalidad impulsiva o de búsqueda de la novedad antes del inicio de la EP (Weintraub et al., 2010; Weintraub and Claassen, 2017).

A pesar de las importantes complicaciones que suponen en la vida personal, familiar y social de los pacientes (S.-Y. Lim et al., 2008), la fisiopatología subyacente al TCI en la EP no está bien definida. El conocimiento que se tiene en la actualidad proviene principalmente de estudios clínicos y de neuroimagen en pacientes (Aracil-Bolaños and Strafella, 2016; Jiménez-Urbieta et al., 2015; Rizos et al., 2016; Weintraub et al., 2010), que indican que una excesiva estimulación dopaminérgica del estriado ventral en pacientes con EP podría provocar cambios funcionales en diferentes áreas de los ganglios basales y la corteza implicados en los circuitos asociativo y límbico. Los estudios de neuroimagen también señalan que el TCI está relacionado con un mayor grado de depleción dopaminérgica del estriado ventral, aunque también se han descrito alteraciones en el estriado dorsal, por lo que el patrón de degeración dopaminérgica que predispone mas a TCI sigue sin estar bien definido (Smith et al., 2016; Voon et al., 2014; Vriend et al., 2014).

Además, la naturaleza de la impulsividad es multidimensional y compleja, pudiéndose distinguir dos dominios principales, la impulsividad motora y la impulsividad de decisión (Dalley et al., 2011; Antonelli et al., 2014; Voon and Dalley, 2015; Robbins and Dalley, 2017), que tiene rasgos comunes con la compulsividad (Dalley et al., 2011). Las acciones motoras incluyen a la impusividad de espera (*waiting impulsivity*) y alteraciones en la inhibición de respuesta (*motor action*), mientras que el dominio de las acciones de decisión incluye alteraciones de la gratificación aplazada (*delay discouting*) y la impulsividad de reflexión (*reflection impulsivity*) (Voon and Dalley, 2015).

El desarrollo de modelos animales que reflejen lo más fielmente posible las características del TCI en pacientes de EP resulta crucial para profundizar en su fisiopatología y poder desarrollar nuevos abordajes terapéuticos. En la última década se han realizado algunos estudios utilizando diferentes modelos de parkinsonismo (diferentes patrones de depleción dopaminérgica en el estriado), test comportamentales y fármacos dopaminérgicos con esta finalidad (Cenci et al., 2015), pero se hacen imprescindibles nuevos estudios por diferentes motivos. Los test comportamentales utilizados hasta la fecha (analizan un único rasgo de la impulsividad (paradigma de refuerzo *diferencial* de bajas tasas de *respuesta* (*differential reinforcement of low rates of responding*; DRL) y paradigma de refuerzo de número fijo consecutivo (*fixed consecutive number*;FCN) (Engeln et al., 2016), y en la mayoría de las veces hacen uso de refuerzos artificiales (estimulación eléctrica de la amígdala; Rokosik and Napier, 2012; Holtz et al., 2016; Tremblay et al., 2017) en vez de refuerzos naturales como la comida o el sexo, alejándose así del contexto clínico.Por ello, el uso de paradigmas que puedan medir simultáneamente diferentes aspectos de los comportamientos impulsivos-compulsivos y que empleen refuerzos naturales se hace particularmente necesario. En este sentido, destacan los paradigmas *5-Choice Serial Reaction Time Task* (5-CSRTT) y *Variable Delay-to-Signal* (VDS). El 5-CSRTT mide simultáneamente el control atencional, la compulsividad y la impulsividad de espera (Robbins, 2002), la cual parece crítica para el desarrollo de adicciones a sustancias que se sabe que comparten características con las adicciones conductuales en la EP (Jiménez-Urbieta et al., 2015; Voon et al., 2014). Por otro lado, el paradigma VDS está basado en diferentes tareas (5-CSRTT, DRL y programas de reforzamiento con intervalos fijos o variables), aunque con una reducción de la carga atencional y del tiempo de aprendizaje requerido (Leite-Almeida et al., 2013). Además, mide diferentes rasgos impulsivos (impulsividad motora e intolerancia al retraso en la recompensa) al mismo tiempo. Por último, respecto a los fármacos dopaminérgicos y regímenes de administración (dosis; agudo o crónico) usados en estos trabajos existe una gran variabilidad (Carvalho et al., 2017; Dardou et al., 2017; Engeln et al., 2016; Holtz et al., 2016; Rokosik and Napier, 2012; Tremblay et al., 2017). Por otra parte, en relación al tipo de fármacos

usados, los datos apuntan a una alta asociación entre la impulsividad y el uso del agonista dopaminérgico Pramipexol (PPX), como ocurre en pacientes de EP con TCI (Grall-Bronnec et al., 2018).

Por otra parte, la determinación de alteraciones moleculare en el estriado asociadas al TCI podría contribuir a conocer mejor la fisiopatología de este trastorno. A nivel molecular, la expresión del factor de transcripción FosB y su forma truncada ΔFosB se encuentra elevada en el CPu de ratas tratadas con sustancias psicoestimulantes (Nestler et al., 2001) y en ratas que presentan adicción por comida (Velázquez-Sánchez et al., 2014), lo que sugiere unn papel relevante en adicciones a sustancias y adicciones conductuales. Estudios previos indican que ratas parkinsonizadas tratadas crónicamente bajo paradigmas de autoadministración de PPX muestran un aumento estriatal de FosB/ΔFosB (Engeln et al., 2013a; Loiodice et al., 2017), pero no está claro si estos cambios se relacionan con un aumento de la impulsividad o si el aumento de la expresión se debe exclusivamente al fármaco, por lo que es necesario profundizar su estudio en modelos animales de impulsividad de la EP.

Nuestra hipótesis es que un patrón de depleción dopaminérgica no restringido solo al área motora del estriado unido al tratamiento agudo o crónico de agonistas dopaminérgicos similará el comportamiento impulsivo que se observa en los pacientes de EP con TCI. Además, especulamos que el paradigma VDS será efectivo para medir diferentes tipos de impulsividad (impulsividad motora e intolerancia al retraso) reduciendo la carga tencional requerida en el paradigma 5-CSRTT. Por último, hipotetizamos que la impulsividad estará asociada a un incremento de la expresión de FosB/ΔFosB en el estriado.

Por todo lo expuesto, el objetivo principal de esta tesis doctoral es el desarrollo de un modelo animal que refleje la mayor cantidad de caracteristicas del TCI inducido por agonistas dopaminérgicos en pacientes con EP, para poder estudiar los cambios patológicos subyacentes. Para ello, se han establecido los siguientes objetivos específicos:

1. Estudiar si el diferente patrón de depleción dopaminérgica en el estriado inducido en dos modelos de parkinsonismo bilateral (sobreexpresión de α-sinucleína humana con la mutación A53T (A53T-hα-syn) en SNc o inyección de 6-OHDA en la región dorsolateral (DL) del Caudado Putamen (CPu)) provoca una diferente propensión a desarrollar un comportamiento impulsivo tras tratameinto con PPX. A su vez, estudiar si la impulsividad de los animales antes y después de la inducción de la lesion dopaminérgica es un indicador de un mayor riesgo de desarrollar impulsividad inducida por PPX en los modelos animales de parkinsonismo.

2. Establecer la validez de dos paradigmas comportamentales (5-CSRTT and VDS) para estudiar comportamientos impulsivos y compulsivos y diferentes tipos de impulsividad en los modelos animals de parkinsonismo descritos.
3. Determinar el potecial para inducir impulsividad de dosis agudas bajas y altas de PPX así como del uso crónico de dosis bajas
4. Analizar si la expression striatal de FosB/ΔFosB está correlacionada con la impulsividad inducida por PPX.

A continuación de describirán y discutirán los resultados obtenidos separados en tres secciones de experimentos.

Experimento 1. Efecto del tratamiento crónico con 0,25 mg/kg de PPX en ratas con parkinsonismo bilateral obtenido mediante la sobreexpresión de A53T-hα-syn en la SNc sobre el comportamiento impulsivo-compulsivo usando el paradigma 5-CSRTT.

Se obtuvo un modelo en ratas parkinsonianas con lesión dopaminérgica bilateral inducida por la sobreexpresión en SNc de A53T-hα-syn mediada por vectores virales adenoasociados (AAV) a las que se les administró crónicamente PPX (0,25 mg/kg/día durante 4 semanas) usando el paradigma 5-CSRTT para el estudio conductual. La lesión dopaminérgica por sí misma indujo un aumento de la impulsividad de espera respecto a las ratas del grupo Control. Además, esta impulsividad se exacerbaba en el estado ON medicación a lo largo de las 4 semanas de tratamiento. Es importante indicar que existe una correlación positiva entre la impulsividad tras la lesión dopaminérgica y tras el tratamiento con PPX en los animales lesionados y no en los animales control.

Al realizar los estudios histológicos y determinar correlaciones con el comportamiento, se comprobó que la impulsividad desarrollada por los animales bajo el efecto de PPX correlacionaba positivamente con la extensión de la depleción dopaminérgica estriatal, siendo por tanto mayor en aquellos animales que mostraban una mayor depleción dopaminérgica en el CPu medida por la expresión del transortador de dopamina (DAT). Esta lesión dopaminérgica era proporcional en todas las regiones estriatales estudiadas (DL, dorsomedial (DM), ventromedial (VM), ventrolateral (VL)). Por otro lado, en los animales parkinsonianos tratados con PPX se observó, comparados conel grupo Control, un aumento de la expresión de FosB/ΔFosB en el DL CPu de forma bilateral y en el núcleo accumbens (estriado ventral/límbico) derecho, zona asociada al control inhibitorio (Aron *et al*, 2004), sin que hubiera una correlación significativa con el comportamiento impulsivo.

Estos resultados indican que el desarrollo de impulsividad es inducido por el tratamiento crónico con PPX a bajas dosis en este modelo de parkinsonismo y es tanto mayor cuanto mayor

es el grado de depleción dopaminérgica estriatal en todas las regiones del CPu, involucrando tanto áreas asociativas y límbicas. Además, la impulsividad producida por la pérdida dopaminérgica podría servir como predictor de la impulsividad tras recibir el tratamiento con PPX.

Experimento 2. Efecto del tratamiento agudo con 0,25 mg/kg y 3 mg/kg de PPX en ratas con parkinsonismo bilateral obtenido mediante la inyección del neurotóxico 6-OHDA en la región motra del estriado (DL CPu) y estudio del comportamiento impulsivo-compulsivo usando el paradigma VDS.

Se obtuvo un modelo en ratas parkinsonianas con lesión dopaminérgica bilateral inducida por inyecciones bilaterales en la región DL del CPu (región motora) del neurotóxico 6-OHDA que fueron tratadas con dosis baja (0,25 mg/kg) y alta (3 mg/kg) de PPX y evaluadas mediante el paradigma VDS. Contrariamente al experimento 1, la lesión por sí misma no indujo ninguna alteración en el comportamiento de los animales. Sin embargo, la administración aguda de PPX indujo un aumento de la impulsividad en ratas parkinsonizadas de forma dosis dependiente, estando la dosis de 0.25 mg/kg de PPX asociada a un aumento de la impulsividad motora y la dosis de 3 mg/kg a un aumento tanto de la impulsividad motora como de la intolerancia al retraso en la recompensa. Cabe destacar que la impuslividad motora se mantuvo parcialmente 24h después de haber recibido la dosis alta de PPX (cuando ya no existía ningún beneficio motor observable), por lo que el efecto en la impulsividad estaría disociado del efecto motor del fármaco. Esto coincide con los resutlados de estudios en humanos que sugieren que pacientes con EP presentan alteraciones en el control inhibitorio, la inhibición de respuestas, la toma de decisiones intertemporales y presentan una aversión al retraso (Al-Khaled et al., 2015; Antonelli et al., 2014; Canário et al., 2019; Milenkova et al., 2011; Nombela et al., 2014; Obeso et al., 2011), estando muy incrementada la intolerancia al retraso en la recompensa en aquellos pacientes con EP y TCI comparado con aquellos pacientes con EP sin TCI (Voon et al., 2010; Housden et al., 2010; Leroi et al., 2013), de modo permanente una vez desarrollado el problema e independientemente de si están bajo el efecto de los fármacos dopaminérgicos. En ratas controles (n=12) la dosis de 3 mg/kg de PPX también produjo un aumento de la impulsividad motora, sugiriendo que, al igual que ocurre en algunas personas sin EP bajo tratamiento con agonistas dopaminérgicos, una sobreactivación dopaminérgica en animales sanos también podría provocar la aparición de comportamientos impulsivos patológicos (Holman, 2009; Cornelius et al., 2010).

Por último, los análisis de correlación muestraron que en el grupo de animales parkinsonianos tratados con PPX la impulsividad bajo el efecto agudo de la dosis alta de 3 mg/kg

de PPX correlacionaba con la impulsividad observada en los mismos animales antes y después de la lesión dopaminérgica antes de recibir ningún tratamiento. Sin embargo, no se ha encontrado una correlación signficativa entre la impulsividad y el grado de denervación dopaminérgica en todo el CPu o en sus diferentes áreas.

En cualquier caso, los resultados de estos dos primeros estudios muestran que la tendencia impulsiva de los animales como "rasgo de personalidad" o la asociada a la perdida dopaminérgica son un factor de riesgo para el desarrollo de trastornos de impulsividad bajo tratamiento con PPX, de forma equivalente a lo que ocurre en pacientes de EP (Rizos et al., 2016; Zadeh et al., 2018). Además, el paradigma VDS ha resultado útil para la evaluación de diferentes fomas de impulsividad en un tiempo relativamente reducido en comparación con el paradigma 5-CSRTT. Además, aunque se han usado dos modelos animales de parkinsonismo diferentes, los resultados muestran que la impulsividad puede darse tanto por una administración crónica de bajas dosis de PPX como por una dosis alta administrada de forma aguda dando validez al modelo.

Experimento 3. Efecto del tratamiento crónico con 0,25 mg/kg en ratas con parkinsonismo bilateral obtenido mediante sobreexpresión de A53T-hα-syn en la SNc y estudio del comportamiento impulsivo-compulsivo usando el paradigma VDS.

Teniendo en cuenta los resultados obtenidos en los experimentos anteriores, se realizó un nuevo estudio utilizando el mismo modelo de lesión dopaminérgica que en el experimento 1 por ser el que producia un incremento de impulsividad tras la lesión dopaminérgica y el paradigma comportamental VDS del experimento 2 por resultar valido para la medición de distintos tipos de impulsividad y mas corto en su implementación.

Al igual que en el primer estudio, la lesión dopaminérgica produjo un leve aumento de la impulsividad de las ratas lesionadas. Puesto que en los dos experimentos previos existe una correlacion entre la impulsividad tras tratamiento con PPX y la existente antes y/o después de la lesión dopaminérgica, en este estudio se decidio analizar por separado las ratas mas y menos impulsivas tras el tratamiento con el fin de conocer diferencias que pudieran ayduar a entender la fisiopatología del desarrollo de este trastorno tal como ocurre en los pacientes con EP, que solo un porcentaje dearrolla una impulsividad patológica. Asi, al ser tratadas crónicamente con PPX, las ratas lesionadas se dividieron en dos subgrupos de animales con un comportamiento claramente diferenciado en el estado ON medicación: un grupo de 4 animales con una impulsividad muy alta (cuartil 1) y un grupo de 13 animales con baja impulsividad (cuartiles 2, 3 y 4) que además mostraban un aumento en el porcentaje de omisiones respecto a las ratas lesionadas de alta impulsividad. Esto refleja el hecho de que no todos los pacientes con EP

desarrollan TCI al ser tratados con agonistas dopaminérgicos, ya que se ha estimado que la prevalencia de este trastorno en la EP varia entre 14 y 30% (Weintraub et al., 2010; Weintraub et al., 2015; de Guzman et al., 2015; Papay et al., 2011; Sharma et al., 2015). No hubo diferfencias en otras variables del estudio de compartamiento entre los grupos excpeto en el porcentaje de omisiones que fue mayor en las ratas con baja impulsividad reflejando posiblemente que puede existir una disminución de la atención inducida por el PPX que es sobreseído por la intesidad del trastorno de impulsividad en el grupo de ratas mas impulsivas.

Por otro lado, se analizó la expresión de DAT y FosB/ΔFosB en el estriado de estos animales. Al contrario que en el experimento 1, la impulsividad no correlacionó significativamente con el grado de depleción dopaminérgica en el estriado ni hubo diferencias entre los grupos de alta y baja impulsividad en estas variables. Respecto a la expresión de FosB/ΔFosB, no hubo diferencias estadísticamente significativas entre ambos grupos en ninguna de las áreas estriatales analizadas. Por tanto, la expresión de este marcador en el sistema dopaminérgico nigrostriatal probablemente no sea un marcador óptimo de la impulsividad en modelos animales de EP.

Las conclusiones más importantes de la presente tesis doctoral son las siguientes:

1) La lesión dopaminérgica bilateral inducida por sobreexpresión de A53T-hα-syn en la SNc induce por sí misma un aumento de la impulsividad tanto en el paradigma 5-CSRTT como en el paradigma VDS, al contrario que la lesión en el DL CPu por 6-OHDA en el paradigma VDS. Esto sugiere que una denervación estriatal más extensa y no restringida exclusivamente al área más motora del estriado (como se ha postulado en pacientes con EP) serían factores determinantes para el desarrollo de la impulsividad.

2) La impulsividad generada por la administración de PPX es dependiente de la dosis y del tiempo de tratamiento. Una única dosis de PPX a dosis altas es suficiente para provocar un aumento significativo de la impulsividad en animales parkinsonianos, principalmente alterando la intolerancia al retraso en la recompensa tal como ocurre en pacientes. Por tanto, este modelo remeda este aspecto del TCI en la EP.

3) Independientemente del modelo de lesión utilizado, los rasgos de impulsividad de animales parkinsonizados antes del desarrollo de la lesión dopaminérgica o una vez desarrollada ésta, parecen ser un marcador para identificar asujetos con mayor riesgo de padecer impulsividad anormal inducida por PPX, como se ha sugerido que ocurre en pacientes.

4) La expresión estriatal del factor de transcripción FosB/ΔFosB en ratas con depleción dopaminérgica causada por sobreexpresión de A53T-hα-syn en la SNc y tratados crónicamente con PPX mostró resultados inconclusos respecto a las ratas Control, y no correlacionó con el

comportamiento en ningún caso. Por tanto, no parece ser un buen marcador de la impulsividad en este modelo de EP.

5) Los modelos animales usados en esta tesis doctoral muestran rasgos similares a los que muestran los pacientes de EP y TCI. Particularmente, el modelo de parkinsonismo progresivo mediante sobreexpresión de A53T-hα -syn con el tratamiento crónico a dosis baja y el comportamiento analizado por el paradigma VDS parece el más idóneo para desarrollar futuros estudios.

I. Introduction

1. The history of Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease worldwide, affecting about 1% of people aged 65 and 3% of people in their 80s (de Lau and Breteler, 2006). In Spain, it has been estimated that the disease currently affects about 300,000 people (García-Ramos et al., 2016).

PD was formally first described in the book entitled "An essay on the shaking palsy" published in 1817 by James Parkinson (1755-1824). He described motor disturbances in six people that he observed in his daily walks in the city of London, reporting that these subjects suffered from "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward and to pass from a walking to a running pace. The senses and intellect remain uninjured".

Later on, Jean-Martin Charcot (1825-1893), along with Armand Trousseau (1801-1867), added a number of signs to the initial description, emphasizing the muscular rigidity, the slowness of movement and the cognitive decline that these patients showed (Micheli, 2006). They also postulated that the term *paralysis agitans* should be removed from the description of the disease and attributed the name "Parkinson's disease" to the illness (Goetz, 1986).

One of the most important advances in the knowledge of PD was made in 1919 by Constantin Tretiakoff who, in his doctoral thesis, noted the loss of the pigmented cells in the SNc of PD patients (Lees et al., 2008). Afterwards, Oleh Hornykiewicz suggested in 1959 that the pathogenesis of the disease was associated with striatal dopamine depletion (Lees et al., 2015), an hypothesis that led to the development of the first clinical trials to treat PD patients with the dopamine precursor L-Dopa or levodopa in the following decade (Birkmayer and Hornykiewicz, 1961; Cotzias et al., 1969).

2. Neuropathology of Parkinson's disease

As previously stated, a progressive loss of neurons in the substantia nigra *pars compacta* (SNc) and the subsequent reduction of striatal dopaminergic innervation, mainly in the dorsolateral (DL) and posterior part of the putamen nucleus, cause the cardinal motor signs (tremor, rigidity and bradkykinesia) of PD (Hornykiewicz and Kish, 1987). In *post-mortem* tissue from PD patients, the remaining cells in the SNc show hyalinic and acidic intra-cytoplasmic inclusions in both the soma, described by Fritz Heinrich Lewy and named as Lewy bodies (LB), and the neuronal process (known as Lewy neurites) (Holdorff et al., 2013) (Figure 1).

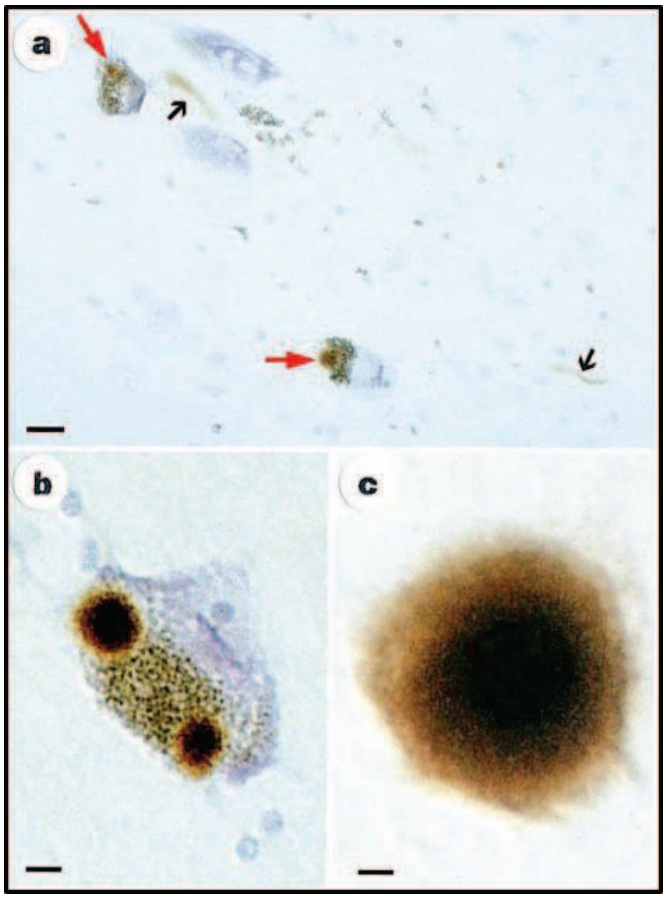

Figure 1. Sections of substantia nigra *pars compacta* (SNc) from patients with Parkinson's disease immunostained for α-synuclein (α-syn). (A) Two pigmented nerve cells, each containing an α-syn-positive Lewy body (red arrows). Lewy neurites (black arrows) are also immunopositive (scale bar, 20 mm). (B) A pigmented nerve cell with two α-syn-positive Lewy bodies (scale bar, 8 mm). (C) An α-syn-positive, extracellular Lewy body (scale bar, 4 mm) (from Spillantini et al., 1997).

The LB are spherical structures that show three different eosinophilic layers (nucleus, body and halo) and are composed of misfolded or aggregated proteins that cellular degradation and reparation systems are not able to eliminate. Thus, the main components of LB are α-synuclein (α-syn), ubiquitin and other proteins such as Tau (Love et al., 1988; Spillantini et al., 1997, 1998).

Other brain nuclei can also show LB pathology and neuronal loss: the nucleus basalis of Meynert, locus coeruleus, dorsal raphe nucleus, dorsal motor nucleus of vagus and pedunculopontine nucleus (PPN) (Sulzer and Surmeier, 2013). In contrast to the dopaminergic system that is mainly associated with motor impairments, the alterations in these nuclei cause cholinergic, serotoninergic and adrenergic deficits that are thought to be related to the development of non-motor symptoms (see below).

3. Aetiology

Although age constitutes the most important risk factor for the development of PD (Collier et al., 2011), the mechanisms behind the neuronal degeneration in PD are still unknown (Obeso et al., 2010). It is currently considered that the aetiology of PD is probably a combination of aging and factors that lead to several abnormalities in the cellular function, such as mitochondrial dysfunction, oxidative stress, etc (Collier et al., 2011; Malkus et al., 2009; Obeso et al., 2010; Sulzer, 2007) (Figure 2).

In the last decades, the study of genetic mutations has gained special attention. Although the genetic forms of PD only represent a small fraction of all the diagnosed cases (<10%), their study has provided valuable knowledge about possible mechanisms underlying the loss of cellular homeostasis in PD. Thus, mutations (Polymeropoulos et al., 1997) as well as duplications and triplications (Singleton et al., 2003) in the gene of α-syn have been described to cause familiar autosomic dominant forms of young onset PD. The function of α-syn is still unknown, but it is thought that it participates in the regulation of neurotransmitters release (Logan et al., 2017).

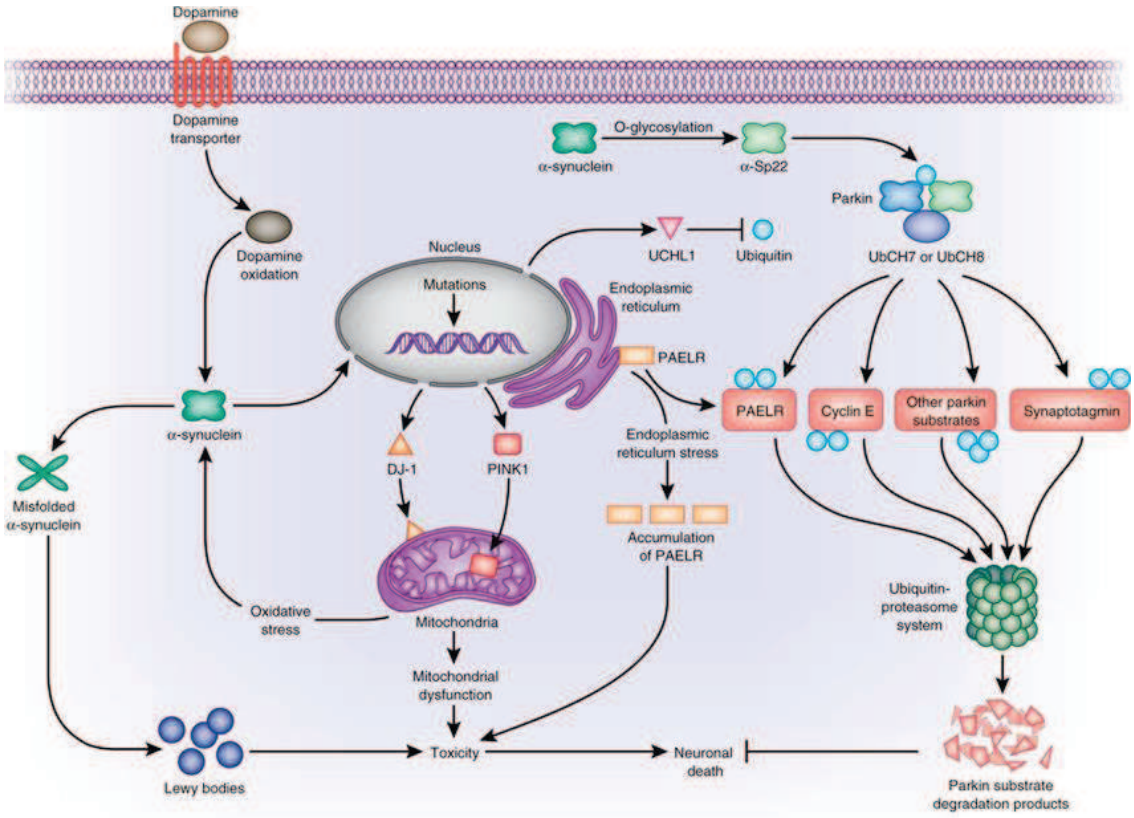

Figure 2. Possible intracellular alterations leading to the loss of cellular homeostasis: protein misfolding and aggregation, oxidative stress, mitochondrial dysfunction and anomalous protein degradation. Mutations in several genes are associated with these mechanisms impairment (from Obeso et al., 2010).

Most common dominant genetic forms of PD comprise mutations in the gene coding for the protein Leucine-Rich Repeat Kinase 2 (LRRK2), also known as *dardarin*. These mutations cause PD with variable penetrance, age of onset and neuropathology, despite patients show the same motor manifestations observed in idiopathic cases (Khan et al., 2005). Mutations in the LRRK2 protein coding gene are particularly frequent in some populations, including Ashkenazi Jews (G2019S substitution) and Basque (R1441G substitution) families, as they represent up to 40-50% of all the familiar cases of PD (Paisán-Ruiz, 2009). LRRK2 is a large multi-domain protein that is known to participate in several cellular functions such as dopamine homeostasis and vesicle trafficking, phosphorylation of proteins (i.e. α-syn), regulation of mitochondrial dynamics and morphology, regulation of cytoskeletal homeostasis, etc (Esteves et al., 2014).

On the other hand, the most common recessively inherited cause of PD are mutations in the Parkin protein coding gene (Kitada et al., 1998). These mutations cause young onset PD, but with no presence of α-syn aggregates nor LB in *post-mortem* tissue in most of the cases. Parkin is an E3-ligase that takes part in both the ubiquitin-proteasome protein degradation system (UPS) and mitophagy, and appears inactivated in sporadic PD due to nitrosative, oxidative and dopaminergic stress (Dawson and Dawson, 2010).

The second most frequent recessive forms of juvenile PD are those that comprise mutations in PINK1 and DJ1 protein coding genes. Along with Parkin, PINK1 is implicated in the regulation of mitochondrial degradation by mitophagy (Matsuda et al., 2010) and DJ1 is linked to different functions, such as chaperone, protease and mitochondrial regulation, protection against oxidative stress and transcription regulation (Ariga et al., 2013).

Other genes that can be mutated in familiar PD are those coding for ATP13A2, FBXO7, PLA2G6 and SYNJ1, and VPS35 among others (Marras et al., 2016; Mastrangelo, 2017), proteins that take part in mitochondrial functions, cellular protein and organelle recycling or degradation system (UPS, mitophagy and autophagy). In keeping with some genetic forms of PD in which the mitochondrial homeostasis is somehow affected, between 30-40% of PD patients show a decreased mitochondrial complex I activity (Schapira, 2008). Moreover, epidemiological studies have shown that there is an increased risk of developing PD in rural environments related to the employment of certain pesticides, herbicides, other neurotoxic agents (e.g. carbon monoxide), which can affect both the mitochondrial function and UPS, leading to an increase in cellular radical oxygen species (ROS) and the subsequent risk of cell death. In line with this, the idea of an environmental risk factor for PD was further supported by the fact that subjects who were exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) developed a parkinsonian syndrome. This toxic, a sub-product of the synthetic opiate manufacturing processes, inhibits the complex I of the mitochondrial respiratory chain and causes neuronal death in the SNc and

the subsequent development of motor signs similar to those observed in idiopathic PD (Langston et al., 1984).

Interestingly, mutations in the glucocerebrosidase gene (GBA), which encodes a lysosomal enzyme, are a well-established PD-associated risk factor. At homozygosity, mutations cause Gaucher disease, but subjects with only one affected allele have an increased risk (30% at the age of 80) to develop PD, with an odds ratio (OR) of 5.43% (Anheim et al., 2012; Sidransky et al., 2009). In contrast to other genetic forms of PD, GBA mutations are relatively common in general population with a prevalence ranging between 2.3 to 9.4% (Sidransky and Lopez, 2012).

4. Clinical features of Parkinson's disease

Since the original studies at the beginning of the 20th century, the spectrum of clinical signs of PD has been enriched notably and nowadays several non-motor symptoms are also recognized to be related to the neurodegenerative process of the disease. However, the diagnose of PD still relays on the cardinal motor signs originally described: resting tremor, rigidity and bradykinesia (Jankovic, 2008).

4.1. Motor signs

The progressive loss of the dopaminergic neurons of the SNc leads to the development of the above-mentioned classic motor signs of the disease. In most of the patients, these motor signs affect primarily one limb and, as the neurodegeneration progresses, they spread to the other limb of the same hemi-body and then to the limbs of the contralateral side.

Resting tremor is present in about 70% of PD patients at the time of diagnosis, although most patients will develop this motor sign during the progression of the disease (Rajput et al., 1991). This tremor in PD occurs at rest, when the majority of patients shows a typical movement of the fingers known as "pill-rolling" that consists on a tendency to join the thumb and index and perform semi-circular movements (Jankovic, 2008). Tremor increases with distraction manoeuvres and disappears with the execution of voluntary movements with the affected limb or during sleep.

Rigidity is the persistent resistance and difficulty for passive movement of the joints of limbs, caused by an increased muscular tone or an excessive and continuous muscular contraction (Jankovic, 2008). The rigidity of limbs can be uniform or show increased or decreased tone, which is known as "cogwheel rigidity". Rigidity increases when other body parts are moved (Froment sign) or when talking.

Hypokinesia (reduction of movements) and bradykinesia (slow execution of movements) are the motor signs that mostly impair PD patients, as they interfere with the activities that require a precise movement control (Berardelli, 2001). Thus, hypokinesia and bradykinesia consist of a difficulty to perform the whole movement process, from planning to execution, and encompass the loss of facial expressiveness, decreased arm swing when walking, or reduction of voluntary or automatic movements.

4.2. Non-motor symptoms

Non-motor symptoms are also present in PD patients. Among them, autonomic dysfunction (Allcock, 2004; Hirayama, 2006; Jankovic, 2008), cognitive decline (Delgado-Alvarado et al., 2016; Hely et al., 2008; Litvan et al., 2011), neuropsychiatric disturbances such as mood disorders (depression and apathy) (Aarsland et al., 2009; Pagonabarraga et al., 2015) Reijnders et al., 2008) and hallucinations (Rabey, 2009), sleep-wake cycle disorders (Chahine et al., 2016) and sensitive disorders (Jankovic, 2008) are the most common. Although in the past they were not considered to be features of the disease, these non-motor alterations have gained attention in the last decades as they can be prodromal markers of the disease (Postuma and Berg, 2016) and directly contribute to the deterioration of the quality of life of PD patients (Barone et al., 2009).

5. Treatment of Parkinson's disease

5.1. Pharmacological treatment and related complications

Currently, there is no an available treatment to stop, slow down or revert the neuronal death in PD. Thus, the current pharmacological treatments are aimed to counteract the decreased dopaminergic tone by the administration of the dopamine precursor L-Dopa or dopaminergic agonists that directly stimulate dopamine receptors (Connolly and Lang, 2014; Jenner, 2015). Among them, L-Dopa in combination with drugs that inhibit its peripheral or central metabolism (by catechol-O-methyltransferase (COMT) or monoamine-oxidase-B (MAO-B)) is the most effective treatment to alleviate motor signs in PD. However, up to 70% of PD patients develop motor complications after 6 years of L-Dopa treatment such as motor fluctuations ("wearing off" or ON-OFF and dyskinesias (involuntary purposeless irregular movements of trunk, limbs and face) (Fabbrini et al., 2007; Schrag, 2000). They are mainly due to fluctuations in plasmatic levels of dopamine related to either a pulsatile administration of L-Dopa or pharmacodynamical problems (Olanow et al., 2006). Thus, since the 1990s, dopaminergic agonists have been implemented to get more continuous striatal dopamine

receptor stimulation, although the improvement achieved is usually partial and transient and their long-term use is associated with the appearance of aberrant impulsive behaviours or other psychiatric complications such as hallucinations.

5.1.1. Non-motor complications: impulse control disorders and other impulsive-compulsive behaviours

These abnormal behaviours can be defined as the failure to resist an urge to perform acts that can be harmful to either oneself or others (Grant et al., 2010). They include classic impulse control disorders (ICD) (pathological gambling, hypersexuality, binge-eating and compulsive buying) as well as other impulsive-compulsive behaviours (ICB) such as punding (i.e. abnormal repetitive non-goal oriented behaviours), hobbyism (excessive focus to perform hobbies such as internet use, music playing, etc), walkabout (excessive aimless wandering) hoarding (Voon and Fox, 2007; Weintraub et al., 2010; Weintraub et al., 2015), and excessive dopaminergic drug intake (dopamine dysregulation syndrome (DDS) (Cilia et al., 2014; O'Sullivan et al., 2009). Overall, it is estimated that the prevalence in PD is about 3-4% for DDS, 1.4-14% for punding and about 14% for classic ICD (Weintraub et al., 2010; Weintraub et al., 2015) although more recently, with the development of specific scales, it has raised up to 30% (de Guzman et al., 2015; Papay et al., 2011; Sharma et al., 2015). Although it is well known that the use of dopaminergic agonists is a risk factor directly associated with classic ICD emergence with an OR of 2.72, these behaviours can also be triggered by L-Dopa, particularly in DDS (Evans et al., 2009; Molina et al., 2000; Weintraub et al., 2010).

Regarding other risk factors for ICD, these include young age at PD onset, sex (male), a pre-PD history of previous substance use disorder or ICD, family history of substance abuse or gambling, and either impulsive or novelty-seeking personality (Weintraub and Claassen, 2017; Zhang et al., 2014).

Currently, it is not fully understood if ICB are similar to classic ICD in terms of their neural substrates, but it is well accepted that both are similar to substance use disorders with respect to risk factors, clinical features, neurobiological substrates, diagnostic criteria, genetic variance and treatment approaches (Potenza, 2006; Weintraub et al., 2015). In line with this fact, both ICD and ICB in PD are nowadays considered together in a broader conceptual network as "behavioural addictions" or "disinhibitory psychopathologies" (Dagher and Robbins, 2009; Holden, 2001; Okai et al., 2011; Potenza, 2006; Voon et al., 2011a).

Currently, there is no pharmacological treatment able to reduce or suppress these abnormal behaviours. The only approach is the reduction of the dose of dopaminergic drugs

(mainly dopaminergic agonists), at the expense of worsening of the parkinsonism and causing a dopaminergic agonist withdrawal syndrome (DAWS) in 30% of cases. This DAWS is characterized by the expression of symptoms that mimic those of withdrawal syndrome in drug abusers including anxiety, panic, agoraphobia, dysphoria, fatigue, diaphoresis and orthostatic hypotension (Cunnington et al., 2012; Pondal et al., 2013; Rabinak and Nirenberg, 2010). Moreover, some patients can still have ICD even after discontinuation of dopaminergic agonists (Mamikonyan et al., 2008).

Therefore, ICD and ICB are severe complications related mainly to dopaminergic agonists that often lead to disastrous consequences for the subjects or their social environment such as financial ruin, loss of employment, divorce and increased health risks (including suicide) (Bharmal et al., 2010; Voon et al., 2011a).

6. Pathophysiology of Parkinson's disease

6.1. Anatomo-functional organization of the basal ganglia

The basal ganglia are an interconnected group of grey matter nuclei located in the deep encephalon, from diencephalon to the mesencephalic tegmentum (Figure 3), compromising the striatum (caudate, putamen and nucleus accumbens (NAc) subdivided in core (NAcC) and shell (NAcS)), globus pallidus (GP) with its external (GPe) and internal/medial (GPi) segments, subthalamic nucleus (STN), substantia nigra (SN) with its *pars compacta* (SNc) and *pars reticulata* (SNr) portions and the ventral tegmental area (VTA).

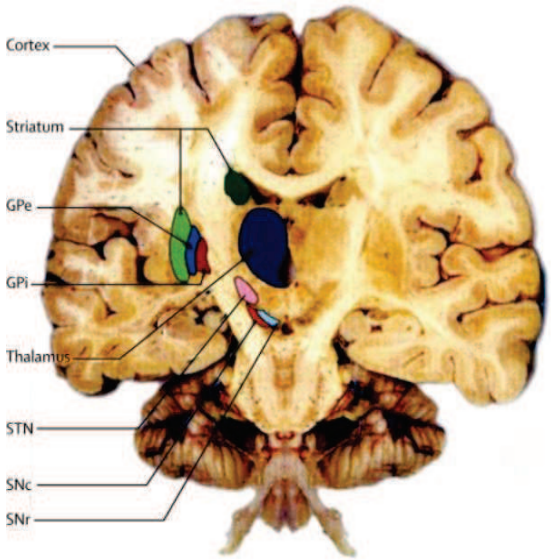

Figure 3. Schematic representation of the localization of the nuclei of basal ganglia in the human brain. Abbreviations: GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus; SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata* (from Obeso et al., 2014).

