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Study of neuronal networks and mechanisms implicated in locomotor reactivity and Parkinson's disease pathogenesis in the *Drosophila* model

Jun Sun

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ÉCOLE DOCTORALE CERVEAU-COGNITION-COMPORTEMENT

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Laboratoire Plasticité du Cerveau, CNRS UMR 8249

Thèse de Doctorat de Neurobiologies

**Study of neuronal networks and mechanisms
implicated in locomotor reactivity and Parkinson's
disease pathogenesis in the *Drosophila* model**

par **Jun Sun**

dirigée par le Dr. Serge Birman

Présentée et soutenue publiquement le 10 juillet 2018

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却顾所来径，苍苍横翠微

----李白

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List of abbreviations

2-HE	2-hydroxyethidium
α -syn	α -synuclein
α -tub	α -tubulin
aa	amino acid
AAAT	Aromatic amino acid transporter
AADC	Aromatic amino acid decarboxylase
AD	autosomal dominant
AGO	Argonaute
ALP	autophagy-lysosome pathway
AMP	adenosine monophosphate
APL	anterior paired lateral
AR	autosomal recessive
ARM	anaesthesia-resistant memory
Atg	Autophagy-related gene
ATP	Adenosine triphosphate
ATP13A2	Cation-transporting ATPase 13A2
BBB	brain blood barrier
CA	corpus allatum
CA	cell-autonomous
NCA	non-cell autonomous
CC	central complex
Cdk5	Cyclin-dependent protein kinase 5
Cdk5rap1	Cdk5 regulatory subunit-associated protein 1
<i>Ce</i>	<i>Caenorhabditis elegans</i>
CMA	chaperone-mediated autophagy

CNS	central nervous system
COMT	Catechol-O-methyltransferase
<i>CYP2E1</i>	Cytochrome P450 2E1
CRE	crepine
DA	dopamine
d a. E.	day after eclosion
DA-Q	dopamine-quinone
damb	Dopamine 1-like receptor 2 (Dop1R2)
DAN	dopaminergic neuron
DAT	DA transporter
DDC	Dopa decarboxylase
DDR2	Dopamine 2-like receptor (Dop2R)
DHE	Dihydroethidium
<i>Dm</i>	<i>Drosophila melanogaster</i>
DN	descending neurons
Dnmt1	DNA methyltransferase 1
DOPAC	3,4-dihydroxyphenylacetic acid
DopEcR	Dopamine/Ecdysteroid receptor 1
DopR	Dopamine 1-like receptor 1 (Dop1R1)
DPM	dorsal paired medial
dumb	Dopamine 1-like receptor 1 (Dop1R1)
EB	ellipsoid body
EHMT2	euchromatic histone lysine N-methyltransferase 2
EIF4G1	eukaryotic translation initiation factor 4 gamma 1
ENS	enteric nervous system
ER	endoplasmic reticulum
FBXO7	F-box only protein 7

FSB	fan-shaped body
GABA	γ -Aminobutyric acid
Gad	Glutamic acid decarboxylase 1
GBA	Glucocerebrosidase
GFP	Green fluorescent protein
GP	globus pallidus
GPe	external segment of GP
GPi	internal segment of GP
GRASP	GFP reconstitution across synaptic partners
Grx	glutaredoxin
GSH	glutathione
GSSG	glutathione disulfide
H ₂ O ₂	hydrogen peroxide
H3K9	Histone 3 lysine 9
H3K9me2	H3K9 dimethylation
H3K9me3	H3K9 trimethylation
HDAC	Histone deacetylase
HO•	hydroxyl radical
HP1a	Heterochromatin protein 1a
<i>Hs</i>	<i>Homo sapiens</i>
Hsc70	Heat-shock cognate protein 70
Hsp70	Heat-shock protein 70
HVA	homovanillic acid
IFN	Interferon
IL	Interleukin
ISC	intestinal stem cell

JH	Juvenile hormone
KC	Kenyon cell
LAD	lamin-associated domain
LAL	lateral accessory lobes
LAMP2A	lysosome-associated membrane protein type 2A
LexAop	LexA operator
LINE1	Long interspersed element 1
LPS	lipopolysaccharide
LRRK2	Leucine rich repeat kinase 2
LTM	long term memory
LTR	long terminal repeats
MAO	Monoamine oxidase
MARK	Microtubule affinity-regulating kinase
MB	mushroom body
MBON	MB output neuron
miRNA	microRNA
MD	mediodorsal
MPP ⁺	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	methyl-phenyl-tetrahydropyridin
msGFP	mCD8::GFP, nsyb::GFP
NAc	nucleus accumbens
NAC	non-amyloid component
NAD	nicotinamide adenine dinucleotide
NAT	N-acetyltransferase
NC	negative control
O ₂ ⁻	superoxide anion

OA-VPM	octopamine-ventral paired medial
OCT3	organic cation transporter-3
PAL	protocerebral anterior lateral
PAM	protocerebral anterior medial
PAZ	Piwi-Argonaute-Zwille
PB	protocerebral bridge
PBST	PBS + Triton
PD	Parkinson's disease
PGC1- α	Peroxisome proliferator receptor gamma coactivator 1 α
PI	performance index
PIb	pars intercerebralis
piRNA	piRNA-like non coding RNA
PINK1	PTEN-induced putative kinase 1
piRNA	PIWI-interacting RNA
PNS	peripheral nervous system
PPL	protocerebral posterior lateral
PPM	protocerebral posterior medial
PTGS	posttranscriptional gene silencing
Pu	putamen
RBR	RING-between RING
REP	Dinucleotide repeat sequence
RISC	RNA induced silencing complex
roGFP	redox-sensitive GFP
RNS	reactive nitrogen species
ROS	reactive oxygen species
rsGFP	reconstructed split GFP

SEZ	subesophageal zone
shi ^{ts1}	temperature-sensitive shibire
SING	startle-induced negative geotaxis
SIP	superior intermediate protocerebrum
siRNA	small interfering RNA
SLP	superior lateral protocerebrum
SMP	superior medial protocerebrum
SN	substantia nigra
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNpc	SN pars compacta
SNpr	SN pars reticulata
SOD	superoxide dismutase
SOG	subesophageal ganglion
Sp	<i>Shizosaccharomyces pombe</i>
spGFP	split GFP
STM	short-term memory
STN	subthalamic nucleus
SYNJ1	synaptotagmin 1
TBS	Tris-buffered saline
TBST	TBS Tween
Tdc	Tyrosine decarboxylase
TE	transposable elements
TG	thoracic ganglion
TGS	transcriptional gene silencing
TH	tyrosine hydroxylase
Th	thalamus

TNF α	tumor necrosis factor α
TNT	tetanus toxin light chain
TOM	translocation of the outer membrane
TRP	Transient receptor potential
Trx	Thioredoxin
UAS	Upstream activating sequences
UCP	Uncoupling protein
UPS	ubiquitin-proteasome system
UTR	untranslated region
VA	ventral anterior
VGCC	Voltage-gated calcium channel
VGlut	Vesicular glutamate transporter
VL	ventrolateral
VMAT	Vesicular monoamine transporter
VNC	ventral nerve cord
VPM	ventral paired medial
VPS35	Vacuolar protein sorting-associated protein 35
VTA	ventral tegmental area

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Introduction

Introduction

Chapter I Dopamine and the dopaminergic system

Dopamine (DA) was identified as a neurotransmitter in brain by Arvid Carlsson about 60 years ago. From the seminal observation that DA could revert reserpine-induced akinetic state, he suggested that DA may be related to Parkinson's disease (PD). Then Oleh Hornykiewicz following this suggestion, found that striatal DA is deficient in PD patients and suggested applying L-DOPA (the direct precursor of DA) to treat PD. George Cotzias later showed that L-DOPA therapy at high dose was safe and efficient to treat PD, and this has been applied till now.

1. History of DA discovery

This brief glance at the earlier DA studies is mainly based on memory reports from of Hornykiewicz (Hornykiewicz, 2002), Carlsson (Carlsson, 2002) and review from Fahn (Fahn, 2008).

In 1910, DA was first synthesized by George Barger and James Ewins (Hornykiewicz, 1986), and then given by Henry Dale its current name "dopamine". In 1938, Peter Holtz et al discovered the enzyme aromatic-L-amino-acid decarboxylase (AADC) in mammalian tissues, converting L-DOPA to DA. Later, the hypothesis was proposed independently by Holtz and Hermann Blaschko, that L-DOPA and DA are intermediates in the biosynthetic pathway of catecholamines, epinephrine and norepinephrine (Blaschko, 1942). Three decades later, DA could be detected in many peripheral tissues (heart, kidney...) and it was found that it could lower blood pressure in animals (Hornykiewicz et al., 1958). In 1964, Toshiharu Nagatsu identified the enzyme tyrosine hydroxylase (TH) that converts L-tyrosine to L-DOPA in beef adrenal medulla, this catalytic process was proved to be the rate-limiting process in DA synthesis later on.

Based on DA biosynthesis, further work had been carried out meanwhile to understand the physiological function of DA. Before 1957, DA remained to be just one kind of catecholamine, but the seminal observation by Arvid Carlsson that L-DOPA application to reserpined animals led to recovery from the akinetic state broke the consensus that reserpine sedative action was due to its effect on brain serotonin or noradrenaline. From this work, Carlsson suggested a role for DA as a neurotransmitter involved in movement control in the central nervous system (CNS) (Pletscher et al., 1955; Carlsson, 2002), independently of its role as a precursor in the synthesis of noradrenaline and adrenaline. This suggestion was

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rejected based on the the firmly entrenched idea that synaptic transmission in the CNS was electrical rather than chemical at a meeting held in London in 1960 (reviewed by Iversen and Iversen, 2007). Inspired by the idea that parkinsonism-like symptoms produced by reserpine could be due to lack of DA in the striatum (Bertler and Rosengren, 1959; Carlsson, 1959), Hornykiewicz began to investigate the autopsied brain from PD patients, and then discovered a marked reduction in DA levels (Goetz, 2011). Later on, he found that L-DOPA treatment to PD patients resulted in a temporary improvement of akinesia (Birkmayer and Hornykiewicz, 1961). Cotzias's efforts introduced high dose L-DOPA therapy that has been utilized as clinical treatment since then (Cotzias et al., 1969). Numerous works during the following 50 years have focused on the study of dopaminergic neurons (DANs), DA receptors and their role in neurophysiology, behaviour control and related diseases, as will be briefly discussed below.

2. The DA neurotransmission system in mammals

2.1 DA dynamics

DA synthesis: As shown in Figure 1, DA is derived from L-tyrosine, an amino acid that is abundant in dietary proteins. Blood-borne tyrosine is taken up in the brain and transported into DANs. L-tyrosine is converted into L-DOPA by the cytosolic enzyme TH using the cofactor tetrahydrobiopterin and this is the rate limiting step in DA biosynthesis (Nagatsu et al., 1964). Cytosolic L-DOPA is then converted to DA by the enzyme aromatic amino acid decarboxylase (AADC), also named DOPA decarboxylase (DDC).

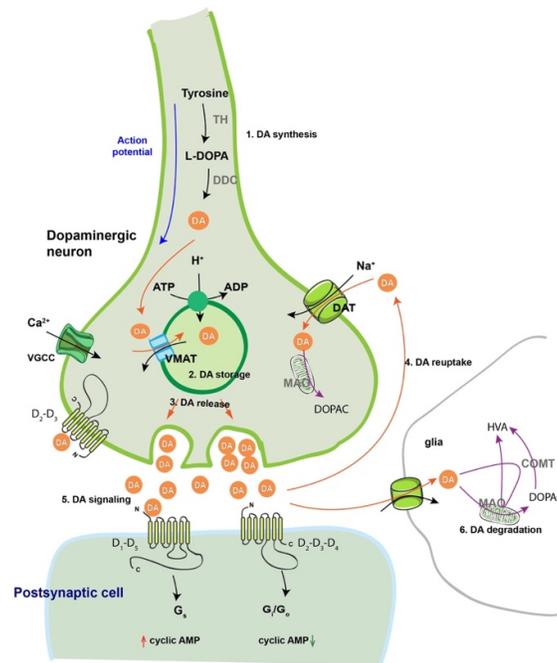


Figure 1 Overview of the DA neurotransmission system.