The anatomo-functional organization of the basal ganglia, as well as the pathophysiology of several diseases related to these nuclei, started to be understood thanks to several anatomical and functional studies performed in the 1980's (Alexander et al., 1986; Albin et al., 1989). These works described the existence of five parallel circuits that have a similar structure as they originate in a cortical area that projects to the striatum, which in turn, connects to the output nuclei (GPi and SNr) that project to the thalamus. Finally, the thalamus encloses the circuit projecting back to the cortical areas (Figure 4).

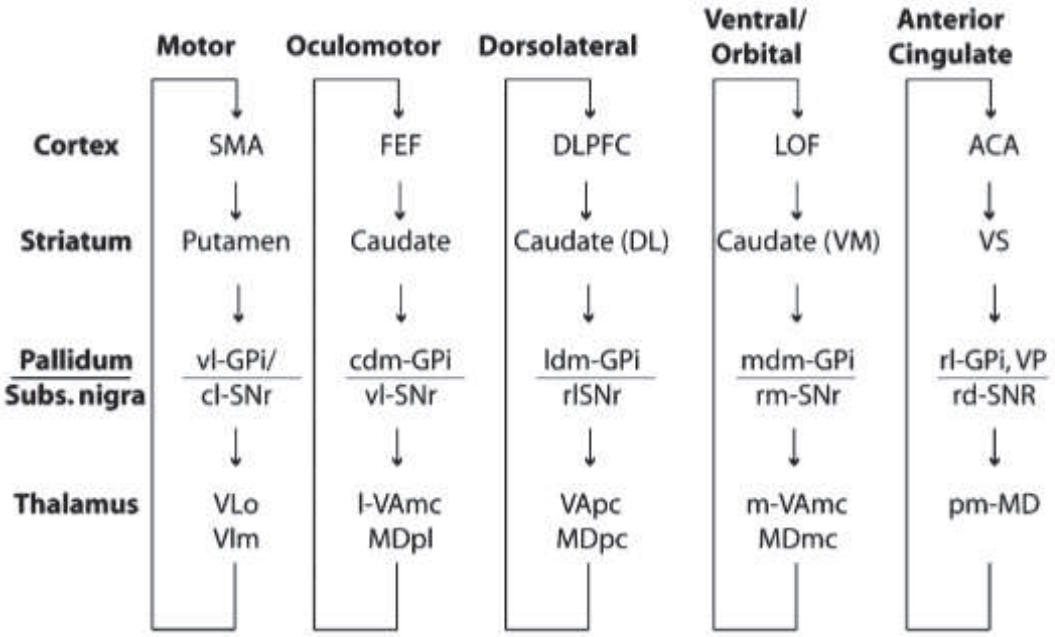

Figure 4. Representation of parallel circuits of basal ganglia proposed by Alexander and colleagues (Alexander et al., 1986). The scheme shows the 5 parallel circuits that originate from different cortical areas, connecting basal ganglia nuclei and thalamus, and finally end in the same cortical area (from Grahn et al., 2009). Abbreviations: ACA, anterior cingulate area; DLPFC, dorsolateral prefrontal cortex; FEF, frontal eye fields; GPi, internal globus pallidus; LOF: lateral orbitofrontal cortex; SMA, supplementary motor area; SNr, substantia nigra *pars reticulata;* Vlm, ventral lateral nucleus *pars medialis*; VLo, ventral lateral nucleus *pars oralis*; VAmc, ventral anterior nucleus *pars magnocellularis*; VApc, ventral anterior nucleus *pars parvollularis*; MD; mediodorsal nucleus; MDpc, mediodorsal nucleus *pars parvollularis*; MDpl, mediodorsal nucleus *pars paralamellaris*; MDmc, mediodorsal nucleus *pars magnocellularis*; VP, ventral pallidum; VS, ventral striatum; cdm-, caudal dorsomedial; cl-, caudolateral; DL, dorsolateral; l-, lateral; ldm-, lateral dorsomedial; m-, medial; mdm-, medial dorsomedial; pm-, posteromedial; rd-, rostrodorsal; rl-, rostrolateral; rm-, rostromedial; vl-, ventrolateral; VM, ventromedial.

Considering their cortical origin, they include motor, oculomotor, DL prefrontal, lateral orbitofrontal and anterior cingulate circuits. Besides, according to their functions, these anatomic loops can be clustered into motor (motor and oculomotor circuits), associative (dorsolateral prefrontal and orbitofrontal circuits) and limbic (anterior cingulate circuit) circuits (Figure 5). The motor circuit is implied in the refinement of motor functions, the associative circuit is involved in executive function, decision-making and in adding a subjective value to goal-directed behaviours, and the limbic circuit is involved in the regulation of emotional aspects of behaviours. Importantly, different areas within each nucleus of the basal ganglia are differentially implicated in the three functional circuits (Figure 5). Thus, DL areas are implicated in motor functions, medial zones are related to associative processes and the ventral ones to limbic functions (Rodriguez-Oroz et al., 2009).

6.1.2. Dopaminergic pathways: regulation of the basal ganglia

The functions of the basal ganglia are regulated by dopamine through three main dopaminergic pathways: nigrostriatal, mesolimbic and mesocortical (Arias-Carrión et al., 2010; Björklund and Dunnett, 2007) (Figure 6).

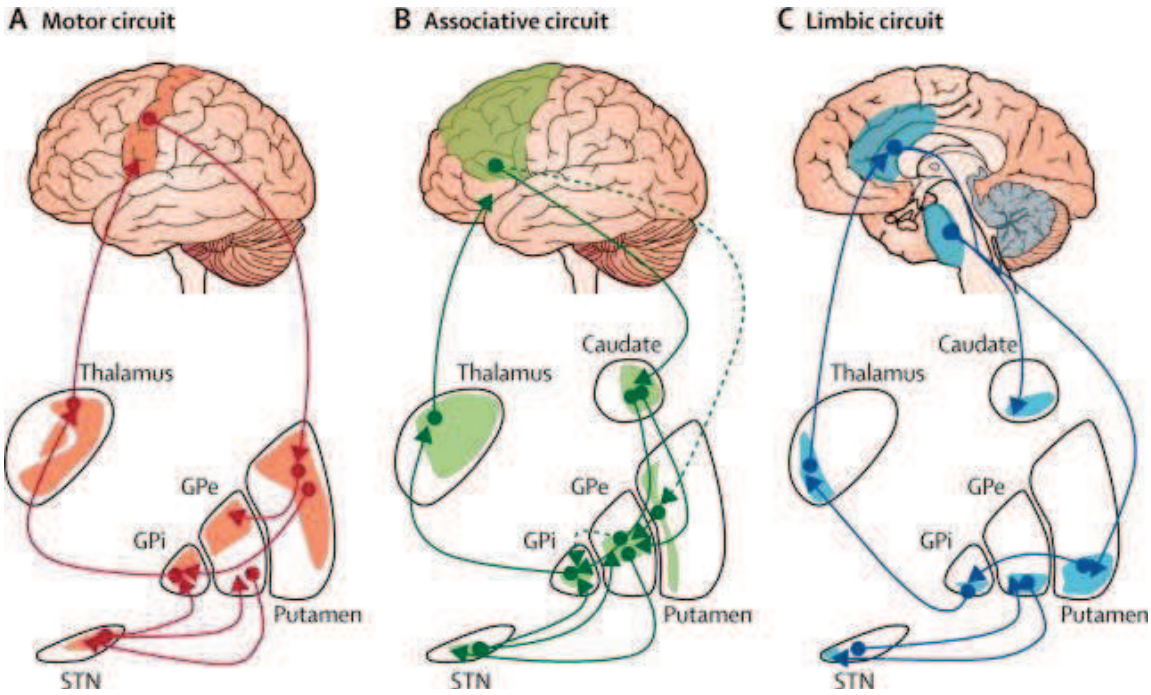

Figure 5: Anatomic-functional distribution of different areas of the basal ganglia nuclei involved in motor (red), associative (green) and limbic (blue) circuits and their main connections (from Rodriguez-Oroz et al., 2009). Abbreviations: GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus.

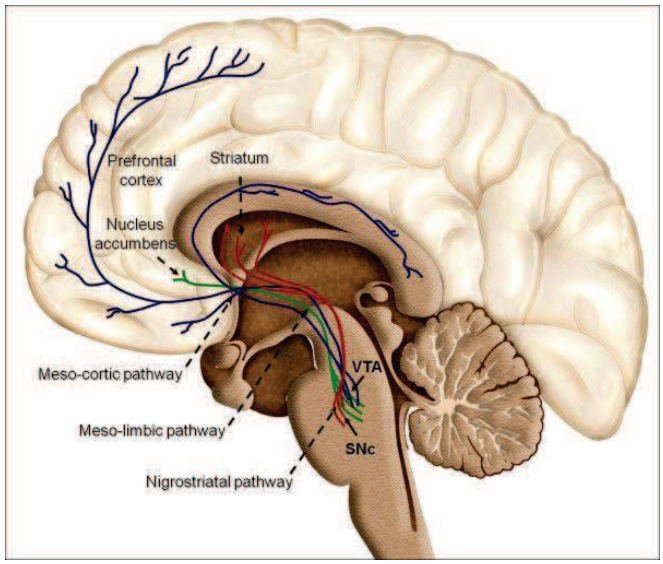

Figure 6: Schematic representation of the nigrostriatal, mesolimbic and mesocortical dopaminergic pathways in the human brain (from Arias-Carrión et al., 2010).

The nigrostriatal pathway originates in the SNc and projects to the caudate and putamen nuclei. It is engaged in voluntary and automatic motor control (mainly putamen), associative learning (anterior putamen and caudate) and in reward-related processes (ventral striatum). In the mesolimbic pathway, dopaminergic cells are present in the VTA, projecting to the ventral striatum and regulating reward-related processes such as incentive salience, pleasure response to certain stimuli and positive reinforcement. In the mesocortical pathway, dopaminergic cells are in the VTA and project to prefrontal, cingulate and perirhinal cortices, being responsible for the regulation of executive functions such as working memory and attention control.

6.1.2. Basal ganglia nuclei

6.1.2.1. Striatum (caudate, putamen and nucleus accumbens)

The striatum is the main nucleus of the basal ganglia and is crucial for the selection and initiation of movements and actions, as well as for the acquisition of habits and abilities (Graybiel et al., 1994; Nicola, 2007). In humans, the striatum is comprised by two nuclei conforming the dorsal part, the caudate and putamen that converge in the most rostroventral area, and the NAc in the ventral part (ventral striatum), which can be subdivided into lateral-rostral (putative core) and medial-caudal (putative shell) (Baliki et al., 2013). In rodents, the striatum corresponds to a unique entity, where the dorsal area is occupied by the caudate and putamen and the ventral area by the NAc, where the core (NAcC) and shell (NAcS) can also be identified (Le Moine and Bloch, 1996; Zahm and Heimer, 1988).

The medium spiny neurons (MSN) are the principal neuron type, representing about 90-95% of total neurons in the striatum, and their action is inhibitory as they use γ-amminobutiric acid (GABA) as neurontransmitter (Alexander and Crutcher, 1990; Kemp and Powell, 1971; Kendall et al., 2000). The second neuronal population are interneurons, among which cholinergic interneurons are the most abundant (Kawaguchi et al., 1995).

Regarding striatal afferences, the most important structures that project to the dorsal striatum are the cortex, thalamus, amygdala, SNc, VTA, and dorsal raphe nuclei (Lanciego et al., 2012). Importantly, among dopaminergic nigrostriatal fibers, the ventrolateral (VL) region of the SNc projects mainly to rostral and dorsal areas of the putamen (motor striatum), while caudomedial (cm) SNc neurons projects to the ventromedial (VM) part of putamen (associative and limbic striatum) (Carpenter and Peter, 1972; Cenci et al., 2015) (Figure 7).

In relation to the NAc, afferences arise from the VTA and centromedial SNc but also from the prelimbic and agranular insular cortices and distinct regions in the hippocampus and basolateral amygdaloid nucleus (Berendse et al., 1992; Groenewegen, 1988; Groenewegen, 1999; Zahm and Brog, 1992).

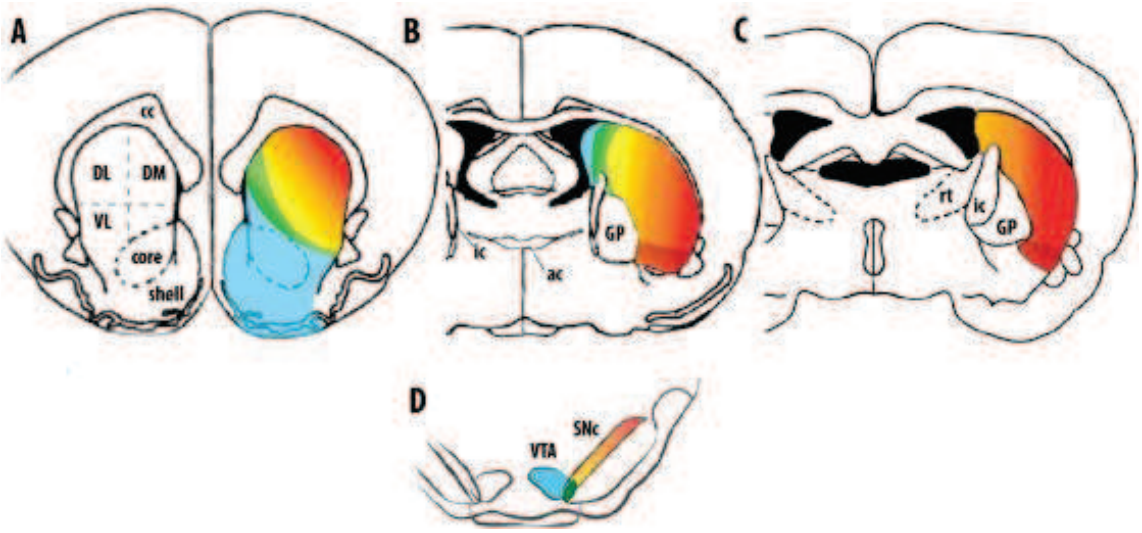

Figure 7. Dopaminergic projections from substantia nigra *pars compacta* and ventral tegmental area to different striatal subregions (from Cenci et al., 2015). The draws illustrate three rostro caudal levels of the rat striatum (A, B, C) and one level at midbrain (D). A pseudo-colour scale is used to depict the distribution of dopaminergic projections originating from different midbrain cell groups, where blue–green–yellow–orange–red indicate progressively more lateral locations. Abbreviations: ac, anterior commissure; core, nucleus accumbens core; cc, corpus callosum; DL, dorsolateral caudate putamen; DM, dorsomedialcaudate putamen; GP, globus pallidus; ic, internal capsule; rt, reticular nucleus of the thalamus; shell, nucleus accumbens shell; SNc, substantia nigra *pars compacta*, VL, ventrolateral caudate putamen; VTA, ventral tegmental area.

With regard to efferences, two subpopulations of MSNs of the dorsal striatum may be defined according to their axonal projections, dopaminergic receptors hold in their membranes and the expression of neuropeptides (Gerfen and Wilson, 1996). Thus, one subgroup expresses D1 receptors (D_1R), containing substance P and dynorphin and projecting to GPi and SNr (named direct pathway). In contrast, the second subgroup expresses D_2R, containing enkephalin and projecting to GPe (named indirect pathway).

Similarly, two segregated pathways emerge from NAc (Heimer et al., 1991; Nauta et al., 1978; Usuda et al., 1998; Zahm and Heimer, 1993). The NAcC preferentially innervates the DL ventral pallidum (VP), GPi and SNr, whereas NAcS mainly projects to medial VP, lateral hypothalamus, SNc, peribrachial area, periaqueductal gray matter and PPN.

6.1.2.2. External and internal globus pallidus

The GP contains GABAergic neurons and is divided into GPe and GPi portions. The GPi is also named entopeduncular nucleus (EP) in non-primates (Beckstead and Cruz, 1986; Nagy et al., 1978).

In relation to their connections, the neurons in the GPe receive an excitatory input from the STN along with an inhibitory input from the striatum, while they project to the STN, striatum, GPi and SNr (Kita, 2007). The GPi receives inhibitory inputs from the striatum and GPe and excitatory input from the STN. This nucleus is considered, together with the SNr, the major output of the basal ganglia (Nambu, 2007), mainly projecting to the lateral region of the thalamus and PPN.

6.1.2.3. Substantia nigra compacta and reticulata

The SN is divided in two regions, the dorsal (SNc) and the ventral (SNr). The SNr is formed mainly by GABAergic neurons and shows both functional and structural similarities with GPi. It receives GABAergic inputs from the GP and striatum and glutamatergic inputs from STN (Celada et al., 1999; Hatzipetros and Yamamoto, 2006). The efferences inhibit neurons in thalamus and superior colliculus, as well as in the SNc (Celada et al., 1999; Chevalier et al., 1981). Regarding the SNc, it contains dopaminergic neurons enriched in neuromelanin, a natural pigment that gives a black colour to this nucleus. It receives inhibitory striatal inputs and projects back to the striatum enclosing the nigrostriatal pathway modulating the activity of MSN (Beckstead et al., 1979).

6.1.2.4. Ventral tegmental area (VTA)

The VTA contains dopaminergic and GABAergic neurons. The dopaminergic neurons are also naturally pigmented by neuromelanin (Margolis et al., 2006), while the GABAergic neurons act as interneurons regulating dopaminergic cells (Creed et al., 2014). Recently, some glutamatergic neurons have been described within the VTA but their function has to be elucidated (Yamaguchi et al., 2011). The dopaminergic neurons drive the main output of VTA, projecting to amygdala, cingulate gyrus, hippocampus, NAc, olfactory bulb and prefrontal cortex (PFC), areas that in turn project back to the VTA (Oades and Halliday, 1987).

6.1.2.5. Subthalamic nucleus

Neurons in the STN use glutamate as a neurotransmitter, making excitatory projections to the SNr, GPi, GPe, PPN and thalamus (Hamani, 2004). The main afferences arise from the cortex, SNc and GPe (Carpenter et al., 1981; Hamani, 2004; Rico et al., 2010).

6.1.3. Motor circuit of the basal ganglia

The motor circuit was formulated mainly to understand the physiopathology of movement and it is based on the direct and indirect striatum-pallidal projections (Albin et al., 1989; DeLong, 1990). It connects the primary motor (M1) and sensory (S1) cortices with the posterolateral striatal MSN of the striatum, the posterolateral regions of basal ganglia nuclei and goes back to the cortical areas via the lateral nucleus of the thalamus.

In detail, the activation of striatal GABAergic MSNs of the direct pathway inhibits the GPi/SNr activity, inducing a pause of neuronal firing at this level, a lack of thalamic inhibition and an activation of the motor cortex that is associated with the occurrence of a motor action (Figure 8). On the other hand, the activation of MSNs of the indirect pathway first inhibits GPe neurons, followed by the disinhibition of the STN, which in turns excites GPi/SNr neurons leading to a thalamic inhibition and lack of activation of the cortices that is associated with stopping of movements (Figure 8).

The release of dopamine at the striatum from nigrostriatal neurons plays a differential modulatory effect on the projecting MSNs at the origin of both direct and indirect pathways (Gerfen, 2000) (Figure 9). Thus, the dopaminergic input exerts a facilitatory effect on MSN containing D_1R receptors (direct pathway), while it promotes an inhibitory effect on MSN containing D_2R receptors (indirect pathway), with a net effect of increasing facilitatory inputs to the motor cortex to allow the execution of the desired movement (Obeso et al., 2002) (Figure 8).

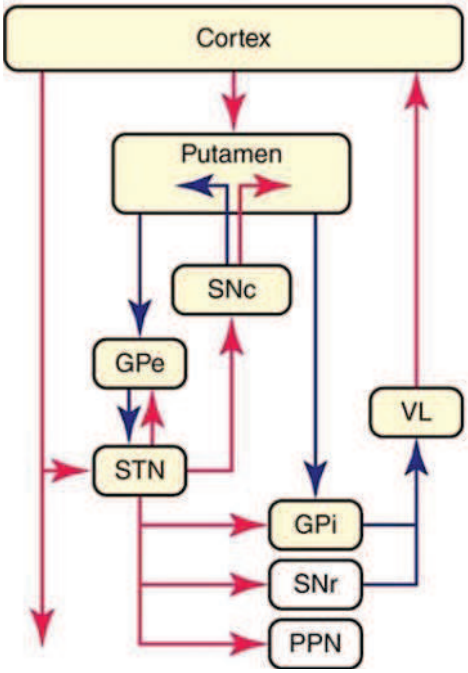

Figure 8. Model of the organization of the basal ganglia in the normal or physiological state. Inhibitory connections are shown as *blue arrows* and excitatory connections as *red arrows* (from Obeso et al., 2002). Abbreviations: GPe, external globus pallidus; GPi, internal globus pallidus; PPN, pedunculopontine nucleus; SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*; STN, subthalamic nucleus; VL, ventrolateral thalamus.

6.1.4. Deregulation of motor circuit in Parkinson's disease

The loss of striatal dopaminergic innervation, by the degeneration of the nigrostriatal pathway, leads to an imbalance of the direct and indirect pathways of the motor circuit (Gerfen et al., 1990; Gerfen, 2000) (Figure 9). Thus, it causes a decrease of the inhibition of the indirect pathway, reducing the activity of GPe and increasing the activity of STN, which in turn hyper excites the GPi and SNr. In addition, the direct pathway is hypoactivated increasing the activity of both GPi and SNr. The hyperactivity of these nuclei leads to an increased inhibition of the thalamus, reducing the stimulation of the motor cortex.

Interestingly, the treatment with dopaminergic drugs, as well as the surgical lesion or deep brain stimulation of the STN, restore in some way most of these abnormal changes, improving the motor signs of the disease (Bergman et al., 1990; Limousin et al., 1995).

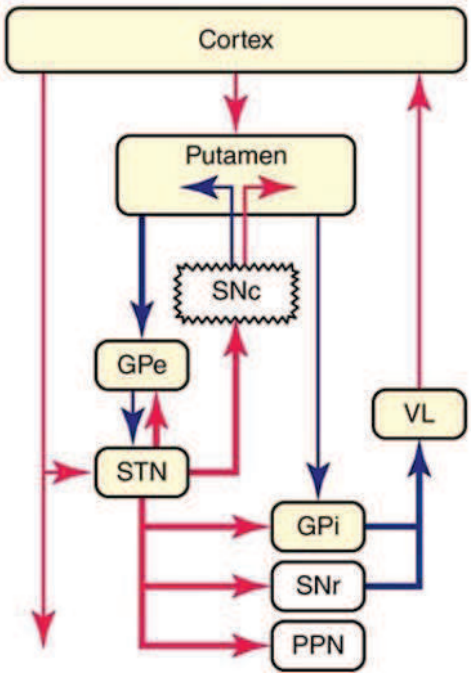

Figure 9. Model of the organization of the basal ganglia in Parkinson's disease (PD) state. Inhibitory connections are shown as *blue arrows* and excitatory connections as *red arrows*. The loss of dopaminergic input in the striatum favours indirect pathway over the direct pathway, which results in an increased neuronal firing in the STN and GPi/SNr, a subsequent thalamic inhibition, and thus in a lack of enough motor cortex activation, impairing the process of movement initiation (from Obeso et al., 2002). Abbreviations: GPe, external globus pallidus; GPi, internal globus pallidus; PPN, pedunculopontine nucleus; SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*; STN, subthalamic nucleus; VL, ventrolateral thalamus.

Beyond the motor circuit, the anatomo-functional organization of the basal ganglia is particularly important to understand the pathophysiological mechanisms underlying the expression of clinical signs of PD. The initial loss of neurons in the VL tier of SNc, that leads to a striatal dopaminergic denervation predominantly in the DL putamen (motor area), spreads progressively so the dopaminergic denervation reaches more medial and ventral areas (associative and limbic areas) (Figure 5). For this reason, motor deficits are the first signs of the disease, but executive dysfunctions or some neuropsychiatric disorders found in PD patients at different disease stages can be explained, at least partially, by the progressive dopaminergic deficit gradually altering both associative and limbic loops (Rodriguez-Oroz et al., 2009). Moreover, both the functional organization of basal ganglia nuclei and the gradual loss of the dopaminergic projections are also crucial when trying to understand the mechanisms underlying the side effects caused by dopaminergic drugs used for the treatment of motor signs in PD.

6.1.5. Anatomo-functional organization of the limbic system

The limbic system has a primordial role in the regulation of emotion and motivation for action and in the process of learning and memory. Throughout history, there have been several attempts to understand the nuclei and their connections involved in the control of emotional behaviour. Paul Broca (1850s), James Papez (1930s), Paul MacLean (1950s) are some of the researchers that have contributed to determine the specific brain networks underlying the limbic circuit (Roxo et al., 2011; Rolls, 2015). Their theories and results suggest the existence of two different but interconnected limbic networks in which the amygdala, medial prefrontal cortex, orbitofrontal cortex (OFC), cingulated gyrus, hippocampus, fornix, thalamus, hypothalamus, mammillary bodies and nucleus accumbens play a key role (Catani et al., 2013; Rolls, 2015; Roxo et al., 2011; Sesack and Grace, 2010) (Figure 10). According to these theories, the cognitive/memory circuit is based on the hippocampus and the emotional one is based on the amygdala.

Thus, the circuit originally described by Papez in 1937 is a network that consists of the parahippocampal gyrus of the cortex, subiculum (vSub; ventral area of hippocampus), formix, mammillary body (hypothalamus), anterior nuclei of the thalamus and cingulate cortex (Rolls, 2015) (Figure 10). The vSuv receives projections from the parahippocampal gyrus and connects via fornix with the mammillary bodies and the anterior thalamic nuclei (both interconnected by the mammillothalamic tract). The thalamus projects to the cingulated cortex, which also receives inputs directly from the hippocampus, and the cingulated cortex projects back to both anterior thalamic nuclei and parahippocampal gyrus (Figure 11).

Later on, it was proposed that the amygdala, orbital and medial prefrontal cortex, insula and anterior temporal lobe also play a crucial role in the limbic system, regulating emotional experience and learning (Rolls, 2015) (Figure 10). The amygdala is a key structure in this emotional circuit. It receives sensorial inputs mainly from different temporal cortical areas, anterior cingulate cortex (ACC) and OFC directly or via insula, thalamus or hypothalamus. It projects back to OFC and ACC, thalamus, hypothalamus, hippocampus and NAc (Figure 11).

Therefore, through different inter-connected nuclei, the limbic system subserves emotions, drives, and memory and contributes to integrate our voluntary and involuntary actions. It is involved in emotional learning, expression and experience: it works computing the reward value of primary (unlearned) stimuli and reinforces in decision-making process (selection or choice), habit learning and the evaluation of context-dependent processes by learning associations between previously neutral stimuli (i.e., objects or individuals' faces) with primary reinforces (Rolls, 2015; Sesack and Grace, 2010).

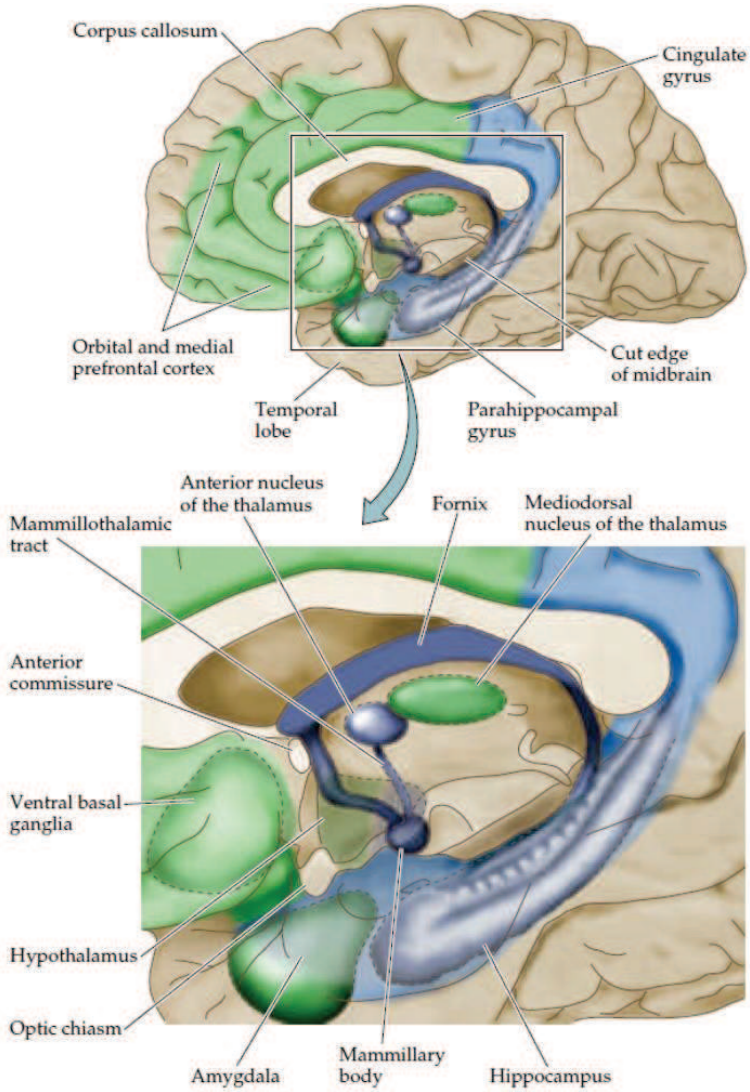

Figure 10. Simplified schematic diagram showing the main nucleus included in the networks for experience and expression of emotions (green) and processing of emotions (blue) of the limbic system (from Purves and Williams, 2001).

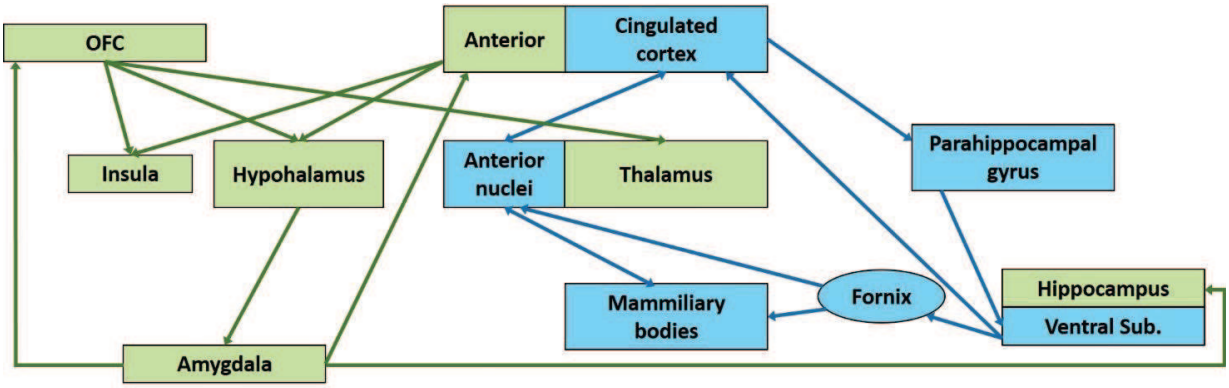

Figure 11. Schematic diagram of the main connections of the brain nucleus that included in the networks of the limbic system for experience and expression of emotions (green) and processing of emotions (blue).

Importantly, neural substrates of the limbic system connect with the basal ganglia-cortex-thalamus limbic circuit through the NAc (Rolls, 2015; Sesack and Grace, 2010) (Figure 12). The NAc receives inputs from the hippocampus, PFC, basolateral nuclei of the amygdala (BLA), VP and MD nucleus of the thalamus, and projects back to the hypothalamus and VP. Thus, itis a critical region that integrates the information about the environmental context and higher cognitive processes (Haber and Knutson, 2010; Rolls, 2015; Sesack and Grace, 2010). As it happens in the motor circuit, the activity of the NAc, and therefore of the limbic system, is critically regulated by dopamine as will be detailed in the next section.

6.1.6. Dopamine regulation of the limbic circuit

Importantly, the activity of the limbic system and of the limbic cortex-basal ganglia-thalamus loops are regulated by dopamine through the mesolimbic dopaminergic projections from the VTA to the NAc (Sesack and Grace, 2010). This pathway is known as the reward or reinforcement system as novel stimuli, natural reinforces (food, sex, …) and non-natural reinforces, such as substances of abuse, lead to its activation, generating a phasic release of dopamine in the NAc (Everitt and Robbins, 2005).

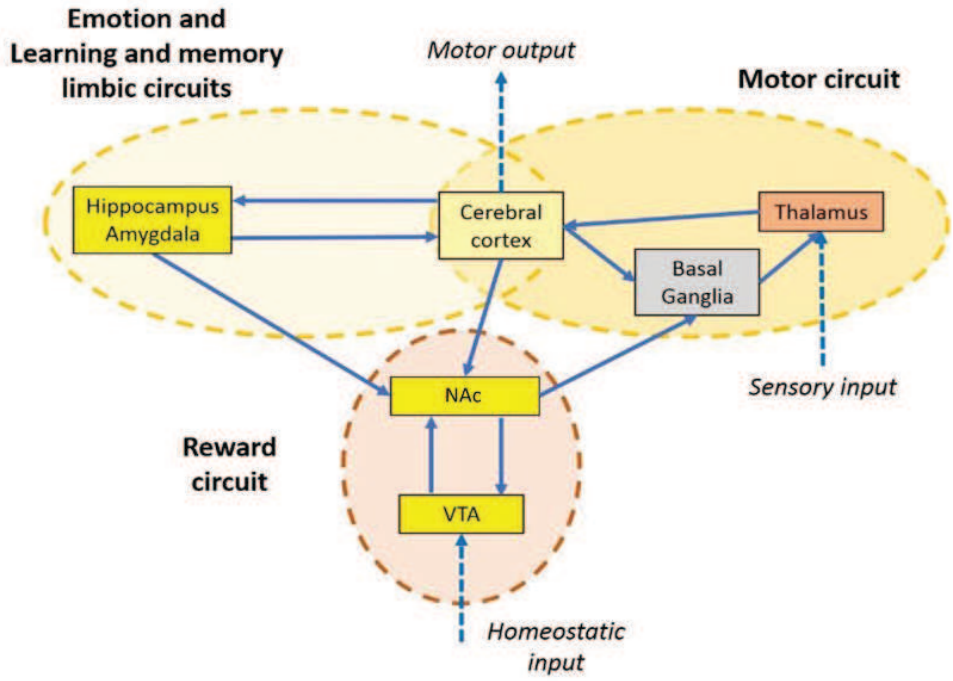

Figure 12. The limbic system participates in two of the three basic functional loops of brain function (learning and memory and the reinforcement system). Simplified scheme illustrating the interaction of the limbic circuits with the basal ganglia circuits through the ventral tengemental area (VTA) and nucleus accumbens (NAc) (modificed from Cardinali, 2018).

As in dorsal striatum, dopamine in the NAc markedly inhibits neurons when it acts on D_2R (Lin et al., 1996; O'Donnell and Grace, 1996; White and Wang, 1986), while increasing cell excitability by stimulating D_1R (Cepeda et al., 1998; Chergui and Lacey, 1999; West and Grace, 2002). Moreover, the dopamine released in the NAc also modulates the synaptic convergence of ventral hippocampus (vSub), amygdala (basolateral amygdala, BLA) and PFC on the same set of NAc neurons, which contributes to shape the goal-directed behaviour (French and Totterdell, 2002; French and Totterdell, 2003; O'Donnell and Grace, 1995) (Figure 13).

Thus, the input from vSub, which is thought to be involved in selecting a reward-related behaviour, is directly favoured by the release of dopamine as it promotes a D_1R-mediated potentiation of the ventral Sub-NAc transmission (Sesack and Grace, 2010). Interestingly, the input from the medial PFC (mPFC) that regulates switching strategies is potently and selectively attenuated by D_2R activation (O'Donnell and Grace, 1994; West et al., 2002; Goto and Grace, 2005). Finally, the BLA inputs are potentiated by D_1R stimulation (Charara and Grace, 2003; O'Donnell and Grace, 1995).

Therefore, a rewarded behaviour would lead a dopamine release from the VTA to the NAc, which potentiates the D_1R-mediated Sub-NAc drives to reinforce ongoing behaviour and attenuates a behavioural switching by the D_2R-mediated mitigation of mPFC-NAc drive (Goto and Grace, 2008) (Figure 14). In contrast, when an ongoing behaviour becomes ineffective obtaining

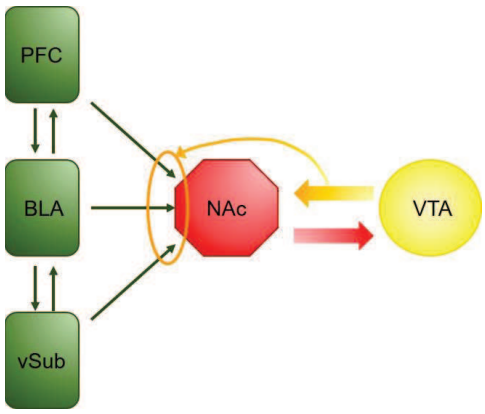

Figure 13. Simplified scheme of the dopaminergic regulation of NAc and its input from the limbic structures prefrontal cortex (PFC), amygdala and hippocampus (ventral subiculum). Inhibitory connections and structures are shown in red and excitatory connections and structures are shown in green. Yellow indicates the modulatory role of dopamine (modified from Sesack and Grace, 2010). Abbreviations: BLA, basolateral amygdala; PFC, prefrontal cortex; NAc, nucleus accumbens; vSub, ventral subiculum of the hippocampus; VTA, ventral tegmental area.

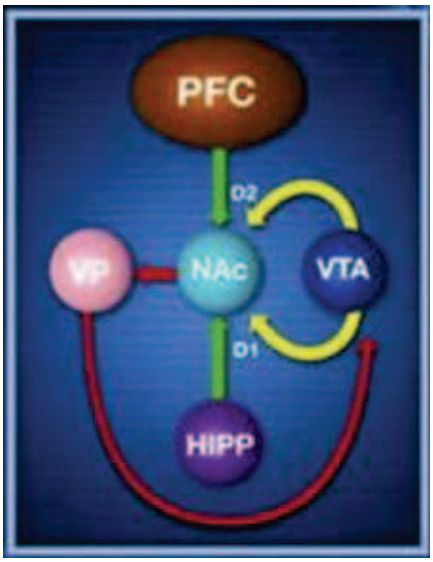

Figure 14. Diagram of the inputs and outputs of the nucleus accumbens, and its modulation by dopamine, involved in reward processing in physiological condition (modified from Napier et al., 2015). Abbreviations: D1, D1 receptor; D2, D2 receptor; HIPP, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; VP, ventral pallidum; VTA, ventral tegmental area.

a reward, a reduction in the dopaminergic input within the NAc occurs (Hollerman and Schultz, 1998; Schultz and Dickinson, 2000), attenuating the Sub-mediated drive of the ongoing behaviour and disinhibiting the mPFC-mediated drive, favouring the behavioural flexibility and allowing a switch from the established responding strategy to the testing of other different responding approaches (Goto and Grace, 2008). When a new strategy becomes effective in obtaining a reward, the limbic dopaminergic system will strengthen this new behaviour mitigating again the mPFC input and promoting vSub input in the NAc in order to continue the activity for the maintenance of the activity (Figure 14) (Goto and Grace, 2008).

Finally, apart from its pivotal role regulating the limbic system, the NAc is an interface with motor circuitry regulating appropriate goal-directed behaviour and habit formation through its connections with the dorsal striatum (Groenewegen et al., 1996; Mogenson et al., 1980; Nicola et al., 2000; Wise, 2004; Zahm, 2000). Thus, although, as explained, the initial reinforcement of a rewarding stimuli activates NAc, the activation of more dorsal striatal structures is observed after a repetitive exposure of such stimuli thanks to the interconnected loops of midbrain-striatum-midbrain projections detailed in figure 15 (Robbins and Everitt, 2002; Yin et al., 2008). This process of an habit formation is also under frontal cortical control, which exerts a cognitive influence over adaptive decision-making (Berke, 2003).

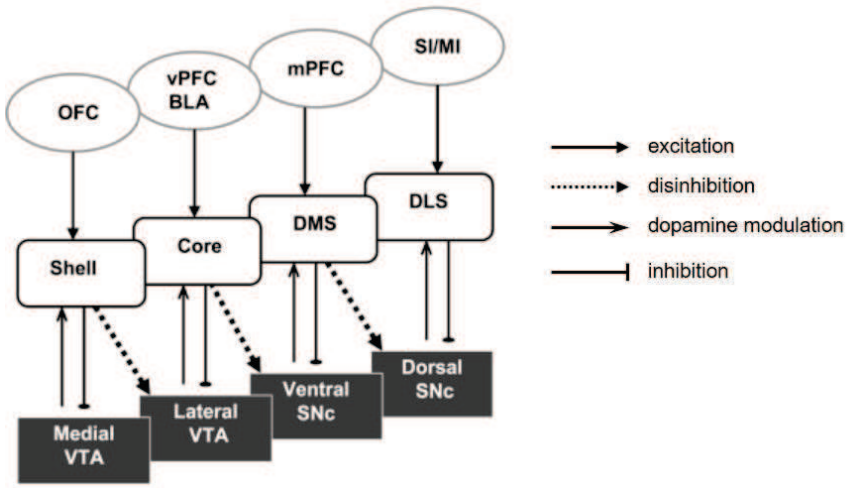

Figure 15. Cortico-basal ganglia networks placing emphasis on the spiralling midbrain-striatum-midbrain projections (dotted lines), which allows information to be propagated forward in a hierarchical manner within striatum. Major corticostriatal and dopaminergic projections are also represented, but pallidal, thalamic and other structures are omitted (from Yin et al., 2008). Abbreviations: BLA, basolateral nuclei of the amygdala; core, nucleus accumbens core; DLS, dorsolateral striatum; DMS, dorsomedial striatum; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex; shell, nucleus accumbens shell; SI/MI, primary sensory and motor cortices; SNc, substantia nigra *pars compacta*; vPFC, ventral prefrontal cortex; VTA, ventral tegmental area.

6.1.7. Deregulation of the limbic system in Parkinson's disease

Acting towards a desired goal is known as motivation (Kuhl and Atkinson, 1986) and the lack of it is broadly known as apathy. Apathy is one of the most common psychiatric symptoms of PD patients (60% of subjects depending on the stage of the disease) and its manifestation can be related to behaviour reward deficiency, depression, decrease in cognitive interests (executive dysfunction) or absence of spontaneous activation of mental processes (also known as auto-activation deficit) (Pagonabarraga et al., 2015).

Although the pathophysiological mechanisms underlying apathy in PD are still poorly understood, it is linked to the dopaminergic degeneration, as several facts suggest. Even though the loss of dopaminergic cells in PD mainly affects the nigrostriatal system, the mesolimbic pathway is also affected. Besides, studies in animal models of parkinsonism and neuroimage studies in PD patients suggest that apathy is likely due to the dysfunction of the dopaminergic mesocorticolimbic circuits (mesolimbic and mesocortical). As these circuits are implicated in the regulation of executive functions, motivation and reward-related learning, their degeneration would cause a lack of emotional resonance preventing them from attaching motivational values to stimuli, broadly known as a reward deficiency syndrome in vulnerable PD patients (Chagraoui et al., 2018; Groenewegen et al., 1997; Mega et al., 1997; Pagonabarraga et al., 2015).

7. Pathophysiology of impulse control disorders in PD

As it was stated before, the emergence of ICD is linked to the chronic use of dopaminergic drugs, mainly dopaminergic agonist (Grall-Bronnec et al., 2018). The mechanisms underlying these behaviours are still not understood although in the last decade, several *in vivo* studies have revealed functional and structural abnormalities.

Importantly, dopaminergic agonists show higher affinity for D2 family of dopamine receptors (D_2R, D_3R, D_4R) than for D1 family of receptors (D_1R, D_5R). The abnormally persistent activation of the D2-like receptors would disrupt the functional balance between the inputs of the hippocampus and PFC within the NAc, hyperactivating the vSub-NAc drive and over-inhibiting the mPFC-NAc drive, within the relatively well preserved limbic circuit in PD (Dagher and Robbins, 2009; Kish et al., 1988; Napier et al., 2015) (Figure 14). On the other hand, neuroimaging studies in PD patients suffering ICDs points toward a higher dopaminergic denervation and higher dopamine release after levodopa intake or the execution of rewarding tasks in the ventral striatum (see following sections).

Anyhow, the existence of an increased dopaminergic input within the limbic circuit in PD patients under dopaminergic treatment, presumably altering different inputs and outputs throughout the whole system, can be considered. This would promote an excessive perseveration on tasks or responses, even if they were no longer rewarding or even in the context of negative rewards or outcomes, leading to the emergence of ICD and ICBs in vulnerable subjects.

7.1. Positron Emission Tomography and Single Photon Emission Computed Tomography imaging studies

Positron emission tomography (PET) studies using different D_2R/D_3R radioligands ([^{11}C]-Raclopride, [^{11}C]-(1)-propyl-hexahydro-naphthooxazin ([^{11}C]-PHNO), [^{11}C]-FLB-457) showed lower radioligand binding in the ventral striatum in PD patients with ICD, suggesting either a minor D_2R/D_3R density or an enhancement of the dopaminergic tone in this striatal region (Joutsa et al., 2015; Payer et al., 2015; O'Sullivan et al., 2011; Wu et al., 2015) (Figure 16). Another PET study conducted to analyse extra-striatal dopamine levels with the D_2R/D_3R radioligand [^{11}C]-FLB-457 also shown that patients with pathological gambling had reduced dopamine concentrations in the ACC during a control task, but not during the execution of a gambling task under the effect of a dopaminergic agonist (Ray et al., 2012) (Figure 16). More recently, a study has shown that PD patients with ICD have reduced D_2R/D_3R radioligand [^{18}F]fallypride binding potential in the ventral striatum and putamen (Stark et al., 2018)

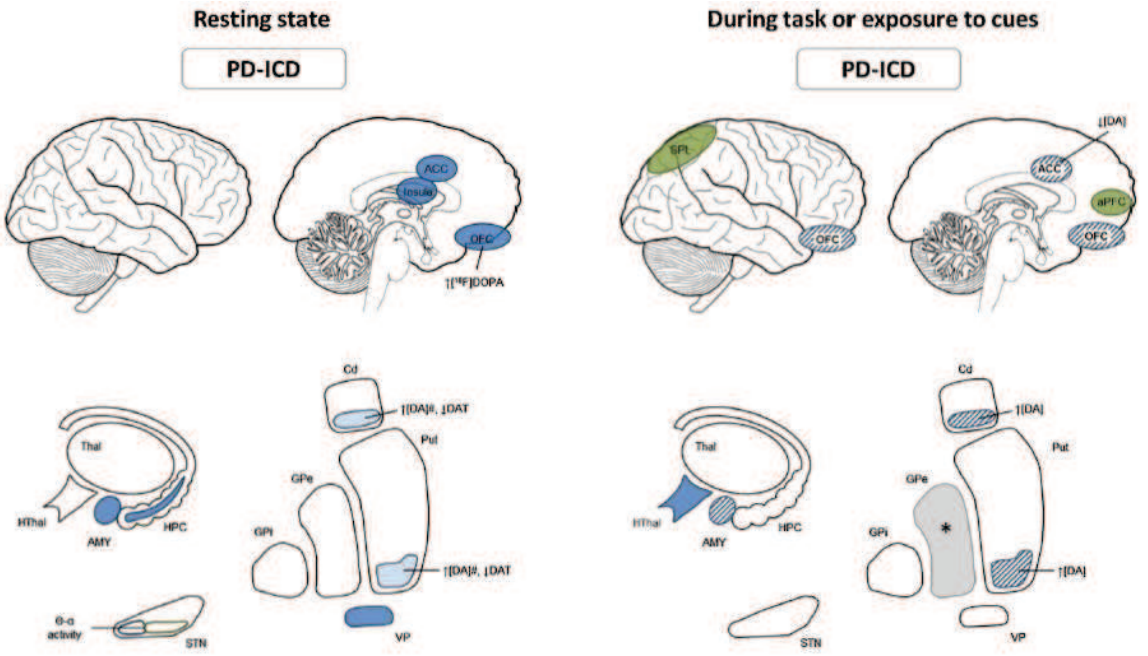

Figure 16. Schematic representation of brain areas involved in PD with ICD according to the results obtained from imaging (fMRI, SPECT and PET) and physiological studies in patients, in resting state or during either the performance of a task or the exposure to cues. Associative areas are represented in green and limbic ones in blue. The intensity of colours represents increased (dark) or reduced (light) activation of the brain areas and controversial findings are represented as striped areas (functional image studies). Encircled areas indicate physiological (local field recordings) features or changes in the dopaminergic system (from Jiménez-Urbieta et al., 2015). (#) Change observed in response to a dopaminergic challenge. (*) Increase of activity in the GPe (no functional division provided). Abbreviations: AMY, amygdala; ACC, anterior cingulated cortex; aPFC, anterior prefrontal cortex; Cd, Caudate nucleus; GPe, external globus pallidus; GPi, internal globus pallidus; HPC, hippocampus; HThal, hypothalamus; OFC, orbital frontal cortex; Put, Putamen; SPL, superior parietal lobe; STN, subthalamic nucleus; Thal, thalamus; VP, ventral pallidum.

On the other hand, PD patients with classic ICD showed greater reduction of the expression of dopamine reuptake transporter (DAT) than patients without ICD in the ventral striatum (Cilia et al., 2010), right striatum (putamen and caudate) (Voon et al., 2014), left both putamen and inferior frontal gyrus (Premi et al., 2016) or NAc (Hammes et al., 2019), meaning a greater dopaminergic denervation. Drug-naive PD patients who developed ICD after the dopaminergic treatment showed also a reduced DAT availability in the right striatum (ventral striatum and anterodorsal and posterior putamen) (Vriend et al., 2014). However, a PET study with [^{18}F]-2-fluoro-5-hydroxy-l-tyrosine ([^{18}F]Fluorodopa), a fluorinated form of L-Dopa, did not show changes in the striatum, although it did identify an increased binding in the medial OFC in

PD patients with different types of ICD after the withdrawal of the dopaminergic treatment (Joutsa et al., 2012a).

Few approaches have also been performed with Single photon emission computed tomography (SPECT) and PET imaging techniques aiming to study metabolic and blood flow changes in PD patients with ICD, with contradictory results (Figure 16). Thus, a [^{15}O]H_2O PET study performed on PD patients with pathological gambling while they played with computerized card selection game, revealed a significant apomorphine-induced reduction of the regional cerebral blood flow (rCBF) in the lateral OFC, amygdala, GPe and rostral cingulate zone (van Eimeren et al., 2010). In contrast, a rCBF-SPECT study in PD patients with pathological gambling under their usual medication showed hyperactivity in the right OFC, hippocampus, amygdala, insula, and VP (Cilia et al., 2008).

The [^{18}F]fluorodeoxy-D-glucose ([^{18}F]FDG) imaging of regional cerebral glucose uptake has been widely and consistently used to assess resting-state patterns of metabolic activity in PD, such as 'PD-related motor pattern' (Eidelberg et al., 1994; Huang et al., 2007;Ma et al., 2007; Ma et al., 2015) and 'PD cognition-related pattern' (Huang et al., 2007; Niethammer et al., 2013; Mattis et al., 2016). When PD subjects were classified according to their level of impulsivity in the Barratt Impulsivity Scale, those with higher impulsivity scores showed higher [^{18}F]-FDG metabolism in OFC and ACC (Tahmasian et al., 2015). More recently and using the same scale a positive association between the impulsivity scores and metabolism in the medial part of the right superior frontal gyrus, right middle frontal gyrus, and ACC in PD patients was found (Schwartz et al., 2018). Our group has also shown that PD-ICD patients had a significantly reduced DaT binding in the VS compared to PD-noICD patients, which accounts for dysfunction in a complex cortico-subcortical network that involves areas of the mesolimbic and mesocortical systems (Navalpotro-Gomez et al., 2019). In addition, other study has shown that PD patients with ICDs have increased metabolism in the right middle and inferior temporal gyri compared to those without ICDs (Verger et al., 2018). Moreover, other authors have reported that PD patients with newly diagnosed ICD show higher glucose metabolism in widespread areas comprising prefrontal cortices, both amygdalae and default mode network hubs when compared to ICD-free subjects. (Marín-Lahoz et al., 2020).

Overall, neuroimaging studies indicate that in patients with ICD the expression of DAT is probably reduced in the ventral striatum (Cilia et al., 2010; Navalpotro-Gomez et al., 2019; Steeves et al., 2009; Vriend et al., 2014), dorsal striatum (Joutsa et al., 2015; Premi et al., 2016; Smith et al., 2016) or in the whole striatum (dorsal and ventral) (Voon et al., 2014) mostly on the right hemisphere. Besides, functional studies point towards a dysfunction of different areas of the limbic circuit, particularly within the right hemisphere (Cilia et al., 2008; van Eimeren et al.,

2010; Schwartz et al., 2018), although hypo and hyperfunction have been reported. On this line, the right hemisphere is allegedly more involved in "response inhibition network" than the left hemisphere (Aron et al., 2004), which could be relevant to the pathophysiology of ICD.

7.2. Magnetic resonance imaging (MRI) studies

Different magnetic resonance imaging (MRI) studies have highlighted the involvement of different areas of limbic circuitry in the physiopathology of ICD in PD.

In structural MRI studies, PD patients with and without ICD have reduced volume in the NAc, amygdala and hippocampus respect to controls (Biundo et al., 2015; Pellicano et al., 2015). Interestingly, PD patients with ICD and punding have also shown increased cortical thinning in several regions implicated in frontal-striatal circuitry such as rostral ACC, left rostral middle frontal region and bilateral caudal middle frontal región and OFC (Biundo et al., 2015; Pellicano et al., 2015; Tessitore et al., 2016; Yoo et al., 2015). Importantly, this cortical thinning has been seen to correlate with the severity of impulsive symptoms (Biundo et al., 2015; Tessitore et al., 2016), suggesting a critical role of the frontal cortical region in the emergence of impulsive behaviours in PD. In contrast, Structural imaging the only longitudinal structural MRI study in PD patients so far has failed to identify morphological features associated with the development of ICB (Ricciardi et al., 2018).

Functional MRI (fMRI) studies have shown that PD patients with different ICD, on their dairy medications, showed a reduced activity of the ventral striatum (Rao et al., 2010; Voon et al., 2011b) as well as in the OFC and ACC (Voon et al., 2011c), both at resting condition and during risk-taking tasks. Patients with PD and hypersexuality, when exposed to sexual visual cues during fMRI image acquisition, showed an increased activation of the ventral striatum, OFC, cingulate cortex, anterior PFC, superior parietal lobule, amygdala and hypothalamus, regardless of their dopaminergic status (with and without the effect of dopaminergic drugs) (Politis et al., 2013). Similarly, a path modelling analysis based on MRI evidenced that there was a significant negative correlation between gambling severity and resting-state activity (rCBF) in the right VLPFC, ACC, posterior cingulate cortex, mPFC, rostral SMA, parahippocampal gyrus and anterior superior temporal gyrus, left striatum and bilateral anterior insula, being all them brain areas involved in the estimation of risks and inhibition of inappropriate reward-seeking behaviours. Interestingly, this study also reported that PD patients with pathological gambling exhibited a disconnection striatum-ACC (Cilia et al., 2011). More recently, the presence of ICD in patients with PD has also been associated with functional disconnection between the left anterior putamen and both the left inferior temporal gyrus and the left anterior cingulate gyrus, as well as a trend toward a functional disconnection between several motor and associative striatal

regions and limbic, associative, and motor cortical regions (Carriere et al., 2015). Patients with PD and without ICDs did not differ from healthy controls in corticostriatal connectivity (Carriere et al., 2015). In a more recent work, elevated ventral striatal connectivity to the anterior cingulate gyrus, orbitofrontal cortex, insula, putamen, globus pallidus, and thalamus was observed in patients with ICB respect to non-ICD patients (Petersen et al., 2018). Similarly, during a reward-based task, PD patients with impulse control disorders have shown a hyperactivation in a right-lateralized network of regions including the subthalamic nucleus, being this activation strongly associated with impulse control disorder severity. In these patients, the right ventral striatum in particular played a critical role in modulating the functional dynamics of right-lateralized inhibitory-control frontal regions when facing penalties (Paz-Alonso et al., 2020). Besides, PD-ICD patients, in contrast to PD-noICD and HC subjects, were engaged across time in a brain configuration pattern characterized by a lack of between-network connections at the expense of strong within-network connections (State III) in temporal, frontoinsular and cingulate cortices, all key nodes of the salience network. Moreover, this increased maintenance of State III in PD-ICD patients was positively correlated with the severity of impulsivity and novelty seeking (Navalpotro-Gomez et al., 2020)

Finally, it has been reported that PD patients with ICBs had left precentral and superior frontal cortical thinning, together with motor and extramotor white-matter tract damage. Moreover, the severity and duration of ICBs modulated the functional connectivity between sensori-motor, visual, and cognitive networks, indicating more severe involvement of frontal, mesolimbic and motor pathways, with increasing psychiatric symptoms, ICB duration, and motor impairment (Imperiale et al., 2018). In summary, MRI studies have showed that regions of the brain involved in response inhibition and reward processing such as the ventral striatum, cortical regions (different zones of the PFC and ACC), and hippocampus show structural, connectivity and functional changes in PD patients with ICDs, highlighting the importance of a correct balance of these areas and their connections for a proper processing of the behavioural outcome (Figure 16).

7.3. Local field potential recording studies

Another approach to study ICB pathophysiology is the recording of local field potentials through implanted electrodes for deep brain stimulation of the STN in PD patients. It was demonstrated that in PD patients with ICD there was an increment in the power of the theta-alpha band activity in the ventral STN that shows a coherence with cortical activity of the pre-motor area/DL PFC (Rodriguez-Oroz et al., 2011), which supports the idea that ICD are associated

with abnormal function in the associative-limbic circuits of cortex-basal ganglia-thalamus loops (ventral subthalamic area/pre-motor frontal cortex) (Figure 16).

8. Animal models of Parkinson's disease

Animal models of parkinsonism have allowed researchers to study mechanisms underlying the pathphysiology of PD and drug-induced dyskinesias. The most commonly used are rodents (mice and rats) and primates. The main approaches used to mimic the dopaminergic depletion of PD have been the utilization of neurotoxic agents, transgenic animals of the genes linked to PD and overexpression of human α-syn (hα-syn) mediated by adeno-associated viral vector (AVV).

8.1. Animal models by neurotoxic agents

Neurotoxic models have been largely used to study the cellular and molecular events associated with the degeneration of dopaminergic neurons and for the development of treatments. However, they have some important limitations to be noted: the induced dopaminergic degeneration is mainly acute (i.e. within hours-days) and usually massive and the animals do not show intracellular protein aggregates as those observed in idiopathic PD patients. The most frequently used neurotoxins are 6-OHDA and MPTP (see next sections). Other toxins include rotenone, paraquat and maneb, which induce the over-production of ROS, subsequently causing neurotoxicity and neuronal death in SN (Nisticò et al., 2011; Roede et al., 2011). However, although these last models of PD recapitulate some features of PD they are not easy to replicate and they have not been widely used in research.

8.1.1. 6-OHDA

The 6-OHDA is a neurotoxin analogous to both dopamine and noradrenaline and is mainly used to cause nigrostriatal dopaminergic denervation in rats (Ungerstedt, 1968). It cannot cross the blood-brain-barrier, so the induction of parkinsonism is achieved by direct injection of 6-OHDA into SNc, medial forebrain bundle (MFB) or striatum by stereotaxic surgery (Blandini et al., 2008) The 6-OHDA is intercepted by DAT in dopaminergic neurons and although the exact mechanism involved in the toxicity of 6-OHDA is not well-known, the current understanding suggests that 6-OHDA exerts its toxicity through a combination of oxidative stress and mitocondrial dysfunction. (Blum et al., 2001; Glinka et al., 1997).

Importantly, low doses of 6-OHDA induce a partial and heterogeneous dopaminergic lesion, while a higher dose is needed to induce a complete dopaminergic depletion (Blandini et

al., 2008). It has been widely used to induce unilateral models of parkinsonism to study L-Dopa-induced dyskinesias after chronic L-Dopa treatment (Huot et al., 2013).

Several experimental designs have been tried to develop a bilateral 6-OHDA model, but the injections within the SNc or MFB (Ungerstedt, 1971) cause an intense bradykinesia, aphagia (with subsequent weight loss) and adipsia, eliciting high morbidity and mortality rates (Blandini et al., 2008; Sakai and Gash, 1994). Bilateral striatal administration of 6-OHDA in the dorsal motor area exhibit a marked bilateral dopaminergic depletion in this area but with less morbidity (Blandini et al., 2007; Przedbroski et al., 1995). Along the same lines, the intraventricular injection of 6-OHDA has been used as an approach to get a progressive PD model (Rodríguez Díaz et al., 2001, Quiroga-Varela et al., 2017). However, due to the high mortality rates of rats and the difficulties associated to the injection of the chronic 6-OHDA by a ventricular canulae, the model has not been widely used.

8.1.2. MPTP

Mice and monkeys treated with MPTP are used as models for PD, but not rats as the dopaminergic neurons of these animals show certain resistance to this toxin (Chiueh et al., 1984; Tieu, 2011). It crosses the brain blood barrier so different dose and administration procedures (intraperitoneal (ip), intraventricular (icv), subcutaneous (sc)) have been implemented (Jackson-Lewis and Przedborski, 2007; Blesa et al., 2012; showing profound bilateral striatal and nigral dopaminergic depletion and relative preservation of VTA neurons (Blesa, 2011; Dauer and Przedborski, 2003; Langston et al., 1983).

MPTP inhibits the complex I of the mitochondrial respiratory chain (Przedborski et al., 2004; Richardson et al., 2007), leading to a failure in the adenosine trisphosphate (ATP) production and an increment of intracellular ROS, changing the cellular homeostasis and inducing the apoptotic cell death (Novikova et al., 2006; Tatton and Kish, 1997).

One of the main advantages of MPTP models is the achievement of a bilateral and relatively progressive parkinsonism when administered chronically at low doses, this being very useful for studying the emergence of motor signs. However, in addition to the common limitation, these models also have some weak points: in mice (less vulnerable than monkeys to MPTP) the motor alterations are often subtle and can only be detected with tests when the dopaminergic deficit is profound (Taylor et al., 2010); and monkeys show a high variability in the achievement of dopaminergic depletion (due to variable dose-response effect) (Blesa et al., 2012), which hinders the obtainment of a progressive model and makes the model difficult to replicate.

8.2. Transgenic animal models

To date, several transgenic mice or rat models of parkinsonism have been developed, focused mainly on the overexpression of wild-type or mutated forms of proteins associated with familiar cases of PD. Most of the studies have been focused on α-syn and LRRK2, and to a lesser extent, on Parkin, Pink1, DJ1 and ATP13A2.

8.2.1. α-Synuclein transgenic models

The effects on the dopaminergic system of the overexpression of either wild type (hα-syn), truncate forms (hα-syn(1-120)) or mutate species of α-syn (A53T-hα-syn, A30P-hα-syn), and the use of different promoters (prion protein (PrP), Thymocyte differentiation antigen 1 (Thy1), platelet-derived growth factor (PDGF)-β; tyrosine hydroxylase (TH) and Pitx3) have been reported (Herzig et al., 2011; Ikeda et al., 2009; Lin et al., 2012; Masliah et al., 2000; Oaks et al., 2013; Ono et al., 2009; Paumier et al., 2013; Rockenstein et al., 2002; Sotiriou et al., 2010; Thiruchelvam et al., 2003; Tofaris et al., 2006; Wakamatsu et al., 2008). Several of these transgenic mice show decreased striatal levels of TH or dopamine, but the majority have not reported significant nigrostriatal degeneration and motor disabilities (Visanji et al., 2016) and therefore are not used in behavioural studies.

8.2.2. Other transgenic models

Some other approaches have also been attempted to obtain transgenic animal models of PD. These include transgenic mice for proteins LRRK2 (Herzig et al., 2011; Lin et al., 2009; Ramonet et al., 2011; Tong et al., 2009; Tsika et al., 2014), PINK1 (Akundi et al., 2011; Gautier et al., 2008; (Gispert et al., 2009; Martella et al., 2009), Parkin (Martella et al., 2009; Perez and Palmiter, 2005; Van Rompuy et al., 2014; Zhu et al., 2007) and DJ-1 (Goldberg et al., 2005; Kim et al., 2005; Pham et al., 2010), but they have obtained limited results.

Besides, transgenic animals lacking more than one gene related to genetic forms of PD (see Blesa and Przedborski, 2014 for review) have been developed. However, in most cases, the nigral neuronal loss and the subsequent dopaminergic depletion in the striatum is null or negligible and fail to recapitulate the motor signs of the disease.

8.3. Over-expression of α-synuclein by adeno-associated viral vectors

Parkinsonian models have also been developed based on the inoculation within the SN of recombinant viral vectors that mediate over-expression of hα-syn (Meredith et al., 2008; Ulusoy et al., 2008). Importantly, several studies have reported that the transgene expression

and overall dopaminergic depletion achieved by adeno-associated viral vectors (AAV) is substantially higher than the depletion achieved by other vectors such as lentiviruses (Oliveras-Salvá et al., 2013; Ulusoy et al., 2010), mainly due to the high concentration of viral particles that can be achieved in small volumes and to the neuronal tropism that some of the serotypes show (Oliveras-Salvá et al., 2013). Thus, this makes AAV mediated induced over-expression of native or mutated h-α-syn the most used non-toxic tool to study PD-like neurodegeneration in laboratory animals (Dehay and Fernagut, 2016). However, the vast number and variety of vectors, expression cassettes, viral serotypes and titters used in literature hampers comparisons between studies (Table 1). Among all the serotypes, the AAV2 serotype has been the most studied, but other serotypes have been also implemented such as AAV2/1, AAV2/5, AAV2/6, AAV2/7, AAV2/8 y AAV2/9 that have shown even better transduction and dissemination of α-syn expression (Koprich et al., 2010; Lundblad et al., 2012; McFarland et al., 2009). Particularly, the serotype 2/9 has been demonstrated to be the most efficient in α-syn transduction (Bourdenx et al., 2014).

In relation to synuclein species, AAV have been used to over-express native h-α-syn as well as the mutated forms A53T and A30P. Thus, first studies showed that these vectors caused a long-lasting over-expression of the transgenes, which caused premature but progressive loss of dopaminergic neurons within the SNc, a concordant loss of dopaminergic terminals within the striatum and subsequent development of locomotor deficits (Kirik et al., 2002; Klein et al., 2002). In that way, the neuronal loss within the SNc reaches 30-80% at four months (Dehay and Fernagut, 2016; Kirik et al., 2002; Lo Bianco et al., 2002). Importantly, the A53T form seems more toxic than the native form in rodents (Lu et al., 2015; Oliveras-Salvá et al., 2013). Of note, the use of AAV2/9 vectors for A53T-hα-syn over-expression effectively induced dopaminergic neurodegeneration and synucleinopathy in mice, rats and marmosets, making translational comparisons among species particularly interesting (Bourdenx et al., 2015).

Finally, although the expression of canonical LB is not observed, these animals usually show some reminiscent features, such as the cytoplasmic accumulation of phosphorylated synuclein and dystrophic neurites (Dehay and Fernagut, 2016; Eslamboli et al., 2007; Kirik et al., 2003).

Therefore, animal models of PD achieved by AAV-mediated over-expression of hα-syn, specially the A53T-hα-syn, show many of the features observed in PD patients, such as the progressive neuronal loss within the SNc, neuropathological hallmarks resembling the LB pathology or progressive development of locomotor deficits.

9. Animal models of impulsive-like behaviours in Parkinson's disease

Impulsivity and compulsivity are heterogeneous behavioural constructs that encompass many different behavioural traits. However, although there is a clear overlap between both of them, both concepts are distinct: impulsivity refers to goal-oriented actions that are not well planned, meanwhile compulsivity encompasses behaviours that are inappropriately and persistently repetitive, with not obvious relationship to the overall goal (Dalley et al., 2011; Robbins et al., 2012; Rochat et al., 2018).

Impulsivity is currently considered a construct with a multidimensional nature, with different psychological mechanisms underlying each dimension (Rochat et al., 2017). Similarly to the general population, the nature of the impulsivity construct is also multidimensional in PD patients (Nombela et al., 2014). This complexity of the behavioural construct, the lack of knowledge about the role of each specific trait in the emergence of ICDs and the lack of a well-established animal model of parkinsonism and impulsivity nowadays, have not facilitate the study of the underlying mechanisms and the testing of therapies for ICD, so an effort towards this is needed.

In this regard, the first studies in the field analysed the reinforcing properties of dopaminergic drugs in parkinsonian rodents. In rats with partial bilateral parkinsonism induced by the injection of 6-OHDA in the nigrostriatal dopaminergic pathway (50% and 75% of neuronal loss within the VTA and SNc respectively), L-Dopa did not show any reinforcing effect but dopaminergic agonists produced receptor subtype- and dose-mediated rewarding effects: the D_1R agonist SKF81297 was associated with conditioned place aversion (CPA); the D_2R agonist bromocriptine induced conditioned place preference (CPP) or conditioned place aversion (CPA) depending on the dose used; the D_3R agonist PD128907 induced CPP (Zengin-Toktas et al., 2013). These results obtained in this study were confirmed in other studies using rats with either bilateral dorsolateral striatal (Riddle et al., 2012) or posterior VTA (Ouachikh et al., 2013) lesions. In contrast, the administration of L-Dopa did produce CPP in a rat model obtained by the bilateral nigral overexpression of A53T-hα-syn (by 30-40% of neuronal loss within SNc with spared VTA) (Engeln et al., 2013b). In regard to studies using self-administration paradigms, a rewarding effect of the D_2R/D_3R agonist Pramipexole (PPX) in a subset of both normal and parkinsonian rats (icv injection of 6-OHDA) has been reported (Engeln et al., 2013a), indicating a differential sensitivity to the drug rewarding-effect in different subjects. Besides, these parkinsonian animals that self-administered PPX showed a heightened expression of FosB/ΔFosB in the dorsal striatum (Engeln et al., 2013a), transcription factors whose increased levels in the striatum has been linked to drug (psychostimulant drugs; Nestler et al., 2001) and behavioural (food addition-

like behaviour; Velázquez-Sánchez et al., 2014) addictions, as well as to the L-Dopa-induced dyskinesias (Andersson et al., 1999; Cenci et al., 1999).

9.1. Types of impulsivity studied in animal models of parkinsonism

As stated before, impulsivity is multifaceted and could subsume different types of impulsive actions, decision and motor actions, which show overlapping yet dissociable neural substrates (Antonelli et al., 2014; Dalley et al., 2011; Dalley and Robbins, 2017; Voon and Dalley, 2015) . Moreover, it is widely accepted that decision actions includes delay discounting as well as reflection impulsivity, while motor actions includes waiting impulsivity and response inhibition (motor impulsivity) (Voon and Dalley, 2015). In operational terms, the two main domains of impulsivity have been classically measured in rodents using a variety of behavioural tasks: 1) decision impulsivity, which is related to an inability to inhibit or defer affectively charged actions, includes both decision-making under risky conditions and reflection impulsivity and can be measured by decision-making tasks (both delay and probabilistic discounting task and gambling task) (Dalley et al., 2011; Evenden, 1999; Verdejo-García et al., 2008; Voon and Dalley, 2015); 2) motor impulsivity, which consists in the inability to withold a movement that is required to be performed to obtain a reward at a given time, includes waiting impulsivity and response inhibitions and can be measured mainly by a stop signal task, Go-NoGo task and 5-CSRRT (Dalley et al., 2011; D'Amour-Horvat and Leyton, 2014; Voon and Dalley, 2015).

In the last decade, several studies have been undertaken to analyse the mentioned impulsive domains, employing several behavioural paradigms in different parkinsonian rat models.

9.1.1. Decision impulsivity

In relation to decision impulsivity, the delay-discounting (delay-related decision-making) and probability discounting (risk-related decision-making) paradigms have been used. The delayed-discounting tasks provide behavioural models of 'non-planning', impulsive decision-making and are based on an inability to prioritize future bigger gratifications over more immediate but smaller rewards (Winstanley, 2011). In contrast, the probabilistic discounting task is similar to the delay-discounting but it adds a risk component: if the probability of obtaining a large reward after a certain action (i.e., lever press) is high, subjects would tend to press the lever that delivers the bigger reward meanwhile if the probability of obtaining this big reward is progressively decreased, subjects will be driven to switch their behaviour and choose actions associated with smaller but more probable rewards (Green et al., 2014). Thus, delay

discounting can be defined as the decrease in the subjective value of an outcome as the time until its occurrence increases, whereas probability discounting is defined as the decrease of the subjective value of an outcome as the likelihood of obtaining it decreases (Green et al., 2014). Importantly, decreased tolerance for delayed gratification measured by the Salience Attribution Test was observed in PD patients with impulsive-compulsive spectrum behaviours (Housden et al., 2010).

The research group headed by Celeste Napier (Rush University, Chicago, EEUU) developed a gambling-like, delayed discounting test that uses the intracranial self-stimulation (ICSS) in the lateral hypothalamus as positive reinforcer (Tedford et al., 2014). Animals have to discriminate between a small ICSS current presented immediately after lever pressing and a large ICSS current presented following a 0 to 15 s delay upon pressing. The bilateral injection of 6-OHDA in the DL CPu induced a significantly decreased preference for larger reinforce as the delay was increased (discounting effect) (Tedford et al., 2015).

On the other hand, the same research group has also developed a probabilistic discounting paradigm using the ICSS as positive reinforcer and has tested it in rats with bilateral parkinsonism (6-OHDA injections in the DL CPu) chronically treated (ip) with PPX (Rokosik and Napier, 2012). In contrast to the results with the delay discounting task, no basal differences were observed between 6-OHDA-lesioned and sham rats and the administration of PPX increased discounting (preference for the large reinforcer) in both groups of animals. The lack of differences between groups was attributed by the authors to the relatively high dose of PPX administered (2 mg/kg). A most recent study has shown that the chronic administration of PPX via osmotic minipumps increases discounting in lesioned animals, with 67% of the animals meeting the criteria for “high risk-taking” (Holtz et al., 2016), but no control rats were studied. Similarly, a study using the rodent betting task (analogous to the probabilistic-discounting but with double-or nothing reward available in the lever with greater gratifications) has shown that two-thirds of intact animals under chronic 5mg/kg ropinirole treatment (D_2R/D_3R agonist) for 28 days dramatically increased selection of the uncertain option regardless of baseline preferences, a result also observed in rats with bilateral DL striatal 6-OHDA lesions (Tremblay et al., 2017).

9.1.2. Motor impulsivity

There are several tasks for rodents to test either response inhibition (stop-signal, go/no-go, fixed consecutive number (FCN) scheduled tasks) or waiting impulsivity (differential low-rates of responding (DRL) task or the 5-choice serial reaction time-task (5CSRTT)). In the DRL, animals are trained to wait a minimum amount of time between consecutive trials in order to obtain a reward (usually food) (Kirshenbaum et al., 2008), while in the 5-CSRTT animals are

trained to respond to brief visual stimulus shown after a period of waiting to earn food rewards. Therefore, anticipatory responses that occur prior to the established timing between responses in the DRL or before the onset of the visual stimulus in the 5-CSRTT are termed premature responses. Thus, although both tasks have similar requirements to motor tasks at the point of response selection, in the 5-CSRTT premature responses arise as a consequence of the animals expecting reward-related cues. Regarding the brain circuits implicated in the emergence of impulsivity in both tasks, there is also a discrepancy as 5CSRTT depends on ventromedial and DRL depends upon ventrolateral frontal cortices (Dalley et al., 2011; Jentsch et al., 2014; Robbins and Dalley, 2017; Voon et al., 2014).

In spite of the task variety, the effect of dopaminergic agonists on either response inhibitions or waiting impulsivity on parkinsonian rats has been scarcely examined until now. In fact, only one study (Engeln et al., 2016) has tested the effect of a progressive dopaminergic lesion (by overexpression of A53T-hα-syn) and chronic PPX treatment using two different tasks, fixed consecutive number (FCN; response inhibition) and differential reinforcement of low rates of responding (DRL; waiting impulsivity). The results indicated that a nigrostriatal lesion by itself increased both impulsive actions and waiting impulsivity while the PPX induced an increase of waiting impulsivity that depended upon the combination of nigral degeneration and a pre-existing impulsivity and an increase of impulsive responses independently of a dopaminergic lesion or basal impulsivity.

Behavioural tasks that could measure different types of impulsivity are of special interest. Regarding this, the variable-delay to signal (VDS) task was developed by Leite-Almeida and colleagues (2013), task that reduces both the attentional load and the pretraining period of the 5-CSRTT and delay discounting tasks and allows to measure compulsive-like behaviours. Besides, the dimensionality of the construct of impulsivity measured is more heterogeneous as it measures decisionand motor impulsivities, providing a unique advantage with regard to all the other previous tasks (Leite-Almeida et al., 2013). This task has been recently used to study impulsive traits in rats with 6-OHDA-induced bilateral lesions in the SNc and VTA (Carvalho et al., 2017a), and has found that the chronic but not acute administration of L-Dopa causes a slight increase in impulsivity.

9.2. Compulsive-like behaviours in animal models of parkinsonism

Up to now, only one experimental study has tested the effects of a dopaminergic agonist on compulsive-like behaviours in parkinsonian animals. Thus, the chronic treatment with PPX induced a compulsive-like behaviour in rats with a dopaminergic lesion of both SNc and VTA (75% and 50% of cell loss, respectively) in the post-training signal attenuation task (PTSA),

paradigm originally designed to study obsessive-compulsive-like disorders (D. Dardou et al., 2017). Besides, the compulsive-like behaviour was associated with an overexpression of FosB/ΔFosB in both dorsal and ventral striatum and orbitofrontal cortex.

In summary, in the present decade several approaches have been performed to obtain an animal model of parkinsonism and impulsive behaviour. Thus, different patterns of striatal dopaminergic depletion and neuronal loss in the SNc were used. The 6-OHDA injection in the DL stiatum generates an acute lesion mainly in the motor circuit of the basal ganglia, meanwhile the overexpression of A53T-hα-syn in SNc induces a progressive parkinsonism. Besides, each behavioural paradigm used have advantages and disadvantages: the FCN only measures response inhibition, delayed discounting and probability discounting tasks have been used in a context of a non-natural reinforcer (ICSS) and test only the decision impulsivity construct, and DRL only measures waiting impulsivity but does not easily provide information regarding multiple aspects that can influence the emergence of impulsivity such as attention, motivation, processing speed etc, as the 5-CSRTT or the VDS do (Winstanley et al., 2006). Moreover, the most used dopaminergic agonist in different studies is the PPX, but the dose and administration paradigms used are heterogeneous. Besides, it is not clear whether the parkinsonian state increases impulsivity by itself in animal models of PD or if an interaction with dopaminergic drugs is needed for the development of abnormal impulsive behaviours. Moreover, it remains to be elucidated if all these factors equally or differently affect diverse impulsive traits included within the behavioural construct of impulsivity.

Therefore, further studies have to be undertaken to determine which would be the optimal dopaminergic lesion pattern, dopaminergic drug treatment (dose and time of treatment) and behavioural paradigm. Besides, the studies already carried out have rarely analysed the underlying mechanisms of abnormal impulsive behaviours. Regarding this, the striatal expression of FosB/ΔFosB is associated with substance and behavioural addictions, but this has to be unravelled in the model of parkinsonism and impulsivity.

II. Hypothesis

The behavioural addictions known as ICD in PD are an important side effect of anti-parkinsonian medication. The development of an animal model that would express impulsivity induced by dopaminergic treatment resembling features observed in PD patients with ICD is critical.