Function of the DA neurotransmission system in mammals requires: 1) DA synthesis: L-tyrosine transported into DANs is converted by TH to L-DOPA, which is then converted to DA by DDC. 2) DA storage: cytosolic DA is imported into synaptic vesicles by VMAT driven by the H^+ -ATPase pump to fight against the concentration gradient. 3) DA release: responding to the action potential and Ca^{2+} ion entry through VGCC, DA is released into the synaptic cleft. 4) DA reuptake: through DAT, DA is captured back by DANs and recycled to vesicles or degraded. 5) DA signaling: DA could act on the postsynaptic DA receptors or the presynaptic DA autoreceptors. 6) DA degradation: DA is degraded by MAO or COMT into HVA and then excreted into urine. AAAT: aromatic amino acid transporter; COMT: catechol-O-methyltransferase; DA: dopamine; DANs: dopaminergic neurons; DAT: DA transporter; DDC: dopa decarboxylase; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid, MAO: monoamine oxidase; TH: tyrosine hydroxylase; VMAT: vesicular monoamine transporter; VGCC: Voltage-gated calcium channel.

DA storage: Cytosolic DA easily reacts with protein, lipids and nucleic acids, forming toxic oxygen radicals (Sulzer and Zecca, 2000). To avoid this toxic reaction, DANs possess multiple mechanisms to protect themselves from cytosolic DA stress, e.g. sequestering DA into synaptic vesicles through the vesicular monoamine transporter 2 (VMAT2) and so keeping it in very acid surroundings. Vacuolar-type H^+ -ATPase (V-ATPase) in synaptic vesicles generates a proton electrochemical force that allows vesicular accumulation of DA against its concentration gradient, leading to a fivefold higher concentration of vesicular DA compared to its cytosolic level (Nakanishi-Matsui and Futai, 2006; Sulzer, 2007; Sulzer, 2016).

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DA release and reuptake: Following neuronal stimulation and calcium influx, according to mammalian studies, DA release takes place both from the axons and somatodendrites (Rice et al., 1997; Phillips and Stamford, 2000; Cheramy et al., 1981). Its signalling depends on DAN firing rate and patterns that vary between low frequency “tonic” firing and brief higher frequency (-200 ms) ‘phasic’ bursts of action potentials (Grace and Bunney, 1983; Grace 2016). DA released into the synaptic cleft is halted by its diffusion away from the synapse and reuptake by the DA transporter (DAT) into the presynaptic neurons or glia (Nirenberg et al., 1997; Uhl et al., 2003). Several psychotherapeutic drugs, and drugs of abuse, such as cocaine and amphetamine, bind to DAT with high affinity. The DA taken-up into the presynaptic cells can be either recycled into vesicles for further use in neurotransmission or degraded by the action of the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) into homovanillic acid (Meiser et al., 2013).

2.2 DA receptors

DA receptors belong to the large G protein-coupled receptor (GPCR) superfamily. Based on structural similarity, the five mammalian DA receptor subtypes could be divided into two major subclasses: D₁-like group (D₁ and D₅) and D₂-like group (D₂, D₃ and D₄) (Missale et al., 1998; Callier et al., 2003). The D₁ receptors are structurally characterized by a short third cytoplasmic loop and a very long C-terminal tail. They are coupled to the G_{os} class of G proteins and, accordingly, activate adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) as a second messenger. In contrast, the D₂ receptors bear a long third cytoplasmic loop and a short cytoplasmic C-terminal end. They are coupled to G_{ai/o} protein to inhibit cAMP synthesis. The D₁-like receptor activation can be either excitation or inhibition, while the ultimate effect of D₂-like activation is usually an inhibition of target neurons (Ayano et al., 2016).

According to their respective location in the CNS, DA receptors are implicated in different physiological and pathological processes (Beaulieu and Gainetdinov, 2011; Hisahara and Shimohama, 2011). In the striatum, D₁ and D₂ are expressed by different neuronal populations and they act in concert to mediate DA control of locomotion. In the basal ganglia, D₂ and D₃ locate in DAN nerve terminals serving as autoreceptors that modulate locomotor output. In the frontal cortex, D₁, D₂, D₄ and D₅ mediate various physiological functions of DA, like its effect on cognition and working memory. Finally, D₁ and D₂ play a fundamental role in the rewarding mesolimbic pathway.

2.3 Anatomy of the neural dopaminergic system

Anatomical organization and projection pattern of DANs in the adult rodent brain are displayed in Figure 2 with the majority of DAN cell bodies located in the dopaminergic areas A8 (retrosubstantia nigra), A9 (substantia nigra, SN) and A10 (ventral tegmental area, VTA) (Bentivoglio and Morelli, 2005). ‘A’ refers to aminergic (DA and noradrenaline), according to the nomenclature introduced by Dahlström and Fuxe (1964), in which A1-A7 neurons express the neurotransmitter norepinephrine, A8 to A14 DA, B is for serotonin and C for adrenaline.

The brain dopaminergic system is divided into three pathways: 1) the nigrostriatal pathway from the SN pars compacta (SNpc) (A9) to the dorsal striatum, 2) the mesolimbic pathway from the VTA (A10) to the nucleus accumbens and olfactory tubercle, 3) the mesocortical pathway from the VTA (A10) to the prefrontal cortex (Iversen and Iversen, 2007). Diminished nigrostriatal pathway signalling results in dysfunctional communication in the basal ganglia circuitry and is responsible for the motor symptoms in PD patients. Loss of nigrostriatal projections was attributed to the specific susceptibility of their thin poorly myelinated or unmyelinated axonal fibers travelling a long distance to the striatum to degeneration (Sulzer, 2007). The respective function of the different dopaminergic pathway will be illustrated in next section.

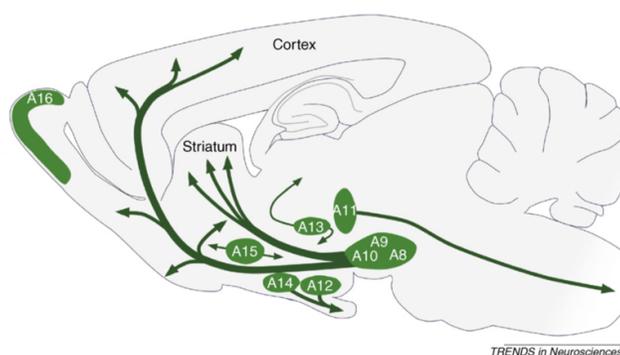


Figure 2 Dopaminergic system in the adult rodent brain.

The DANs in the mammalian brain are localized in 9 distinct cells groups (nuclei), distributed from the mesencephalon to the olfactory bulb, as illustrated schematically from Björklund and Dunnett (2007). The principal projections of the DA cell nuclei are indicated by arrows.

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2.4 DA function

2.4.1 Basal ganglia structure

In order to understand the motor control loops, it is necessary to present an overview of the basal ganglia components and circuits (Bentivoglio and Morelli, 2005, Lanciego et al., 2012). The main structure of the basal ganglia in the mammalian brain includes the striatum, the globus pallidus (GP), the subthalamic nucleus (STN), and the SN, shown in Figure 3. The globus pallidus is divided by the internal medullary lamina into a lateral or external segment (GPe) and a medial or internal segment (GPi). The striatum comprises the caudate nucleus, the putamen and the ventral striatum. The caudate nucleus, the putamen, and the nucleus accumbens are considered as input nuclei receiving information from the cortical and thalamic area. The output comprises the GPi and SN pars reticulata (SNpr) conveying to the thalamus, targeting in particular the ventral tier thalamic nuclei (Ventral anterior (VA)/Ventrolateral (VL)), which project via the thalamocortical system to frontal cortical areas that gives origins to cortical descending pathways. The intrinsic nuclei include GPe, STN and SNpc located between the input and output nuclei in the information relay.

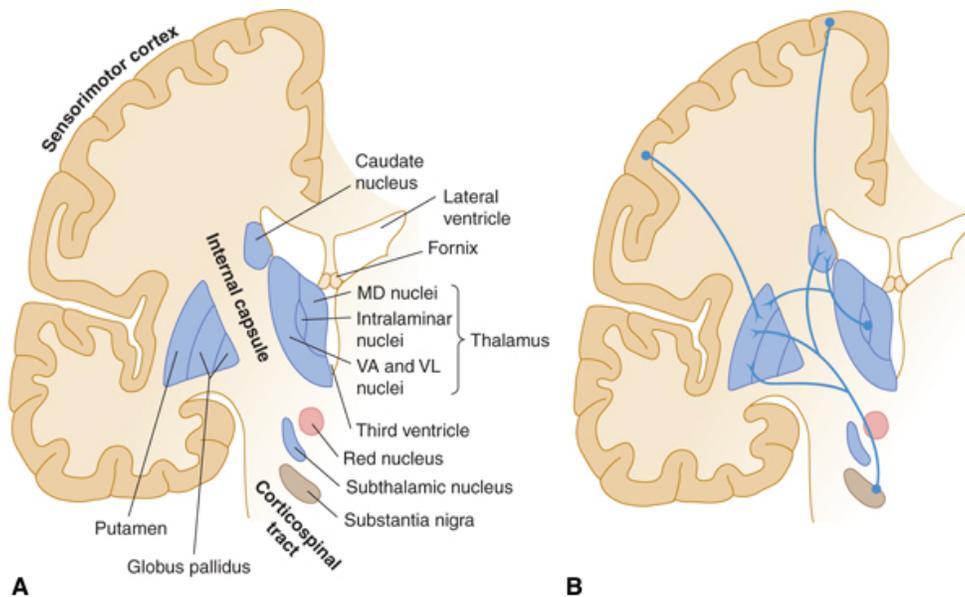


Figure 3 Basal ganglia structure in the human brain

(A) Localization of the different components of the basal ganglia, which comprises the striatum, the subthalamic nucleus (STN) and the substantia nigra (SN). The striatum includes the caudate nucleus, putamen and ventral striatum. (B) Representation of the neuronal connections implicated in motor control. The different pathways are schematically depicted in Figure 4. VA nuclei: Ventral anterior

nuclei; VL nuclei: Ventrolateral nuclei; MD: mediodorsal (Source: <https://neupsykey.com/control-of-movement-2/>).

2.4.2 Function of the DA nigrostriatal pathway in basal ganglia circuitry

The nigrostriatal pathway refers to the dopaminergic projections that connects the SNpc to the dorsal striatum (caudate nucleus and putamen) and are involved in movement control. Neuronal loss in SNpc causes enhanced inhibitory output feeding back to the cortex that leads to canonical Parkinsonian syndromes such as rigidity, akinesia and resting tremor. The pathology underlying nigrostriatal degeneration will be addressed in Chapter two.

The basal ganglia circuitry is constituted of two major projection systems schematically depicted in Figure 4. The *direct* pathway arises from GABAergic neurons in the striatum that send their monosynaptical projection to the GPi/SNpr (Gpi and SNpr are grouped together and share a number of cyto- and chemoarchitectural characteristics and to some extent similar types of afferent and efferent systems). The *indirect* pathway includes GABAergic striatal neurons sending information to the GPi/SNpr via sequential interconnection with the GPe and STN (Bentivoglio and Morelli, 2005).