Over the past few years, several research groups have attempted to obtain an optimal rat model, but the results about which dopaminergic lesion pattern, behavioural test or dopaminergic drug treatment paradigm should be employed, are not clear enough.

We first hypothesized that a more widespread, striatal dopaminergic lesion induced by the overexpression of A53T-hα-syn will be a better model than more localized bilateral lesion of the DL CPu (motor striatum) induced by 6-OHDA as it resembles more effectively what happens in PD.

We also hypothesize that the treatment with PPX will induce the development of an abnormal impulsive behaviour in animals with parkinsonism, especially with either acute high doses or a chronic administration, simulating scenarios where abnormal behaviours are commonly expressed in PD patients.

The 5-CSRTT is a behavioural paradigm to evaluate attention control, waiting impulsivity and compulsive-like behaviour. The VDS task provides a rapid and simultaneous assessment of both subtypes of impulsivity (motor and decision impulsivities) reducing both the attentional load and pretraining period. Thus, we also speculate that the VDS task will also be an effective behavioural paradigm in terms of efficacy, impulsivity measure and compulsivity measure.

Finally, the increased striatal expression of FosB/ΔFosB constitutes a common feature in animal models of substance addictions. Thus, given the similarities between substance and behavioural addictions, we hypothesize that the expression of this early gene would be increased in different regions of the striatum in the animal model of parkinsonism and behavioural impulsivity allowing to identify the striatal regions involved in this disorder.

III. Objectives

The main objective of this doctoral thesis is the development of a rat model that would mimic the largest possible features of parkinsonism and express abnormal impulsive behaviour induced by the dopaminergic agonist PPX, so the associated pathological changes underlying these disorders could be studied.

The following specific objectives were established:

1. To study if different models of bilateral parkinsonism (overexpression of A53T-hα-syn in SNc, 6-OHDA in DL CPu) have different propensity to develop PPX-induced abnormal impulsivity due to a specific pattern of dopaminergic depletion in the striatum.
2. To establish the outcome specificity of different behavioural paradigms (5-CSRTT and VDS) to study abnormal impulsivity and compulsivity in the animal models of parkinsonism.
3. To determine the potential to induce abnormal impulsivity of high and low acute doses as well as chronic administration of a low dose of the dopaminergic agonist PPX.
4. To study if impulsivity of the animals before and after the induction of the dopaminergic lesion is a marker of increased risk of developing PPX induced abnormal impulsivity in animal models of PD.
5. To analyse the striatal expression of FosB/ΔFosB and its correlation with the PPX-induced abnormal impulsivity.

VI. General Methodology

1. Animals

Sprague-Dawley male rats (250 g at the beginning of behavioural training) were obtained from Harlan (Barcelona, Spain) and Charles River (Saint-Germain-Nuelles, France). Animals were housed in pairs under inverted 12h light-dark cycle at controlled humidity and temperature conditions (70% humidity, 22ºC), with food and water available ad libitum. When required, food availability was restricted with a periodical weight monitoring to prevent losses 15% below of initial weight. All animals were habituated to handling for three consecutive days. All aspects of testing and feeding were carried out during the dark phase.

The experimental procedures were approved by Committee on Animal Research and Ethics at Biodonostia Health Research Institute (San Sebastián, Spain) and were carried out in accordance with the guidelines of the Spanish Government (RD 53/2013) and the European Union Directive (2010/63/EU). Proceedings were cautiously performed to avoid and/or alleviate animal suffering.

2. Induction of dopaminergic degeneration

Two rat models of parkinsonism were used in three different experiments in order to define the optimal dopaminergic lesion that is associated with the development of impulsivity after dopaminergic treatment. In experiments 1 and 3, a widespread bilateral striatal lesion induced by overexpression of A53T-hα-syn in the SNc was employed, while in experiment 2 a bilateral lesion restricted to the DL CPu was induced by striatal injections of 6-OHDA.

2.1. Bilateral recombinant AAV vector-mediated overexpression of human α-synuclein with A53T mutation in the SNc

Under a mixture of oxygen-isoflurane anaesthesia, rats were placed in a stereotactic frame (Stoelting Co., Wood Dale, IL). The AAV2-9 coding for either A53T-hα-syn (Lesion group) or green fluorescent protein (GFP) (Control group) were bilaterally inoculated in two points of the SNc in both hemispheres. The coordinates used were: 1) anteroposterior (AP): -4.9 mm, lateral (L): ±2.2 mm, ventral (V): -7.7 mm; 2) AP: -5.4 mm, L: ±2.0 mm, V: -7.7 mm, from Bregma and dura. The tooth bar was located at -3.3mm below the horizontal (Bourdenx et al., 2015; (Engeln et al., 2013b; Paxinos and Watson, 2007). The viral vectors were inoculated 1 µl per site at a rate of 0.5 µl/min using an infusion pump and a Hamilton syringe 75RN 5.0µL (Hamilton Company, Reno, NV), which upon completion of inoculation was maintained in the place for 2 minutes per point to prevent the solution diffusion away from the injection site. The AAV2-9 were custom ordered from the Production Platform of Adeno-Associate Viral Vectors in

University of Bourdeaux (Bordeaux, France) encoding A53T-hα-syn or GFP under control of the cytomegalovirus (CMV) promoter.

2.2. Bilateral injections of 6-OHDA in the dorsolateral caudate putamen

Rats were injected with desipramine (i.p. 25 mg/kg in 0.9% NaCl; Sigma-Aldrich, St. Louis, MO) 45 min before surgery to prevent loss of noradrenergic neurons and ensure the specific lesion of dopaminergic neurons. Afterwards, under a mixture of oxygen-isoflurane anaesthesia, rats were placed in a stereotactic frame (Stoelting Co., Wood Dale, IL). Animals were injected with 6-OHDA (Lesion groups; 7.5 µg in 2 µl per side dissolved in 0.2% ascorbic acid; Sigma-Aldrich, St. Louis, MO) or vehicle (Sham groups; 0.2% ascorbic acid dissolved in 0.9% NaCl) in the DL CPu of both hemispheres (Riddle et al., 2012). The following coordinates were used: AP: +1 mm, L: ±3.4 mm, V: 4.7 mm from Bregma and dura and tooth bar placed at -3.3mm below the horizontal (Paxinos and Watson, 2007). The 6-OHDA or vehicle were injected at a rate of 0.2 µl/min using an infusion pump and a syringe 75RN 5.0µL(Hamilton Company, Reno, NV), which were maintained in place for 2 minutes to not to allow the solution to diffuse away from the injection site.

3. Motor assessment: adjusting stepping test

The adjusting stepping test was used in all the experiments for *in vivo* monitoring of the motor impairment induced by dopaminergic degeneration (Bido et al., 2017). On testing days, each rat was held by the experimenter with one hand fixing the hind limbs and slightly raising the hind part above the surface. With the other hand, the experimenter fixed one of the upper limbs and animals were slowly moved sideways over a distance of 0.9 m in approximately 5 s, firstly in the forehand and then in the backhand directions. The sequence of testing was always the right paw forehand and backhand adjusting stepping followed by the left paw in the forehand and backhand directions. The test was repeated twice for each animal each session and the average values of the number of adjusting steps in both directions (adduction and abduction) with each forepaw were considered in the analysis.

4. Evaluation of impulsivity and compulsive-like behaviours

Two behavioral paradigms were employed: the 5-CSRTT paradigm was chosen in experiment 1 and the VDS paradigm was used in experiments 2 and 3.

In both paradigms, the premature responses are considered as the expression of impulsivity while perseverative responses reflect compulsive-like behaviour. The difference lies

in the fact that the 5-CSRTT measures only waiting impulsivity and the VDS discriminate between motor impulsivity and intolerance to delay (decision impulsivity). Omissions in both paradigms can be influenced by several features such as attention and motivation. Besides, in the 5-CSRTT the latencies for both correct response and reward retrieval can reflect processing speed, attentional deficits and/or severe motor impairment, so their interpretation usually depends upon the context (Amitai and Markou, 2011; Asinof and Paine, 2014).

4.1. Behavioural Chambers

Three standard operant chambers placed in sound attenuated and ventilated boxes (Med Associates Inc., St. Albans, VT) were utilized for both 5-CSRTT and VDS paradigms (Figure 17). Each chamber (25 cm x 25 cm x 25 cm) presents 5 square apertures (2.5×2.5×2.5 cm) arranged horizontally on a curved wall 2.5 cm above the grid floor, with a LED light bulb and infrared photobeams to detect movements in each of them. The opposite wall presents the food delivery magazine, an aperture equipped with a LED light bulb and photobeams connected to a food dispenser. Above this aperture, the house-light that illuminates the chamber.

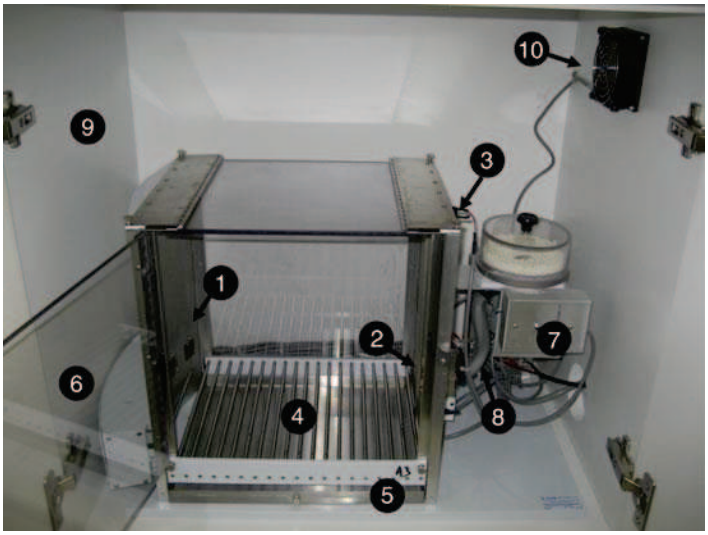

Figure 17. Main components of the employed standard operant chambers (from Bari et al., 2008). On the front wall of the chamber (1) there are five square holes equipped with infrared detectors. Infrared photobeams are also present inside the food magazine (2) fitted in the middle of the opposite wall, where the houselight (3) is also located. The floor of the chamber (4) is made by stainless steel rods and at the bottom there is a removable tray (5), which is filled by clean sawdust at the beginning of each day of training or testing. The hinged door (6) consists on a transparent polycarbonate plate. The pellet dispenser (7) is located outside of the box and automatically delivers food pellet to the magazine through a plastic tube (8). Each apparatus is enclosed within a sound-attenuating cubicle (9) fitted with an electric fan (10) providing ventilation and low background noise.

The scheduling and data collection of experimental events in the 5-CSRTT paradigm were automatically controlled by Med-PC software (Med Associates Inc., St. Albans, VT) and in the VDS paradigm by a custom-designed program using Med-PC IV software (Med Associates Inc., St. Albans, VT) (Annex II). Dust-free grain-based rodent tablets (5TUM 45 mg, TSE Systems) were used as rewarding food pellets and were obtained from Cibertec, S.A. (Spain).

4.2. 5-Choice Serial Reaction Time-Task paradigm

In the 5-CSRTT paradigm, animals were trained to respond (nose-poke) to brief flashes of light randomly presented in one of five spatial locations. The employed protocol was based on a previous published procedure (Bari et al., 2008), in which several phases are distinguished:
- Habituation phase: animals were habituated to the apparatus in two consecutive days for 20 min/session. In these sessions, all cue lights and houselight were switched on and 20 food pellets were placed in the magazine and 2 in each response aperture.
- Pre-training phase I: all cue lights and houselight were switched on during the session. A nose-poke in any of the five holes resulted in a food pellet delivery in the magazine entry. Rats were trained until all animals earn 100 pellets within 30 min in three consecutive daily sessions.
- Pre-training phase II: houselight was switched off during the session. Rats were trained to detect brief flashes of light (30s of stimulus duration (SD)) randomly presented in any one of the five response holes. A nose-poke response into the illuminated hole (correct response) was rewarded by a food pellet delivery in the food magazine. Incorrect responses (nose-poke response in a different aperture from the one in which the stimulus was presented) were not punished. The procedure was repeated until all animals earned 100 pellets in 3 consecutive sessions. The inter-trial interval (ITI) was fixed in 6 seconds.
- Training: rats were trained 6 days a week (Monday to Saturday) to reach criterion performance (accuracy >80%, omissions <20%) with a SD of 1s. The SD was reduced from 30s to 6, 5, 2.5, 2, 1.75, 1.5, 1.25 and 1 s over training stages, allowing the animals to progress through stages by reaching criterion performance in at least three consecutive sessions. Each daily session consisted of 100 discrete trials that commenced with the illumination of both the chamber by the house light and the food magazine, in which a food pellet was delivered (Figure 18). The collection of this pellet was followed by a fixed ITI of 5 s. At the end of the ITI, a visual stimulus with the duration corresponding to each training phase was presented randomly at one of the apertures at the rear. Responses in this aperture within 5 s (limited hold, (LH)) were recorded as correct responses and were rewarded by a food pellet delivery in the magazine feeder. Response errors were recorded as omissions (failures to respond within the LH),

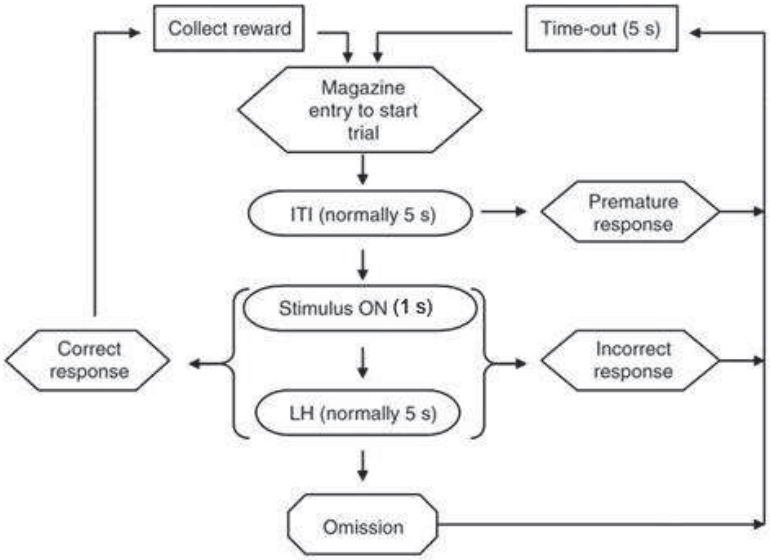

Figure 18. Scheme of the trial sequences in the 5-CSRTT paradigm (modified from Bari et al., 2008). Abbreviations: ITI, intertrial interval; LH, limited hold.

incorrect responses (responses made at the wrong location) and premature responses (responses made before the presentation of the visual stimulus in any of the five apertures). These response errors were all punished with a 5-s period of darkness (time-out) during which no food was delivered. Perseverative responses (additional responses in an aperture after a correct response) produced no time-out punishment period. After the retrieval of the food pellet from magazine aperture or after the time-out period, the next trial was initiated. A response in the food magazine after a premature response restarted the same trial. Each session finished after 100 trials or 30 min. To facilitate acquisition of the task, the SD was progressively shortened from 60 to 1 s over 4 weeks.

- Test: each test consisted of 100 discrete trials fixing the stimulus duration to 1 s, the LH to 5 s, the ITI to 5 s and the time-out to 5 s (Figure 18).

The performance measures recorded were as follows: number of correct responses, number of incorrect responses, total number of trials responded (correct responses+ incorrect responses), accuracy (percentage of correct responses; [(correct responses/(trials responded)] × 100), percentage of omissions ([number of omissions/(trials responded + omissions)] × 100), number of premature responses, number of perseverative responses, latency to correct responses (latency from stimulus presentation to a correct nose-poke response) and latency to reward (latency to food collection after a correct response). As the response in each session varies in terms of the number of trials (see experiment 1 in the results section), the rate of premature and perseverative responses related to responded trials (premature or perseverative

responses/trials responded) were used for analysis (Amitai and Markou, 2011; Pezze et al., 2007).

4.3. Variable Delay-to-Signal paradigm

In the VDS paradigm, animals are required to respond to a brief flash of light always presented only in the central response aperture (Leite-Almeida et al., 2013). Several phases are distinguished:

- Pre-training: rats were habituated to behavioural chambers twice a day for two days. In these sessions, 5 food pellets were placed on the central response aperture and 20 of them in the reward aperture.

- Training: rats were trained in behavioural chambers twice a day for five consecutive days (Monday to Friday). Daily training sessions were separated by 5 hours. Each session started by turning on the house light and the delivery of one pellet in the food magazine. Afterwards, a trial started, consisting of a delay period of a 3 s with only the house light on followed by a response period adding the lightning of the central response aperture for 60 s (LH). Nose pokes in this aperture were either punished if performed during the delay period (premature responses) with a 5 s time-out period in complete darkness or rewarded with the delivery of a pellet if performed during the response period (correct response). Omitted responses were also punished with a 5 s time-out period. Sessions finished after 100 trials or 30 minutes.

- Test: rats were tested only once a day. It consisted of 120 trials, divided into 3 blocks (Figure 19), similar to those in the training phase, with several exceptions: i) the delay period lasted 3 s in the first (3si block) and the last (3sf block) 25 trials, and randomly either 6 or 12 s in the middle 70 trials (6-12s block); ii) premature responses did not initiate a time out punishment; iii) multiple nose pokes after a correct response (perseverative responses) were allowed during the delay period (compulsive-like behaviour) and did not initiate a light-off punishment period. Thus, the main differences in VDS test with the 5-CSRTT are that only central response aperture is illuminated as a signal, that the LH lasts 60s, and that premature responses during the test are not punished by changes in the light of the chamber.

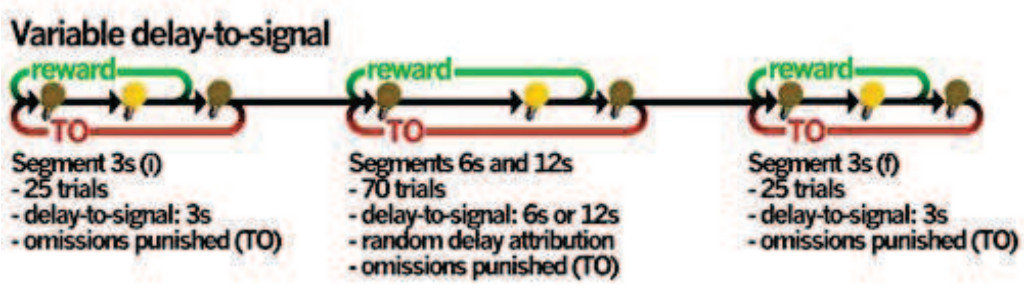

Figure 19. Scheme of the trial sequences of testing in the VDS paradigm (from Leite-Almeida et al, 2013).

In each test session we recorded: percentage of omissions [(number of omissions/trials)*100]; premature responses (PMR; responses made during the delay period); and perseverative responses (PSR; repetitive responses after a correct response and before the retrieval of the rewarding food pellet). Then, we calculated the PMR rate for the 3si and 3sf blocks, defined as PMR per minute of total delay ([number of premature responses/(number of trials in either 3si or 3sf block x 3s)] x 60), as in the previous work by Leite-Almeida and colleagues (2013).

Due to the fact that the software we used to automatically control boxes and analyse data differed from that used by Leite-Almeida and colleagues (TSE Operant Behaviour, TSE Systems GmbH, Germany), we could not detect whether there was a PMR in the first second of the delay period in the 3si block. We therefore interpreted changes in the PMR 3si rate as an expression of motor impulsivity. Similarly, we could not distinguish between PMR after 6 or 12 s of delay, so we obtained only the total number of PMR in this block. Therefore, PSR thus reflects compulsive-like behaviour, while PMR and PMR rates are considered to be an expression of impulsivity, with PMR 3si and PMR 3sf rates representing motor impulsivity and PMR 6-12s indicating delay intolerance.

5. Pramipexole administration

The PPX dihydrochloride (A1237; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% NaCl. Its effects were behaviourally evaluated either acutely (s.c.; 0.25 mg/kg and 3 mg/kg; unique dose) or chronically (sc; 0.25 mg/kg; once a day during 4 weeks). The doses were chosen based on previous studies (Engeln et al., 2013a; Maj et al., 1997).

V. Experiments: specific methodology, results and discussions

Experiment 1

In the experiment 1, we have analysed the waiting impulsivitity and compulsive-like behaviour of rats with a bilateral dopaminergic depletion induced by the overexpression of A53T-hα-syn in the SNc chronically treated with PPX (0.25 mg/kg/day for 4 weeks) using the 5-CSRTT.

1. Specific methodology

1.1. Experimental design

Before 5-CSRTT training, rats were gradually food-deprived (20 to 10 g/rat/day) until they reached approximately 90% of their free-feeding body weight, thereafter establishing their intake at 15 g/rat/day. Only animals who reached the performance criteria of the 5-CSRTT on 5 consecutive days (accuracy >80%, omissions <20%; ≈60 sessions within 12 weeks of training; presurgery condition; n=26) were randomly assigned to either the Control (n=9) or Lesion (n=17) groups and underwent stereotactic surgery. Bradykinesia was assessed using the adjusting stepping test 1 week before surgery and every 2 weeks after surgery until week 17, based on previous studies that show an established dopaminergic lesion at this point in time (Figure 20; (Bourdenx et al., 2015).

After surgery, rats were left for 3 days to recover with ad libitum food access before starting food restriction and 5-CSRTT training. In the first 11 weeks after surgery, rats were trained twice weekly in the 5-CSRTT and they were then trained 6 days a week until week 17 at standard conditions (SD of 1s, ITI of 5s, LH of 5s) (Figure 20). At this point, animals were tested for 3 consecutive days (pretreatment condition). The next day, all Control and Lesion rats were treated chronically with PPX for 4 weeks, administered in a single daily dose at 0.25 mg/kg/d (Figure 20). The behavioural evaluation in the ON state was undertaken 1 hour after drug administration and the OFF state evaluation was undertaken before drug administration.

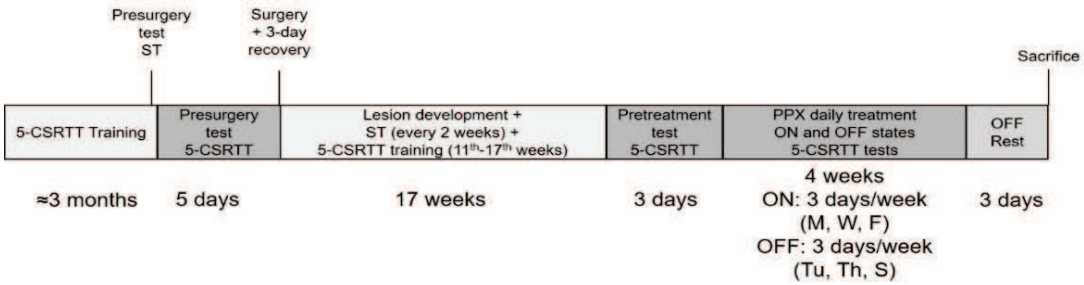

Figure 22. Experimental design of the experiment. Abbreviations: 5-CSRTT, 5-choice serial reaction time task; ON, ON medication state, OFF, OFF medication state PPX, Pramipexole; ST, adjusting stepping.

Thus, the behaviour in the 5-CSRTT was evaluated under the following conditions: basal state (presurgery; mean of 5 days); 17 weeks after surgery (pretreatment; mean of 3 days); and ON and OFF medication state at 1, 2, 3, and 4 weeks after treatment onset. The OFF and ON medication states were evaluated every other day during the treatment to avoid excessively long evaluations on the same day. The average of the measurements obtained each week for the ON and OFF medication states was analyzed. The animals were sacrificed for histological studies 3 days after the last 5-CSRTT test session in OFF medication.

1.2. Viral vectors

Concentration of viral particles were $6.7x10^{13}$ genome containing particles (cgp)/ml for AVV-GFP and $9.6x10^{12}$ gcp/ml for AVV-A53T-hα-syn.

1.3. Fixed tissue collection

Rats were anesthetized with a mixture of oxygen isoflurane (5%) and perfused transcardially with 4% paraformaldehyde. The rat's brain was removed and postfixed in the same fixative for 24 hours, and then cryoprotected in 30% sucrose. Serial coronal sections (40-mm thick) were obtained on a freezing microtome (SM2010R; Leica Biosystems, Nussloch, Germany) and stored at -20ºC in a cryoprotectant solution (see annex I).

1.4. Immunochemistry

Immunohistochemistry was performed on coronal sections containing the SNc and striatum to detect TH and DAT expression respectively, in order to evaluate the dopaminergic innervation in the nigrostriatal pathway, and on coronal sections containing the striatum to detect FosB/ΔFosB, in order to evaluate the expression of a transcription factor that could be a biomarker of impulsivity.

In detail, tissue was washed in 0.1 M PBS and subsequently incubated in 3% H_2O_2 (Sigma-Aldrich, St. Louis, MO) to block the endogenous peroxidase and in a blocking solution (4% normal rabbit serum for DAT and FosB/ΔFosB; 4% normal horse serum for TH; Vector Laboratories, Inc., Burlingame, CA). Afterwards, sections were incubated overnight at room temperature with one of the following primary antibodies: goat anti-DAT (1:100; sc-1433, Santa Cruz Biotechnology, Inc., Dallas, TX), mouse anti-TH (1:1000; MAB-5280, EMD Millipore HQ, Billerica, MA) and rabbit anti-FosB (1:500; sc-48, Santa Cruz Biotechnology, Inc. Dallas, TX) that detects both FosB and ΔFosB. The DAT labelled sections were then incubated with biotinylated rabbit anti-goat (1/100; Vector Laboratories, Burlingame, CA) and the TH labelled sections with biotinylated horse anti-

mouse IgG (1/500; Vector Laboratories, Burlingame, CA) for 1h at room temperature, followed by a 1h incubation with an avidin-biotin-peroxidase complex (1:100; Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Signal was revealed with a 3-3'-diaminobenzidine (DAB)/H_2O_2 solution (Sigma-Aldrich, St. Louis, MO). For FosB/ΔFosB labelling, sections were incubated with HRP-labelled polymer anti-rabbit kit and revealed following manufacturer instructions (DAKO Envision Kit, Agilent technologies, Santa Clara, CA). Afterwards, all sections were mounted onto slides, air-dried overnight, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped with Eukitt mounting medium (Sigma-Aldrich, St. Louis, MO).

1.5. Unbiased stereological counting of SNc dopaminergic TH-immunoreactive neurons

The TH+ neurons present in the SNpc were counted by unbiased design-based stereology on a Bx61 microscope (Olympus, Hicksville, NY, USA) equipped with a DP71 camera (Olympus NY, USA) and with a stage connected to a xyz stepper (H101BX, PRIOR) driven by CAST Visiopharm software (Hoersholm, Denmark). Stereological counting was performed using the optical fractionator method (Fernández-Suárez et al, 2014) every fourth section throughout the entire rostro-caudal extent of the SNpc between -4.30 mm and -6.72 mm relative to Bregma (Paxinos and Watson, 2007). Thus, the SNc was outlined and delimited correctly using external structures at low magnification (4X) and the optical dissector height was set at 10 µm to count 150-200 cells per animal, using a sampling frame of 4838 μm^2 and sampling steps of 213 µm x 213 µm (dx,dy). Only whole somas falling within the borders or encountering inclusion borders but not touching the exclusion borders was counted. The total number of TH+ neurons (N) was calculated using the following equation:

$$N = \sum Q^{-} \frac{t}{h} \frac{1}{asf} \frac{1}{ssf}$$

where ΣQ- is the total number of particles counted, t is the mean section thickness, h is the height of the optical dissector, asf is the area sampling fraction, and ssf is the section sampling fraction.

1.7. Quantification of striatal DAT and FosB/DFosB immunoreactivity

Microphotographs of striatal immunostained sections were obtained on a Nikon Eclipse 801 microscope (Nikon Corporation, Tokyo) (DAT n = 9 sections/animal, 1X objective; FosB/ΔFosB n = 6 sections/animal, 10x objective). Images were background corrected and the optical density (O.D.) of DAT immunoreactivity was obtained using ImageJ (National Institute for

Health, NIH) in 4 subregions of the CPu (DL, VL, DM, VM) as described previously (Quiroga-Varela et al., 2017). The FosB/DFosB immunoreactive neurons in the CPu (DL, VL, DM, VM) and nucleus accumbens (NAc; core and shell) were quantified using an automatic triangle thresholding method in ImageJ as previously described (Gago et al, 2011). A region of constant size (0.56 mm^2) was used as a reference area.

1.8. Variables and statistical analysis

Statistical analyses were carried out using SigmaStat software (version 3.5, SPSS, Inc., Chicago, IL) and were performed for all the behavioural and histological variables to determine differences within groups at each condition (presurgery, pretreatment, ON medication state (weeks 1, 2, 3, 4) and OFF medication state (weeks 1, 2, 3, 4) and between groups. Normality was assessed using the Kolmogorov-Smirnov test and variance equality by Levene's test.

The differences in the number of adjusting stepping test between Control and Lesion groups was analyzed using the Mann-Whitney U test and within groups was analysed using Friedman repeated ANOVA followed by Tukey *post hoc* test. Variables derived from the 5-CSRTT were accuracy, % of omissions, premature responses rate, perseverative responses rate, latency for correct response, latency for reward. For these measures, differences within each group were analyzed using Friedman repeated measures ANOVA followed by a *post hoc* Tukey´s test for multiple comparisons. Differences between Control and Lesion groups at each condition were set by Mann-Whitney U test. Changes in the expression of histological biomarkers between groups and between the CPu sub-regions within each group were analyzed using the Mann-Whitney U test. Correlation analysis were performed using the non-parametric Spearman test. Statistical significance was set at $p<0.05$.

2. Results

2.1. Motor impairment and dopaminergic lesion

The number of adjusting steps fell significantly and progressively in the lesioned animals relative to the Control rats from the 7th (14% reduction, $p<0.01$) until the 17th week after surgery (47% reduction, $p<0.001$; Figure 21 and Table 1). In the Lesion group there was a 64% reduction in striatal (CPu) DAT compared to the Control rats ($p<0.01$; Table 1) and this reduction was significant in both hemispheres of the whole CPu (right $p<0.01$; left $p<0.001$), as well as in the distinct striatal sub-regions studied (right $p<0.01$ each region; left $p<0.001$ each region; Figure 22 and Table 1). Moreover, DAT expression was significantly lower in the medial (DM+VM) than in the lateral (DL+VL) CPu of Lesion animals ($p=0.046$). Stereological analysis of

TH+ neurons in the SNc of the Lesion rats indicated a significant 43% reduction of dopaminergic cells relative to the Control rats ($p<0.001$), which was also significant in both hemispheres (right $p<0.01$; left $p<0.001$; Figure 23 and Table 1).

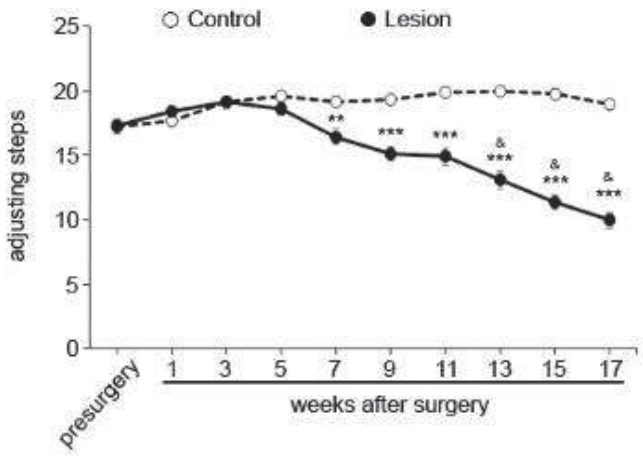

Figure 21. Effect of bilateral dopaminergic depletion on motor function. Stepping test was assessed before (presurgery) and every two weeks after surgery before the beginning of PPX treatment. Values represent the average number of adjusting steps of both forelimb and are expressed as mean ± SEM. **p<0.01, ***p<0.001 vs Control, $^{\&}$p<0.05 vs presurgery.

Table 1. Summary of statistical analyses of adjusting stepping test and histological studies (DAT and TH expression).

Study	Measurement	Statistical test	Comparison	Statistics	p	Degrees of freedom
number of adjusting steps	at 7th week at 17th week	Mann-Whitney U test	Control vs Lesion	U=25.5 U=5	<0.01** <0.001***	
	at 7th week at 17th week	Friedman repeated measures ANOVA followed by *post hoc* Tukey's test	Lesion vs presurgery	X^2=126.40	<0.001***	9
	at 7th week at 17th week		Control vs presurgery	X^2=24.40	<0.01**	
DAT O.D. values	whole CPu right whole CPu left whole CPu right DL CPu right DM CPu right VL CPu right VM Cpu left DL CPu left DM CPu left VL CPu left VM Cpu	Mann-Whitney U test	Control vs Lesion	U=17 U=22 U=13 U=20 U=26 U=26 U=28 U=14 U=15 U=15 U=17	<0.001*** <0.01** <0.001*** <0.01** <0.01** <0.01** <0.01** <0.001*** <0.001*** <0.001*** <0.001***	
	medial (DM+VM) vs lateral (DL+VL) dorsal (DL+DM) vs ventral (VL+VM)		Lesion	U=86 U=182	<0.05* >0.05	
number of TH+ neurons	whole SN right SN left SN	Mann-Whitney U test	Control vs Lesion	U=13 U=23 U=15	<0.001*** <0.01** <0.001***	

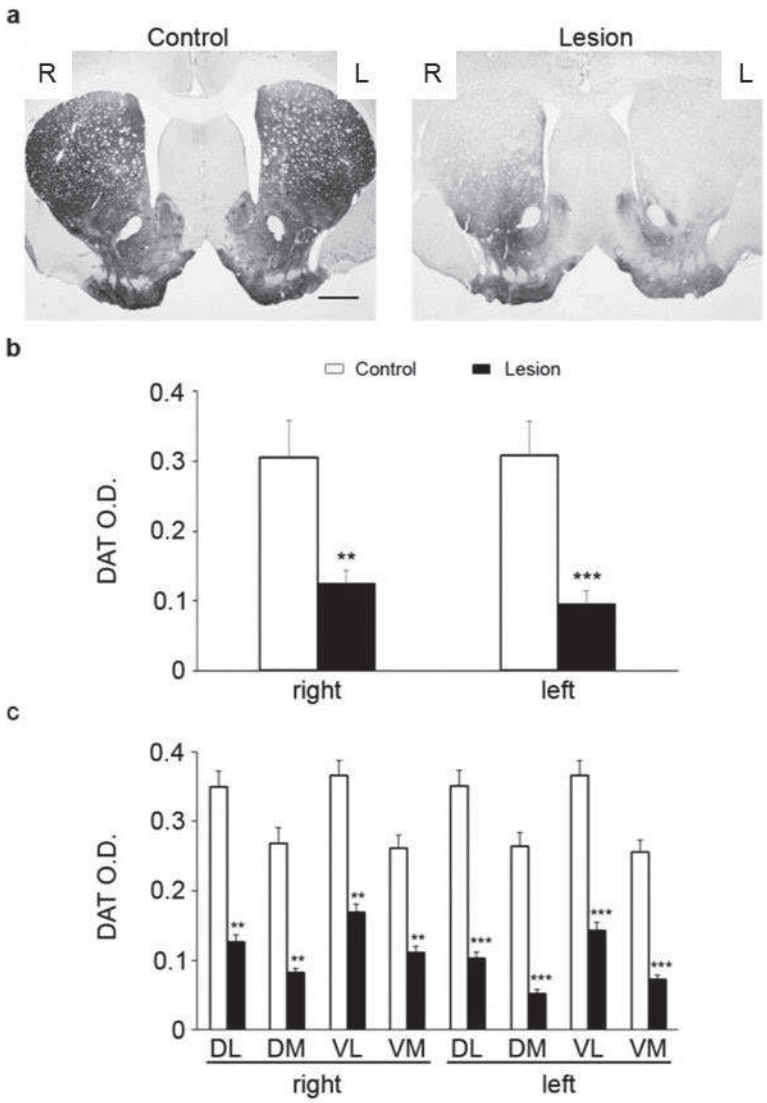

Figure 22. Dopamine transporter (DAT) expression in the striatum. (a) Representative photomicrographs of coronal striatal sections from Control and Lesion animals immunolabeled for DAT (scale bar, 1 mm). (b and c) Relative optical density (O.D.) of DAT in the whole Caudate putamen (CPu) (b) and in its 4 striatal subregions (c). The values are expressed as the mean ± SEM (n=9 slices/animal: Control n=9, Lesion n=17). **p<0.01, ***p<0.001 vs Control. Abbreviations: L, left; R, right; DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial.

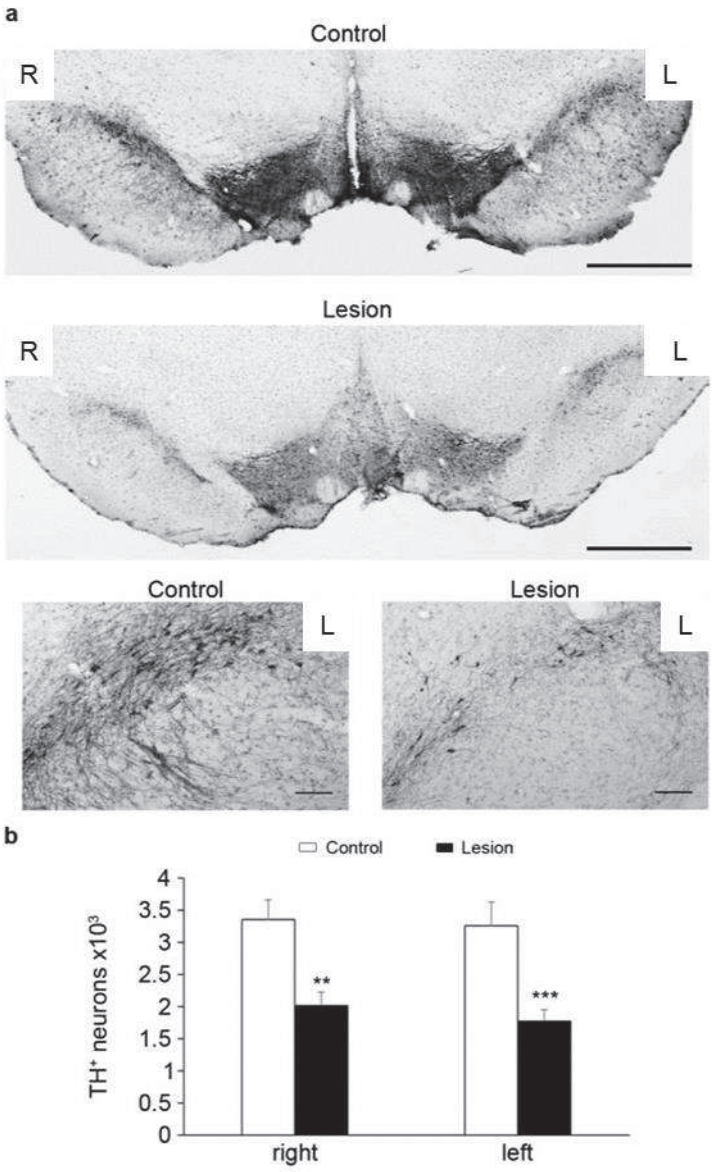

Figure 23. Neurons expressing tyrosine hydroxylase (TH) in the substantia nigra *pars compacta* (SNc). (a) Representative photomicrographs of coronal TH-immunostained nigral sections of Control and Lesion animals (scale bars, 1 mm and 100 μm). (b) Stereological quantification of the number of TH^+ neurons in Control and Lesion rats, expressed as the mean ± SEM (n=7 slices/animal: Control n=9, Lesion n=17). **p>0.01, ***p<0.001 vs Control. Abbreviations: L, left; R, right; TH, tyrosine hydroxylase.

2.2. Behavioural measurements

2.2.1. Changes in 5-CSRTT induced by dopaminergic lesion and PPX treatment: Effect of the dopaminergic lesion

No significant differences in any variable were evident between the two groups presurgery (Figures 24 and 25, Table 2). By contrast, 17 weeks after surgery (pretreatment) there was a significant reduction in accuracy ($p<0.01$), a higher percentage of omissions ($p<0.05$), a higher rate of premature responses ($p<0.05$), and longer latencies for correct responses ($p<0.01$) and reward retrieval ($p<0.05$) in Lesion rats (Figures 24 and 25, Table 2). Conversely, the rate of perseverative responses was no different between the two groups (Figure 24d and Table 2).

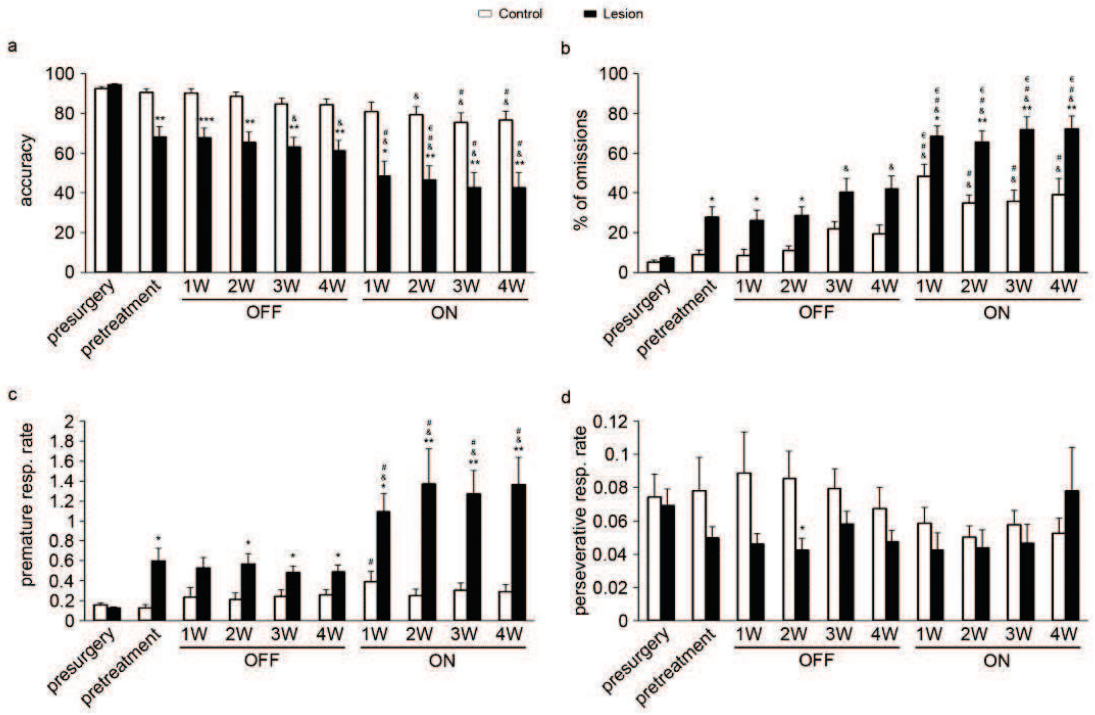

Figure 24. Effect of mild bilateral dopaminergic depletion and chronic PPX treatment on the five-choice serial reaction time task (5-CSRTT). Behavioural parameters of Control and Lesion animals were measured presurgery, pretreatment and weekly throughout the chronic PPX treatment (4 weeks), in the ON and OFF medication state: (a) accuracy, (b) percentage of omissions, (c) rate of premature responses (premature responses/responded trials), (d) perseverative responses rate (perseverative responses/responded trials). Data are expressed as the mean ± SEM (Control n=9, Lesion n=17). Abbreviations: On, ON medication state; OFF, OFF medication state. *p<0.05, **p<0.01 vs Control, &p<0.05 vs presurgery, #p<0.05 vs pretreatment, €p<0.05 vs OFF.

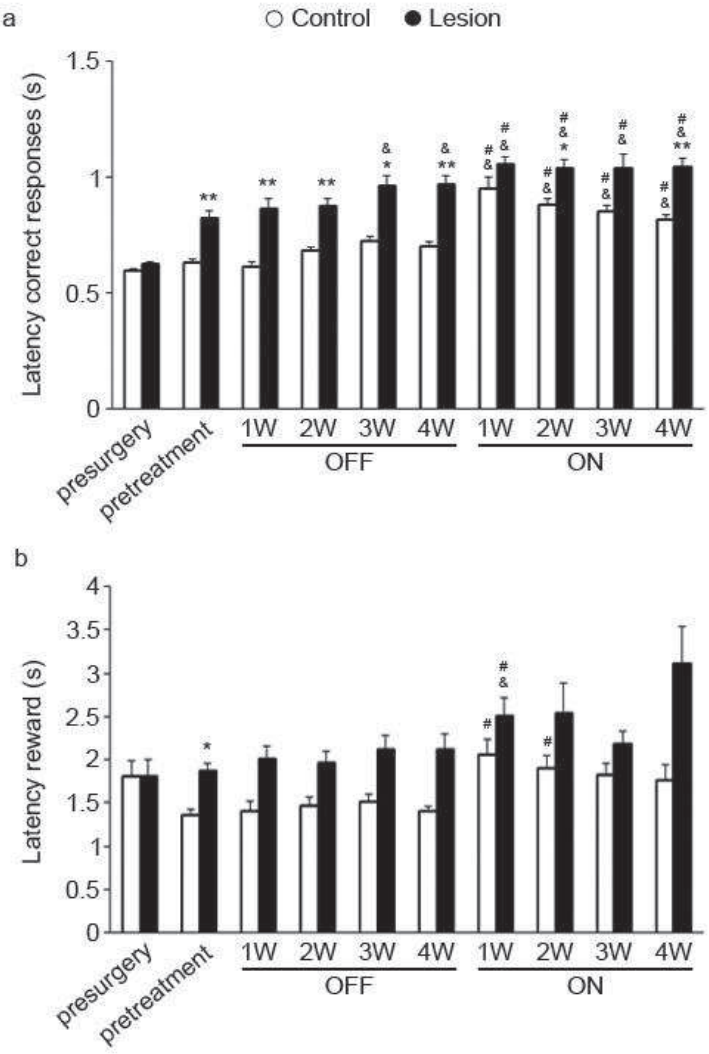

Figure 27. Effect of mild bilateral dopaminergic depletion and chronic PPX treatment in the five-choice serial reaction time task (5-CSRTT). Behavioural parameters of Control and Lesion animals were measured presurgery, pretreatment and weekly throughout the chronic PPX treatment (4 weeks), in the ON and OFF medication state: (a) latencies of correct responding and (b) latencies for reward retrieval.Behaviour Data are expressed as mean values ± SEM (Control n=10, Lesion n=17). Abbreviations: On, ON medication state; OFF, OFF medication state. *p<0.05 **p<0.01 vs Control, $^{\&}$p<0.05 vs presurgery, $^{\#}$p<0.05 vs pretreatment.

Table 2. Summary of statistical analyses of 5-CSRTT behavioural measurements.

Measurement	Statistical test	Comparisons	Statistic	p	Degrees of freedom
Accuracy	Mann-Whitney U test	Control vs Lesion: presurgery time-point	U=92	>0.05	
% of omission			U=95	>0.05	
Premature responses rate			U=50	>0.05	
Perseverative responses rate			U=70	>0,05	
Latency for correct response			U=89.5	>0.05	
Latency for reward			U=79	>0.05	
Accuracy		Control vs Lesion: pretreatment time-point	U=28	<0.01**	
% of omission			U=116.5	<0.05*	
Premature responses rate			U=120	<0.05*	
Perseverative responses rate			U=53	>0.05	
Latency for correct response			U=126	<0.01**	
Latency for reward			U=116.5	<0.05*	
Accuracy		Control vs Lesion: week 1 OFF	U=17	>0.01**	
% of omission			U=120.5	<0.05*	
Premature responses rate			U=111	>0.05	
Perseverative responses rate			U=52	>0.05	
Latency for correct response			U=131	<0.01**	
Latency for reward			U=111.5	>0.05	
Accuracy		Control vs Lesion: week 2 OFF	U=26	<0.01**	
% of omission			U=121.5	<0.05*	
Premature responses rate			U=114	<0.05*	
Perseverative responses rate			U=29	<0.05*	
Latency for correct response			U=124.5	<0.01**	
Latency for reward			U=104.5	>0.05	
Accuracy		Control vs Lesion: week 3 OFF	U=25	<0.01**	
% of omission			U=106.5	>0.05	
Premature responses rate			U=115	<0.05*	
Perseverative responses rate			U=53	>0.05	
Latency for correct response			U=121	<0.05*	
Latency for reward			U=104.5	>0.05	
Accuracy		Control vs Lesion: week 4 OFF	U=24	<0.01**	
% of omission			U=112	>0.05	
Premature responses rate			U=114	<0.05*	
Perseverative responses rate			U=51	>0.05	
Latency for correct response			U=125.5	<0.01**	
Latency for reward			U=113	>0.05	
Accuracy		Control vs Lesion: week 1 ON	U=32	<0.05*	
% of omission			U=117.5	<0.05*	
Premature responses rate			U=123	<0.05*	
Perseverative responses rate			U=52.5	>0.05	
Latency for correct response			U=102	>0.05	
Latency for reward			U=92	>0.05	

Measurement	Statistical test	Comparisons	Statistic	p	Degrees of freedom
Accuracy	Mann-Whitney U test	Control vs Lesion: week 2 ON	U=24	<0.01**	
% of omission			U=129	<0.01**	
Premature responses rate			U=132	<0.01**	
Perseverative responses rate			U=61	>0.05	
Latency for correct response			U=108.5	<0.05*	
Latency for reward			U=92	>0.05	
Accuracy		Control vs Lesion: week 3 ON	U=25	<0.01**	
% of omission			U=127.5	<0.01**	
Premature responses rate			U=128	<0.01**	
Perseverative responses rate			U=53.5	>0.05	
Latency for correct response			U=86.5	>0.05	
Latency for reward			U=85	>0.05	
Accuracy		Control vs Lesion: week 4 ON	U=26	<0.01**	
% of omission			U=126	<0.01**	
Premature responses rate			U=122	<0.05*	
Perseverative responses rate			U=73.5	>0.05	
Latency for correct response			U=107	<0.01**	
Latency for reward			U=93	>0.05	
Accuracy	Friedman repeated measures ANOVA followed by Tukey's post hoc test	Control through time-points	X^2=47.958	<0.001***	9
% of omission			X^2=63.766	<0.001***	9
Premature responses rate			X^2=18.745	<0.05*	9
Perseverative responses rate			X^2=10.430	>0.05	9
Latency for correct response			X^2=64.033	<0.001***	9
Latency for reward			X^2=30.214	<0.001***	9
Accuracy		Lesion through time-points	X^2=83.481	<0.001***	9
% of omission			X^2=118.641	<0.001***	9
Premature responses rate			X^2=61.353	<0.001***	9
Perseverative responses rate			X^2=9.796	>0.05	9
Latency for correct response			X^2=63.782	<0.001***	9
Latency for reward			X^2=32.255	<0.001***	9

2.2.2. Changes in 5-CSRTT induced by dopaminergic lesion and PPX treatment: Effect of chronic PPX administration

2.2.2.1. Lesion versus Control groups

In both the ON and OFF states, there was a significant decrease in accuracy in lesioned animals relative to the Control rats (Table 2) throughout the treatment period ($p<0.05$, $p<0.01$; Figure 26a). In addition, in the ON state, in the lesioned rats there was a significant increase in the percentage of omissions ($p<0.05$, $p<0.01$; Figure 26b) and in the premature responses ($p<0.05$, $p<0.01$; Figure 26c) during the 4 weeks of treatment, and in the latency for correct responses in the 2nd ($p<0.05$) and 4th weeks ($p<0.01$; Figure 27). In the OFF state, a significant increase in the percentage of omissions was observed in the first two weeks ($p<0.05$; Figure 26b)

and in the premature responses rate from the 2nd week ($p<0.05$; Figure 26c), as well as a significant reduction in the perseverative responses rate in week 2 ($p<0.05$; Figure 26d).

2.2.2.2. Effect throughout the treatment in each group

In the animals of the Lesion group, there was a significant reduction in accuracy ($p<0.05$) and more omissions ($p<0.05$), as well as a higher rate of premature responses ($p<0.05$; Figure 26a-c) and a longer latency for correct responses ($p<0.05$; Figure 27 and Table 2) throughout the treatment relative to the presurgery and pretreatment times. The reward retrieval latency was only higher after 1 week of treatment ($p<0.05$; Figure 27) and no differences were evident in the rate of perseverative responses with respect to the presurgery and pretreatment conditions (Figure 26d). In the OFF state, there was a significant reduction in accuracy ($p<0.05$) and an increase in the omissions ($p<0.05$; Figure 26a-b) and in the latency for correct responses during the 3rd and 4th weeks of treatment ($p<0.05$) relative to the performance presurgery (Figure 27). Comparing both states, the accuracy in the ON state was worse at 2 weeks and the percentage of omissions was higher at four weeks (Figures 26 and 27).

In the Control group (Table 2), there was a significant reduction in accuracy in the ON state from the 2nd week of treatment ($p<0.05$) relative to presurgery, and in the 3rd and 4th weeks relative to pretreatment ($p<0.05$; Figure 26a). There was also an increase in omissions ($p<0.05$) and of the latency to a correct response during the entire treatment relative to both the presurgery and pretreatment values ($p<0.05$; Figures 26 and 27). An increase in the rate of premature responses after the first week of treatment was also evident relative to the pretreatment period ($p<0.05$; Figure 26c). The reward retrieval latency was longer after 1 and 2 weeks than pretreatment ($p<0.05$; Figure 27). By contrast, no significant difference was observed for any variable in control rats in the OFF state (Figures 26 and 27). Comparing both states, the only difference was in the higher proportion of omissions at week 1 in the ON state (Figures 26 and 27).

2.3. Striatal FosB/ΔFosB expression

The FosB/ΔFosB expression was stronger in the whole CPu (Lesion 1611 nuclei/mm^2, Control 1288 nuclei/mm^2; $p<0.05$) and in the NAc (Lesion 1936 nuclei/mm^2, Control 1654 nuclei/mm^2; $p<0.05$) of the Lesion rats than in the Control animals (Figure 26). In terms of the distinct sub-regions, the FosB/ΔFosB expression was significantly higher in the right DL ($p<0.01$) and VL ($p<0.05$), NAc core ($p<0.05$) and NAc shell ($p<0.05$; Figure 26b and Table 3), as well as in the left DL ($p<0.01$) and VL ($p<0.05$; Figure 26c and Table 3) of the Lesion rats.

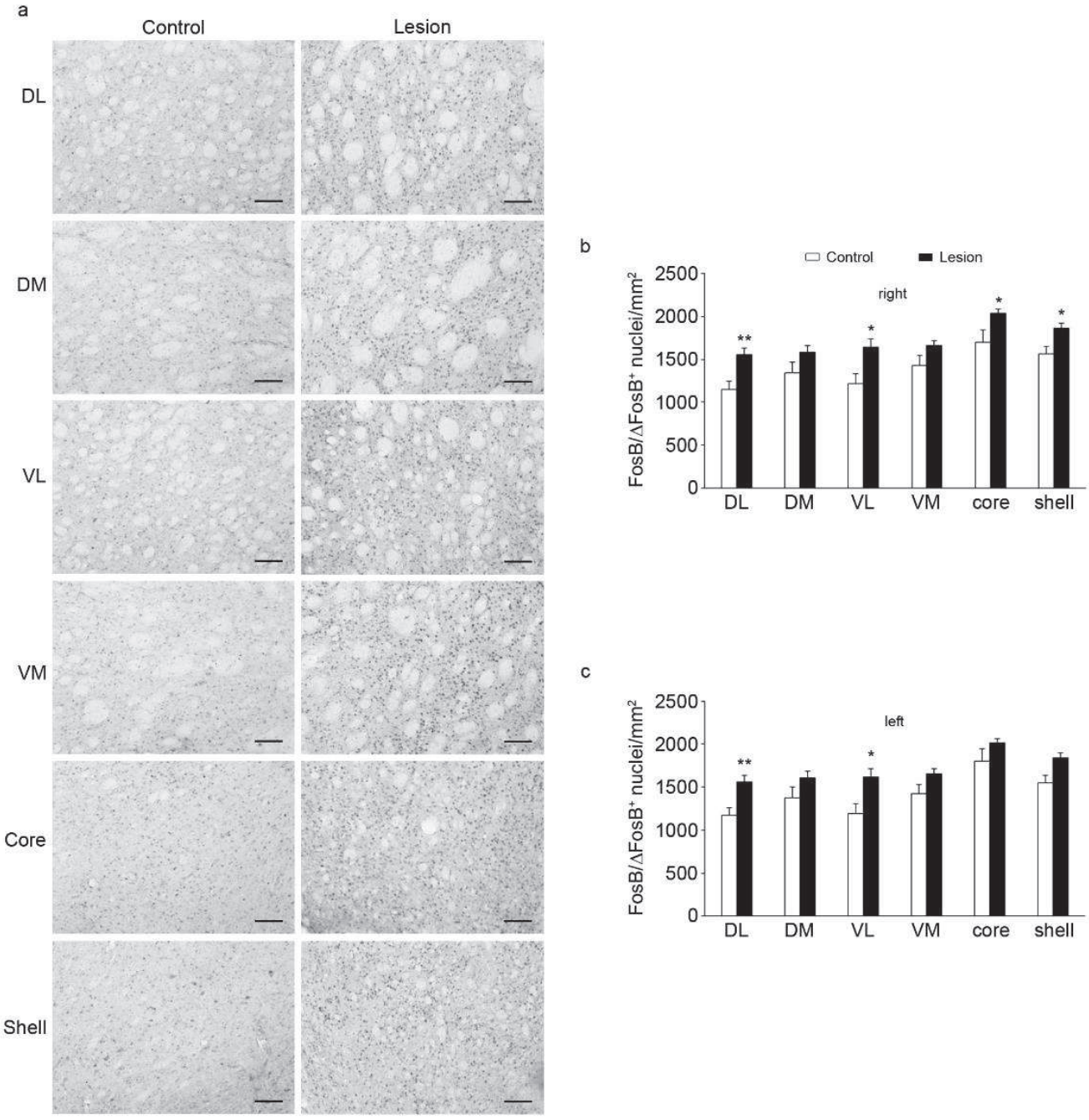

Figure 26. The FosB/ΔFosB expression in the striatum. (a) Representative photomicrographs of FosB/ΔFosB-immunoreactive nuclear expression in the different striatal sub-regions of Control and Lesion animals (scale bars, 100 µm). (b) Quantification of the density of FosB/ΔFosB-immunoreactive nuclear profiles (number of nuclei profiles/mm2) in the different sub-regions of the Caudate Putamen (CPu) and Nucleus Accumbens (NAc; core and shell) in Control and Lesion rats (n=6 slices/animal: Control n=9, Lesion n=17). *p<0.05, ***p<0.001 vs Control. Abbreviations: DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial.

Table 3. Summary of statistical analyses of FosB/ΔFosB expression.

Measurement	Statistical test	Comparison	Statistics	p
Whole Cpu	Mann-Whitney U test	Control vs Lesion	U=113	<0.05*
Whole Nac			U=109	<0.05*
right DL CPu			U=121	<0.01**
right DM CPu			U=102	>0.05
right VL CPu			U=73	<0.05*
right VM Cpu			U=102	>0.05
left DL CPu			U=122	<0.01**
left DM CPu			U=95	>0.05
left VL CPu			U=116	<0.05*
left VM Cpu			U=91	>0.05
right NAc core			U=122	<0.05*
right NAc shell			U=114	<0.05*
left NAc core			U=79	>0.05
left NAc shell			U=107	>0.05

2.4. Correlations between the behavioural and histological parameters

Distinct correlation analyses were performed. In parkinsonian rats (lesion group), a spearman correlation analysis showed that the rate of premature responses at the pretreatment time-point was positively correlated with the rate of premature responses in both the ON and OFF states during the whole treatment (OFF state, $p<0.001$ at each week; ON state, $p<0.01$ weeks 1, 2, 3, $p<0.05$ week 4; Table 4).

In addition, there was a significant negative correlation between the striatal DAT O.D. and the rate of premature responses at the pretreatment time-point ($p<0.01$), as well as that in the OFF state during the whole experiment ($p<0.01$ teach week; Table 4). No significant correlation was evident between the number of TH+ neurons in SNc and the rate of premature responses at any time-point (Table 4). In the ON state, this correlation was only significant in week 3 ($p<0.05$) whereas there was a tendency towards significance in week 4 ($p=0.06$; Table 4). The FosB/ΔFosB expression in the whole CPu or in each of its sub-regions was not correlated with the rate of premature responses in either the Lesion group or when the two groups of animals were considered together (data not shown). However, the striatal FosB/ΔFosB expression was negatively correlated with the DAT values in the whole CPu ($p<0.05$) and in each hemisphere (right $p<0.05$; left $p<0.05$) when the Control and Lesion groups were considered together (Table 4). In terms of the CPu sub-regions, this inverse correlation with DAT density was also observed in the DL ($p<0.01$) and VL ($p<0.01$) of the right hemisphere, and in the four sub-regions of the left hemisphere (VL $p<0.01$; DL, DM, VM $p<0.05$; Table 4). Perseverative responses were not correlated with any histological or behavioural parameter (data not shown).

Table 4. Summary of correlation studies.

Measurement	Statistical test	Comparison	Statistics	p
Lesion group: premature response rate at different time-points	Spearman	pretreatment vs basal	ρ=-0.20	>0.05
		pretreatment vs week 1 OFF	ρ=0.93	<0.001***
		pretreatment vs week 2 OFF	ρ=0.84	<0.001***
		pretreatment vs week 3 OFF	ρ=0.77	<0.001***
		pretreatment vs week 4 OFF	ρ=0.77	<0.001***
		pretreatment vs week 1 ON	ρ=0.63	<0.01**
		pretreatment vs week 2 ON	ρ=0.69	<0.01**
		pretreatment vs week 3 ON	ρ=0.60	<0.01**
		pretreatment vs week 4 ON	ρ=0.55	<0.05*
Lesion group: DAT O.D. values and premature response rate		basal	ρ=0.28	>0.05
		pretreatment	ρ=-0.66	<0.01**
		week 1 OFF	ρ=-0.75	<0.001**
		week 2 OFF	ρ=-0.67	<0.01**
		week 3 OFF	ρ=-0.64	<0.01**
		week 4 OFF	ρ=-0.68	<0.01**
		week 1 ON	ρ=-0.33	>0.05
		week 2 ON	ρ=-0.35	>0.05
		week 3 ON	ρ=-0.53	<0.05*
		week 4 ON	ρ=-0.46	>0.05
Lesion group: TH+ neurons in SNc (bilateral) and premature response rate		basal	ρ= 0.29	>0.05
		pretreatment	ρ= 0.13	>0.05
		week 1 OFF	ρ= -0.042	>0.05
		week 2 OFF	ρ= 0.054	>0.05
		week 3 OFF	ρ= -0.091	>0.05
		week 4 OFF	ρ= -0.15	>0.05
		week 1 ON	ρ= 0.069	>0.05
		week 2 ON	ρ= 0.056	>0.05
		week 3 ON	ρ= 0.15	>0.05
		week 4 ON	ρ= 0.12	>0.05
Control + Lesion groups: FosB/ΔFosB nuclei and DAT O.D. values		whole CPu	ρ= -0.49	<0.05*
		right CPu	ρ= -0.46	<0.05*
		left CPu	ρ= -0.47	<0.05*
		right DL CPu	ρ= -0.52	<0.01**
		right DM CPu	ρ=-0.34	>0.05
		right VL CPu	ρ= -0.54	<0.01**
		right VM Cpu	ρ=-0.31	>0.05
		left DL CPu	ρ=-0.42	<0.05*
		left DM CPu	ρ=-0.48	<0.05*
		left VL CPu	ρ=-0.55	<0.01**
		left VM Cpu	ρ=-0.40	<0.05*

3. Discussion

We have analyzed the impulsive behaviour induced by a mild bilateral dopaminergic lesion simulating early PD (Rodriguez-Oroz et al, 2009) and following the chronic administration of PPX, this resembling the clinical situation of de novo PD patients treated with D_2R/D_3R dopaminergic agonists (see Figure 27 for the summary of the results).

a

WITHIN GROUPS VS PRESURGERY CONDITION

		accuracy	% of omissions	premature resp. rate	perseverative resp. rate	correct resp. latency	reward latency
LESION	ON	↓1-4W	↑1-4W	↑1-4W	↔	↑1-4W	↑1W
	OFF	↓3-4W	↑3-4W	↔	↔	↑3-4W	↔
CONTROL	ON	↓2-4W	↑1-4W	↔	↔	↑1-4W	↔
	OFF	↔	↔	↔	↔	↔	↔

WITHIN GROUPS VS PRETREATMENT CONDITION

		accuracy	% of omissions	premature resp. rate	perseverative resp. rate	correct resp. latency	reward latency
LESION	ON	↓1-4W	↑1-4W	↑1-4W	↔	↑1-4W	↑1W
	OFF	↔	↔	↔	↔	↔	↔
CONTROL	ON	↓3-4W	↑1-4W	↑1W	↔	↑1-4W	↑1-2W
	OFF	↔	↔	↔	↔	↔	↔

LESION VS CONTROL

condition	accuracy	% of omissions	premature resp. rate	perseverative resp. rate	correct resp. latency	reward latency
pretreatment	↓	↑	↑	↔	↑	↑
ON	↓1-4W	↑1-4W	↑1-4W	↔	↑2,4W	↔
OFF	↓1-4W	↑1-2W	↑2-4W	↓2W	↑1-4W	↔

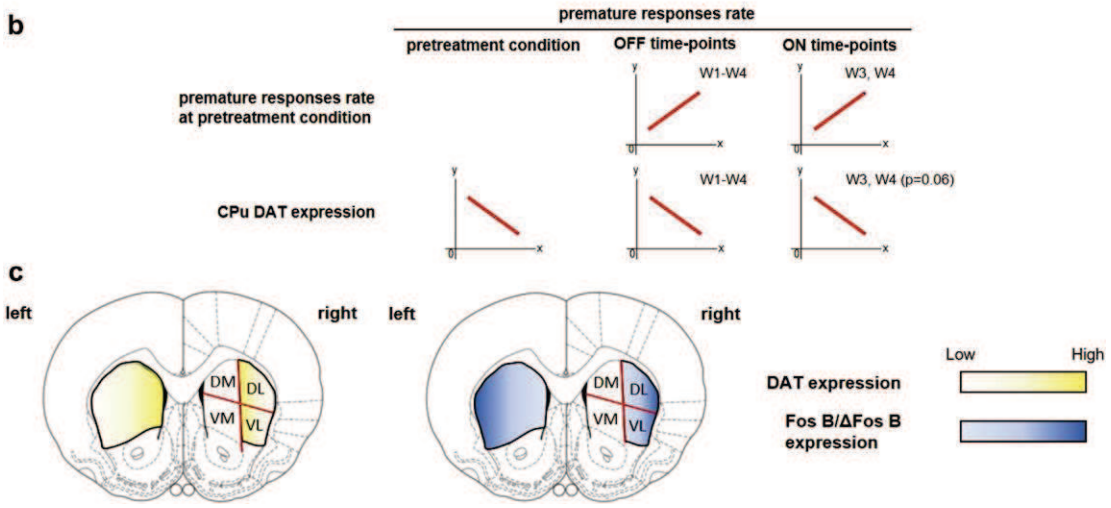

Figure 27. Summary of the changes in the behavioural parameters measured in the 5-CSRTT (A) and the results of the correlation analysis (B-C). (A) Increase (↑) or decrease (↓) in the behavioural parameters recorded in each group relative to the presurgery (top row) and pretreatment values (middle row), and between groups (bottom row). (B) In parkinsonian rats, the rate of premature responses in the pretreatment state positively correlates with the rate of premature responses in both the ON (weeks 3 and 4) and OFF (weeks 1e4) states. The DAT expression in the CPu negatively correlated with the rate of premature responses at pretreatment and in the ON (week 3, week 4) and OFF state (1-4 weeks). (C) Considering the control and lesion groups together, the FosB/DFosB expression correlated negatively with the DAT expression in the entire CPu and in the 4 subregions (DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial) of the left hemisphere, and the whole CPu and in the DL and VL subregions of the right hemisphere. Abbreviations: 5-CSRTT, 5-choice serial reaction time task; CPu, caudate putamen.

The mild bilateral striatal dopaminergic denervation (64%) induces an increase in waiting impulsivity (the rate of premature responses), in keeping with another recent study in this model (Engeln et al, 2016) and animals with bilateral 6-OHDA lesions in the DL CPu (Tedford et al, 2015) that performed different behavioural tasks. This is also consistent with the fact that although impulsivity is a multifactorial construct modulated by several neurotransmitters (the serotonergic, noradrenergic, opioid, and GABAergic systems), dopamine plays a pivotal role in this phenomenon (Cumming and Borghammer, 2011; D'Amour-Horvat and Leyton, 2014). However, impulsive-like behaviour does not develop in a parkinsonian model induced by bilateral injection of 6-OHDA into the SN and ventral tegmental area (VTA) (Carvalho et al, 2017) or into the lateral CPu (Baunez and Robbins, 1999). Differences may reflect the severity and topography of the dopaminergic lesion produced as well as distinct tests used to evaluate impulsivity (VDS, probability discounting task or rodent beting task (rBT)). These data suggest that the pattern and severity of dopaminergic denervation in the CPu are relevant to the impulsive behaviour in parkinsonian rats. In this sense, it is important to note that the dopaminergic neurons in the ventral tegmental area are preserved in the model used in this study (Engeln et al, 2013b; Maingay et al, 2006). This preservation is in keeping with recent studies showing that not only ventral (Cilia et al, 2010; Vriend et al, 2014) but also dorsal striatum may play a role in pathological gambling (Boileau et al, 2013; van Holst et al, 2010) as well as in ICDs in PD (Premi et al, 2016).

A higher degree of striatal denervation appears to be linked with the development of impulsive behaviour because of lesion and PPX administration in the final weeks of treatment. Thus, PD patients with stronger nigrostriatal denervation at the moment of diagnosis might produce a sub-clinical impulsive trait and it would be more prone to develop into abnormal ICD when treated with a D_2R/D_3R dopaminergic agonist. Indeed, while the prevalence of ICDs is similar in untreated PD patients and healthy controls (Antonini et al, 2011; Cilia et al, 2011; Weintraub et al, 2013), untreated PD patients display a stronger inter-temporal choice (a preference for sooner but smaller rewards rather than later but larger ones), indicating that dopaminergic denervation itself can alter behavioural processes relevant to ICD (Al-Khaled et al, 2015; Milenkova et al, 2011). In addition, striatal dopaminergic denervation at diagnosis is stronger in patients that develop ICD after dopaminergic treatment than in those that remain free of ICD symptoms (Vriend et al, 2014).

Waiting impulsive behaviour was exacerbated under the effect of chronic PPX from the first week of treatment, indicating that dopaminergic agonists are indeed the main risk factor for ICD development in PD patients (Weintraub and Claassen, 2017). Interestingly, we also found that from the second week of treatment onwards, the enhanced waiting impulsivity in

parkinsonian rats in the OFF medication state was similar to that pretreatment, which may reflect the premature response induced by dopamine depletion. This finding resembles the fact that ICD in PD patients is not only evident under the acute effect of medication but rather as a continuum. Moreover, impulsivity in both the ON and OFF states is better correlated with the impulsivity induced by the dopaminergic lesion alone and not with basal impulsivity. As such, dopaminergic denervation can apparently affect impulsivity profoundly and subsequent chronic treatment with dopaminergic agonists may enhance the impulsive behaviour in the most vulnerable parkinsonian rats.

The increase in omissions and the longer latencies, as well as the decrease in accuracy after bilateral dopaminergic lesion, which is exacerbated by PPX, could be caused by a deficit in attention and/or motivation, or lesion-induced bradykinesia. However, this latter possibility could be dismissed as the PPX dose chosen was efficient in improving motor deficits (Sandra L Rokosik and Napier, 2012). This is consistent with studies in which this behaviour was related to the severity and topography of the striatal denervation in rats with bilateral CPu dopaminergic depletion (Baunez and Robbins, 1999; Favier et al, 2014). Indeed, attention and impulsivity are interrelated, particularly given that sustained attention is needed to suppress drug-seeking behaviours in addiction (De Wit, 2009) and impulsive individuals that score low in sustained attention tasks (Bakan task; (Rusted et al, 1991). Moreover, PPX reduces short-term verbal memory, verbal fluency, and attentional-executive functions (Brusa et al, 2003). In our experiments, the administration of PPX may reduce attention in lesioned animals, a reflection of the interaction between dopaminergic depletion and the dopaminergic drug, which could impair the accuracy, increase the omissions, and induce longer latencies. Attention and motivation account for omissions, as well as incorrect responses, such that we corrected the raw premature and perseverative responses using rates that take this fact into account. Further studies should be undertaken to explore the influence of PPX on attention and motivation during the performance of these tasks.

Interestingly, we did not observe any relationship between perseverative responses (compulsive-like behaviour) and PPX treatment in parkinsonian rats. Although there is a component of perseveration in gambling and punding, and they are currently considered as impulsivity-compulsivity spectrum disorders (Diagnostic and Statistical Manual of Mental Disorders, 5th ed. DSMe5, American Psychiatric Association, 2013), they share the impulsivity trait with the rest of abnormal impulsive behaviours (ICDs) in patients with PD treated with dopaminergic agonists. Hence, the lack of a correlation between PPX and perseveration could be due to the fact that the impulsive trait of punding and gambling is induced by PPX, as opposed to the perseverative/compulsive trait. Moreover, the results of a recent meta-analysis suggest

that the compulsive tendencies of pathological gamblers are not directly related to the gambling behaviour itself but rather, to both the development and the maintenance of gambling symptoms (van Timmeren et al, 2018).

Given their relevance to addiction (Cooper et al, 2017; Nestler, 2001), we analyzed FosB/ΔFosB expression and interestingly, these transcription factors were more strongly expressed in the lateral CPu (both right and left) and in the right NAc (both core and shell) of parkinsonian animals, indicating a stronger involvement of the right hemisphere. This is in keeping with several studies showing that regions of the right hemisphere participate in impulsivity in PD. Indeed, right subthalamotomy is associated with greater risk of impulsivity and disinhibition (Obeso et al, 2017) and higher dopaminergic denervation in the right dorsal and ventral striatum has been described in patients with PD with an ICD (Voon et al, 2014) (Vriend et al, 2014). However, we obtained no significant correlation between FosB/ΔFosB expressions in any striatal subregion with waiting impulsivity, although the levels of these transcription factors in the CPu were negatively correlated with the severity of striatal dopaminergic denervation in the right lateral CPu (motor CPu) and in the left 4 CPu subregions. Elsewhere, enhanced FosB/ΔFosB expression in the medial regions of the CPu of parkinsonian rats was correlated with the rewarding effects of PPX (Engeln et al., 2013a). The discrepancy in the CPu region that correlates with the effects of PPX could be due to the model used, as bilateral depletion was obtained by intracerebroventricular injections of 6-OHDA in that study and the animals were allowed to self-administer PPX intravenously, in contrast to the progressive SNc degeneration we provoked. Importantly, L-DOPA administration causes an increment in FosB/ΔFosB expression in the CPu (Cenci et al, 1999), which is positively correlated with the severity of dyskinesia (Andersson et al, 1999). Thus, further studies will be necessary to determine if the striatal expression of FosB/ΔFosB is associated to the expression of nonmotor behavioural complications or if it is simply because of dopaminergic treatment, and whether this is associated with a specific pattern of dopaminergic depletion.

In summary, we have demonstrated that a mild bilateral dopaminergic lesion, similar to that in patients with early PD, is linked to a more severe impulsive trait prompted by PPX administration, which seems to be related to the severity of dopaminergic depletion. All in all, we believe that the impulsivity trait associated with the dopaminergic lesion and its interplay with therapeutic D_2R/D_3R dopaminergic agonist administration makes the present model a useful tool to study how dopaminergic agonists affect the induction of pathological impulsivity in PD.

Experiment 2

In the experiment 2, we analyzed the impulsivitity and compulsivity in the VDS of rats with a bilateral dopaminergic depletion induced by 6-OHDA in the DL CPu and acutelly treated with PPX (0.25 mg/kg and 3 mg/kg).

1. Specific methodology

1.1. Experimental design

For three consecutive days, animals were habituated to handling and were food deprived as previously described (General methodology; Figure 28). Then, rats were trained in the VDS task twice a day for 5 days. The following day, basal values for the adjusting stepping and VDS tasks were obtained (presurgery condition). Rats were randomly divided into 4 groups: 6-OHDA+vehicle, 6-OHDA+PPX, sham+vehicle, sham+PPX (n=12 each group) and the next day underwent stereotactic surgery. At the time of analysis, two animals were excluded from the 6-OHDA+vehicle group due to misplaced lesions. Animals were allowed to recover with ad libitum food access for 3 days, followed by another 3 days of food restriction (Figure 28). Thereafter, they were retrained in the VDS task twice daily for 5 days before the effect of the lesion was tested (12th day after surgery) in both the adjusting stepping and VDS tests (pretreatment condition). They were then retrained twice daily for 5 days, and the acute effect of 0.25 mg/kg and 3 mg/kg of PPX was tested on two different days following a Latin square design (Figure 28). Both tests were separated by a 72h washout period that included a day of retraining to avoid habituation of animals to testing conditions (Figure 28). Finally, the day after the last PPX administration, all animals were tested using the VDS and adjusting stepping tests to analyse the residual effect of treatments (post-treatment condition) (Figure 28). Thus, the VDS test was performed under the following conditions: basal state (presurgery); 6 days after surgery (pretreatment), under the effect of 0.25 mf/kg PPX, under the effect of 3 mg/kg PPX and one day after the last PPX injection (posttreatment).

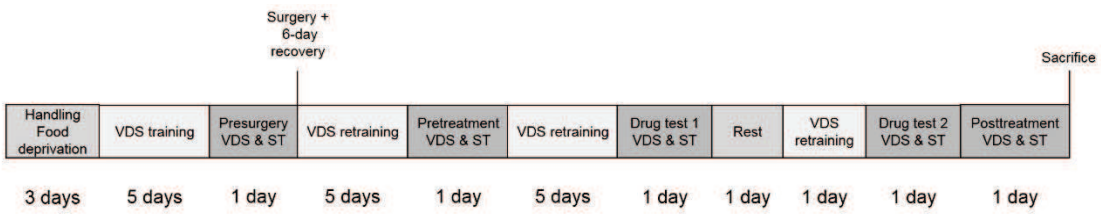

Figure 28. Experimental design. Abbreviations: PPX, pramipexole; ST, adjusting stepping test; VDS, variable delay-to-signal;

Bradikinesia was assessed with the adjusting stepping test each day of behavioural testing, being always performed after the completion of the VDS testing sessions. The animals were sacrificed one day after posttreatment test day for histological studies.

1.2. Fresh frozen tissue collection

Rats were sacrificed and brains were rapidly removed and frozen in dry ice. Serial coronal sections (14 µm thick) were obtained using a cryostat (CM 1950, Leica Biosystems, Germany), thaw-mounted on SuperFrost Plus™ slides (Thermo Fisher Scientific, Waltham, MA), and stored at -80ºC.

1.3. Immunohistochemistry

Immunohistochemistry was performed on coronal sections containing the striatum to detect DAT expression. In detail, sections were incubated subsequently in 0.3% H_2O_2, blocking solution (4% normal serum; Vector Laboratories, Inc., Burlingame, CA), and goat anti-DAT antibody (overnight at 4ºC; 1:100 in PBS; Santa Cruz Biotechnology, Inc., Dallas, TX). Afterwards, sections were incubated with the secondary biotinylated rabbit anti-goat Ig-G (1:1000 in PBS; Vector laboratories, Inc., Burlingame, CA) for 30 minutes, followed by 1h incubation with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA). Signal was revealed with a DAB/H_2O_2 solution. Afterwards, all sections were mounted onto slides, air-dried overnight, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped with Eukitt mounting medium (Sigma-Aldrich, St. Louis, MO).