The ganglion circuitry involved in the control of voluntary limb movements arises from the motor cortices (primary motor cortex, supplementary motor area and premotor cortex) that send glutamatergic and excitatory projections to the somatomotor territories of the basal ganglia. Convergence of motor projection occurs in the striatum, which also receive nigrostriatal inputs. DA release regulates the activity of striatal neurons involved in both the direct and indirect pathways. Direct pathway striatal neurons express preferentially D₁ receptors and the neuropeptide substance P, while indirect pathway neurons express D₂ receptors and enkephalin (Przedborski, 2017). Targeting their respective receptors, DA leads to stimulation of the direct pathway via excitatory D₁-receptors, while simultaneously inhibiting the indirect pathway via inhibitory D₂-receptor activation.

The STN, which is the excitatory glutamatergic nucleus of the basal ganglion, also receives direct projections from the cortex through the corticosubthalamic pathway bypassing the striatum, named the *hyperdirect* pathway (Bentivoglio and Morelli, 2005) (Figure 4).

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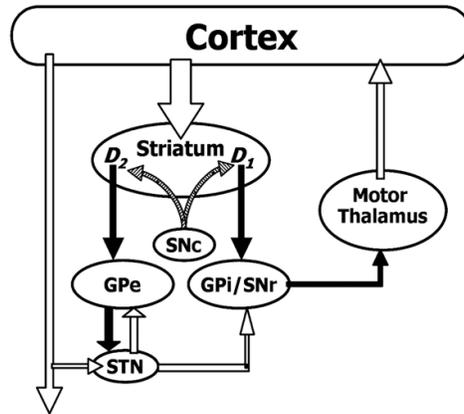


Figure 4 Standard model of basal ganglion circuitry for locomotion.

The black arrows indicate the inhibitory connection, the white arrows correspond to excitatory connections, and the grey arrow represent the modulatory dopaminergic innervation of the striatum. The *direct* pathways refers to the striatum-GPi/SNr-thalamus tract, the *indirect* pathway to the striatum-GPe-STN-Gpi/SNr-thalamus pathway, and the *hyperdirect* pathway to the frontal cortex connections to the STN-GPi-thalamus tract that bypasses the striatum. Source: (Rubchinsky et al., 2003).

2.4.3 Other dopaminergic pathways and related functions

The mesolimbic pathway refers to projections of DANs located in the VTA to the nucleus accumbens (NAc), limbic system-amygdala, and hippocampus. The NAc is considered as a limbic-motor interface that plays a role in motivation and reward, translating information about reward into proper behavioural responses (Haber and McFarland, 1999), while the limbic system is associated with emotion and memory formation (Ledoux et al., 1993). Given the nature of the cognitive sensation, the mesolimbic pathway is also involved in addiction and depression (Berke and Hyman, 2000; Ungless et al., 2010). Dysfunction in this system results in a range of psychological process and neuropsychiatric diseases, such as schizophrenia, addiction, attention deficit hyperactivity disorder, and depression (Alcaro et al., 2007; Dichter et al., 2012).

The mesocortical pathway originates from the neurons located throughout the SNpc and VTA. These neurons send branch axons within the cortex reaching more than one cortical area (Bentivoglio and Morelli, 2005). The dopaminergic innervation of the prefrontal cortex has been implicated in the modulation of normal cognitive processes, working memory related to age-related memory decline and alterations, neurodegenerative disorders including PD, as

well as psychiatric diseases (Manoach, 2003; Seamans and Yang, 2004; Durstewitz and Seamans, 2008).

3. DA study in *Drosophila*

Before presenting the DA system in *Drosophila*, a brief introduction to *Drosophila* neurobiology study should be given to highlight its prominent contribution to neuroscience research. As reviewed by Bellen et al. (2010), powerful impacts of fly work pioneered mechanisms underlying nervous system development such as *Notch* identification, characterized synaptic transmission at the larval neuromuscular junction, and initiated neurogenetics studies to understand the neuronal circuitry of behaviour and provide insights into neurodegenerative mechanisms.

3.1 Advantages of the *Drosophila* model in neurobiology

A combination of several key factors makes the fly a uniquely powerful animal model neuroscience research and better understand human neurodegenerative diseases. First, compared to other model organisms, flies have quite a short life cycle and produce a large number of descendants, and they are relatively inexpensive to maintain. Second, nearly 75% of human genes have functional homologues in flies (Rubin et al., 2000; Reiter et al., 2001). However, *Drosophila* has a smaller number of genes (around 14,000 protein-coding genes) compared to humans (around 20,000-25,000). Owing to less redundancy, this reduced genome complexity allows easier interpretation of loss-of-function studies. Although humans and flies may not look very similar, numerous developmental pathways and physiological process are conserved across evolution (Rubin and Lewis, 2000). Third, as to the nervous system, flies have a real brain with neurons and glial cells, protected by a blood-brain barrier, and complex neuronal networks utilizing similar neurotransmitters as in vertebrates for behavior modulation (memory, sleep, locomotion, mating, etc). Furthermore, defined regions of the vertebrate and fly brains appear to share genetic and structural homologies, like the vertebrate basal ganglia and *Drosophila* central complex (Strausfeld and Hirth, 2013; Fiore et al., 2015).

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Unsurpassed advantages of *Drosophila* in neurobiology compared to any other organism result from the various genetic tools that are available. The *Gal4/UAS* expression system made from yeast (*Saccharomyces cerevisiae*) components, is very reliable and useful. This system is based on tissue-specific expression of the transcription factor Gal4 that binds to specific upstream activating sequences (UAS) to initiate transcription of downstream genes (Brand and Perrimon, 1993). Gal4 expression pattern can be refined and selectively suppressed in a subset of neurons by combination with a thermosensitive Gal80 repressor (McGuire et al., 2003). Other *in vivo* expression systems have been designed since then such as the *LexA/LexAop* system derived from bacterial (*Escherichia coli*) components, in which the LexA transcription factor binds to LexA operator (LexAop) sequences (Pfeiffer et al., 2010).

Thousands of Gal4- and LexA-transformed lines are currently available to develop neurobiology studies in *Drosophila*, in which the enhancers from different genes allow targeted expression of the exogenous transcription factors in selective neuronal or glial subtypes. *UAS* or *LexAop* sequences fused with the gene or RNA interference (RNAi) sequence of interest allow their Gal4-driven expression at desired time and in specific neurons clusters or even in a single pair of neurons, which provide better understanding of gene function in specific neuronal population. Particularly for neurobiological studies, different effector or reporter transgenes driven by *Gal4/UAS* or *LexA/LexAop* permit neuronal visualization (fluorescent proteins), recording (GCaMP), silencing (temperature sensitive Shibire (Shi^{ts1})), or activation (thermosensitive channels dTrpA1/TRPM8, light activated channels ChR2-XXL (Dawydow et al., 2014) or ChR2/CsChrimson (Owald et al., 2015b)). The use of *LexA/LexAop* as a complementary binary system combined with *Gal4/UAS* has many application, such as the identification of potential synaptic connections by the green fluorescent protein (GFP)-reconstitution across synaptic partners (GRASP) method (Feinberg et al., 2008; Macpherson et al., 2015) and the analysis of neuronal circuitry by monitoring activity in neurons of interest while activating or inhibiting the feedback or upstream neurons which project on them (Cohn et al., 2015).

3.2 Structure of the brain mushroom bodies and central complex.

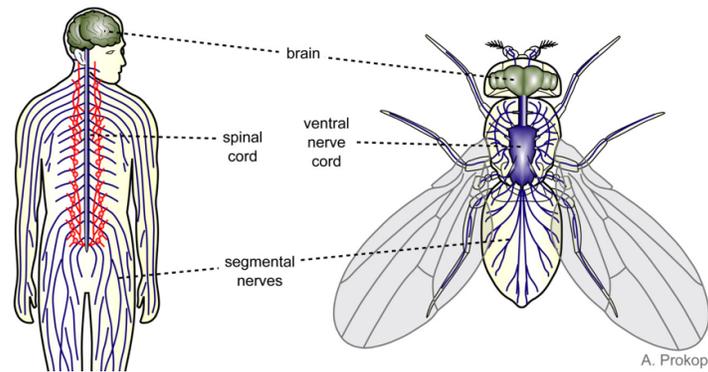


Figure 5 Comparison of the human and *Drosophila* nervous system

The nervous systems in the fly and humans, which comprise brain, ventral nerve cord (VNC) and segmental nerves, appears quite similar in their organization. Source: <https://droso4schools.wordpress.com/organs/>

A 3D model of defined synaptic neuropils was established by Kei Ito's group (Ito et al., 2013) that gives a whole view of the *Drosophila* brain. Among those complex neuropils, the mushroom body (MB) attracted overwhelming attention (Nighorn et al., 1994). The MB is one of the most clearly distinguishable neuropils in the insect brain (Strausfeld et al., 1998). This complex structure is composed of about 2,200 neurons per hemisphere that include 7 subtypes of intrinsic neurons or Kenyon cells (KCs) representing ~2000 neurons, 20 subtypes of afferent DANs which modulate each MB compartment and 21 subtypes of MB output neurons (MBONs) conveying information outside the MB (Aso et al., 2014a) (Figure 6).

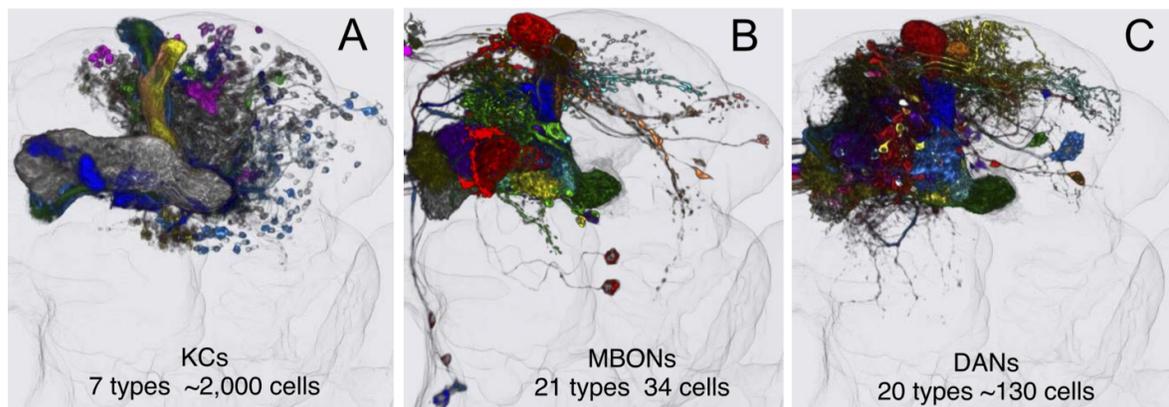


Figure 6 Display of the three types of neurons in the MBs

(A) KCs have their cell bodies and dendrites in the posterior region of the brain and send their axons forming the horizontal and vertical lobes of the MBs. (B) MBONs arborize on the MB lobes and send

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their axons reaching to the SMP, SIP, SLP, and CRE regions. (C) Dendrites of MB-associated DANs lie in the CRE, SMP, SLP and SIP and tile the MB calyx and lobes. CRE: crepine; MB: mushroom body; SMP: superior medial protocerebrum; SIP: superior intermediate protocerebrum; SLP: superior lateral protocerebrum. Source: (Aso et al., 2014a).

KCs form the fundamental structure of the MBs. Their cell bodies constitute a large cluster in the dorsal posterior brain; their dendritic branches make up the calyx and their axons bundle up in the peduncles. At the end of the peduncles, the axons bifurcate dorsally and medially to form the vertical (α and α') and horizontal (β , β' and γ) lobes, respectively. The MB contains three types of KCs that are named according to the lobes in which they send axonal projections: $\alpha\beta$, $\alpha'\beta'$ and γ KCs (Lee et al., 1999; Tanaka et al., 2008). The DANs are the most prevalent modulatory neurons in the MB (Pech et al. 2013b). They were thought to release DA to regulate information at the KC>MBON synapses. However, recent studies also identified KC>DAN and DAN>MBON synapses in the adult MB (Cervantes-Sandoval, et al., 2017; Takemura et al., 2017). In addition to being a center for olfactory learning and memory formation, retrieval and consolidation, the MB has been reported to be involved in locomotor activity control, sleep, courtship and other functions (Martin et al., 1998; Besson and Martin, 2005; Sitaraman et al., 2015a, b; McBride et al., 1999; Haynes et al., 2015; Bouzaiane et al., 2015).

In addition to the well-investigated MBs, another structure that attracted attention is the central complex (CC) straddling the middle of the protocerebrum in the centre of the brain (Wolff et al., 2015). Its four components are the protocerebral bridge (PB), fan-shaped body (FSB), ellipsoid body (EB) and noduli. The CC is involved in coordinating locomotor behaviour, including flight and walking activity, visual function and various types of memory in flies (Martin et al., 1999; Strauss, 2002; Kahsai et al., 2010; Liu et al., 2006).

3.3 Anatomy of the dopaminergic system in the *Drosophila* CNS

DANs in *Drosophila* are involved in the control of many behaviours (Riemensperger et al., 2011; Yamamoto and Seto, 2014). Comparably to DAN nuclei (A8-A16) in rodents (shown in Figure 2), *Drosophila* DAN system could be divided into 15 clusters consisting of around 300 TH-positive neurons (Nässel and Elekes, 1992). As shown in Figure 7, in the anterior brain, there are 5 protocerebral anterior lateral (PAL) neurons, a small group located in the lateral and medial part of the subesophageal zone (SEZ) and

about 100 protocerebral anterior medial (PAM) neurons (Friggi-Grelin et al., 2003a; Mao and Davis, 2009; Liu et al., 2012a; Niens et al., 2017). The posterior DAN clusters in the adult brain are protocerebral posterior lateral 1 and 2 (PPL1 and PPL2), protocerebral posterior medial 1/2 and 3 (PPM1/2 and PPM3), a group of small neurons located lateral to the SEZ and a group of large neurons in the medial part of the SEZ. In the VNC, dense innervation descending from the CNS projects to all three segments, together with giant neurons and dense clusters of DANs in the abdominal ganglia.

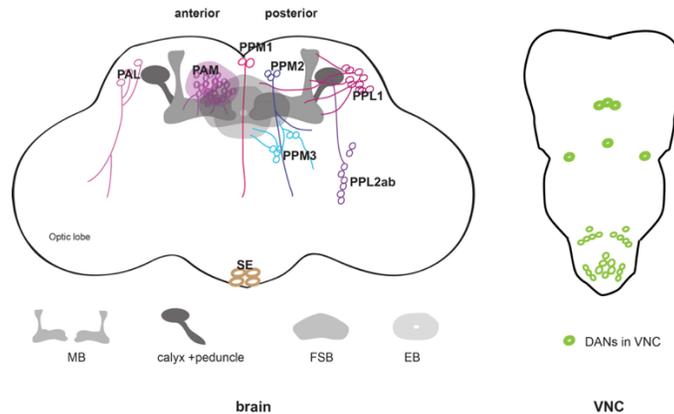


Figure 7 DAN systems in brain and VNC

This scheme depicts the localization of DAN clusters in the brain and VNC. In the anterior brain, PAM projects to the MB medial lobes, and PAL clusters mainly project to the optic lobes. Clusters PPLs and PPMs locate in the posterior brain with their axons reaching to the MB vertical lobes, FSB, EB and other neuropils in the brain. The known efferent neuropil of DANs in brain are shown in grey. In the VNC, around 6 giant DANs locate between the segments and groups of dense DANs lie in the abdominal ganglia. DAN: dopaminergic neurons, EB: ellipsoid body, FSB: fan shaped body, MB: mushroom body, PAL: protocerebral anterior lateral, PAM: protocerebrum anterior medial, PPL: protocerebrum posterior lateral, PPM: protocerebral posterior medial, VNC: ventral nerve cord.

In the fly brain, as introduced in this section, the PAM together with PPL1 neurons, and one PPL2ab neuron send projection onto the MB lobes and calyx, respectively, while some neurons of PPL1 and PPM3 tiles the CC neuropil. Besides, long projections from the other clusters of DANs reach to the optic lobes (PAL), subesophageal ganglion (SOG) (PPM2) and inferior/superior/lateral/medial protocerebrum (PAL and PPL2) (Mao and Davis, 2009; Liu et al., 2012b). The DANs innervating the MBs and CC are described in more details below.

PPL1. The PPL1 cluster of DANs (12 per hemisphere) are labelled by the DA driver *TH-Gal4* (Friggi-Grelin et al., 2003a) and their projections tile the MB vertical and γ lobes, as well as the FSB. Based on the different areas they target, three PPL1 DANs were named MB-

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MP1 (γ 1 ped), MB-V1 (α 2 α '2) and MB-MV1 (γ 2 α '1). Other PPL1 neurons target to the α 3 α '3 compartment and the FSB (Tanaka et al., 2008, Aso et al., 2012, Liu et al., 2012b). Dendrites of PPL1 neurons mainly lies in the superior medial protocerebrum (SMP) and a minority arborize in the crepine (CRE) and superior intermediate protocerebrum (SIP), and a tiny bit in the superior lateral protocerebrum (SLP) (Aso et al., 2014a). A function for PPL1 DANs has been described for learning and forgetting, sleep and locomotion control (Aso et al., 2010; Aso et al., 2012; Berry et al., 2012; Uneo et al., 2012; Liu et al., 2012b; Plaçais et al., 2012; Pathak et al. 2015; Sitaraman et al., 2015b; Vaccaro et al., 2017; Artiushin and Sehgal, 2017).

PPM3. The 6-8 PPM3 DANs are all labelled by *TH-Gal4* and were found to innervate the CC, which is described as a higher centre for locomotion control (Strauss, 2002; Mao and Davis, 2009; White et al., 2010). PPM3 neurons projecting to the dorsal FSB and the EB are involved in ethanol-stimulated locomotion, aggressive behaviour, arousal and sleep, et al. (Kong et al., 2010, Liu et al., 2012b; Ueno et al., 2012; Alekseyenko et al., 2013; Artiushin and Sehgal, 2017).

PPL2ab. The cell bodies of PPL2ab neurons are located on the lateral edge of the protocerebrum near the optic lobes. The PPL2ab contain 4 to 6 cells. Two of these neurons were observed to target the MB calyx and tile the lateral horn and others send broad projections to the protocerebral area (Mao and Davis, 2009). Due to few specific drivers available for targeting this cluster, available recent work suggest that PPL2ab neurons may not affect motor activity and sensory processing, but regulate the male courtship behaviour (Kuo et al., 2015; Chen et al., 2017).

PAM. PAM are the last born DANs during metamorphosis with smaller somata that form a dense cluster of around 100 neurons located in the anterior brain (Liu et al., 2012a; Riemensperger et al., 2013). These DANs send projections towards the medial lobes of the MBs, tile the tips of the γ lobes and the β ' and β lobes and their dendrites arborize in the CRE, SIP, SLP, and SMP (Pech et al., 2013a; Aso et al., 2014a). The PAM clusters is labelled by *R58E02-Gal4* in which the enhancer is derived from the first intron of the *Drosophila* DA transporter gene (*DAT*) (Liu et al., 2012a). This cluster is involved in appetitive olfactory learning (Liu et al., 2012a; Waddell, 2013). It was also reported to have wake-promoting effect (Sitaraman et al., 2015b) and to be involved in caffeine-induced arousal (Nall et al., 2016) and the control of startle-induced climbing and air puff-stimulated flight (Riemensperger et al., 2013; Bou Dib et al., 2014; Agrawal and Hasan, 2015).

3.4 Comparison of DA dynamics in *Drosophila* and mammals

DA appears to be an evolutionarily old neurotransmitter system that was recruited like most classical transmitters in the common cnidarian/bilaterian ancestor (Moroz and Kohn, 2016). As systematically reviewed by Yamamoto and Seto, 2014, the organization of DA neurotransmission is highly conserved between *Drosophila* and mammals. The genes involved in DA synthesis, transport, secretion, signal transduction have their homologs in flies, e.g. *TH*, *Ddc*, *DAT*, *VMAT* and *DA receptors*. Consistent to their effects in mammals, flies respond similarly to pharmacological agents targeting the DA system, e.g. modafinil, cocaine, caffeine, amphetamines. (Hendricks et al., 2003; Birman, 2005; Andretic et al., 2005; Shaw et al., 2000; Chang et al., 2006).

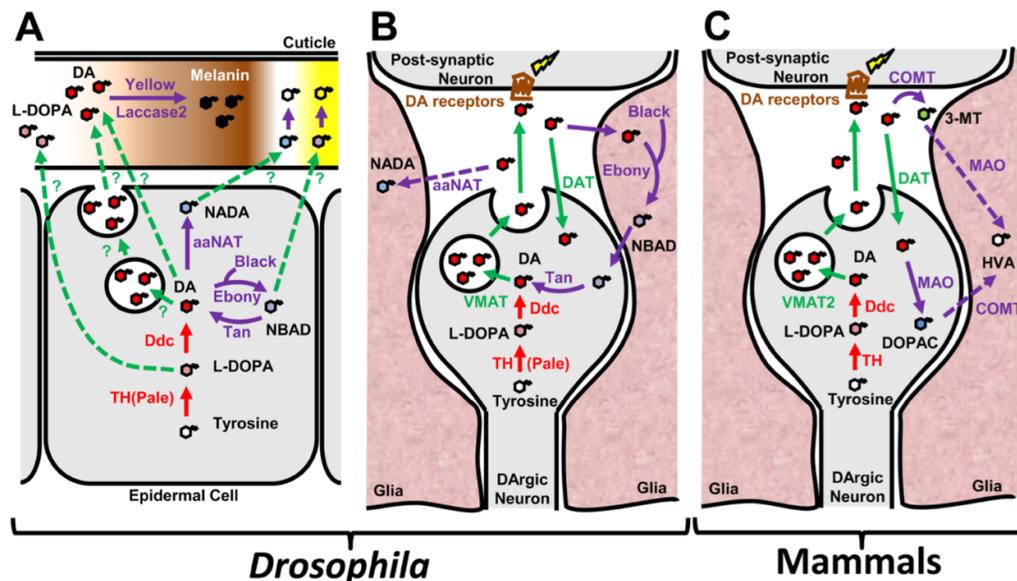


Figure 8 Schematic diagrams of DA dynamics and signalling.

A) *Drosophila* hypoderm and cuticle B) *Drosophila* brain and C) mammalian brain. In *Drosophila*, DA synthesis takes place not only in the CNS but also in the hypoderm, and utilizes in both tissues the enzymes TH and Ddc encoded from alternatively spliced genes (Morgan et al, 1986; Birman et al., 1994). DA or L-DOPA secreted from hypodermal cells are oxidized into melanin and contributes to pigmentation and sclerotization of the insect cuticle. Ebony, Black, Tan, and aaNAT in *Drosophila* are involved in DA metabolism. Except for DA degradation, DA synthesis, storage, release and reuptake share similar mechanisms between the fly and mammals, and the genes that function in *Drosophila* has homologs in mammals. aaNAT: arylalkylamine N-acetyltransferase. Source: (Yamamoto and Seto, 2014)

However, discrepancies lie that, first, the major enzymes involved in DA metabolism appear quite different between flies and mammals. Nearly no COMT activity and a low MAO

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activity were detected in *Drosophila*, while N-acetyltransferase (NAT) activity (whose mammalian homolog is involved in the biosynthesis of melatonin), was described to be 50-fold greater than the MAO activity and 500-fold greater than COMT suggesting that this acetylation pathway is the major route for DA metabolism. The corresponding aaNAT enzyme was identified later in 1996 (Wright, 1987; Hintermann et al., 1996). More details of the comparison between the DA system in *Drosophila* and mammals are shown in Figure 8.