1.4. Quantification of striatal DAT immunoreactivity

Microphotographs of striatal immunostained sections were obtained with a Nikon Eclipse 801 microscope (Nikon Corporation, Tokyo) (n=9 sections/animal, 1X objective). Images were background corrected and optical density (O.D.) values of DAT immunoreactivity were acquired using ImageJ (NIH) from the 4 subregions of the CPu (DL, VL, DM, VM) and 2 subregions of the NAc (core and shell) (see experiment 1).

1.5. Variables and statistical analysis

Statistical analyses were carried out using SigmaStat software (version 3.5, SPSS, Inc., Chicago, IL) and the R statistical software (R Core Team, 2017) and were performed for all the behavioural and histological variables to determine differences within groups at each condition (presurgery, pretreatment, ON medication state (0.25 mg/kg or 3 mg/kg) and posttreatment)

and between groups. The normality of the data was assessed using the Kolmogorov-Smirnov test, and variance equality was assessed with Levene's test. Given non-normal data distribution and variance heterogeneity for the variables total PMR, PMR 3si rate, PMR6-12s, PMR 3sf rate, PSR and percentage of omissions, the effects of the factors condition (presurgery, pretreatment, 0.25 mg/kg, 3 mg/kg, post-treatment) and group (sham+vehicle, sham+PPX, 6-OHDA+vehicle, 6-OHDA+PPX) were determined by an omnibus test of a mixed between- x within-subjects model using the Welch-James test, implemented in the welchADF package (Villacorta, 2017). For all the analysis, post-hoc Mann-Whitney U (unpaired data) or Wilcoxon T (paired data) tests were utilised to specify the origin of the interaction effect (Aguirre et al., 2019; Schulz et al., 2019). The within factor was condition (presurgery, pretreatment, 0.25 mg/kg, 3 mg/kg and post-treatment) and the between factor was group (sham+vehicle, sham+PPX, 6-OHDA+vehicle, 6-OHDA+PPX). Differences in treated sham and 6-OHDA+PPX animals at post-treatment based on the last PPX dose received were determined by the Mann-Whitney U test. Variations in DAT O.D. values between groups in each striatal region were set using a Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc analysis. Correlations between variables were established by Spearman correlation analysis. Statistical significance was set at $p<0.05$.

2. Results

2.1. Behavioural effects of the dopaminergic lesion and PPX administration in the adjusting stepping test

We examined the effects of experimental group and condition on adjusting stepping test outcomes (group: WJ (3, 20.51)=47.78, $p<0.001$; condition: WJ(4, 25.61)=35.67, $p<0.001$; group x condition: WJ(12, 23.78)=25.38, $p<0.001$). In detail, twelve days after surgery, the dopaminergic depletion caused motor impairment as both groups of 6-OHDA animals showed a significant reduction in the number of adjusting steps with respect to presurgery ($p<0.001$ 6-OHDA+vehicle; $p<0.01$ 6-OHDA+PPX) and sham groups ($p<0.001$ 6-OHDA+vehicle and 6-OHDA+PPX vs sham+vehicle and sham+PPX). This reduction was reversed by the two doses of PPX as the number of adjusting steps increased significantly respect to the pretreatment condition ($p<0.001$ 6-OHDA+ 0.25 mg/kg PPX vs pretreatment and $p<0.001$ 6-OHDA+3 mg/kg PPX vs pretreatment) (Figure 29).

2.2. Behavioural effects of the dopaminergic lesion and PPX administration in the VDS paradigm

We evaluated PMR across the whole and within each block of the VDS test.

2.2.1. Effects on total PMR

The overall analysis indicated a significant effect of the factor condition and an interaction between the two factors (experimental group and condition) (Group: WJ(3, 22)=2.853, p=0.06; Condition: F(4, 24)=13.460, p<0.001; Group x Condition: F(12, 24)=3.017, p<0.05). We observed no differences between experimental groups at pretreatment (p=0.921, 6-OHDA+PPX vs 6-OHDA+vehicle; p=0.707, 6-OHDA+PPX vs sham+PPX) (Figure 30). 6-OHDA rats treated with 0.25 mg/kg PPX showed the same number of PMR as the other experimental groups (p=0.530 vs 6-OHDA+vehicle; p=0.507 vs sham+PPX). However, 6-OHDA animals treated with 3 mg/kg PPX showed a significant increment in PMR with respect to sham+vehicle (p<0.001), sham+PPX (p<0.01) and 6-OHDA+vehicle (p<0.001), as well as 6-OHDA+PPX (0.25 mg/kg) (p<0.01) groups. This effect disappeared at post-treatment (p<0.001) (Figure 30).

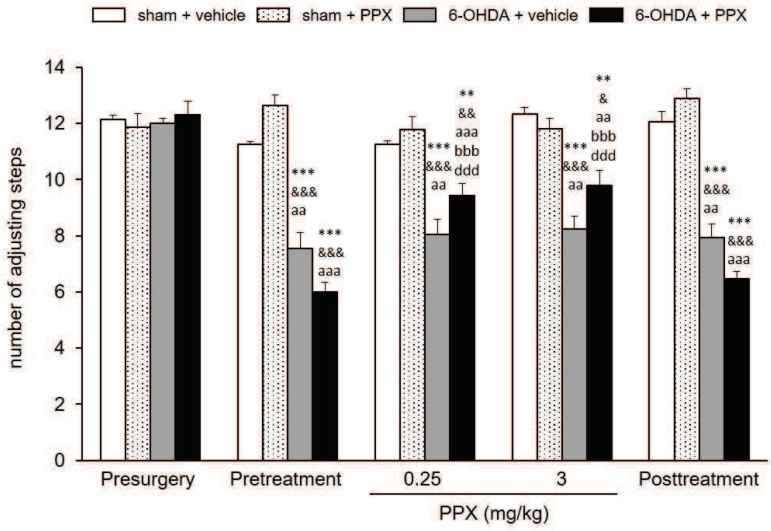

Figure 29. Average of total number of adjusting steps for each experimental group at different time-points. Abbreviations: PPX, Pramipexole. **p<0.01, ***p<0.001 vs sham + vehicle; $^{\&}$p<0.05, $^{\&\&}$p<0.01, $^{\&\&\&}$p<0.001 vs sham+PPX; ^{aa}p<0.01, ^{aaa}p<0.001 vs presurgery; ^{bbb}p<0.001 vs pretreatment; ^{ddd}p<0.001 vs posttreatment.

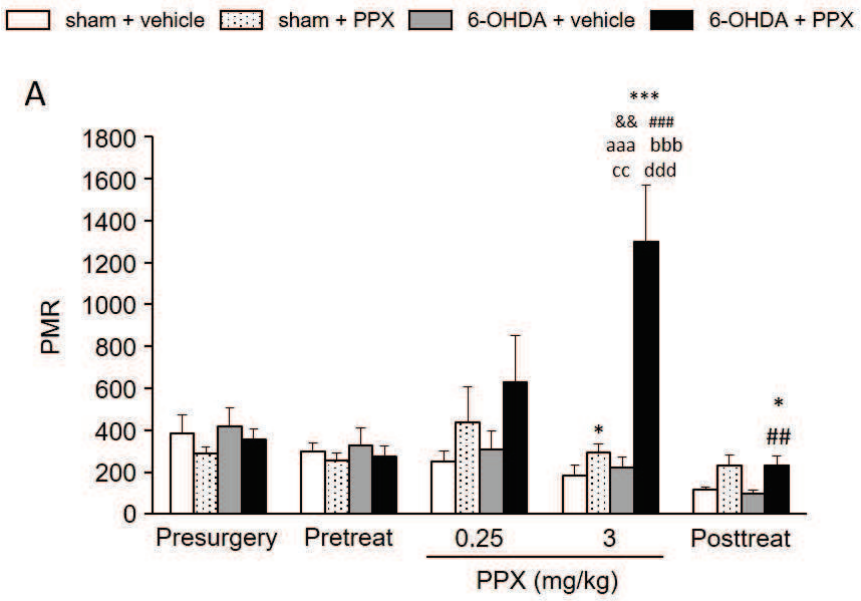

Figure 30. Behavioural measurements in the VDS. Premature responses (PMR) in the experimental groups at different conditions. Abbreviations: Pretreat, pretreatment; Posttreat, posttreatment; PPX, Pramipexole. *p<0.05, ***p<0.001 vs sham+vehicle; $^{\&\&}$p<0.01 vs sham+PPX; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs 6-OHDA+vehicle; ^{aaa}p<0.001 vs presurgery; ^{bbb}p<0.001 vs pretreatment; ^{cc}p<0.01 vs 0.25 mg/kg PPX; ^{ddd}p<0.001 vs posttreatment.

2.2.2. Effects on PMR 3si rate

The overall analysis showed a significant effect on PMR 3si rate due to the factor condition and an interaction between the two factors (experimental group and condition) (group: WJ(3, 22)=2.354, p=0.09; condition: WJ(4, 24)=11.250, p<0.001; group x condition: WJ(12, 25)=2.253, p<0.05). Experimental groups were not different at pretreatment (p=0.262, 6-OHDA+PPX vs 6-OHDA+vehicle; p=0.225, 6-OHDA+PPX vs sham+PPX). The administration of 0.25 mg/kg PPX to 6-OHDA rats did not induce significant changes in PMR 3si rate compared to either sham+PPX (p=0.236) or 6-OHDA+vehicle (p=0.307) (Figure 31A). The 6-OHDA rats treated with 3 mg/kg showed an increased PMR 3si rate with respect to both 6-OHDA animals treated with vehicle (p<0.01) and 6-OHDA animals treated with 0.25 mg/kg PPX (p<0.5), an effect that disappeared at post-treatment (p<0.01) (Figure 31A). Sham animals under the effect of 3 mg/kg displayed an increase in PMR 3si rate with respect to vehicle-treated sham animals (p<0.01; Figure 31A). No differences between 6-OHDA+PPX (3 mg/kg) and sham+PPX (3 mg/kg) animals were observed (p=0.248).

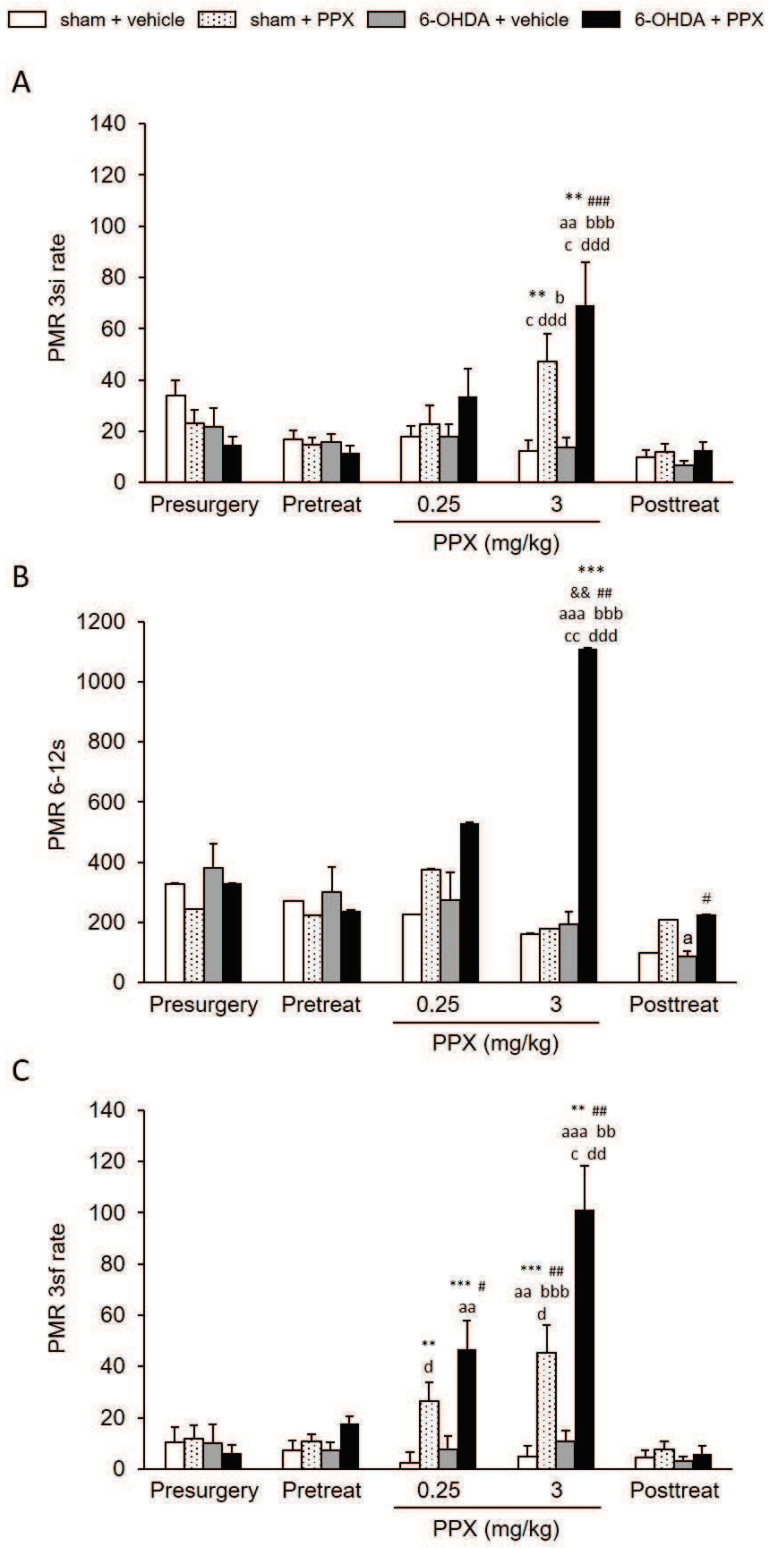

Figure 31. Premature response (PMR) rates in the 3si (A) and 3sf (C) blocks and PMR in the 6-12s block at different conditions. Abbreviations: Pretreat, pretreatment; Posttreat, posttreatment; PPX, Pramipexole. **p<0.01, ***p<0.001 vs sham+vehicle; $^{\&\&}$p<0.01 vs sham+PPX; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs 6-OHDA+vehicle; ^{a}p<0.05, ^{aa}p<0.01, ^{aaa}p<0.001 vs presurgery; ^{b}p<0.05, ^{bb}p<0.01, ^{bbb}p<0.001 vs pretreatment; ^{c}p<0.05, ^{cc}p<0.01 vs 0.25 mg/kg PPX; ^{d}p<0.05, ^{dd}p<0.01, ^{ddd}p<0.001 vs posttreatment.

2.2.3. Effects on PMR 6-12s values

The statistical analysis indicated a significant effect on 6-12s due to the factor condition and the interaction between the two factors (group and condition) (group: WJ(3, 22)=2.731, p=0.07; condition: WJ(4, 23)=10.944, p<0.001; group x condition: WJ(12, 24)=2.834, p<0.05). No differences between groups were observed at pretreatment (p=0.921, 6-OHDA+PPX vs 6-OHDA+vehicle; p=0.544, 6-OHDA+PPX vs sham+PPX). Treatment with 0.25 mg/kg PPX in 6-OHDA animals did not induce significant changes in PMR 6-12s values compared to either sham+PPX (p=0.525) or 6-OHDA+vehicle (p=0.692) (Figure 31B). In contrast, 6-OHDA rats treated with 3 mg/kg PPX showed increased PMR 6-12s values with respect to 6-OHDA+vehicle (p<0.01), sham+PPX (3 mg/kg) (p<0.001), and 6-OHDA+PPX (0.25 mg/kg) (p<0.01) groups (Figure 31B).

2.2.4. Effects on PMR 3sf rate

The overall statistical analysis indicated a significant effect on PMR 3sf rate due to both factors (experimental group and condition) and their interaction (group: WJ(3, 20)=9.652, p<0.001; condition: WJ(4, 24)=11.481, p<0.001; group x condition: WJ(12, 24)=3.393, p<0.01). No differences were detected between groups at pretreatment (p=0.894, 6-OHDA+PPX vs 6-OHDA+vehicle; p=0.770, 6-OHDA+PPX vs sham+PPX) (Figure 31C). In 6-OHDA rats, the 0.25 mg/kg dose significantly increased PMR 3sf rate values compared to 6-OHDA+vehicle animals only (p<0.05) (Figure 31C). This effect was significantly higher for 6-OHDA animals treated with 3 mg/kg (p<0.05 vs 0.25 mg/kg) (Figure 31C) and disappeared at post-treatment (Figure 31C). Sham animals, under the effect of both 0.25 and 3 mg/kg PPX displayed an increase in PMR 3sf rate with respect to sham+vehicle animals (p<0.01 at 0.25 mg/kg condition; p<0.001 at 3 mg/kg condition; Figure 31C). No differences were observed between 6-OHDA+PPX (3 mg/kg) and sham+PPX (3 mg/kg) animals (0.25 mg/kg condition p=0.470; 3 mg/kg condition p=0.194).

2.3. Effects on PMR at post-treatment according to the last PPX dose received

As animals were treated following a Latin square design, we compared the behaviour of animals that received either 0.25 mg/kg or 3 mg/kg PPX as the last dose in both sham+PPX and 6-OHDA+PPX animals. 6-OHDA animals which had received 3 mg/kg as last dose displayed higher PMR in the 3sf block (p<0.05) than 6-OHDA animals treated with 0.25 mg/kg as last dose of PPX (Table 5). No differences were observed in the sham groups.

Table 5. PMR at post-treatment of sham+PPX and 6-OHDA+PPX rats according to the last PPX dose received (0.25 mg/kg or 3 mg/kg)

	sham+PPX 0.25 mg/kg	sham+PPX 3 mg/kg	6-OHDA+PPX 0.25 mg/kg	6-OHDA+PPX 3 mg/kg
PMR total	229 ± 44.7	209.8 ± 0.7	153 ± 41.6	317 ± 57.5
PMR 3si rate	14.1 ± 5.1	9.3 ± 3.9	6.7 ± 3.0	16.9 ± 4.5
PMR 6-12s	194.6 ± 35.9	193.8 ±81.6	153.8 ± 50.9	276.7 ±54.0
PMR 3sf rate	13.3 ± 10.5	3.2 ± 1.1	1 ± 0.7	9.5 ± 5.1[c]

Abbreviations: PPX, Pramipexole. [c]p<0.05 vs 0.25 mg/Kg PPX.

2.4. Effects on PSR

The overall statistical analysis indicated a significant effect due to both factors (group and condition) and their interaction (group: WJ(3, 20.86)=4.902, p<0.01; condition: WJ(4, 21)=6.769, p<0.01; group x condition: WJ(12, 24)=2.811, p<0.05). However, the post hoc analysis did not show any changes in the total value of PSR due to either the dopaminergic lesion or PPX treatment when 6-OHDA+PPX were compared to either 6-OHDA+vehicle or sham+PPX animals (Table 6).

Table 6. Behavioural measurements in the VDS. Perseverative responses (PSR) and percentage of omissions in the experimental groups at different conditions.

		Sham+ vehicle	Sham+ PPX	6-OHDA+ vehicle	6-OHDA+ PPX
PSR	Presurgery	4.1±0.7	4.6±0.6	5.6±2.6	3.4±0.9
	Pretreatment	7.4±1.4	5.1±0.9	12.3±3.2	6.9±1.3
	0.25 mg/kg	4.2±0.8	11.2±2.2	14.6±3.4	13.0±3.1
	3 mg/kg	5.7±1.0	12.1±1.9	14.9±5.0	11.5±2.5
	Posttreatment	5.0±0.8	3.8±0.9	10.3±2.2	9.8±1.7
% of omission	Presurgery	0.6±0.4	0.4±0.3	0.1±0.1	0±0
	Pretreatment	0.5±0.3	0.1±0.1	1.2±0.7	0.1±0.1
	0.25 mg/kg	0±0	1.9±1.4	0±0	5.8±3.6
	3 mg/kg	0±0	5.1±2.0	0.2±0.2	6.1±2.9
	Posttreatment	0.3±0.3	0±0	0.2±0.2	0.3±0.3

Abbreviations: 6-OHDA, 6-hydroxidopamine; PPX, Pramipexole

2.5. Effects on percentage of omissions

No changes in the percentage of omissions were induced by either the group or condition factors or their interaction (group: WJ(3, 19.92)=2.060, p=0.14; condition: WJ(4, 24.45)=2.216, p=0.09; group x condition: WJ(12, 23.9)=1.625, p=0.15) (Table 6).

2.6. Striatal dopaminergic depletion induced by 6-OHDA injection in the DL CPu

Significant differences were observed between the four experimental groups in the striatal areas of the right (DL: H=31.448, p<0.001; DM: H=9.896, p<0.05; VL: H=3.850, p>0.05; VM: H=8.850, p<0.05) and left (DL: H=27.572, p<0.001; DM: H= 19.098, p<0.001; VL: H= 10.597, p<0.05; VM: H= 11.937, p<0.01) hemispheres (Figure 32). The expression of DAT in the DL CPu of both hemispheres in the 6-OHDA groups was significantly reduced (50%) compared to sham groups (p<0.05) (Figure 32). No differences were found between the 6-OHDA groups except in the VM region where 6-OHDA+PPX animals showed a bilateral decrease compared to 6-OHDA+vehicle rats (p<0.05) (Figure 32). No significant differences between groups were observed in either the core or shell regions of the NAc in either hemisphere (Figure 32).

2.7. Correlation analyses

In the 6-OHDA+PPX group, there was a positive correlation between PMR 3si rates at presurgery and pretreatment (p<0.05). There was also a positive correlation between PMR 6-12s values at presurgery and PMR 3si rate after 3 mg/kg PPX (p<0.05). No significant correlations were found between any measures of PMR at pretreatment or and after 0.25 mg/kg PPX. However, there was a positive correlation between all PMR values at pretreatment and both the number of PMR in the 6-12s block (p<0.05) and the total number of PMR (p<0.05) under the effect of 3 mg/kg PPX. There was a positive correlation between all PMR measures (total PMR number, PMR 3si rate, PMR 3sf rate, and PMR 6-12s) in both the pretreatment, 0.25 mg/kg, and 3 mg/kg conditions (p<0.05, 0.01, 0.001).

There was no significant correlation between either DL or total CPu DAT optical density values in each hemisphere or between PMR values in any condition.

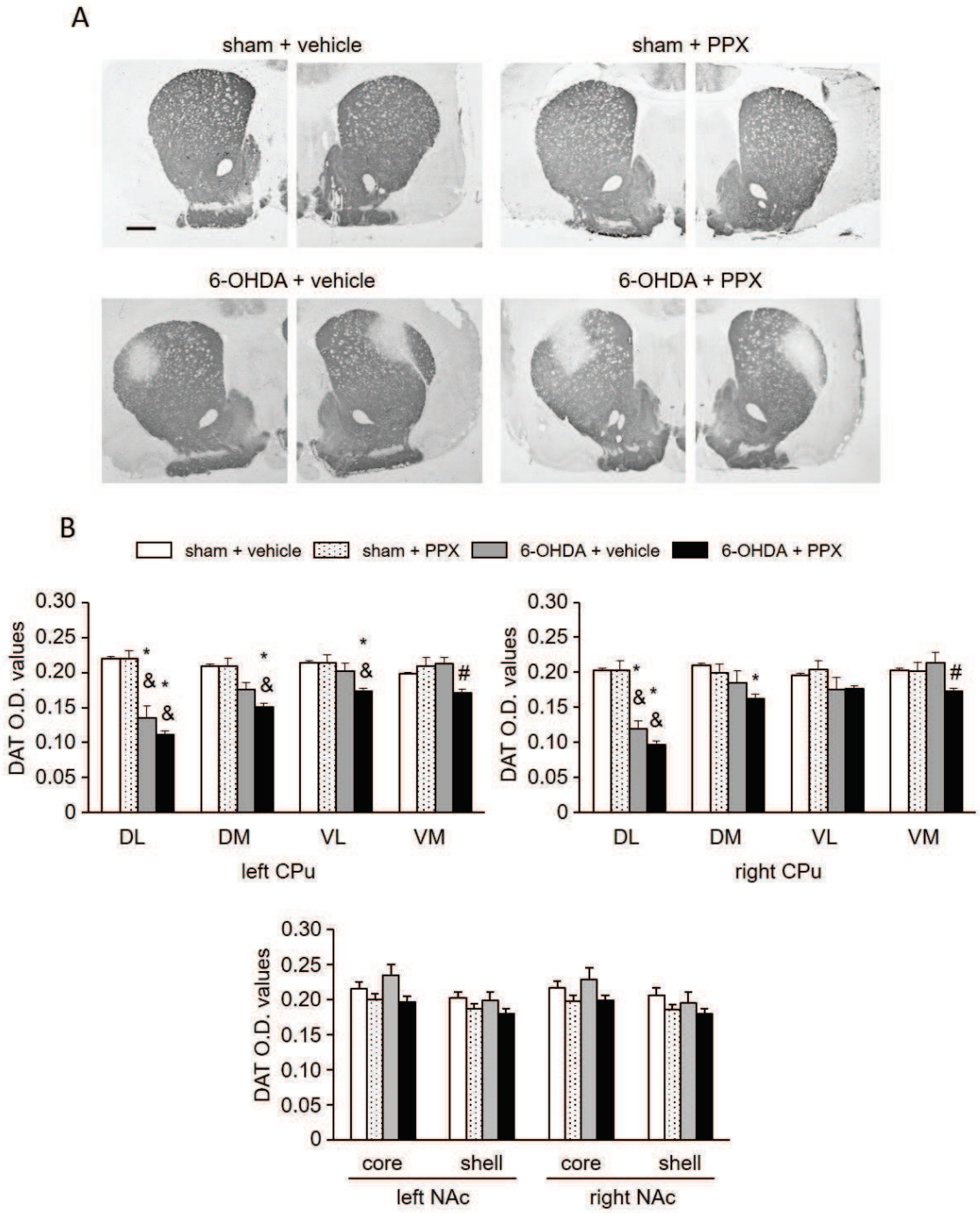

Figure 32. Representative photomicrographs of DAT immunohistochemistry in the striatum of all the experimental groups (A) and the optical density (O.D.) values in the 4 subdivisions of the caudate putamen and the core and shell of the nucleus accumbens (B). Abbreviations: CPu, caudate putamen; DL, dorsolateral; DM, dorsomedial; NAc, nucleus accumbens; VL, ventrolateral; VM, ventromedial. *p<0.05 vs sham+vehicle; &p<0.05 vs sham+PPX; #p<0.05 vs 6-OHDA+vehicle. Scale bar, 1 mm.

3. Partial discussion

We show that acute treatment with PPX triggers both motor impulsivity and delay intolerance in a dose dependent manner in a rat model of parkinsonism with moderate bilateral dopaminergic depletion in the DL striatum.

Studies in patients with PD suggest that dopaminergic depletion prior to dopaminergic treatment may induce some degree of impulsivity (Smith et al., 2016), especially decision impulsivity (Al-Khaled et al., 2015; Antonelli et al., 2014), although this issue remains under discussion (De Micco et al., 2018). Furthermore, it is currently debated if there is a pattern of striatal dopaminergic denervation that predisposes patients to develop ICDs; some studies have found higher dopaminergic denervation in the ventral striatum (Cilia et al., 2010; Navalpotro-Gomez et al., 2019; Steeves et al., 2009; Vriend et al., 2014) or dorsal striatum (Joutsa et al., 2015; Premi et al., 2016; Smith et al., 2016). It has long been recognized that the dorsal striatum is mostly associated with action control and motor impulsivity (Bari and Robbins, 2013; Robbins and Dalley, 2017). However, some studies have linked the dorsal striatum to choice impulsivity in PD patients (Joutsa et al., 2015; Szamosi et al., 2013) as well as both healthy and drug-dependent subjects (Kim and Im, 2019). Therefore, we first evaluated impulsivity elicited by the bilateral dopaminergic lesion in the dorsal striatum. The parkinsonian animals showed mild dopaminergic denervation (around 50%) in the DL CPu (a predominantly motor area), causing bradykinesia as measured by the adjusting stepping test. This resembled the dopaminergic denervation of early PD in both severity and topography, at the time that the disease is generally diagnosed and treated with dopaminergic agonists in PD patients (Stowe et al., 2008). In our study, this dopaminergic depletion pattern did not induce any subtype of impulsivity. This finding is in keeping with a number of studies using animals with the same pattern of dopaminergic depletion which have found no changes in probabilistic discounting (Magnard et al., 2018; Rokosik and Napier, 2012), but not with other experimental work showing increased delay discounting (Stephanie E. Tedford et al., 2015). This discrepancy could be due to differences in the extension of dopaminergic lesions that may underlie different compensatory mechanisms in the striatum as well as to the use of different tasks which employ different reinforcers (sucrose solutions or intracranial self-stimulation) to evaluate impulsivity. In contrast, animals with dopaminergic depletion in both dorsal and ventral striatum showed enhanced waiting impulsivity (Jiménez-Urbieta et al., 2019), suggesting that the involvement of the ventral striatum is relevant for the development of an impulsivity trait in PD patients even before dopaminergic treatment. Future studies testing animal models with different

topographies of dopaminergic depletion undergoing the same behavioral tasks would inform this debate.

We next analyzed the effect of two acute doses of PPX, finding a dose-dependent effect in 6-OHDA animals. The low dose (0.25 mg/kg) only induced motor impulsivity in the 3sf block, while the high PPX dose (3 mg/kg) increased total PMR as well as motor impulsivity (3si and 3sf blocks) and delay intolerance (6-12s block). Although the relevance of the dose of dopaminergic agonists in the development of ICD (Corvol et al., 2018; Cossu et al., 2018; Maloney et al., 2017; Voon et al., 2017; Weintraub et al., 2010; Weintraub and Claassen, 2017) remains controversial, our results suggest that there is a dose-response effect whereby a low dose leads to a marginal increase of motor impulsivity in 3sf whereas a higher dose induces a consistent increment in motor impulsivity and delay intolerance.

Our results are in keeping with previous studies that described an increase in probabilistic discounting under chronic treatment with PPX in the same animal model as that used in this study (Holtz et al., 2016; Sandra L. Rokosik and Napier, 2012) as well as an increase in motor impulsivity under acute (Engeln et al., 2016) and chronic (Jiménez-Urbieta et al., 2019) administration of PPX in animals with bilateral depletion induced by A53T α-synuclein. Interestingly, in sham rats the administration of 0.25 mg/kg PPX induced an increase in PMR 3sf, and 3 mg/kg of PPX enhanced motor impulsivity (3si and 3sf blocks) but did not increase delay intolerance in the 6-12s block, indicating that a high dose of dopaminergic agonists may promote abnormal impulsivity even in a normal nigrostriatal system, as observed in subjects without PD receiving high doses of dopaminergic agonists (Cornelius et al., 2010; Holman, 2009). Besides, as delay intolerance appeared only in 6-OHDA+PPX animals, this behaviour must be due to the interaction between the dose of PPX and the dopaminergic lesion and mimic the intolerance for delayed gratification expressed in PD patients with ICD (Housden et al., 2010; Leroi et al., 2013; Voon et al., 2010).

Importantly, the impulsivity elicited by PPX in the present study was dissociated from its antiparkinsonian effect as both doses of PPX effectively and similarly reversed forelimb akinesia in 6-OHDA rats comparing pretreatment with both the 0.25 mg/kg and 3 mg/kg conditions. Moreover, the 3mg/kg dose had a residual effect on impulsivity but not on the parkinsonian state (bradykinesia). This indicates that once impulsivity is triggered by high doses of PPX, such behaviour may be maintained as a trait, in keeping with observations of PD patients in whom ICD does not mirror the antiparkinsonian benefit of the drug (Bastiaens et al., 2013).

On the other hand, we found a positive correlation between impulsivity induced by the dopaminergic lesion and both PMR 6-12s block (delay intolerance) and total impulsivity after the 3 mg/kg PPX dose administration. This resembles PD where it is known that an impulsivity

trait before dopaminergic treatment predisposes patients to develop ICD after exposure to dopaminergic agonists (Weintraub et al., 2010). Moreover, the presurgery PMR 6-12s value also correlated with PMR 3si after the administration of 3 mg/kg PPX, in keeping with the fact that subjects with impulsivity trait before the development of PD are more prone to develop ICD. The determination of basal individual differences among animals should be considered in future studies so as to detect a subset of rats that might be more likely to demonstrate increased impulsive behaviour after PPX administration.

Although ICD in PD is mostly related to dopaminergic agonists (Weintraub et al., 2010), they can also be triggered by levodopa (Ballivet et al., 1973; Barbosa et al., 2018; Molina et al., 2000; Weintraub et al., 2015). The VDS paradigm has previously been used to test the behavioural effects of acute and chronic levodopa treatment in rats with bilateral 6-OHDA injections in the ventral tegmental area (VTA) or the substantia nigra (SN) (Carvalho et al., 2017). A slight increment in impulsive behaviour in long delay trials (6-12s block) was found in 6-OHDA rats only after chronic L-dopa administration. Thus, despite the different topology of dopaminergic depletion in animal models, our results support the idea that dopaminergic agonists play a predominant role in the development of ICDs and further support the utility of the VDS paradigm for testing impulsivity caused by dopaminergic agents.

Finally, compulsive-like behaviour was not observed in the 6-OHDA animals either before or after PPX treatment. Note that a rat model of PD parkinsonism with a SN/VTA lesion showed compulsive behaviour after chronic treatment with 0.3 mg/kg PPX in the post-training signal attenuation (PTSA) task, which specifically measures compulsive-like behaviour (D. Dardou et al., 2017). While this indicates that low doses of PPX may prompt compulsive-like behaviours, the lack of effects in our model may be due to the acute administration paradigm we employed or to the topography of the lesion, which did not include the VTA. Further studies should be carried out to resolve this issue.

In summary, we describe a rat model resembling several aspects of abnormal impulsivity in PD. Our model supports the contention that impulsivity in PD is linked to dopaminergic depletion that includes dorsal striatum and to PPX dosage: 1) the presence of a moderate dorsolateral dopaminergic lesion, which is typical of patients with early PD; 2) treatment with the dopaminergic agonist PPX induced motor impulsivity and intolerance for delayed gratification in a dose-dependent manner, specially in a subset of animals and 3) this dose-dependent effect continued after the dopaminergic drug was suppressed but was dissociated from the motor benefit. Taken together, these features make the present animal model of PD and PPX-induced impulsivity a useful tool to study the pathological mechanisms underlying the development of impulsivity in early PD.

Experiment 3

In this experiment, the VDS paradigm was used to analyse the impulsivity and compulsivity of rats with bilateral dopaminergic depletion induced by A53T-hα-syn overexpression in the SNc that are chronically administered PPX (0.25 mg/kg).

1. Specific methodology

1.1. Experimental design

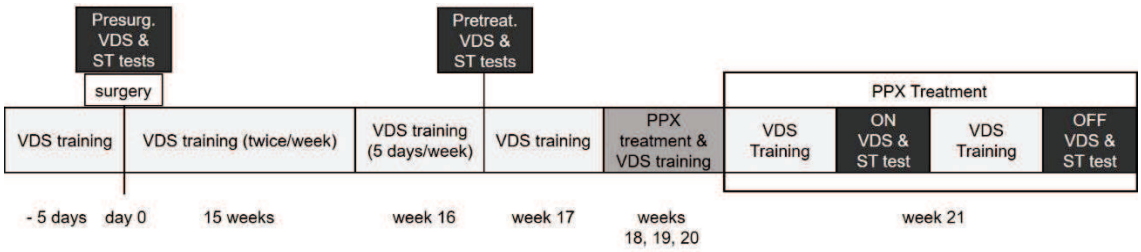

Figure 33. Experimental design. Abbreviations: VDS, variable delay-to-signal task; Presurg, presurgery; ST, adjusting steppings test; Pretreat, pretreatment; PPX, pramipexole; ON, ON medication state; OFF, OFF medication state.

Rats were habituated to handling on three consecutive days to handling and they were food deprived ato 10g/rat/day for three days until they reached approximately 90% of their free-feeding body weight (Figure 33). The ratsn, they were habituated to the operant chambers and trained ion the VDS task twice a day fover 5 days, as previously described previously (see experiment 2). The following day, presurgery values were obtained for for the adjusting stepsping test and for in the VDS paradigm were obtained (Figure 33). Then, animals were then randomly assigned into either the Control (AVV-GFP; n=5) or Lesion (AVV-A53T-hα-syn; n=17) groups and usubjected nderwento stereotactic surgery. Afterwards Subsequently, and until the week 15 post-surgery, the rats were retrained in the VDS task twice a weekly and on. At the week 16 post-surgery, they were retrained for 5 days and tested (pretreatment values:) (Figure 33). Then, rats where then trained again for 5 days (week 17 post-surgery) and all the animals underwent pharmacological treatment with PPX (0.25 mg/kg/day) over. The following weeks (weeks 18-20 after surgery), all the animals underwent pharmacological treatment with 0.25 mg/kg/day PPX, which was administered in the afternoon after the VDS training sessions. The On week 21 post-surgery, the animals were treated with PPX and tested once in either the ON or OFF medication states (ON: Tuesday; OFF: Thursday) (Figure 33). Standard training sessions were included between these tests to avoid the habituation of the rats to testing. PPX was administered 1 h before the testing in the ON session, and right after the test in the OFF session.

Hence, the VDS testing was performed at presurgery, 16 weeks after surgery (pretreatment), and in the ON and OFF medication states relative after the PPX chronic PPX treatment (Figure 33). Bradykinesia was assessed with with the adjusting stepping steps test each day of behavioural testing, always after the completion of the VDS test. Finally, the animals were sacrificed in OFF medication three days later than the last VDS session by intracardial perfusion of saline solution followed by 4% paraformaldehyde, and the brain tissue was obtained for immunohistochemical analysis (Figure 33).

1.2. Viral vectors

The concentrations of AAV-GFP viral particles was ere 6.7 x 1013 gcp/ml for AAV-GFP and of AAV-A53T-hα-syn 9,.6 x 1012 gcp/ml for AAV-A53T-hα-syn.

1.3. Fixed tissue colection

Rats were anesthesizedanesthetized with a mixture of oxygen and isoflurane (5%), and they were then prerfused transcardially with 4% paraformaldehyde (PFA). The rat's brain was removed and post-fixed again in the same fixativePFA for 24 hours, and then cryoprotected in 30% sucrose. Serial coronal sections (40 µm thick) were obtained on a freezing microtome (SM2010R, Leica Biosystems, Nussloch, Germany) and stored at -20 ºC in a cryoprotectant solution (see annex I).

1.4. Immunochemistry

Immunohistochemistry was performed on coronal sections that contained ing the striatum to study the distribution of detect both DAT and FosB/ΔFosB.

In detail, the tissue was first washed in 0.1M PBS and subsequently then incubated in 3% H2O2 (Sigma-Aldrich, St. Louis, MO) to block the endogenous peroxidases. and After, in a blocking non-specific binding in a solution (4% normal rabbit serum solution; (Vector Laboratories, Burlingame, CA). Aftherwards, sections were incubated overnight at room temperature (RT) with one of the following primary antibodies: goat anti-DAT (1:100; sc-1433, Santa Cruz Biotechnology, Inc. Dallas, TX); or and rabbit anti-FosB (1:500; sc-48, Santa Cruz Biotechnology, Inc. Dallas, TX), which that detects both Fos B and ΔFosB. The DAT labelled sections were then incubated for 1h at RT with a biotinylated rabbit anti-goat secondary antibody (1/100; Vector Laboratories, Burlingame, CA) and then for 1h at room temperature for llowed by a 1h incubation with an avidin-biotin-peroxidase complex (1:100; Vectastain ABC kit, Vector Laboratories, Burlingame, CA), visualizing a. ntibody binding Signal was revealed with a

3-3'-diaminobenzidine (DAB)/H_2O_2 solution (Sigma-Aldrich, St. Louis, MO). For FosB/ΔFosB labelling, the sections were incubated with the HRP-labelled polymer anti-rabbit kit and revisualizealed following the manufacturer instructions (DAKO Envision Kit, Agilent technologies, Santa Clara, CA). FinallyAfterwards, all the sections were mounted onto slides, air-dried overnight, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped with Eukitt mounting medium (Sigma-Aldrich, St. Louis, MO).