Second, interestingly, DA synthesis, transport and secretion are not restricted to the CNS in *Drosophila*, as there are DA-expressing cells in the hypoderm. DA is indeed required for cuticle pigmentation and hardening (sclerotization) in insects (Wright, 1987). Genes regulating DA biosynthesis in the *Drosophila* brain, i.e. *TH* and *Ddc*, are also essential for cuticle formation. Null mutations in the *Ddc* and *ple* (*TH*) loci results in unpigmented *Drosophila* embryos that are unable to hatch in part because they have a soft cuticle (Wright 1987; Budnik and White, 1987). Specific upstream DNA regulatory elements and alternatively spliced transcripts uncouple the DA function in the fly CNS and hypoderm (Bray et al., 1988; Morgan et al., 1986; Johnson et al., 1989; Birman et al., 1994; Vié et al., 1999; Friggi-Grelin et al., 2003b ; Davis et al., 2007). DA also has functions in the ovary and in the gut in *Drosophila* (Neckameyer, 1996; Draper et al., 2007).

3.5 DA receptor signalling in *Drosophila*

3.5.1 DA receptors

Four G-protein coupled DA receptors have been identified in *Drosophila*: two D₁-like receptors (*Dop1R1/dumb* and *Dop1R2/damb*), one D₂-like receptor (*Dop2R/DDR2*) and one non-canonical receptor (*DopEcR*) (Feng et al., 1996; Han et al., 1996; Hearn et al., 2002; Kim et al., 2003; Srivastava et al., 2005; Draper et al., 2007). Conserved with mammalian mechanisms, D₁-like receptors act through activation of the cAMP pathway while the D₂-like receptor inhibits the cAMP pathway. Importantly, the D₂-like receptor *Dop2R*, similar to those of mammals, localizes both presynaptically in DANs as an autoreceptor that regulates DA release and postsynaptically (Vickrey and Venton, 2011; Qi and Lee, 2014). The brain localization of the four DA receptors and their respective function in different behaviours are summarized in **Table 1**. Summarized function of DA receptors in different neuropils reflects

the role of DA in different behavioural modulation in *Drosophila*, including sleep, learning, courtship and aggression.

Table 1 *Drosophila* DA receptor and their respective localization and function

Receptor	locate	Involved function	Reference
Dop1R1	MB	Learning, memory, forgetting, temperature preference, wake and sleep	Kim et al., 2007; Andretic et al., 2008; Seugnet et al., 2008; Bang et al., 2011; Berry et al., 2012; Jiang et al., 2016; Plaçais et al., 2017;
	MB $\alpha'\beta'$	LTM, water reward	Lin et al., 2014
	MB gamma	Learning, STM, LTM, courtship learning	Qin et al., 2012; Keleman et al., 2012
	EB	Repetitive startle-induced arousal; ethanol-stimulated locomotion	Kong et al., 2010, Lebestky et al., 2009
	FSB	Night sleep regulation; wake promoting; aggression	Ueno et al., 2012; Liu et al., 2012b; Alekseyenko et al., 2013
	PIb	Sleep	Jiang et al., 2016
	PDF neurons	Sleep/wake arousal	Lebestky et al., 2009
	TG	Hind leg grooming	Pitmon et al., 2016
Dop1R2	MB	LTM, forgetting	Berry et al., 2012; Musso et al., 2015; Plaçais et al., 2017
	FSB	Sleep	Pimentel et al., 2016
	P1	Sex drive	Zhang et al., 2016
	Glutamate neurons	Susceptibility to paraquat and DA toxicity	Cassar et al., 2015
DopEcR	MB	Behavioural sensitization, Courtship memory	Aranda et al., 2017; Ishimoto et al., 2013
	?	Ethanol-induced sedation	Petrucelli et al., 2016
	?	Proboscis extension reflex during starvation	Inagaki et al., 2012
		Modulation of Ca^{2+} dynamics within the MB	Lark et al., 2017
Dop2R	PB+?	Aggression	Alekseyenko et al., 2013
	CA	Juvenile hormone (JH) synthesis	Gruntenko et al., 2012
	?	Locomotor activity	Draper et al., 2007
	MB, APL neurons	ARM	Scholz-Kornehl and Schwarzel, 2016

APL: anterior paired lateral; ARM: anaesthesia-resistant memory; CA: corpus allatum; CC: central complex; EB: ellipsoid body; FSB: fan-shaped body; LTM: long-term memory; MB: mushroom

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bodies; JH: juvenile hormone; PB: protocerebrum bridge; Plb: pars intercerebralis; TG: thoracic ganglion; STM: short-term memory; ?:unknown region

3.5.2 DA control of locomotor behaviour

In fly, previous studies demonstrated that DA plays a central role in the modulation of locomotion and stereotyped movements like grooming or stimuli-induced behaviours (Yellman et al., 1997; Lima and Miesenböck, 2005; Riemensperger et al., 2011; Sadaf et al., 2015; Pitmon et al., 2016). Optogenetic stimulation of DA release led to an instant increase in fly walking activity (Lima and Miesenböck, 2005). Brain DA-deficient flies exhibited a reduction in spontaneous locomotion (walking speed and covered distance) (Riemensperger et al., 2011) and tetanus toxin light chain (TNT)-induced inactivation of synaptic transmission in a large number of DANs strongly reduced locomotion as well (Alekseyenko et al., 2013). In contrast, flies showing already high motor activity exerted transient arrest upon optogenetically-induced DA release (Lima and Miesenböck, 2005), which could be related to the inverted U-shaped of DA dose-effect relation on behaviour and arousal (Birman, 2005). The identity of DANs that control spontaneous locomotion is currently unknown, but at least part of them could be those innervating the MBs and CC. Indeed, previous study showed that the MBs suppress locomotor activity (Huber, 1967; Heisenberg et al., 1985; Martin et al., 1998; Helfrich-Förster et al., 2002), while the CC is required for locomotor maintenance (Strauss and Heisenberg, 1993; Martin et al., 1999; Martin et al., 2001; Strauss, 2002).

In the work of Friggi-Grelin et al., expressing tetanus toxin (TNT) in DANs targeted by *TH-Gal4* caused lower performance index in the startle-induced negative geotaxis (SING) climbing test, paradoxically resulting from an increased excitability (arousal) that prevented the flies from climbing (Friggi-Grelin et al., 2003a). The role of brain DA in tempering exogenously-generated arousal was subsequently confirmed in another group by monitoring locomotor hyperactivity induced by repetitive air puffs (Lebestky et al., 2009). These authors demonstrated that inhibiting signalling from DANs that innervates the EB neurons in the CC (R2/R4 and R3) resulted in elevated arousal indicated by increased fly velocity. Interestingly, the R2/R4 EB neurons and activity of one pair of PPM3 DANs that innervate them are also required for ethanol-induced hyperactivity (Kong et al., 2010).

As shown in the study of White et al., 2010, walking activity increases in aged flies while the

startle-induced climbing ability in contrast progressively decreases with age indicating that these two different locomotor activities (spontaneous locomotion and locomotor reactivity) are regulated differently and may involve distinct neuronal circuits, in agreement with several anterior observations (Connolly, 1967; Meehan and Wilson, 1987; O'Dell and Burnet, 1988; Martin et al., 1999). Remarkably, brain DA-deficient flies also showed strong deficits in locomotor reactivity, as assessed by the SING test (Riemensperger et al., 2011). Recent results implicated the MBs and associated DANs in the control of startle-induced locomotion and age-related SING decline (Riemensperger et al., 2013 and further work described below in this PhD dissertation). The first evidence came from the observation that α -synuclein-induced degeneration of projections from the PAM DANs to the MB medial lobes was in large part responsible for the accelerated loss of SING under this PD-like condition (Riemensperger et al., 2013). In addition to this, accelerated SING deficit was observed in aging *Clk*^{AR} mutant flies associated with loss of MB-targeting PPL1 DANs (Vaccaro et al., 2017). This topic will be further elaborated in the Results part.

Introduction

Chapter 2 Parkinson's disease (PD)

1. Overview of PD pathology and treatments

PD is one of the most common neurodegenerative disorder, affecting 1 % of the population over the age of 60, and nearly 5% of people over the age of 85 (Reeve et al., 2014). This disease was first medically described by the British physician James Parkinson as “paralysis agitans” in 1817. The essential criteria for the clinical diagnosis of PD is defined as parkinsonism, a movement disorder that is characterized by tremor at rest, bradykinesia, rigidity and postural instability. In addition to these features, non-motor symptoms such as impaired olfaction, disordered sleep, and constipation usually precede the clinical recognition of PD by years or even decades (Tolosa et al., 2009; Kiala and Lang, 2015). The cardinal neuropathological feature of PD is the degeneration or cell death of the DANs in the SNpc, accompanied with cytoplasmic inclusions named Lewy bodies (Lees et al., 2009). The main component of these aggregates is a small protein called α -synuclein (α -syn) which is thought to play a central part in PD. Because DANs contain neuromelanin, degeneration of DANs is visible by midbrain depigmentation in PD patients, as shown in Figure 9.

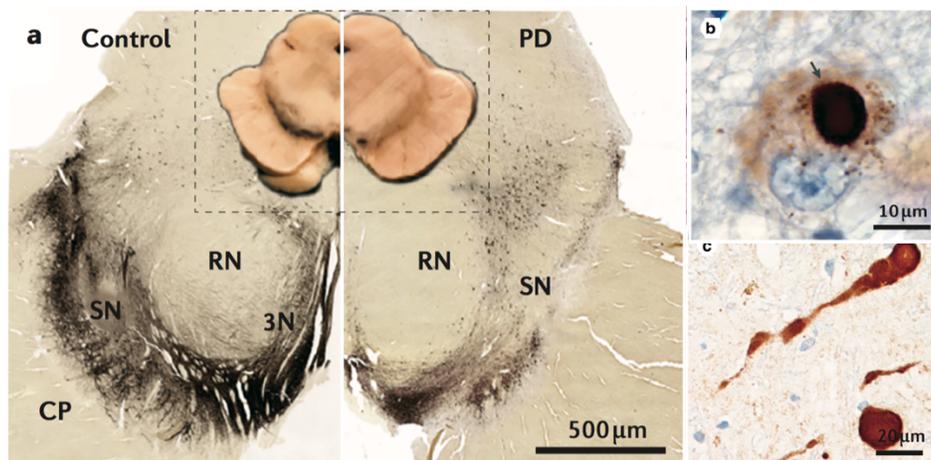


Figure 9 Two key neuropathological feature of PD

Schematic representation of the midbrain from healthy and PD brains. (a) The natural pigmentation of the SNpc caused by neuromelanin within DANs is diminished in PD patients. Lewy bodies (b) and Lewy neurites (c) can be immunostained by α -syn antibodies. SN: substantia nigra, 3N: 3RD nerve fibres, CP: cerebral peduncle, RN: red nucleus; Source: Poewe et al., 2017

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As shown in Figure 10, excitatory signal of the nigrostriatal pathway from the SNpc onto the putamen (Pu) leads to an inhibition of the GPi, subsequently relieving its inhibition on the thalamus (Th) which then activates the motor cortex for the execution of movement. However, in the case of PD, a loss of signalisation from the SNpc (in purple) causes an increase in the inhibitory output from the GPi to the thalamus (in red), thus repressing the initiation of movements (in green). This disturbance in basal ganglia circuitry is believed to be responsible for the motor dysfunction symptoms (Shulman et al., 2011; Poewe et al., 2017).