1.5. Quantification of striatal DAT and FosB/ΔFosB immunoreactivity

Microphotographs of striatal sections immunostained for DAT (n=9 sections/animal, 1x objective) or FosB/ΔFosB (n=6 sections/animal, 10x objective) sections were obtained on a Nikon Eclipse 801 microscope (Nikon Corporation, Tokyo) (DAT n=9 sections/animal, 1x objective; FosB/ΔFosB n=6 sections/animal, 10x objective). The O.D. values of DAT immunoreactivity in 4 sub-regions of the caudate putamen (CPu) was obtained using ImageJ (National Institute of Health, NIH), as described previously (see experiment 1): dorsolateral (DL), Ventrolateral (VL), Dorsomedial (DM) and Ventromedial (VM). The number of FosB/ΔFosB immunoreactive neurons in the same regions in the CPu regions and in the NAc (core and shell) was quantified using an automatic triangle thresholding method in ImageJ. A region of constant size (0.56 mm^2) was used as a reference area (see experiment 1).

1.6. Variables and statistical analysis

All the statistical analyses were carried out using SigmaStat software (version 3.5, SPSS, Inc., Chicago, IL), assessing normality with a Kolmogorov-Smirnov's test and variance equality with a Levene's test. Non-parametric statistical analyses were performed for all the behavioural and histological variables to determine the differences within the groups for each condition (presurgery, pretreatment, week 4 ON medication state and week 4 OFF medication state) and between the groups. Statistical significance was set at $p<0.05$.

The differences in the number of adjusting steps between the control and lesioned animals were determined by Friedman repeated measures ANOVA followed by a Student-Newman-Keuls *post hoc* test for multiple comparisons. Differences between the groups for each condition were set by a Mann-Whitney U test. As in experiment 2, the variables analysed from the VDS test were the PMR (total PMR, PMR 3si rate, PMR 6-12s, PMR 3sf rate), perseverative responses and the percentage omissions. For these measurements, the group of lesioned rats was considered as a unique group or it was divided into two groups based on their premature response in the ON state after chronic PPX treatment. Thus, the quartile of rats with a higher

tendency towards prematurity within the test session in the ON medication state were grouped as the high impulsivity lesioned group (n=4) or low impulsivity lesioned group (n=13). The differences within each group (Control, Lesioned, High impulsivity lesioned or Low impulsivity lesioned) were analysed with a Friedman repeated measures ANOVA followed by Student-Newman-Keuls *post hoc* test for multiple comparisons. For each condition (between groups analysis), differences between the Control and Lesioned groups were analysed using a Mann-Whitney's U test. The variables total PMR, PMR 3si rate, PMR 6-12s and PMR 3sf rate were compared between the controls and each of the lesioned subgroups, avoiding statistical bias produced by quartile division using a Mann-Whitney's U test. For perseverative responses and the percentage omissions, the changes between the control, high impulsivity and low impulsivity lesioned groups were analysed by a Kruskal-Wallis ANOVA followed by Dunn's *post-hoc* test for multiple comparisons. The O.D. values of DAT expression and the number of FosB/ΔFosB positive nuclei in each striatal region were compared between the control and lesioned animals using a Mann Whitney's U test. A correlation analysis between the variables was performed using a non-parametric Spearman test.

2. Results

2.1. Motor impairment and antiparkinsonian effect of PPX

Lesioned animals undertook significantly fewer adjusting steps relative to the control animals ($p<0.001$) and with respect to their presurgery values ($p<0.05$) from week 5 and 8, respectively (Figure 34A). By contrast, the lesioned rats undertook more adjusting steps in the ON medication state than in the OFF medication state ($p<0.05$) or pretreatment ($p<0.05$, Figure 34B). No changes were observed over time in the control animals (Figure 34A-B).

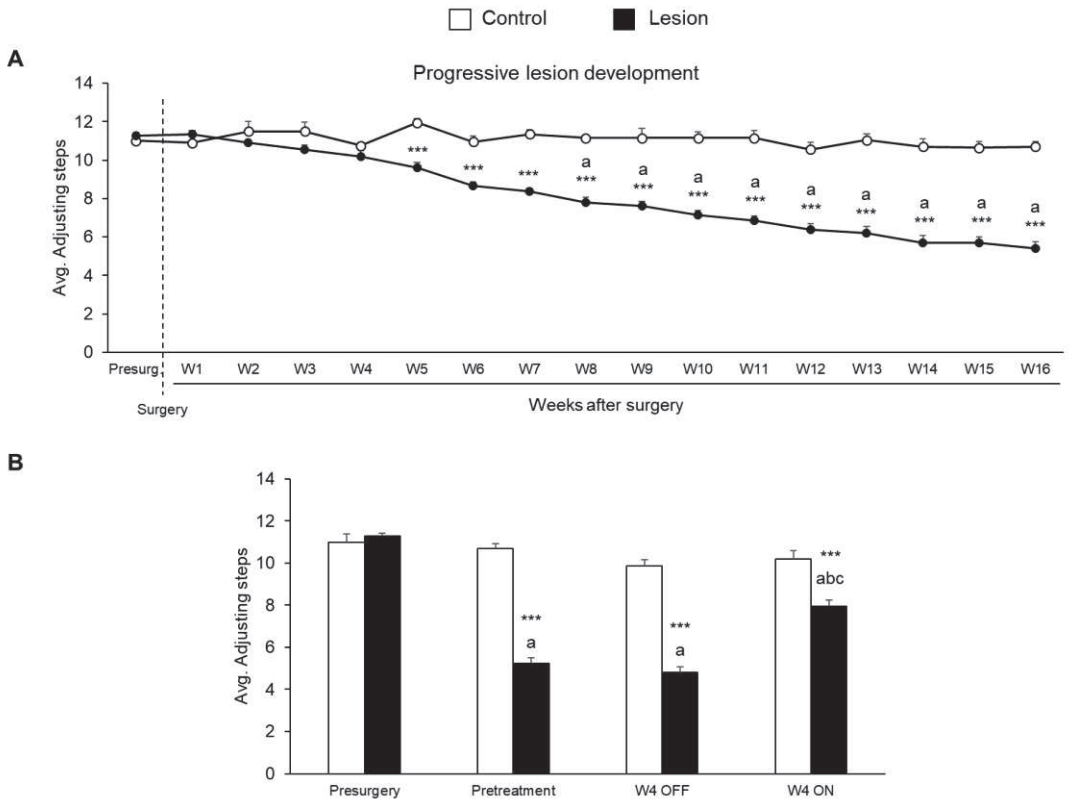

Figure 34. Progressive motor impairment caused by the over-expression of A53T-hα-synuclein over 16 weeks (A) and the anti-parkinsonian effect of PPX chronic treatment (4 weeks, B): ***p<0.001 vs Control; [a]p<0.05 vs presurgery; [b]p<0.05 vs pretreatment; [c]p<0.05 vs W4 OFF. Abbreviations: Presurg, presurgery; W4, week 4 of chronic treatment; OFF, OFF medication state; ON, ON medication state.

2.2. Changes in the VDS parameters after dopaminergic lesion and PPX chronic treatment

2.2.1. Effects on total PMR

Lesioned rats showed an increase in the total PMR pretreatment with respect to presurgery ($p<0.05$, Figure 35A and table 7), which was higher in the ON medication state ($p<0.05$) than in the OFF medication state ($p<0.05$, Figure 35A and table 7). The number of PMRs in the ON medication state appeared to be higher in the lesioned animals than in the control animals, although this difference was not statistically significant ($p=0.583$). In the OFF medication state, both the lesioned and control rats had a lower total PMR than in presurgery ($p<0.05$) and pretreatment ($p<0.05$; Figure 35A and table 7).

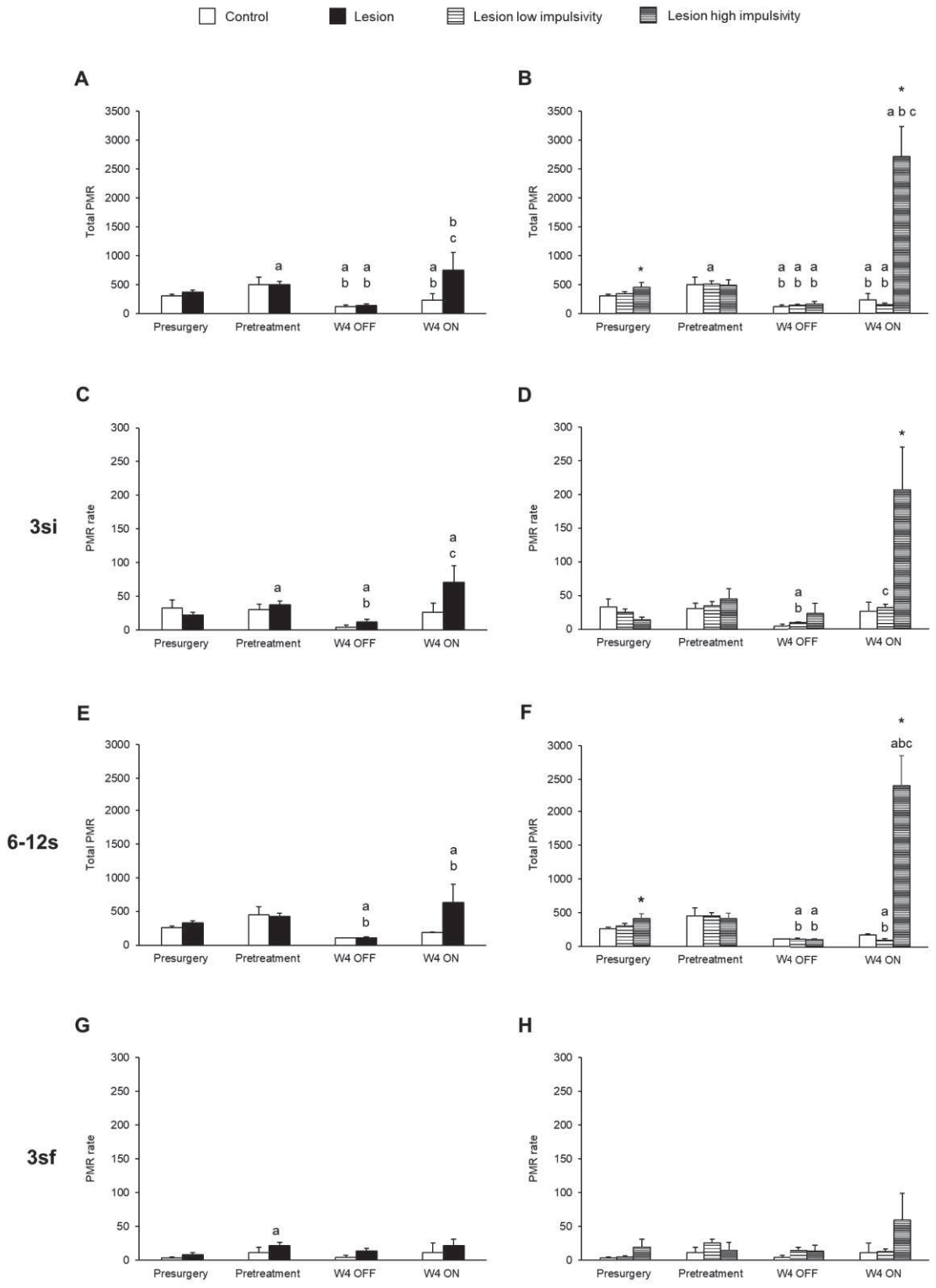

Figure 35. Effect of dopaminergic depletion and chronic PPX administration of PPX on the total premature response (PMR) (A-B), PMR 3si rate (C-D), PMR 6-12s (E-F), PMR 3sf rate (G-F). The results are shown for the lLesioned group (A, C, E, G) and for the lLesioned animals according to divided by their tendency towards a premature response ding in the ON medication state after PPX chronic treatment (B, D, F, H):. *p<0.05 vs Control; ap<0.05 vs presurgery; bp<0.05 vs pretreatment; cp<0.05 vs OFF medication state. Abbreviations: OFF, OFF medication state; ON, ON medication state.

Table 7: Summary of statistical findings in behavioural measurements.

Statistical test	Comparisons	Measurement	Statistic	p	Degrees of freedom
Mann-Whitney U test	Control vs Lesion: presurgery	PMR	U=57.000	>0.05	
		PMR 3si rate	U=35.000	>0.05	
		PMR 6-12s	U=57.000	>0.05	
		PMR 3sf rate	U=50.550	>0.05	
	Control vs Lesion: pretreatment	PMR	U=49.000	>0.05	
		PMR 3si rate	U=52.000	>0.05	
		PMR 6-12s	U=49.000	>0.05	
		PMR 3sf rate	U=57.500	>0.05	
	Control vs Lesion: week 4 OFF	PMR	U=38.000	>0.05	
		PMR 3si rate	U=57.500	>0.05	
		PMR 6-12s	U=38.000	>0.05	
		PMR 3sf rate	U=63.000	>0.05	
	Control vs Lesion: week 4 ON	PMR	U=48.000	>0.05	
		PMR 3si rate	U=61.000	>0.05	
		PMR 6-12s	U=48.000	>0.05	
		PMR 3sf rate	U=32.000	>0.05	
	Control vs Lesion low impulsivity: presurgery	PMR	U=37.000	>0.05	
		PMR 3si rate	U=27.500	>0.05	
		PMR 6-12s	U=37.000	>0.05	
		PMR 3sf rate	U=38.500	>0.05	
	Control vs Lesion low impulsivity: pretreatment	PMR	U=39.5	>0.05	
		PMR 3si rate	U=35.000	>0.05	
		PMR 6-12s	U=37.000	>0.05	
		PMR 3sf rate	U=47.500	>0.05	
	Control vs Lesion low impulsivity: week 4 OFF	PMR	U=34.000	>0.05	
		PMR 3si rate	U=41.000	>0.05	
		PMR 6-12s	U=27.000	>0.05	
		PMR 3sf rate	U=48.500	>0.05	

Statistical test	Comparisons	Measurement	Statistic	p	Degrees of freedom
Mann-Whitney U test	Control vs Lesion low impulsivity: week 4 ON	PMR	U=30.000	<0.05*	
		PMR 3si rate	U=41.000	>0.05	
		PMR 6-12s	U=28.000	>0.05	
		PMR 3sf rate	U=22.000	>0.05	
	Control vs Lesion high impulsivity: presurgery	PMR	U=0.000	<0.05*	
		PMR 3si rate	U=15.500	>0.05	
		PMR 6-12s	U=0.000	<0.05*	
		PMR 3sf rate	U=8.000	>0.05	
	Control vs Lesion high impulsivity: pretreatment	PMR	U=8.000	>0.05	
		PMR 3si rate	U=7.000	>0.05	
		PMR 6-12s	U=8.000	>0.05	
		PMR 3sf rate	U=10.000	>0.05	
	Control vs Lesion high impulsivity: week 4 OFF	PMR	U=8.000	>0.05	
		PMR 3si rate	U=7.500	>0.05	
		PMR 6-12s	U=9.000	>0.05	
		PMR 3sf rate	U=5.500	>0.05	
	Control vs Lesion high impulsivity: week 4 ON	PMR	U=0.000	<0.05*	
		PMR 3si rate	U=1.000	<0.05*	
		PMR 6-12s	U=0.000	>0.05	
		PMR 3sf rate	U=5.000	>0.05	
Friedman repeated measures ANOVA followed by Tukey's post hoc test	Control through time-points	PMR	χ^2=9.367	<0.05*	3
		PMR 3si rate	χ^2=6.840	>0.05	3
		PMR 6-12s	χ^2=6.120	>0.05	3
		PMR 3sf rate	χ^2=4.467	>0.05	3
	Lesion through time-points	PMR	χ^2=17.329	<0.001***	3
		PMR 3si rate	χ^2=18.176	<0.001***	3
		PMR 6-12s	χ^2=17.329	<0.001***	3
		PMR 3sf rate	χ^2=9.761	<0.05*	3

Statistical test	Comparisons	Measurement	Statistic	p	Degrees of freedom
Friedman repeated measures ANOVA followed by Tukey's post hoc test	Lesion low impulsivity through time-points	PMR	χ^2=20.354	<0.001***	3
		PMR 3si rate	χ^2=12.400	<0.01**	3
		PMR 6-12s	χ^2=22.015	<0.001***	3
		PMR 3sf rate	χ^2=7.955	<0.05*	3
	Lesion high impulsivity through time-points	PMR	χ^2=11.100	<0.001***	3
		PMR 3si rate	χ^2=8.100	<0.05*	3
		PMR 6-12s	χ^2=11.100	<0.001***	3
		PMR 3sf rate	χ^2=4.846	>0.05	3
Measurement	**Statistical test**	**Comparisons**	**Statistic**	**p**	**Degrees of freedom**
PSR	Mann-Whitney U test	Control vs Lesion: presurgery	U=34.500	>0.05	
		Control vs Lesion: pretreatment	U=44.500	>0.05	
		Control vs Lesion: week 4 OFF	U=40.500	>0.05	
		Control vs Lesion: week 4 ON	U=45.500	>0.05	
		Control vs Lesion low impulsivity: presurgery	U=26.000	>0.05	
		Control vs Lesion low impulsivity: pretreatment	U=35.500	>0.05	
		Control vs Lesion low impulsivity: week 4 OFF	U=33.500	>0.05	
		Control vs Lesion low impulsivity: week 4 ON	U=35.500	>0.05	
		Control vs Lesion high impulsivity: presurgery	U=11.500	>0.05	
		Control vs Lesion high impulsivity: pretreatment	U=11.000	>0.05	
		Control vs Lesion high impulsivity: week 4 OFF	U=13.000	>0.05	
		Control vs Lesion high impulsivity: week 4 ON	U=10.000	>0.05	
	Friedman repeated measures ANOVA followed by Tukey's post hoc test	Control through time-points	χ^2=1.898	>0.05	3
		Lesion through time-points	χ^2=24.800	<0.001***	3
		Lesion low impul. through time-points	χ^2=20.268	<0.001***	3
		Lesion high impul. through time-points	χ^2=5.526	>0.05	3

Measurement	Statistical test	Comparisons	Statistic	p	Degrees of freedom
% Omission	Mann-Whitney U test	Control vs Lesion: presurgery	U=42.500	>0.05	
		Control vs Lesion: pretreatment	U=41.000	>0.05	
		Control vs Lesion: week 4 OFF	U=32.500	>0.05	
		Control vs Lesion: week 4 ON	U=46.000	>0.05	
		Control vs Lesion low impulsivity: presurgery	U=32.000	>0.05	
		Control vs Lesion low impulsivity: pretreatment	U=31.000	>0.05	
		Control vs Lesion low impulsivity: week 4 OFF	U=26.500	>0.05	
		Control vs Lesion low impulsivity: week 4 ON	U=44.000	>0.05	
		Control vs Lesion high impulsivity: presurgery	U=9.500	>0.05	
		Control vs Lesion high impulsivity: pretreatment	U=10.000	>0.05	
		Control vs Lesion high impulsivity: week 4 OFF	U=14.000	>0.05	
		Control vs Lesion high impulsivity: week 4 ON	U=18.000	>0.05	
	Friedman repeated measures ANOVA followed by Tukey's post hoc test	Control through time-points	χ^2=7.727	>0.05	3
		Lesion through time-points	χ^2=20.761	<0.001***	3
		Lesion low imp. through time-points	χ^2=25.454	<0.001***	3
		Lesion high imp. through time-points	χ^2=3.000	>0.05	3

Lesioned animals were divided into two different groups based on their impulsivity in the ON medication state, yet no differences between presurgery and pretreatment were found between high impulsivity and low impulsivity lesioned animals (Figure 35B and table 7). After chronic administration of PPX, an increase in the total PMR was only evident in high impulsivity lesioned rats when ON medication relative to the pretreatment state ($p<0.05$; Figure 35B and table 7). Moreover, only high impulsivity lesioned rats had higher total PMR at presurgery ($p<0.05$) and in the ON medication state ($p<0.05$) compared to the control animals (Figure 35B and table 7). In the OFF medication state, the controls and both groups of lesioned animals had a lower total PMR than at presurgery ($p<0.05$) and pretreatment ($p<0.05$; Figure 35B and table 7).

2.2.2. Effects on PMR 3si rate

Lesion rats showed aThen increase in the rate of PMR 3si was higher rate at pretreatment in lesioned rats and in the ON medication state with respect to presurgery ($p<0.05$, Figure 35C), and with respect to Control the control animals, although in this latter case the difference did not reach statistical significance ($p=0.158$). In the OFF medication state, Lesion rats had a lower PMR 3si rate than in presurgery ($p<0.05$) and in the ON medication state ($p<0.05$; Figure 35A and table 7).

Neither high nor low impulsivity caused an increase of PMR 3si in the lesioned animals at pretreatment relative to presurgery (Figure 35D and table 7). There was only an increase in the PMR 3si rate of high impulsivity lesioned animals in the ON medication state compared to the control animals ($p<0.05$, Figure 35D table 7), which was not evident in the OFF medication state. In the OFF medication state, both high and low impulsivity lesioned animals had a significantly lower PMR 3si rate than at presurgery and pretreatment, although this phenomenon was only significant for low impulsivity lesioned animals ($p<0.05$; Figure 35D and table 7).

2.2.3. Effects on PMR 6-12s

There were no differences in the PMR 6-12s between pretreatment and presurgery lesioned animals (Figure 35E). However, after chronic PPX administration the PMR 6-12s increased in the ON medication state relative to pretreatment ($p<0.05$, Figure 35E and table 7) and apparently, with respect to the control animals although this latter difference did not reach statistically significance ($p=0.695$). There was a decrease in the PMR 6-12s in lesioned animals in the OFF medication state relative to pretreatment ($p<0.05$) and presurgery ($p<0.05$; Figure 35E and table 7).

There were no differences in PMR 6-12s pretreatment and presurgery between either high or low impulsivity lesioned animals (Figure 35F and table 7). However, chronic PPX administration produced a significant increase in the PMR 6-12s in high impulsivity lesioned animals in the ON medication state compared to pretreatment ($p<0.05$; Figure 35F and table 7). Relative to the control animals, only high impulsivity lesioned rats had a higher PMR 6-12s presurgery ($p<0.05$) and in the ON medication state ($p<0.05$; Figure 35F and table 7). In the OFF medication state, both high and low impulsivity lesioned animals had a significantly lower PMR3si rate than presurgery ($p<0.05$) and pretreatment ($p<0.05$; Figure 35D and table 7).

2.2.4. Effects on PMR 3sf

Lesioned animals had a higher PMR 3sf rate at pretreatment than presurgery ($p<0.05$; Figure 35G and table 7) and no significant changes were observed after chronic PPX administration in either the ON and OFF medication states (Figure 35G and table 7). Moreover, there were no differences evident between lesioned and control animals under any condition (Figure 35G and table 7), and no significant differences were observed between high and low impulsivity lesioned animals relative to the control rats under any condition (Figure 35H and table 7).

2.3. Effects on PSR

At pretreatment, the PSR was higher in lesioned animals relative to presurgery ($p<0.05$), yet no further differences were observed in either the ON or OFF medication states (Figure 36A and table 7). Likewise, there were no significant differences relative to the control animals under any conditions (Figure 36A and table 7). The high impulsivity lesioned animals didn't show significant changes in any condition (Figure 36B and table 7), while there was an increase in PSR at pretreatment and OFF medication in the low impulsivity lesioned rats relative to presurgery ($p<0.05$, Figure 36B and table 7), yet not in the ON medication state (Figure 36B and table 7). No differences were evident in the PSR between either high or low impulsivity lesioned animals and control rats under any condition (Figure 36B and table 7).

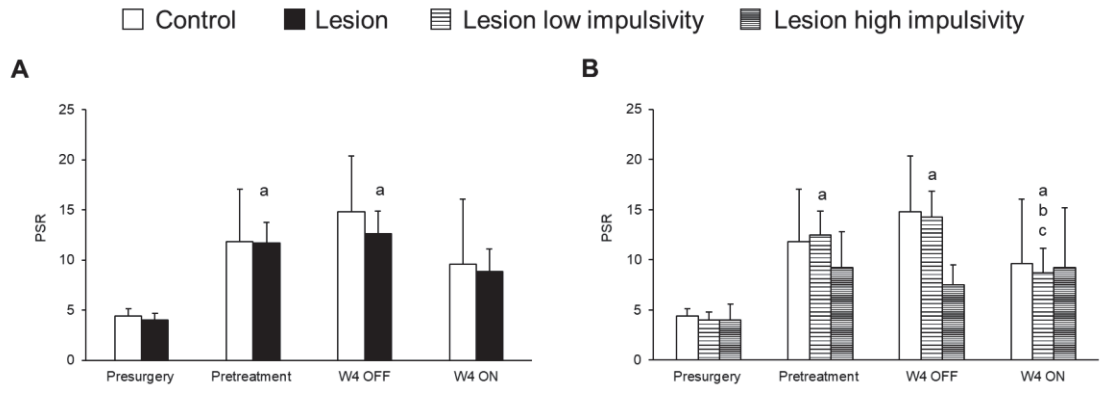

Figure 36. Effect of dopaminergic depletion and chronic administration of PPX on perseverative responses (PSR) in Lesion and Control animals. The results are shown for the total Lesion group (A) and Lesion animal divided by their tendency to premature responding under chronic PPX administration in ON medication state (B). [a]p<0.05 vs presurgery; [b]p<0.05 vs presurgery; [c]p<0.05 vs OFF.

2.4. Effects on omissions

Lesioned animals had a similar percentage of omissions at pretreatment as presurgery (Figure 37A and table 7) and while there was an increase in the ON medication state relative to pretreatment ($p<0.05$), this was not observed in the OFF medication state (Figure 37A and table 7). Moreover, there were no differences relative to the control rats under any condition (Figure 37A). There was a similar percentage of omission in high impulsivity lesioned rats under all conditions (Figure 37B), whereas low impulsivity lesioned rats displayed more omissions in the ON medication state than pretreatment ($p<0.05$; Figure37B and table 7) and than high impulsivity lesioned rats, although these differences were not observed in the OFF medication state. No differences were observed between either high and low impulsivity lesioned rats with respect to the control animals under any condition (Figure 37B and table 7).

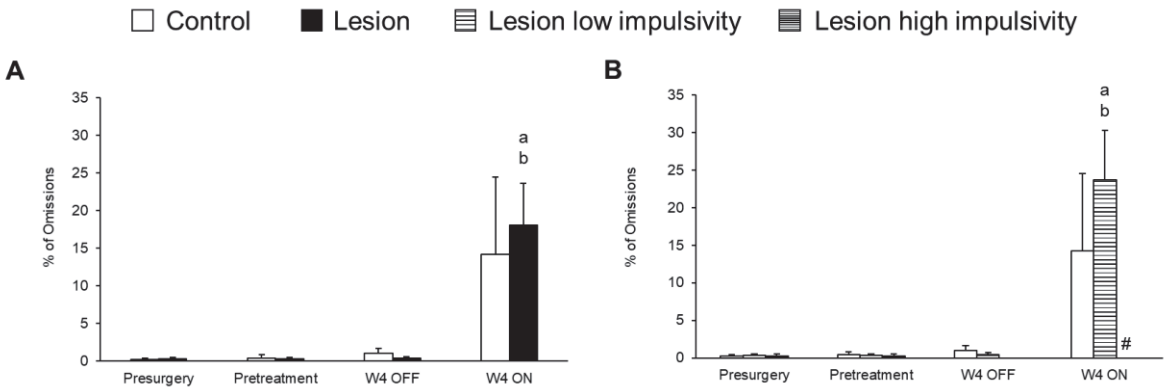

Figure 37. Percentage omissions after dopaminergic depletion and either acute or chronic PPX administration in lesioned or control animals. The percentage omissions are shown for the whole lesioned group (A) and for the lesioned animals relative to their impulsive status in the ON medication state (B): [a]p<0.05 vs presurgery; [b]p<0.05 vs pretreatment; [#]p<0.05 vs lesion low-impulsivity.

2.5. Histology

There were lower O.D. values for DAT expression in lesioned rats both in the right (44.72%, $p<0.01$) and left (59.37%, $p<0.05$) CPu with respect to the control rats. This decrease was evident in the four CPu regions (VL, VM, DM and DL), yet not in the NAc core and shell regions (Figure 38B and table 8). No significant differences were observed between high and low impulsivity lesioned rats in any striatal region. Similarly, there were no significant differences in the number of FosB/ΔFosB positive neurons between control and lesioned animals in any striatal region (Figure 39B and table 8). Likewise, no significant differences were observed between high and low impulsivity lesioned rats in any striatal region.

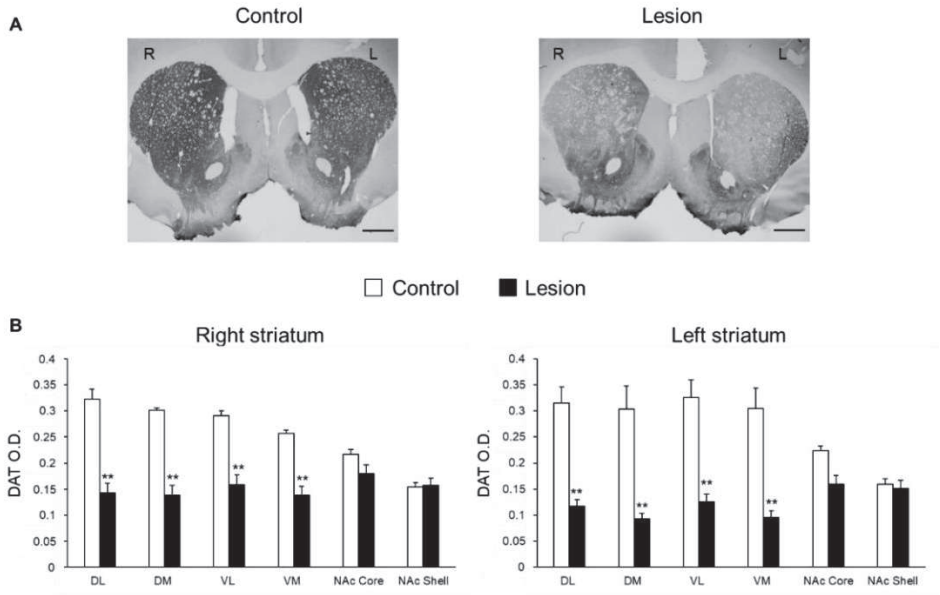

Figure 38. Expression of the dopamine transporter (DAT) in the striatum. (A) Representative photomicrographs of striatal DAT labelling in control and lesioned animals (scale bars, 1 mm). (B) Optical density (O.D.) values of DAT staining in the striatal sub-regions of both hemispheres in control and lesioned animals: *p<0.05, **p<0.01 vs Control. Abbreviations: R, right hemisphere; L, left hemisphere; DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial.

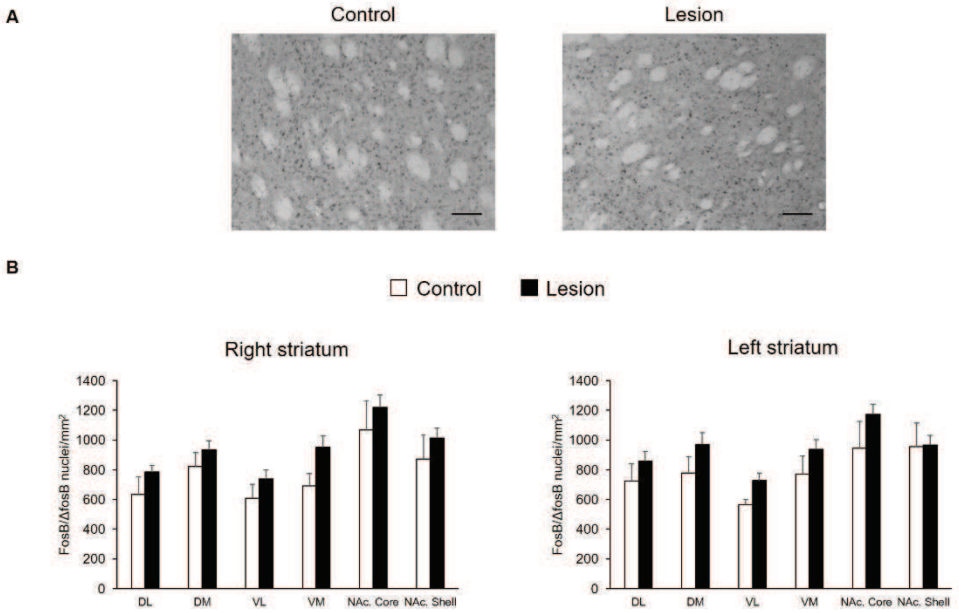

Figure 39. Quantification of FosB/ΔFosB labelled neurons in the striatum. (A) Representative photomicrographs of FosB/ΔFosB immunostaining in the left ventromedial (VM) region of the striatum of control and lesioned animals (scale bars, 100 µm). (B) Quantification of FosB/ΔFosB immunoreactive nuclear profiles (number of nuclear profiles/mm^2) in the different sub-regions of the Caudate Putamen (CPu) and Nucleus accumbens (NAc) in control and lesioned rats. Abbreviations: DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial; Core, NAc core; Shell, NAc shell.

Table 8: Summary of statistical analyses of striatal DAT and FosB/ΔFosB expression. Note: only differences between control and lesion rats are shown.

Study	Measurement	Statistical test	Comparison	Statistics	p
DAT O.D. values	whole CPu	Mann-Whitney U test	Control vs Lesion	U=11	<0.05*
	right whole CPu			U=7	<0.01**
	left whole CPu			U=12	<0.05*
	right DL CPu			U=12	<0.05*
	right DM CPu			U=4	<0.01**
	right VL CPu			U=14	<0.05*
	right VM Cpu			U=7	<0.01**
	left DL CPu			U=11	<0.05*
	left DM CPu			U=11	<0.05*
	left VL CPu			U=12	<0.05*
	left VM Cpu			U=9	<0.05*
FosB/ΔFosB nuclei/mm^2	Whole Cpu	Mann-Whitney U test	Control vs Lesion	U=53	>0.05
	Whole Nac			U=44	>0.05
	right DL CPu			U=56	>0.05
	right DM CPu			U=49	>0.05
	right VL CPu			U=49.5	>0.05
	right VM Cpu			U=58	>0.05
	left DL CPu			U=48	>0.05
	left DM CPu			U=51	>0.05
	left VL CPu			U=56	>0.05
	left VM Cpu			U=50	>0.05
	right NAc core			U=41	>0.05
	right NAc shell			U=46	>0.05
	left NAc core			U=49	>0.05
	left NAc shell			U=35	>0.05

2.6. Correlation studies

Taking into consideration the lesioned rats as a whole or as low and high impulsivity rats separately, there were no significant correlations between any PMR measure (total, PMR 3si rate, PMR 6-12s, PMR 3sf rate) under any condition, or between these behavioural values and the histological striatal biomarkers DAT or FosB/ΔFosB.

3. Discussion

We describe here how progressive bilateral dopaminergic depletion, induced by overexpressing of A53T-hα-syn within the SNc, causes progressive motor impairment, together with an increase of motor impulsivity (PMR i3si and 3sf rates) and compulsive-like behaviour (PSR) in the VDS paradigm with respect to the basal values. When these animals are treated chronically with PPX, they showed both motor impulsivity and delay intolerance (PMR 6-12s) in the ON medication state. Moreover, two distinct behavioural phenotypes were observed under the effect of PPX, with a subset of lesioned rats showing a clear increase in motor impulsivity (PMR 3si rate) and delay intolerance (PMR 6-12s -high impulsivity) and others showing almost no change with respect to the pretreatment values or control animals (low impulsivity lesioned rats). This outcome resembles the situation in PD patients, as only around 15% of PD patients treated with dopaminergic agents develop one or more ICD (Eisinger et al., 2019; Weintraub et al., 2010), suggesting that the model tested here could reflect the clinical scenario.

Relative to non-ICD PD subjects receiving similar dopaminergic treatments, PD patients with ICDs show increased sensitivity to risky choices, independent of the effect of loss aversion (Voon et al., 2011a) and more impulsive choices coupled with a lower tolerance to delayed gratification (Housden et al., 2010; Leroi et al., 2013; Voon et al., 2010). In addition, a pre-PD history of ICD and an impulsive personality trait are well known risk factors for abnormal impulsive behaviours in response to dopaminergic treatment after PD diagnose (Weintraub and Claassen, 2017). Similarly, high impulsivity lesioned rats displayed more impulsive responses in the 6-12s block than control rats in the basal state. This interesting result suggests that animals with pre-parkinsonism delay intolerance have a higher risk of developing treatment-induced impulsivity and thus, that this could be used as a marker to identify particularly vulnerable subjects.

The significant reduction in the PMR observed in the OFF medication state in both lesioned and control animals could be due to the adaptation of animals to the task, since they were trained throughout the weeks of PPX administration. However, this effect was not observed in the ON medication state indicating that PPX governs the animals' behaviour when under its effect. This outcome contradicts the results obtained when originally developing the VDS paradigm, where it appeared that the task is resistant to multiple testing, making it particularly suitable for longitudinal assays (Leite-Almeida et al., 2013). Indeed, this phenomenon may be exclusive to this experiment since task habituation was not observed within the first experiment performed as part of this thesis using the 5-CSRTT paradigm and several test sessions. However, the VDS represents a much easier task in terms of attentional

demand than the 5-CSRTT and thus, it can be used to avoid confounding behavioural factors (such as more omissions). Indeed, the differences in the nature of the tasks probably allows the animals to habituate more readily to the VDS testing conditions, a facet that should be taken into account in future studies employing the VDS. Therefore, this fact should be taking into account for future studies employing the VDS.

Regarding the histological markers, previous results from animal models suggest that the FosB/ΔFosB transcription factors play an important a role in PPX self-administration (Engeln et al., 2013a) and PPX-induced CPP (Loiodice et al., 2017) in parkinsonian rats. We observed enhanced FosB/ΔFosB expression in lesioned animals relative to the control rats after 4 weeks of chronic PPX administration (0.25 mg/kg/day) in the same animal model, although this expression was not correlated with waiting impulsivity in the 5-CSRTT paradigm (experiment 1). By contrast, there were no significant changes in striatal FosB/ΔFosB expression in lesioned rats here, not even when considering low and high impulsivity animals separately. Thus, while awaiting confirmation, striatal FosB/ΔFosB does not appear to be an optimal biomarker of abnormal impulsivity in animal models of PD.

In terms of DAT expression, no correlation was detected here between dopaminergic depletion and impulsivity, in contrast to the inverse correlation found between striatal DAT expression and waiting impulsivity in experiment 1 in the same animal model. This difference could be due to at least two factors. Firstly, in experiment 1 the average value of three measurements was considered for each condition per week (i.e.: 3 days in the ON and 3 days in the OFF medication state), which dramatically reduces the variability in the data and permits statistically significant relationships to be more readily identified. Secondly, the 5-CSRTT and the VDS measure different impulsivity traits, and the pattern of dopaminergic depletion could be related distinctly to the emergence of each trait. Further experiments addressing these issues will help to understand the differences underlying these two studies.

In summary, the animal model of progressive Parkinsonism studied here and the study of chronic PPX treatment in the VDS paradigm reproduces some features of the clinical presentation of ICD in PD. Indeed, chronic treatment with a dopaminergic agonist causes an increase of impulsivity in a subset of lesioned animals (high impulsivity), which show a pre-parkinsonian impulsive status (presurgery condition), although this is not correlated with dopaminergic depletion. The differences with previous experiments performed in this thesis, and in particular with the first experiment (that used the same lesion-type and where animals followed the same PPX administration protocol) could reflect at least two factors. First, while the impulsivity traits analysed in the two experiments might share some features, they may have a different nature, driven by different brain structures and circuits. Alternatively, in the first

experiment average values from three days were considered for each condition (i.e.: ON and OFF medication), as opposed to just one in the third experiment, which may dramatically reduce the variability in the data, and make it easier to detect subtle relationships between different histological markers and behaviours. Therefore, future studies on larger numbers of animals or performing more tests for each condition might shed light on this issue.

Thus, the use of the present animal model of parkinsonism and PPX treatment protocol and VDS as the reference paradigm may represent a useful tool to investigate pathological changes underlying the emergence of ICD in PD and and aid the search for optimal biomarkers.