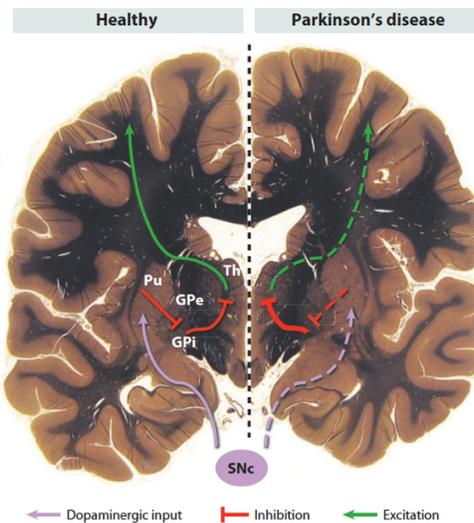


Figure 10 Anatomy and pathophysiology of the PD brain

Normal neuronal circuits for facilitating movement (left) and deranged circuits in PD (right). Excitatory nigrostriatal pathway from the SNpc to the putamen (Pu) normally inhibits the internal globus pallidus (GPi), subsequently relieving its inhibition of the thalamus (Th), which can then transfer excitatory inputs to the motor cortex for the execution of movement. Source: Shulman et al., 2011

Since its introduction in the 1960s, DA replacement therapy by the ingestion of L-DOPA (levodopa) has become the standard treatment for the different motor symptoms of PD. It is combined with other drugs to reduce complications induced by levodopa on the long term, such as motor fluctuation and dyskinesias (Jankovic and Aguilar, 2008). Deep brain stimulation (high frequency stimulating electrodes) targeted to the the subthalamic nucleus (STN) to reduce limb tremor in patients with advanced PD can be effective as well (Hamel et al., 2003; Herzog et al., 2003; Kalia and Lang, 2015). Although currently available PD therapies both delay disability and prolong life expectancy, none has been proven to

significantly alter the ongoing neurodegenerative process of the pathology (Kalia and Lang, 2015; Poewe et al., 2017).

2. Factors involved in PD onset

PD is mostly a sporadic disorder, but about 10-15% of patients have a family history for this disease. The motor defects and the phosphorylated and ubiquitinated forms of α -syn fibrils found in Lewy bodies are present in both dominantly-inherited and sporadic PD cases (Baba et al., 1998; Papapetropoulos et al., 2007; Kiala and Lang et al., 2015). Moreover, PD is a slowly progressing disease with a high incidence in aged people. Although the Lewy bodies and the DAN loss are considered as the two key features of PD, there is now evidence that healthy aged people can also exhibit Lewy bodies and SN degeneration (Rodriguez et al., 2015). This discovery highlights the fact that, besides the ageing factor, a combination of genetic susceptibility and environmental agents could be responsible for PD pathogenesis and caused abnormal DAN loss in the SNpc (Rodriguez et al., 2015; Malkus et al., 2009). Figure 11 summarizes the interrelated molecular events caused by the three main PD onset insults: environmental toxins (green), genetic mutations (purple) and endogenous factors (blue), all contributing to increased oxidative stress, mitochondrial dysfunction and impaired protein degradation (Malkus et al., 2009), which will be more precisely described in the following sections.

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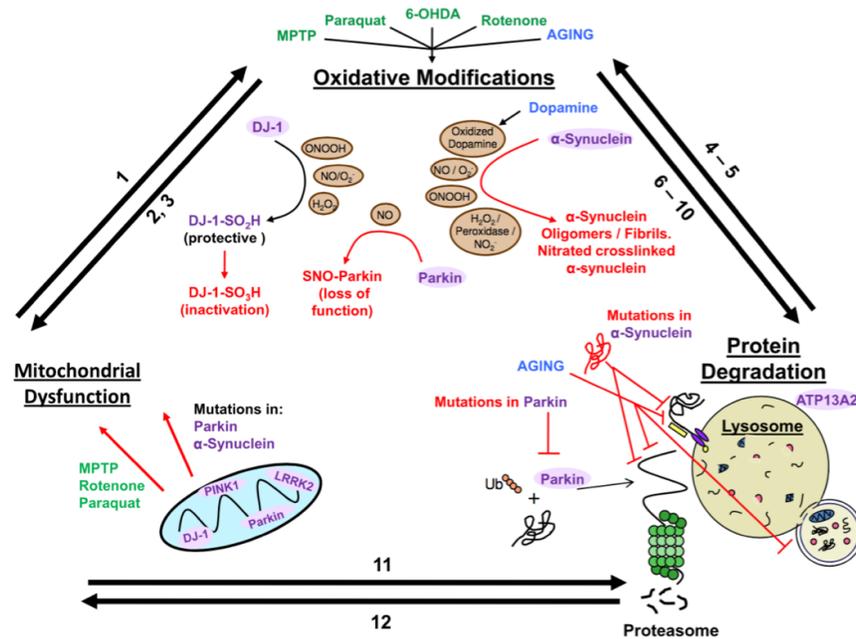


Figure 11 “Bermuda Triangle” of molecular events underlying neuronal death in PD

1. Reactive oxygen species generated by dysfunctional mitochondria. 2, 3. Protection by ROS scavenger systems against mitochondrial toxins. 4, 5. Disrupted protein degradation systems increase oxidative stress. 6-10. Oxidative modification accumulates inactivated, crosslinked and aggregated proteins that are not easily degraded by UPS and CMA, and on the contrary, impair the proteolytic system. 11. Damaged mitochondria is degraded by UPS for the outer membrane proteins, and by the autophagy-lysosome pathway for the rest. 12. Proteasome inhibition causes mitochondrial ROS accumulation and decreases the activity of Complex I and II. Source: Malkus et al., 2009.

2.1. Genetic factors

SNCA, which codes for α -syn, was the first gene found to be linked to PD (Spillantini et al., 1997; Polymeropoulos et al., 1997). Apart from *SNCA*, at least 16 loci and 14 genes have been associated since then with inherited forms of parkinsonism. The most studied are summarized in **Table 2** (Hewitt and Whitworth, 2017). Mutations in *Leucine-rich repeat kinase 2 (LRRK2)*, and *parkin* and *PTEN-induced putative kinase 1 (PINK1)*, are the most common causes of dominant and recessive familial PD, respectively (Corti et al., 2011). Even if PD is mostly associated with old age, a small percentage of patients present symptoms before 60. The majority of cases are caused by mutations affecting the protein degradation system or mitochondrial functions, such as *Park1/SNCA*, *Park9/ATP13A2*, *Park2/parkin*, *Park6/PINK1*, and *Park7/DJ-1*. This suggests that perturbation of these two systems,

associated with an unbalanced oxidative stress, is sufficient to cause PD. The following part will mainly decipher the mechanism of the dominant genetic factor *SNCA* in PD pathology.

Locus	Chrom.	Gene	Inheritance	Mechanism (?)
PARK1/4	4q21	SNCA	AD	Protein aggregation, autophagy, synaptic vesicle dynamics
PARK2	6q26	PARKIN	AR	Mitochondria, autophagy
PARK6	1p36	PINK1	AR	Mitochondria, autophagy
PARK7	1p36	DJ-1	AR	Ox. stress, mitochondria
PARK8	12q12	LRRK2	AD	Vesicles dynamics, autophagy
PARK9	1p36	ATP13A2	AR	Lysosome
PARK14	22q13	PLA2G6	AR	Lipids, mitochondria
PARK15	22q12	FBXO7	AR	Mitochondria, autophagy
PARK16	1q32	RAB7L1	Risk locus	Vesicles dynamics, autophagy
PARK17	16q13	VPS35	AD	Vesicles dynamics, autophagy
PARK18	3q27	EIF4G1	AD	Translation
PARK19	1p32	DNAJC6	AR	Chaperone, vesicles dynamics
PARK20	21q22	SYNJ1	AR	Vesicles dynamics
Unassigned	1q22	GBA	Risk locus	Lysosome

Table 2 PD associated genes

At least 19 genes are currently associated with PD pathogenesis, of which 14 are shown in this table. They are mainly involved in mitochondrial regulation in response to oxidative stress, protein degradation and vesicle dynamics. AR: autosomal recessive; AD: autosomal dominant; GBA: glucocerebrosidase; SYNJ: synatjanin 1; EIF4G1: eukaryotic translation imitation factor 4 gamma 1; VPS35: vacuolar protein sorting-associated protein 35; FBXO7: F-box only protein 7. Source: Hewitt and Whitworth, 2017.

2.1.1 *SNCA*

2.1.1.1 α -syn structure

α -syn was first identified in 1988 as a neuron-specific soluble protein predominantly localized at the presynaptic nerve terminal (Maroteaux et al., 1988). In human, α -syn is encoded by *SNCA* which maps to chromosome 4q21 (Chen et al., 1995; Lavedan, 1998) and was initially identified as the PARK1 locus (Nicholl et al., 2002), involved in both familial and sporadic PD cases. Structurally, full-length α -syn is a 140 amino acid (aa) protein that

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show three domains with distinct structural characteristics: a basic N-terminus (aa 1-60), a central ('non-amyloid genic component') hydrophobic core (aa 61-95), and an acidic C-terminal tail (aa 96-140) (Birman, 2000; Surguchov, 2008; Venda et al., 2010). The highly conserved N-terminal domain encodes for a series of 11 amino acid repeats with a consensus motif of KTKEGV, which in certain conditions forms amphipathic helices. This repeated sequences enable α -syn to form an alpha helix secondary structure or to interact with acidic lipid membranes (Davidson et al., 1998). The central portion of α -syn is highly hydrophobic and explains the aggregate-prone nature of the protein (Cho et al., 2009). The C-terminal domain of the protein remains unbound and available for potential interactions with other proteins (Eliezer et al., 2001) and it could inhibit aggregation. Hence, truncated forms are more prone to aggregate.

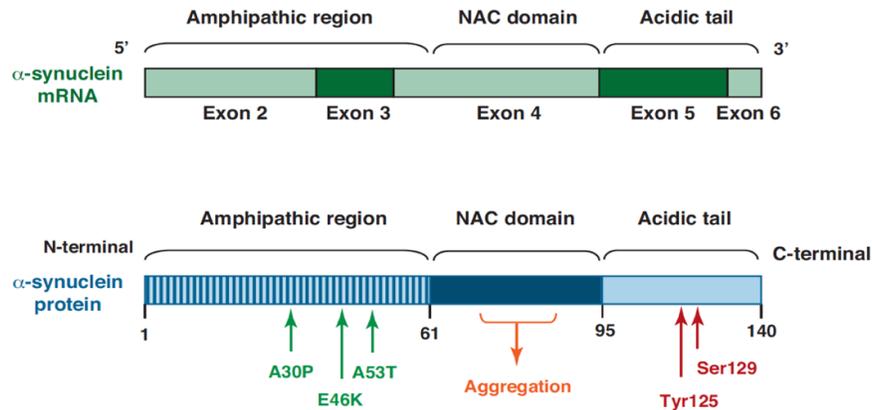


Figure 12 Structure of the α -syn protein

SNCA mRNA comprises seven exons, five of which shown above correspond to the coding region. The α -syn protein has 140 amino acid (aa), which are divided into three domains with distinct structural characteristics: N-terminus (aa 1-60), a central (non-amyloid component, NAC) hydrophobic core (aa 61-95), and an acidic C-terminal tail (aa 96-140). Three missense mutations that are linked to familial PD (A30P, E46K and A53T) lie in the amphipathic region. The NAC domain central hydrophobic core of these mutant proteins shows increasing propensity to form fibrils. The acidic C-terminal tail contains mostly negative charged residues and remains unfolded. Source: Venda et al., 2010.

2.1.1.2 α -syn underlies PD etiology (rare familial case and sporadic)

α -syn association with the etiology of PD was first shown in 1997 with the report describing a missense mutation (A53T) in *SNCA* causing autosomal dominant familial PD (Polymeropoulos et al., 1997). One month later, Lewy bodies in SNpc from PD patients were shown to be α -syn positive (Spillantini et al., 1997). In the next decade, more missense

mutations (A30P, E46K) were identified in other families (Krüger et al., 1998; Zarranz et al., 2004). Clinically, *SNCA* mutations cause late-onset forms of PD. Interestingly, individuals carrying *SNCA* triplication show an early-onset form with more severe non-motor symptoms, more widespread neurodegeneration and a faster disease progression (Singleton et al., 2003; Farrer et al., 2004). Genome-wide associated study (GWAS) studies based on sporadic PD cases have identified mutations in the *SNCA* promoter but failed to identify protein coding variants, suggesting the risk alleles are likely to affect expression level of α -syn rather than its function (Michel et al., 2016). Dinucleotide repeat polymorphism (REP1) within the 5' untranslated region of *SNCA* appears to control α -syn level of expression, an expanded REP1 sequence in some PD patients resulting in increased *SNCA* expression (Cronin et al., 2009; Linnertz et al., 2009).

2.1.2 α -syn aggregation

As shown in Figure 13, α -syn has been suggested to occur as an equilibrium between partially helical tetramers and unfolded monomers (Bartels et al., 2011; Wang et al., 2011a), and this protein has the capacity to assemble into soluble oligomers and fibrils. Evidence suggests that it may be the oligomeric forms of α -syn, rather than the larger intracellular inclusions, that are the most cytotoxic, causing neuronal dysfunction and death (Kazantsev and Kolchinsky, 2008; Karpinar et al., 2009; Martin et al., 2012; Winner et al., 2011; Micheal et al., 2016). Oligomers refer to protofibrils, possessing annular structures that may form pores and cause membrane permeabilization (Volles and Lansbury, 2002). Kanaan and Manfredsson (2012) hypothesised that mutant forms of α -syn have a reduced energy barrier for oligomer and fibril formation. The A53T mutation promotes the formation of fibrils while A30P does not. The toxicity of A30P mutation is mainly due to strongly facilitated oligomeric formation with a slow rate of fibrils accumulation (Conway et al., 2000). Phosphorylation at Ser129 has been reported to enhance α -syn toxicity, possibly by increasing α -syn aggregation (Fujiwara et al., 2002), which could explain why Lewy bodies containing phosphorylated α -syn were also found in sporadic PD cases (Mezey et al., 1998). Antibodies specific to Ser129-phosphorylated α -syn also recognized LBs in other synucleopathies than PD, like dementia with Lewy bodies and multiple system atrophy (Fujiwara et al., 2002).

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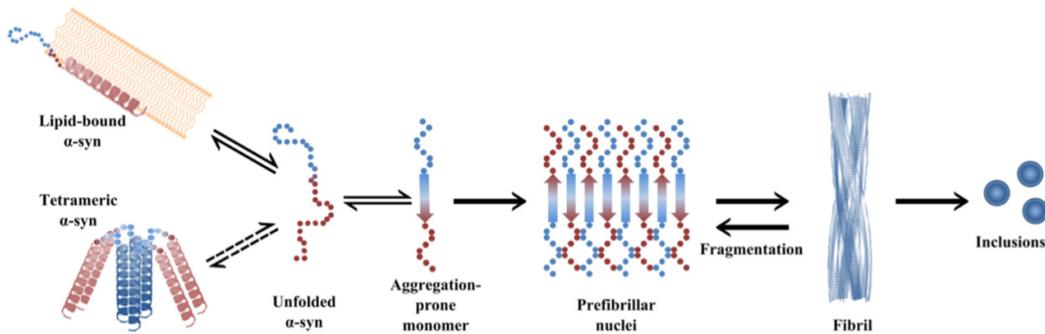


Figure 13 Scheme of α -syn aggregation

In physiological conditions, α -syn exists as an unfolded form or aggregation-prone monomers, which may form stable tetramers or interact with lipids in an α -helical structure shape. Those different forms of α -syn are in equilibrium. In disease-associated conditions, aggregation-prone α -syn monomers form soluble prefibrillar oligomeric nuclei, followed by the elongation of these into mature amyloid fibrils. Fibrillary aggregates may then be sequestered into Lewy bodies in cell bodies and in Lewy neurites along the axons. Source: Cox et al., 2014.

α -syn can be the target of iron and DA in DANs, which would in turn promote α -syn aggregation (Conway et al., 2001). Mice expressing human mutant α -syn A30P are more susceptible to the PD-inducing drug methyl-phenyl-tetrahydropyridin (MPTP) (Nieto et al., 2006; Song et al., 2004), while *SNCA*-null mice are resistant to MPTP (Dauer et al., 2002).

2.1.3 α -syn in DA metabolism and release

Physiologically, α -syn is a presynaptic protein acting as a negative regulator of synaptic transmission. *SNCA* null mice showed decreased synaptic vesicle endocytosis, as well as decreased DA stores and increased DA release, indicating its potential function as a brake on dopaminergic neurotransmission (Abeliovich et al., 2000; Cabin et al., 2002; Vargas et al., 2014). α -syn interacts with and inhibits TH and DDC, two key enzymes involved in DA biosynthesis (Tehrani et al., 2006; Perez et al., 2002; Bridi and Hirth, 2018). By interacting with DAT, α -syn modifies its transporting activity. α -syn is also able to regulate the amount of VMAT in synaptic vesicles (Benskey et al., 2016). Upon neuronal stimulation, α -syn rapidly disperses from the presynaptic terminal (Fortin et al., 2005). As proposed by Benskey et al., 2016, this could potentially disinhibit TH and DDC and allow *de novo* synthesis to replenish DA stores for release during synaptic transmission (Figure 14b). At the termination of synaptic transmission, α -syn would relocate to the synaptic terminals to halt chemical

transmission by impeding the trafficking and docking of synaptic vesicles with the presynaptic membrane and increasing the rate of synaptic vesicles recycling (Figure 14a).

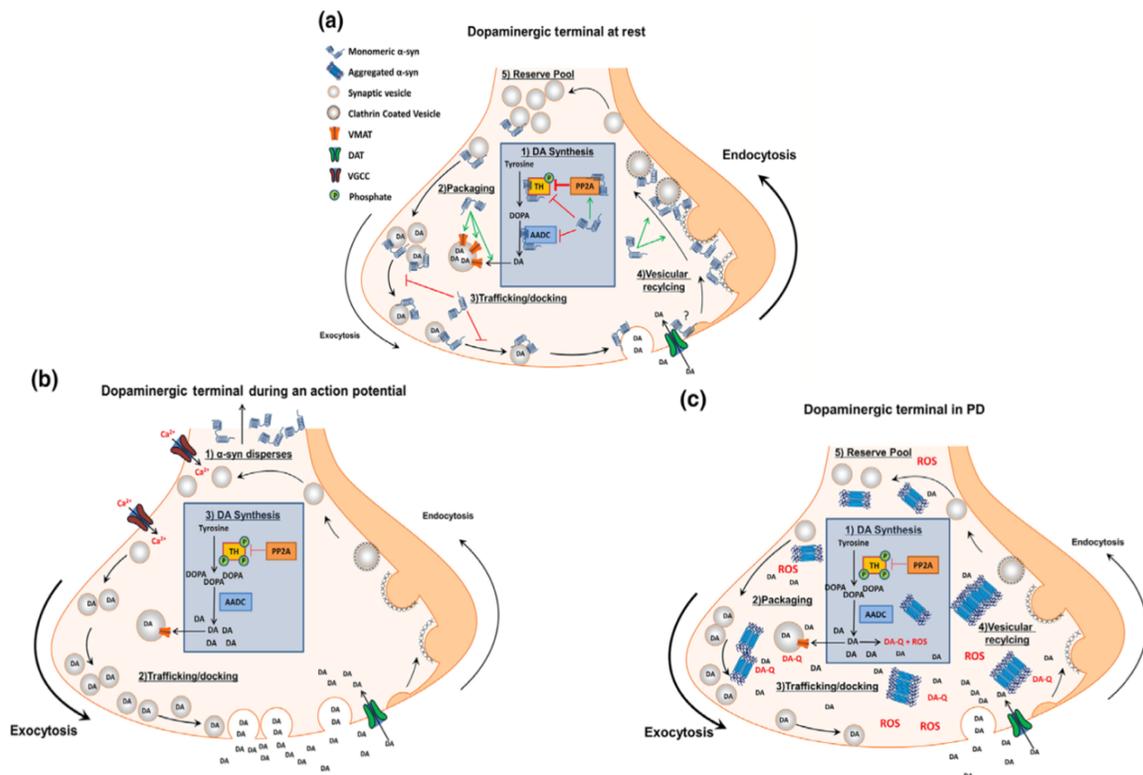


Figure 14 Proposed function of α -syn in dopaminergic neurotransmission

(a) In rest condition, high α -syn in the presynaptic terminal would act as a brake on chemical neurotransmission. 1) α -syn would reduce DA synthesis by inhibiting TH and AADC activity or increasing PP2A activity. 2) α -syn facilitates the sequestration of cytosolic DA by increasing VMAT on vesicles. 3) By interacting with synaptic vesicles and SNARE complex, α -syn would prevent the trafficking and docking of vesicles with presynaptic membrane, thereby inhibiting DA release. 4) α -syn facilitate endocytosis by mediating membrane bending to recycle the vesicles. 5) α -syn helps maintain the number of vesicles in the reserve vesicle pool. (b) During an action potential, α -syn disperses from the terminal synapse. 1) With neuronal stimulation and calcium influx, α -syn rapidly disperses from the presynaptic terminals. 2) Without α -syn, trafficking and docking for exocytosis is efficient for DA release. 3) In the absence of inhibitory α -syn to TH and AADC activity, unimpeded DA synthesis would replenish DA release during synaptic transmission. (c) The aggregation of α -syn inhibits the normal function of monomeric α -syn, leading to subsequent increased cytosolic DA and ROS stress. 1) Aggregated α -syn loses its inhibitory function on TH and AADC and causes increased DA synthesis. 2-3) Loss of function of α -syn would lead to decreased VMAT level and inefficiency for packaging. 3) The released DA caused by upregulation of trafficking and docking is up-taken by DAT, which leads to the elevated DA level in cytoplasm. 4-5) Loss of α -syn function impairs endocytosis and decreases the size of the vesicular pool. The sustained increasing cytosolic DA auto-oxidizes to produce ROS and DA quinones, which promote α -syn aggregation. TH: tyrosine hydroxylase, AADC: aromatic amino acid decarboxylase, DA: dopamine, PP2A: protein phosphatase 2A, VMAT: vesicular monoamine transporter, DAT: dopamine transporter, VGCC: voltage gated

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calcium channel, ROS: reactive oxygen species, DA-Q: dopamine quinone. Source: Benskey et al., 2016.

However, in pathological condition, α -syn aggregation would increase cytosolic DA due to the disinhibition of TH and DDC, decrease in VMAT and unregulated synaptic vesicles trafficking and recycling machinery (Bridi and Hirth, 2018). The sustained high level of DA in cytoplasm would lead to auto-oxidation, producing reactive oxygen species (ROS) and DA quinones which will subsequently oxidize proteins and lipids, triggering severe oxidative stress. This increase in ROS and DA metabolites would promote α -syn aggregation, thus entering a vicious toxic cycle leading ultimately to cell death (Lee et al., 2011b).