VI. General discussion

In this doctoral thesis, we set out to define an animal model that recapitulates the clinical features of PD patients with ICD, by subjecting different animal models of parkinsonism to different regimes of acute and chronic PPX administration, and testing these animals in two behavioural paradigms to measure impulsivity. Thus, we undertook three studies: 1) an animal model of progressive parkinsonism induced by A53T-hα-syn overexpression in the SNc was subjected to chronic low dose of PPX (0.25 mg/kg) and the impulsive behaviour induced was measured in the 5-CSRTT paradigm; 2) an animal model of bilateral acute parkinsonism induced by 6-OHDA injection into the DL striatum was subjected to acute PPX treatment at low and high doses (0.25 mg/kg and 3 mg/kg) and behaviour was measured in a VDS paradigm; 3) an animal model of progressive parkinsonism induced by over-expression of A53T-hα-syn in the SNc was subjected to chronic low dose of PPX (0.25 mg/kg) and their impulsive behaviour was measured in a VDS paradigm. These models present some common features but also some differences, which are summarized in table 9, and all these outcomes will be discussed below.

1. Dopaminergic denervation and impulsivity

An enhancement of subtle impulsive traits has been reported in untreated PD patients relative to healthy controls (Al-Khaled et al., 2015; de Rezende Costa et al., 2016; Milenkova et al., 2011; Stenberg, 2016), suggesting that the loss of dopaminergic tone itself may increase impulsivity. However, other studies report no remarkable presence of pathological impulsivity in drug-naïve PD patients (Ryu et al., 2019) or report similar ICD frequencies to those of general population (Antonini et al., 2011). We found here that rats with a partial dopaminergic depletion (50-65%) in the CPu induced by A53T-hα-syn display enhanced impulsive behaviour in both the 5-CSRTT (Experiment 1) and VDS (Experiment 3) paradigms, consistent with previous studies showing increased impulsivity in the same animal model (Engeln et al., 2016). By contrast, partial striatal lesions (20% loss) mostly restricted to the DL (motor) striatum (40-50% loss) induced by the neurotoxin 6-OHDA does not trigger impulsive behaviour (Experiment 2).

Table 9. Summary of the main results obtained in the three experiments. Abbreviations and symbols: n.s., no significant; ↑↑↑, significant increase; ↓↓↓, significant decrease.

		Experiment 1		Experiment 2		Experiment 3	
	Experimental Group	Control	Lesion	Sham	6-OHDA	Control	Lesion
	Impulsivity: presurgery	n.s.	n.s.	n.s.	↑↑↑motor (6-OHDA+PPX high impulsivity)	n.s.	↑↑↑delay intolerance (Lesion high impulsivity)
	Impulsivity: pretreatment	n.s.	↑↑↑ (Lesion high impulsivity)	n.s.	n.s.	n.s.	↑↑↑motor
Acute PPX	Impulsivity: 0.25 mg/kg ON state			↑↑↑ motor	↑↑↑ motor		
	Impulsivity: 0.25 mg/kg OFF state			n.s.	n.s.		
	Impulsivity: 3 mg/kg ON state			↑↑↑ motor	↑↑↑ motor, delay intolerance		
	Impulsivity: 3 mg/kg OFF state			n.s.	n.s.		
Chronic PPX	Impulsivity: 0.25 mg/kg ON state	n.s.	↑↑↑ (Lesion high impulsivity)			n.s.	↑↑↑ motor, delay intolerance (Lesion high impulsivity)
	Impulsivity: 0.25 mg/kg OFF state	n.s.	↑↑↑			↓↓↓ overall impulsivity	↓↓↓ overall impulsivity (both Lesion low and high impulsivity)
	Omissions: ON state	↑↑↑	↑↑↑	n.s.	n.s.	n.s.	↑↑↑ (Lesion low impulsivity)
	Compulsivity: ON state	n.s.	n.s.	n.s.	n.s.	n.s.	↑↑↑ (Lesion low impulsivity)
	Correlation: presurgery and pretreatment impulsivity	n.s.	n.s.	n.s.	Positive correlation	n.s.	n.s.
	Correlation: ON state and presurgery impulsivity	n.s.	n.s.	n.s.	Positive correlation (3 mg/kg)	n.s.	n.s.
	Correlation: ON state vs pretreatment impulsivity	n.s.	Positive correlation	n.s.	Positive correlation (3 mg/kg PPX)	n.s.	n.s.
	Correlation: impulsivity vs striatal DAT	n.s.	Negative correlation	n.s.	n.s.	n.s.	n.s.
	Correlation: impulsivity vs striatal FosB/ΔFosB	n.s.	n.s.			n.s.	n.s.

The impulsivity induced pretreatment in the α-syn overexpression model but not in the 6-OHDA rats suggests that the pattern and degree of striatal dopaminergic depletion may influence the development of an ICD. Thus, by comparing both patterns of CPu dopaminergic depletion, lesion of associative and limbic areas of the CPu together with the motor area, but not lesions restricted to the dorsal motor region (DL), may induce an impulsive behaviour even before the administration of dopaminergic agents. Moreover, and as observed in PD patients, the pattern of striatal dopaminergic depletion could influence the development of ICDs in PD after the administration of dopamine agonists. Indeed, cross-sectional neuroimaging studies showed that PD patients who develop ICDs had stronger denervation in the ventral striatum (Cilia et al., 2010; Navalpotro-Gomez et al., 2019) than patients without ICD. Moreover, longitudinal studies showed that drug-naive PD patients who developed ICD after dopaminergic treatment also had greater denervation across different striatal territories (ventral striatum and anterodorsal and posterior putamen (Vriend et al., 2014). Here, we found a negative correlation between the dopaminergic denervation in the whole striatum and the impulsivity triggered by chronic PPX (0.25 mg/kg) administration in the 5-CSRTT (Experiment 1), yet no correlation was found with the same animal model (Experiment 3) or in the 6-OHDA animals with a DL lesion in the striatum (motor striatum) (experiment 2), both using the VDS paradigm.

These differences may relay on different factors. First, the variability of the data within the experiment 1 is lower than in the experiment 3 as the average value of three measurements was considered for each condition per week in the former (i.e.: 3 days in the ON and 3 days in the OFF medication state). This restricted variability permits statistically significant relationships to be more readily identified. Second, the pattern of dopaminergic depletion in the experiments 1 and 3 is different than in the experiment 2, with the A53T-hα-syn model displaying a higher and widespread dopaminergic depletion than the 6-OHDA model. Importantly, this different topography and extension of the dopaminergic lesion could differently affect the behaviour of the animals. Third, the constructs of impulsivity measured by each task are different, as waiting impulsivity is detected in the 5-CSRTT (Robbins, 2002) whereas the impulsive outcome of the VDS is multidimensional, involving motor impulsivity and delayed intolerance (Leite-Almeida et al., 2013). Moreover, the cognitive requirements of both tasks, as well as the brain structures implicated in the emergence of impulsivity in each case, probably differ (Robbins et al., 2012).

2. Dopaminergic treatment and impulsivity

ICDs emerge in PD patients under dopaminergic treatment, mainly with dopaminergic agonists (Weintraub et al., 2010). In our experiments, impulsivity only increased in lesioned rats

under the effect of PPX and not in the OFF medication state. This finding resembles the marked increase in sensitivity of PD patients with ICD to risk in a gambling task (Voon et al., 2011a) and an increased impulsive choice only when they are under the effect of dopaminergic drugs (Leroi et al., 2013; Voon et al., 2010).

In addition, several studies have found that high doses of dopaminergic agonists are associated with an increased risk of developing ICDs in PD (Bastiaens et al., 2013; Joutsa et al., 2012; Lee et al., 2010; Limotai et al., 2012; Perez-Lloret et al., 2012; Weintraub et al., 2006). On the other hand, other studies have pointed out the importance of the daily dose of PPX (Grosset, 2008; Valença et al., 2013). Consistent with these findings, we observed that administering a chronic low dose (0.25 mg/kg, Experiments 1 and 3) or an acute high dose of PPX (3 mg/kg, (Experiment 2) triggers abnormal impulsivity in both animal models. Similarly, other studies in which 6-OHDA was used to lesion the rat DL striatum also highlighted the dose-dependent pro-impulsive potential of PPX after both acute and chronic administration (Holtz et al., 2016; Riddle et al., 2012). Hence, the dose and duration of treatment with dopamine agonists could be associated with the emergence of abnormal impulsive behaviours in PD. Interestingly, in Experiment 2 the sham rats also showed an increase in impulsivity when treated with 3 mg of PPX, indicating that a high dose of dopaminergic agonists may promote abnormal impulsivity even in a normal nigrostriatal system, as observed in subjects without PD receiving doses of dopaminergic agonists (Cornelius et al., 2010; Holman, 2009).

In contrast to the effects of chronic PPX administration, there were no changes (experiment 1) or decreased impulsivity (experiment 3) when animals were in the OFF medication state. This difference may reside in the fact that the large number of training sessions implemented in experiment 3 could have caused excessive habituation of the animals to the task. Regarding the subtypes of impulsivity, PPX treatment increases waiting and motor impulsivity in lesioned rats, and it especially delays intolerance, which mimics the increased sensitivity to risky choices and increased intolerance for delayed gratification in PD patients with ICDs relative to those patients without ICDs (Housden et al., 2010; Leroi et al., 2013; Voon et al., 2010).

Other risk factors for ICD development are a personality characterized by impulsivity, higher novelty-seeking and immediate reward preference. In fact, it is recommended that specific ICD questionnaires are completed at the moment of PD diagnose to screen and identify subjects with impulsive traits or history of pre-PD impulsiveness (Evans et al., 2019). This would serve to personalize their treatment and reduce the risk of ICD development. We noticed a positive correlation between impulsivity before treatment and impulsivity under chronic low dose (0.25 mg/kg) PPX administration in the 5-CSRTT paradigm of the hα-syn model (experiment

1), as well as with acute high dose PPX (3 mg/kg) in the VDS paradigm of the 6-OHDA model (experiment 2), similar to the results obtained in the DRL task under acute PPX treatment (Engeln et al., 2016). Moreover, in experiment 2, we found a positive correlation between the impulsivity under high dose PPX and that in the presurgery state. Despite the lack of statistical significance in the correlation, parkinsonian animals with high impulsivity showed higher basal impulsivity than control rats in experiment 3. The failure to reach statistical significance of this correlation could be due to the large difference in the number of animals in the control (n=5) and lesioned (n=17) groups, and the large variability in the results in contrast to the limited variability of the experiment 1.

Therefore, either a personality trait (presurgery) or the impulsivity induced by dopaminergic depletion (pretreatment) favours the development of higher impulsivity under PPX, indicating that there are risk factors for abnormal impulsivity after PPX treatment, which resembles the situation in PD patients (Weintraub et al., 2010). Importantly, abnormal impulsivity can occur in long exposure to a low dose (experiments 1 and 3) or short exposure to high dose (experiment 2).

Finally, in the two first experiments there is a correlation between the impulsivity under the dopaminergic treatment and the premorbid condition (presurgical impulsivity or impulsivity once the dopaminergic lesion is stablished). Similarly, in the third experiment, rats with high impulsivity also showed higher impulsivity at presurgery. This clearly indicates that the developmenn o f impulsivity is a cosnturc t based on the impulsivity trait patients have before dopaminergic intake and the total dose in either form (high acute dose or low chronic dose).

3. Effects on compulsive-like behaviour

Although impulsivity and compulsivity are different constructs, they share some neurological substrates and they may both compromise the emergence of substance and behavioural addictions, as well as other neuropsychiatric disorders (Chamberlain et al., 2018; Robbins et al., 2012). Impulsivity and compulsive-like behaviours do not appear to have been analysed previously in animal models of Parkinsonism and thus, to the best of our knowledge this is the first time in which both these behavioural constructs are analysed. Indeed, in the tasks evaluated we studied perseveration errors (one trait of compulsivity), different impulsivity traits (waiting impulsivity, motor action and delay intolerance) and problem shifting attention (accuracy and omission %) (Grant and Kim, 2014; Robbins et al., 2012).

In the three experiments PPX did not induce compulsive-like behaviour, suggesting that impulsivity is the behavioural construct directly affected by PPX and not compulsivity. However,

the use of a specific task to measure compulsive-like behaviour (post-training signal attenuation task) showed that bilateral lesion of the SNc and VTA with 6-OHDA does not affect compulsivity, although it was enhanced under chronic PPX treatment (0.3 mg/kg twice daily for 14 days) in a previous study (Dardou et al., 2017). This discrepancy could be due to the fact that the VTA is predominantly preserved in our model and that our tests only measure one trait of compulsivity.

4. Effects on other behavioural outcomes

It is known that attention and impulsivity are interrelated, as sustained attention is needed to suppress drug-seeking behaviours in addiction (De Wit, 2009) and impulsive individuals score low in sustained attention tasks (e.g., the Bakan task; Smith et al., 1990). A progressive dopaminergic lesion and PPX treatment caused a mild decrease in attentional performance in the 5-CSRTT, reflected as a loss of accuracy, more omissions and longer latencies (experiment 1). In the VDS paradigm, which was designed to reduce the attentional load of the 5-CSRTT, 6-OHDA-induced striatal dopaminergic depletion but it did not affect performance (experiment 2), although low impulsivity lesioned rats presented more omissions under PPX treatment, probably indicative of low attentional performance (experiment 3). Indeed, this impaired attention is also observed in human beings who have more limited short-term verbal memory, verbal fluency and attentional-executive functions after PPX administration (Brusa et al., 2003). Therefore, we suggest that future studies should use tasks with a mild attentional load, such as the VDS paradigm, to avoid possible confounding factors. Importantly, excessive training in this task may induce habituation of the animals, reducing the number of impulsive responses without pharmacological challenge (i.e.: in the OFF medication state), an aspect that should be carefully considered in future studies performed with a VDS paradigm. Moreover, as motor impulsivity and delay intolerance can be analysed simultaneously with this behavioural test, attention is much less severely affected. In addition, the training period is shorter than in the 5-CSRTT and the vast majority of other behavioural tasks. Hence, the VDS paradigm would appear to be an appropriate task to analyse impulsivity in animal models of Parkinsonism.

5. Histological biomarkers of PPX-induced impulsive behaviour

Regarding the molecular and cellular mechanisms underlying PPX induced impulsive behaviours, several transcription factors (c-Fos, FosB/ΔFosB, CREB, Nur) are thought to be involved in regulating the expression of the genes induced by substances of abuse (Nestler, 2001; Zhou et al., 2014). In particular, FosB/ΔFosB plays a relevant role given its implication in both substance and behavioural addictions (Nestler et al., 2001b; Velázquez-Sánchez et al.,

2014). In fact, in rats with parkinsonism induced by intracerebroventricular injections of a PPX (0.25 mg) self-administration protocol enhanced FosB/ΔFosB expression in the medial striatum of parkinsonian rats in a manner that correlated with drug self-administration (Engeln et al., 2013a).

Considering that its expression in dorsal striatum is associated with the emergence of dyskinesias, the main motor side-effect caused by dopaminergic drugs in animal models and PD patients (Engeln et al., 2016; Lindgren et al., 2010; Pavón et al., 2006), we hypothesized that the striatal expression of FosB/ΔFosB in certain areas could also be related to the development of impulsivity. Indeed, we observed enhanced expression of this transcription factor in all the striatal areas analysed (DL, DM, VL, VM, and the NAc core and shell) in animals with progressive parkinsonism subjected to chronic PPX (0.25 mg/kg/day) treatment in experiment 1, although this increase was not statistically significant in experiment 3. Moreover, a significant negative correlation between striatal DAT and FosB/ΔFosB expression was also only found in experiment 1. However, no correlations were observed between the FosB/ΔFosB expression in the striatum and impulsive or compulsive behaviour in either experiment 1 or 3. In experiment 2, striatal expression of FosB/ΔFosB was not analysed and immunohistochemistry was not performed on the brain sections as the animals were not perfused with a fixative solution but rather, fresh brain tissue was obtained. However, other techniques, such as *in situ* hybridization or polymerase chain reaction (PCR), could be used to determine the FosB/ΔFosB mRNA expression and should be implemented in future studies.

Therefore, it remains unclear if the striatal expression of FosB/ΔFosB is a molecular marker of PPX-induced impulsivity in this animal model of progressive Parkinsonism. The analysis of this transcription factors' expression in other brain areas, such as the prefrontal cortex and/or the subiculum of the hippocampus, should be considered to determine or fully discard its utility as a biomarker for behavioural addictions in animal models of PD.

In summary, according to the features of ICD in PD patients and based on the results obtained in our experiments, we believe a good model to study abnormal impulsivity triggered by dopaminergic agonist treatment in parkinsonian rats would include the following features: i) partial bilateral striatal dopaminergic depletion around 45-60% not restricted to the DL (motor) striatum and induced by A53T-hα-syn expression that induces abnormal impulsivity; ii) chronic, low dose PPX administration; iii) the use of the VDS paradigm to simultaneously measure motor impulsivity and delay intolerance.

VII. Conclusions

1. Partial dopaminergic lesion across the whole striatum induces impulsivity in rats whereas denervation in the DL CP does not. This suggests a dopaminergic depletion in associative, motor and limbic areas of the striatum but not only in rhe motor area is crucial for the emergence of impulsivity prior to the intake of dopaminergic agents.

2. Dopaminergic agonists play a critical role in the induction of abnormal impulsivity in parkinsonian rats both in chronic administration of low doses or acute administration of high doses. In contrast, only acute high doses of dopaminergic agonists induce impulsivity in control animals. Hence, dopaminergic deficiency itself is a risk factor for abnormal impulsivity induced by dopaminergic agonists, although it can also be elicited in subjects with an intact dopaminergic system challenged with high doses of these drugs, as witnessed in humans with other conditions who are treated with dopaminergic agents.

3. Chronic treatment with low doses of dopaminergic agonists or acute high doses triggers all types of impulsivity (waiting impulsivity, motor impulsivity and delay-intolerance) in parkinsonian rats, whereas acute low doses only induce motor impulsivity. Therefore, the total daily dose of dopaminergic agonists, independently of the mode of administration, promote the development of abnormal impulsive behaviours in parkinsonian rats in a dose dependent manner.

4. Impulsivity of the animals either before or after induction of the dopaminergic lesion correlated with the severity of impulsivity after dopaminergic treatment indicating that the basal impulsivity could predict the risk of developing abnormal impulsivity after treatment with dopaminergic agonists. This mimics the fact that a history of ICDs, substance abuse, or a personality trait of high impulsivity prior to PD development are risk factors for impulsivity induced by dopaminergic agents in humans.

5. All the parkinsonian animals are not equally affected by treatment withdopaminergic agonists, with some subjects more sensitive to the behavioural effect of the drug. These results mimic the clinical scenario and suggest that future studies should take into account the different susceptibility of parkinsonian rats to dopaminergic agents instead of treating them uniformly.

6. Compulsive-like behaviour (measured by perseverative responses) is not affected by either the dopaminergic lesion or dopaminergic agonists treatment in any of the experiments.

7. Our results suggest that FosB/ΔFosB expression in the striatum is not relevant in the pathophysiology of abnormal impulsivity triggered by dopaminergic agonists in parkinsonian rats. The analysis of FosB/ΔFosB expression in other brain areas should be assessed in future studies, e.g., in the limbic system.

8. All in all, the animal model of progressive parkinsonism treated with either chronic low doses and acute high doses of dopaminergic agonist and behavioural evaluated with the VDS paradigm may be the most suitable model to study abnormal impulsivity in parkinsonism.

VIII. References

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VIII. Annexes

Annex I

Buffers

PB 0.2 M pH 7.4:	
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4-H_2O)	6.9 gr
Disodium hydrogen phosphate dihydrate (Na_2HPO_4-$2H_2O$)	26.7 gr
Distiled water	1000 ml
Adjust pH to 7,4 with NaOH 10M.	
PB 0.1 M pH 7.4:	
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4-H_2O)	3.45 gr
Disodium hydrogen phosphate dihydrate (Na_2HPO_4-$2H_2O$)	13.35 gr
Distiled water	1000 ml
Adjust pH to 7.4 with NaOH 10M.	
PBS 0.1 M pH 7.4:	
PB 0.1 M pH 7.4	1000 ml
Sodium chloride (NaCl)	9 gr
PFA 8%:	
PFA	80 gr
MiliQ H_2O	1000 ml
PFA 4% 400ml:	
PFA 8%	200 ml
PB 0,2 M pH 7,4	200 ml
Sucrose 30% in PBS 0,1 M pH 7,4:	
Sucrose	300 gr
Sodium azide (NaN_3)	0.2 gr
PBS 0.1 M pH 7.4	1000 ml
Diaminobenzidine (DAB):	
DAB (Sigma-Aldrich, St. Louis, MO)	200 mg
PBS 0m1 M pH 7,4	20 ml

Gelatinized slides:

Gelatine (Sigma-Aldrich, St. Louis, MO)	1 gr
Chromium(III) potassium sulfate $KCr(SO_4)$ (Panreac)	0.1 gr
MiliQ H_2O	200 ml

Preparation: Heat the water. Disolve gelatine completely and add $KCr(SO_4)$. Filter the solution, and store it at 4ºC up to two days at most.

Gelatinization process: Degrease the slides introducing them at EtOH and Eter 1:1 solution for several days. Dry the slides and immerse them in the gelatinizing solution at 60ºC for 1 minute. Finally, dry the slides at 37ºC for 24 hours and store them in a box.

Cryoprotectant solution for perfused tissue sections:

Etilenglycol	300 ml
Glycerol	300 ml
MiliQ H_2O	300 ml
PB 0,1 M pH 7,4	100 ml

Annex II

Custom-designed script for VDS task using Med-PC IV software

```
\ VDS.MPC – Variable delay to signal
\ Ref Leite-Almeida 2013
\ Rats are required to respond to brief flashes of light presented randomly in
\ central response apperture. Testing begins with the onset of the House
\ Light and the Pellet or Dipper Receptacle Light. A Head Entry issues a Free
\ Reward Presentation, initiates the Session Timer. Following the Reward
\ Interval a delay-to-signal is activated for the first Trial. Nose poking during the delay
\ results in a Time Out and is recorded as a Premature Response. Stimulus Time
\ Outs also occur following an Incorrect Response, or following an Error of
\ Omission (failure to respond within the Limited Hold Interval) but are punished with
\ Light.
\\ By default this procedure runs for 120 trials starting with a fixed
\ delay-to-signal of 3 seconds and fixed Stimulus Presentation of 60 seconds.
\ The Time to Respond (Limited Hold) is 60 seconds while Premature
\ Responses add a 5 second Time Out before repeating the delay. Incorrect
\ Responses or Errors of Omission add a 5 second Time Out before starting a new
\ Trial. Responses in Time Out reset the Time-Out Timer and are also considered
\ Premature responses.
\ Nose Poke Inputs and Outputs must be connected in order from Left to Right.
\ Edit Additional Input and Output constants if necessary to match your Hardware.

\ Inputs
^HeadEntry = 6

\ Outputs
^ReceptacleLight = 6
^HouseLight = 7
^RewardOp = 8

\ A() = Control Variables with Assigned Aliases as Defined
Var_Alias Trials to Run  = A(0) \ Default = 120
Var_Alias Response (Limited Hold) Time (sec) = A(1) \ Default = 60 seconds
Var_Alias Time Out (sec)  = A(2) \ Default = 5 seconds
Var_Alias Reward (1=Pellet 2=Dipper) = A(3) \ Default = 1-Pellet
Var_Alias Reward Duration (sec) = A(4) \ Default = 2 seconds
Var_Alias Session Time (min)  = A(5) \ Default = 180 minutes

^Trials = 0
^LimitedHold = 1
^TimeOut = 2
^RewardCode = 3
^RewardDur = 4
^Session = 5
^Latency = 6
^StimulusLoc = 7
^StimulusDur = 8
^ITIDur = 9
```

\ List Data Variables Here
\ D() = Summary Response Data
\ D(0) = Correct Responses
\ D(1) = Incorrect Responses
\ D(2) = Omissions
\ D(3) = Premature Responses
\ D(4) = Perseverant Responses
\ D(5) = Time Out Responses
\ D(6) = Total Receptacle Head Entries
\ D(7) - D(9) = Not Used
\ D(10) = % Correct {D(0) / B(^Trials) * 100}
\ D(11) = % Incorrect {D(1) / B(^Trials) * 100}
\ D(12) = % Omission {D(2) / B(^Trials) * 100}

\ G() = Summary Latency Data
\ G(0) = Average Latency to Correct Response {G(5) / D(0)}
\ G(1) = Average Latency to Incorrect Response {G(6) / D(1)}
\ G(2) = Average Latency to Reward {G(7) / D(0)}
\ G(3) & (4) Not Used
\ G(5) = Total Latency Time to Correct Response
\ G(6) = Total Latency Time to Incorrect Response
\ G(7) = Total Latency Time to Reward

\ K() = Trial by Trial Data (20 Elements Displayed in Two Rows)
\ K(I) = Trial Number
\ K(I+1) = Nose Poke Stimulus Location 1 - 9
\ K(I+2) = First Response to Stimulus (1 - 9)
\ K(I+3) = Correct Response Latency
\ K(I+4) = Incorrect Response Latency
\ K(I+5) = Latency to Reward
\ K(I+6) = Omission Error (No Response)
\ K(I+7) = Perseverant Responses to NP #1
\ K(I+8) = Perseverant Responses to NP #2
\ K(I+9) = Perseverant Responses to NP #3
\ K(I+10) = Perseverant Responses to NP #4
\ K(I+11) = Perseverant Responses to NP #5
\ K(I+12) = Perseverant Responses to NP #6
\ K(I+13) = Perseverant Responses to NP #7
\ K(I+14) = Perseverant Responses to NP #8
\ K(I+15) = Perseverant Responses to NP #9
\ K(I+16) = Premature Responses (ITI & Time Out)
\ K(I+17) = Receptacle Head Entries (All)
\ K(I+18) = Cue Duration
\ K(I+19) = ITI Duration

\ List Working Variables Here
\ B() = Working Variables that Parallel the Control Variables.
\ These are Converted to MED Units or Used as Counters
\ or Elapsed Timers
\ N = List of Stimulus Location
\ P = List of Cue Duration for Stimulus Hole 1
\ Q = List of Cue Duration for Stimulus Hole 2

```
\ R = List of Cue Duration for Stimulus Hole 3
\ S = List of Cue Duration for Stimulus Hole 4
\ T = List of Cue Duration for Stimulus Hole 5
\ U = List of Cue Duration for Stimulus Hole 6
\ V = List of Cue Duration for Stimulus Hole 7
\ W = List of Cue Duration for Stimulus Hole 8
\ X = List of Cue Duration for Stimulus Hole 9
\ Z = List of ITI Durations

DIM A = 5
DIM B = 9
DIM D = 12
DIM G = 7
DIM K = 5000

\ List of Stimulus Locations 1 - 9
\ See SOF-700RA-8 Manual for Running Fewer than 9 Nose Pokes at a Time.
LIST N = 3 \, 4, 5 \, 6, 7, 8, 9

\ List of Stimulus Cue Durations. Note this has been repeated 9 times so that a
\ separate list is used for each Stimulus Location when multiple durations are
\ used. Add as many values as you want. Repeating the values would allow them
\ to potentially repeat before a different value is drawn. To obtain a balanced
\ design, you need to have the Total Number of Trials be an even multiple of the
\ (Number of Stimuli Used) * (# of Times in the List).
LIST P = 0.5 \ Stimulus Duration Time. Fixed at 60 Seconds.
LIST Q = 0.5
LIST R = 60
LIST S = 0.5 \ Use Change Variables or Add Additional Values
LIST T = 0.5 \ See SOF-700RA-8 Manual for Additional Information
LIST U = 0.5
LIST V = 0.5
LIST W = 0.5
LIST X = 0.5

\ ITI Duration Lists
LIST Z = 3 \ Fixed at 3 Seconds.

\ Z-Pulses Used in this Program
^Stimulus = 1 \ Z1 = Signals Stimulus Timer
^Dipper = 2 \ Z2 = Signals Dipper Timer
^Correct = 3 \ Z3 = Signal Correct Response
^Incorrect = 4 \ Z4 = Signal Incorrect Response
^Omission = 5 \ Z5 = Signal Omission Error (No Response)
^Reward_NP = 6 \ Z6 = Signal Reward Control
^EndReward = 7 \ Z7 = Signal End of Reward Cycle
^End = 32 \ Z32 = End of Session

DISKCOLUMNS = 10
DISKFORMAT = 6.2

\************************************************
```

```
\ VDS Schedule
\ S1 - Set Default Values
\ Trials to Run  (100)
\ Response (Limited Hold) Time (5 seconds)
\ Time Out  (5 seconds)
\ Reward  (1-Pellet)
\ Reward Duration (2 seconds)
\ Session Time  (30 minutes)
\************************************************
S.S.1,
S1,
 0.001": SET A(^Trials) = 100, A(^LimitedHold) = 60, A(^TimeOut) = 5;
 SET A(^RewardCode) = 1, A(^RewardDur) = 2, A(^Session) = 30 ---> S2

S2, \ Wait for START command
 #START: CLEAR 1,60;
 SET B(^LimitedHold) = A(^LimitedHold) * 1";
 SET B(^TimeOut) = A(^TimeOut) * 1";
 ON ^HouseLight; Z^Reward_NP ---> S3
 1": SHOW 1,Trials,A(^Trials), 2,Limited Hold,A(^LimitedHold), 3,Time Out,A(^TimeOut);
 SHOW 4,Reward Code,A(^RewardCode), 5,Reward Duration,A(^RewardDur),
6,Session,A(^Session) ---> SX

S3, \ Wait for Completion of Free Reward Cycle
 #Z^EndReward: ON ^HouseLight ---> S5

S5, \ Trial Set Up: Draw Values for Stimulus Value (Location),
 \ Stimulus Duration, and ITI Value
 0.01": ADD B(^Trials);
 SET K(I) = B(^Trials), K(I+20) = -987.987;
 RANDD K(I+1) = N; SET B(^StimulusLoc) = K(I+1);
 IF K(I+1) = 1 [@Draw1Values, @Next]
 @Draw1: RANDD K(I+18) = P, K(I+19) = Z ---> S7
 @Next: IF K(I+1) = 3 [@Draw3Values, @Next]
  @Draw3: RANDD K(I+18) = R, K(I+19) = Z ---> S7
  @Next: IF K(I+1) = 5 [@Draw5Values, @Next]
  @Draw5: RANDD K(I+18) = T, K(I+19) = Z ---> S7
  @Next: IF K(I+1) = 7 [@Draw7Values, @Next]
 @Draw7: RANDD K(I+18) = V, K(I+19) = Z ---> S7
 @Next: IF K(I+1) = 9 [@Draw9Values, @Next]
 @Draw9: RANDD K(I+18) = X, K(I+19) = Z ---> S7
 @Next: ---> S6

S6, \ Used to Draw Stimuli 2, 4, Choice Studies
 0.01": IF K(I+1) = 2 [@Draw2Values, @Next]
 @Draw2: RANDD K(I+18) = Q, K(I+19) = Z ---> S7
 @Next: IF K(I+1) = 4 [@Draw4Values, @Next]
  @Draw4: RANDD K(I+18) = S, K(I+19) = Z ---> S7
  @Next: IF K(I+1) = 6 [@Draw6Values, @Next]
  @Draw6: RANDD K(I+18) = U, K(I+19) = Z ---> S7
  @Next:IF K(I+1) = 8 [@Draw8Values, @Abort]
 @Draw8: RANDD K(I+18) = W, K(I+19) = Z ---> S7
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 @Abort: SET Y = 99999 ---> STOPABORTFLUSH

S7, \ Convert Drawn Values to MED Clock Units
 0.01": SET B(^StimulusDur) = K(I+18) * 1";
 SET B(^ITIDur) = K(I+19) * 1" ---> S8

S8, \ Time ITI - Record Premature Response
 \ Record Nose Pokes & Reset Timer
 \ Record Head Entries
 B(^ITIDur)#T: Z^Stimulus ---> S10 \ See S.S.3 for Stimulus Control
 #R1 ! #R2 ! #R3 ! #R4 ! #R5 ! #R6 ! #R7 ! #R8 ! #R9: OFF ^HouseLight; ADD D(3), K(I+16) ---> S9
 #R^HeadEntry: ADD D(6), K(I+17) ---> SX

S9, \ Time Out to Premature Response
 \ Record Nose Pokes & Reset Timer
 \ Record Head Entries
 B(^TimeOut)#T: ON ^HouseLight ---> S8
 #R1 ! #R2 ! #R3 ! #R4 ! #R5 ! #R6 ! #R7 ! #R8 ! #R9: ADD D(5), K(I+16) ---> S9
 #R^HeadEntry: ADD D(6), K(I+17) ---> SX

S10, \ Wait for Response or End of Limited Hold
 #RK(I+1): SET K(I+2) = K(I+1);
 Z^Reward_NP; Z^Correct ---> S12
 #R1: SET K(I+2) = 1; OFF ^HouseLight; Z^Incorrect ---> S11
 #R2: SET K(I+2) = 2; OFF ^HouseLight; Z^Incorrect ---> S11
 #R3: SET K(I+2) = 3; OFF ^HouseLight; Z^Incorrect ---> S11
 #R4: SET K(I+2) = 4; OFF ^HouseLight; Z^Incorrect ---> S11
 #R5: SET K(I+2) = 5; OFF ^HouseLight; Z^Incorrect ---> S11
 #R6: SET K(I+2) = 6; OFF ^HouseLight; Z^Incorrect ---> S11
 #R7: SET K(I+2) = 7; OFF ^HouseLight; Z^Incorrect ---> S11
 #R8: SET K(I+2) = 8; OFF ^HouseLight; Z^Incorrect ---> S11
 #R9: SET K(I+2) = 9; OFF ^HouseLight; Z^Incorrect ---> S11
 #R^HeadEntry: ADD D(6), K(I+17) ---> SX
 B(^LimitedHold)#T: OFF ^HouseLight; Z^Omission ---> S11

S11, \ Time Out to Incorrect or Omission Response
 B(^TimeOut)#T: ON ^HouseLight ---> S14
 #R1 ! #R2 ! #R3 ! #R4 ! #R5 ! #R6 ! #R7 ! #R8 ! #R9: ADD D(5), K(I+16) ---> S11
 #R^HeadEntry: ADD D(6), K(I+17) ---> SX

S12, \ Wait for Head Entry Detection
 \ Record Perseverant Responses
 #R^HeadEntry: ADD D(6), K(I+17) ---> S13
 #R1: ADD D(4), K(I+7) ---> SX
 #R2: ADD D(4), K(I+8) ---> SX
 #R3: ADD D(4), K(I+9) ---> SX
 #R4: ADD D(4), K(I+10) ---> SX
 #R5: ADD D(4), K(I+11) ---> SX
 #R6: ADD D(4), K(I+12) ---> SX
 #R7: ADD D(4), K(I+13) ---> SX
 #R8: ADD D(4), K(I+14) ---> SX
 #R9: ADD D(4), K(I+15) ---> SX
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S13, \ Wait for Completion of Reward Cycle
 #Z^EndReward: ---> S14

S14, \ Test for End of Session
 0.01": IF (B(^Trials) >= A(^Trials)) OR (B(^Session)/60 >= A(^Session)) [@EndSession,
@NextTrial]
 @End: OFF ^HouseLight; Z^End ---> S15
 @NextTrial: SET I = I + 20 ---> S5

S15, \ Delay for Screen Update
 2": ---> STOPABORTFLUSH

\************************************************
\ RESPONSE LATENCY TIMER
\************************************************
S.S.2,
S1,
 #START: ---> S2

S2, \ Wait for Stimulus Signal Z^Stimulus
 #Z^Stimulus: SET B(^Latency) = 0 ---> S3

S3, \ Response Latency
 \ 1st Statement: Increment Latency w/0.01 sec. Resolution
 \ 2nd Statement: Add Correct Response, Calculate % Correct
 \ Set Trial Latency, Total Latency & Calc Ave Lat.
 \ 3rd Statement: Same as above for Incorrect Response
 \ 4th Statement: Same as above for Omission Error
 0.01": SET B(^Latency) = B(^Latency) + 0.01 ---> SX
 #Z^Correct: ADD D(0); \ Add Correct Response
 SET K(I+3) = B(^Latency); \ Set Correct Latency
 SET G(5) = G(5) + B(^Latency); \ Set Total Correct Latency
 SET G(0) = G(5) / D(0); \ Calculate Average Correct Latency
 SET B(^Latency) = 0 ---> S4 \ Reset Latency Variable
 #Z^Incorrect: ADD D(1);
 SET K(I+4) = B(^Latency);
 SET G(6) = G(6) + B(^Latency);
 SET G(1) = G(6) / D(1) ---> S5
 #Z^Omission: ADD D(2), K(I+6) ---> S5

S4, \ Reward Latency
 #R^HeadEntry: SET K(I+5) = B(^Latency);
 SET G(7) = G(7) + B(^Latency);
 SET G(2) = G(7) / D(0) ---> S5
 0.01": SET B(^Latency) = B(^Latency) + 0.01 ---> SX

S5, \ Calculate % Correct, % Incorrect, & % Omission
 0.01": SET D(10) = D(0) / B(^Trials) * 100;
 SET D(11) = D(1) / B(^Trials) * 100;
 SET D(12) = D(2) / B(^Trials) * 100 ---> S2
```

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\*************************************************
\ STIMULUS DURATION TIMER
\*************************************************
S.S.3,
S1,
 #Z^Stimulus: ON B(^StimulusLoc) ---> S2

S2,
 B(^StimulusDur)#T: OFF 1,2,3,4,5,6,7,8,9 ---> S1
 #Z^Correct ! #Z^Incorrect ! #Z^Omission: OFF 1,2,3,4,5,6,7,8,9 ---> S1

\*************************************************
\  UPDATE DISPLAY
\*************************************************
S.S.5,
S1,
 #START: ---> S2

S2,
 1": SHOW 1,Trial #,B(^Trials), 2,Stimulus #,K(I+1), 3,Correct,D(0);
 SHOW 4,Incorrect,D(1), 5,Omission,D(2);
 SHOW 8,% Correct,D(10), 9,% Incorrect,D(11), 10,% Omission,D(12) ---> S3

S3,
 0.01": SHOW 13,Avg Cor Lat,G(0), 14,Avg Incor Lat,G(1), 15,Avg Rew Lat,G(2);
 SHOW 16,Premature,D(3), 17,Perseverative,D(4), 18,TO_Resp,D(5);
 SHOW 19,Head Entry,D(6) ---> S2

\*************************************************
\ REWARD CONTROL CYCLE
\*************************************************
S.S.6,
S1,
 #START: SET B(^RewardDur) = A(^RewardDur) * 1";
 IF A(^RewardCode) = 1 [@Pellet, @Next]
 @Pellet: ---> S2
 @Next: IF A(^RewardCode) = 2 [@Dipper, @Abort]
  @Dipper: ---> S10
  @Abort: ---> STOPABORTFLUSH

S2, \ Pellet Reward Control
 #Z^Reward_NP: ON ^Houselight, ^ReceptacleLight, ^RewardOP ---> S3

S3, \ Pulse Pellet Dispenser for 50ms
 0.05": OFF ^RewardOP ---> S4

S4, \ Wait for Head Entry Detection
 #R^HeadEntry: ---> S5

S5, \ Time to End of Reward Cycle (Reward Duration)
 B(^RewardDur)#T: OFF ^ReceptacleLight; Z^EndReward ---> S2
```

```
S10, \ Dipper Reward Control
 #Z^Reward_NP: ON ^ReceptacleLight ---> S11

S11, \ Wait for Head Entry Detection
 #R^HeadEntry: ON ^RewardOP ---> S12

S12, \ Time to End of Reward Cycle (Reward Duration)
 B(^RewardDur)#T: OFF ^ReceptacleLight, ^RewardOP; Z^EndReward ---> S10

\************************************************
\  SESSION TIMER
\************************************************
S.S.10, \ This State Set Increments the Elapsed Time
 \ Counter only. Values are tested at the end
 \ of each Trial in S.S.1, S14 above.
S1,
 #START: SHOW 21,Session,B(^Session)/60 ---> S2

S2, \ Wait for First Head Entry to Start Timing
 #R^HeadEntry: ---> S3

S3,
 1": ADD B(^Session); SHOW 21,Session,B(^Session)/60 ---> SX
 #Z^End: ---> S1
```