2.1.4 α -syn and epigenetic modification

Epigenetic modification refers here to gene methylation, histone modification and small RNA-mediated mechanisms that alter gene expression without changing the DNA sequence. Abnormalities in epigenetic modification are associated with dysregulation of *SNCA* expression. A DNA hypomethylation of *SNCA* intron 1 was reported in the SN of sporadic PD patients. Hypomethylation will prevent certain silencing mechanisms, suggesting its role in the upregulation of α -syn expression, potentially promoting α -syn aggregation (Jowaed et al., 2010; Matsumoto et al., 2010). Moreover, certain clusters of miRNA (miRNA-7, miRNA-153) have been reported to target the 3' untranslated region (UTR) of the *SNCA* transcript (Junn et al., 2009b; Doxakis, 2010; Marchese et al., 2017).

On the other hand, α -syn itself could interact with histones in the nucleus (Goers et al., 2003). It has been suggested that α -syn could exert its neurotoxicity by inhibiting histone acetylation (Kontopoulos et al., 2006). Attributed to α -syn-mediated histone deacetylation, peroxisome proliferator receptor gamma coactivator 1- α (PGC1- α) level is reduced in post-mortem PD SNpc, contributing to mitochondrial malfunction (Zheng et al., 2010; Siddiqui et al., 2012). Consistent with these discoveries, in the cell overexpressing α -syn, Sirtuin 2 (a nicotinamide adenine dinucleotide-dependent histone deacetylase (HDAC)) inhibitors reduced α -syn induced toxicity (Outeiro et al., 2007). Overexpression of histone deacetylase 6 (HDAC6) was however reported to protect DA neurons against α -syn toxicity in the *Drosophila* model (Du et al., 2010). Interestingly, under oxidative stress, α -syn can be translocated into the nucleus (Ammal Kaidery et al., 2013). Moreover, in PD patients and mice PD model, α -syn sequesters DNA methyltransferase 1 (Dnmt1) from the nucleus, contributing to the global DNA hypomethylation (Desplats et al., 2011). Transgenic *Drosophila* with ectopic

human α -syn expression showed an elevation in histone H3 lysine 9 dimethylation (H3K9me2) via the induction of euchromatic histone lysine N-methyltransferase 2 (EHMT2). This increased H3K9me2 could affect the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex assembly and hence synaptic vesicle functions (Sugeno et al., 2016).

2.1.5 α -syn affects mitochondrial function

Mitochondrial deficits have largely been described as a crucial pathogenic event underlying the pathogenesis of PD (Schapira, 2008; Schapira and Gegg, 2011; Winklhofer and Haass, 2010; Gao et al., 2017). α -syn was reported to localize at or in mitochondria and connect to the endoplasmic reticulum (ER) through mitochondrial-associated ER membrane (Li et al., 2007; Cole et al., 2008; Devi et al., 2008; Guardia-Laguarta et al., 2014). This mitochondrial localization is consistent with the fact that the accumulation of α -syn leads to mitochondrial dysfunction, as reported in cultured cells, transgenic animal models and sporadic and familial PD cases (Hsu et al., 2000; Devi et al., 2008; Chinta et al., 2010). By directly interacting with mitochondria, α -syn aggregation could disturb mitochondrial fusion and fission (Kamp et al., 2010; Nakamura et al., 2011). Moreover, α -syn overexpression has been involved in complex I inhibition, with, as a consequence, increase in ROS production and oxidative damage to lipids and proteins, finally leading to increased mitophagy (Chinta et al., 2010). In addition, intermediate α -syn accumulation reduces mitochondrial Ca^{2+} retention, which alters mitochondrial membrane potential and nicotinamide adenine dinucleotide hydride (NADH) oxidation (Luth et al., 2014). Finally, overexpression of α -syn was shown to directly inhibit activity of the outer membrane mitochondrial import receptor subunits TOM20 and TOM40, impairing protein import into mitochondria (Bender et al., 2013; Di Maio et al., 2016).

2.1.6 Proteolytic pathways of α -syn degradation

The level of α -syn depends on a dynamic balance between its synthesis and its clearance. α -syn can be degraded by the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway (ALP) (Webb et al., 2003). The UPS mainly degrades short-lived, soluble proteins, while the ALP is in charge of the bulk degradation of longer-lived macromolecules, specific cytosolic components, and dysfunctional organelles (Goldberg, 2003; Xilouri et al., 2013b). The ALP pathway is categorized into three subtypes: microautophagy macroautophagy, and chaperone-mediated autophagy (CMA). CMA is a process that degrades cytosolic proteins containing a particular consensus peptide motif. Microautophagy involves the direct

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engulfment of cytoplasm at the surface of lysosomes or late endosomes. Macroautophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, termed autophagosome.

Different studies gave partly discrepant results on the physiological degradation of α -syn. On the one hand, using different pharmacologic strategies in living mouse brain, a group showed that ALP inhibition (lysosomal acidification and autophagosome fusion blockage) had no effect on α -syn degradation in non-transgenic mouse while UPS inhibitor treatment yielded a strong increase in α -syn level (Ebrahimi-Fakhari et al., 2011). On the other hand, other studies showed that breakdown of α -syn was mainly done via CMA with a smaller proportion being degraded via the UPS (Cuervo et al., 2004; Massey et al., 2006; Martinez-Vicente et al., 2008; Schapira and Tolosa, 2010; Vogiatzi et al., 2008; Xilouri et al., 2013b). The different processes that appear involved in α -syn degradation are illustrated in Figure 15.

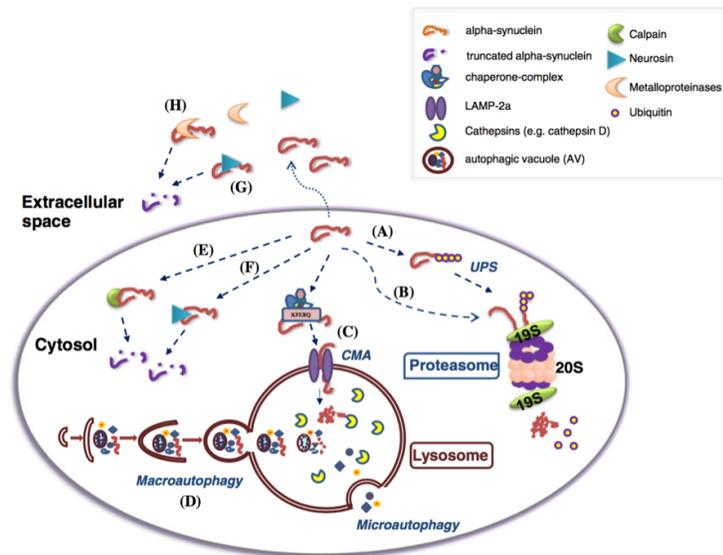


Figure 15 Overview of α -syn degradation pathways

Wild type or mutant α -syn can be degraded by the proteasome through both ubiquitin-dependent (A) and ubiquitin-independent (B) pathways. In mammalian cells, chaperone-mediated autophagy (CMA) (C) is responsible for degradation of monomeric and dimeric wild type α -syn and macroautophagy (D) have been proposed to degrade various other forms of α -syn (wild type, mutant, oligomeric). Moreover, other proteases such as calpains (E) and neurosin (F) have been involved in the cleavage of wild type or aggregated α -syn inside the cell, which may promote the generation of truncated α -syn. In addition, neurosin and metalloproteinases could be secreted to cleave extracellular α -syn, potentially generating pathogenic fragments with increased tendency to aggregate. Source: Xilouri et al., 2013b.

α -syn bears a CMA-targeting motif (KFERQ-like) which is recognized by the heat shock cognate 70 kDa protein (Hsc70) that forms a complex with other chaperone proteins that unfolds and steers α -syn to the lysosome-associated membrane protein 2A receptor (LAMP-2A), through which α -syn is translocated into the lysosomal lumen for degradation (Orenstein and Cuervo, 2010). The A30P and A53T α -syn mutants show high affinity for LAMP-2A, however, they cannot be translocated inside the lumen to be degraded and block this receptor, thus acting as inhibitors of the CMA pathway (Cuervo et al., 2004; Schapira and Tolosa 2010; Xilouri et al., 2013b). DA-modified α -syn also impairs CMA-dependent protein degradation (Martinez-Vicente et al., 2008). Some studies provided the evidence that macroautophagy activation can compensate for MA dysfunction caused by α -syn mutants (Massey et al., 2006). It was also shown, however, that wild-type α -syn can impair macroautophagy by interacting with the small GTPases Rab1a, thus preventing autophagosome formation at an early point (Winslow et al., 2010). Conversely, whole-brain specific loss of Atg7 (a protein required for autophagosome formation) resulted in α -syn accumulation (Friedman et al., 2012). Mutation of PD-associated genes associated to the endosomal/lysosomal system, vacuolar protein sorting (VPS35), type 5 P-type ATPase cation metal transporter (ATP13A2) and glucocerebrosidase (GBA) are also involved in α -syn accumulation underlying PD pathologies (Michel et al., 2016).

2.1.7 Other PD-associated genes

Ageing-related progressive mitochondrial inefficiency or mutations associated with mitochondrial dysfunction all result in increased oxidative stress. Three autosomal recessive (AR) mutations, in PARK2/parkin, PARK6/PINK1 and PARK7/DJ-1, are the most frequent causes of early-onset PD cases (Corti et al., 2011). These proteins are implicated in mitochondrial dynamics and oxidative stress control.

The link between *parkin* mutation and juvenile parkinsonism was discovered in 1998 (Kitada et al., 1998). Parkin is an E3 ubiquitin ligase and an integral component of the UPS, mediating the covalent attachment of ubiquitin moieties needed for substrate protein clearance (Shimura et al., 2000). Because of its cysteine-rich RBR (ring between ring fingers) domain, Parkin is vulnerable to inactivation by severe oxidative stress (Winklhofer et al., 2003; LaVoie et al., 2007; Wong et al., 2007; Schlehe et al., 2008). Its susceptibility to oxidative stress and its complex three-dimensional structure probably explain that oxidized and aggregated Parkin proteins were found in PD patients (Yao et al., 2004; LaVoie et al., 2005; Wang et al., 2005). Note that familial Parkin-related neuropathology does not generally

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present Lewy bodies, and there is no definitive evidence that α -syn directly interacts with Parkin (von Coelln et al., 2006; Ahlskog, 2009).

PINK1 is a serine/threonine kinase, which appears to be crucial for mitochondria function. It was identified in 2004 as a gene responsible for early-onset PD (Valente et al., 2004). PINK1 is a 581 aa protein with an N-terminal mitochondrial targeting sequence, and it is found at the outer and inner mitochondrial membrane and in the cytosol (Lin and Kang, 2008; Narendra et al., 2010; Jin et al., 2010). PINK1-dependent activation of Parkin is considered as a major step of mitophagy, which plays an essential role in mitochondria quality control and homeostasis. The integration of PINK1 into the outer mitochondrial membrane is a prerequisite to Parkin recruitment for mitophagy activation, when mitochondrial membrane potential is disrupted (Exner et al., 2012). Studies in *Drosophila* demonstrated that Parkin and PINK1 interact in a common biological pathway required for mitochondrial homeostasis and that Parkin functions downstream of PINK1, since phenotypes caused by *Drosophila Pink1* inactivation could be rescued by Parkin (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Consistent with this, silencing PINK1 in Hela cells led to abnormal mitochondrial morphology and altered membrane potential and this dysfunction from PINK1 loss of function could be rescued as well with Parkin (Exner et al., 2007).

Mechanism of mitochondrial quality control by Pink1 and Parkin are now well documented (Pickrell and Youle, 2015; Truban et al., 2017; Mouton-Liger et al., 2017). Upon mitochondrial depolarization, translocated PINK1 accumulates in the outer mitochondrial membrane, allowing Parkin recruitment and activation, which initiates the mitophagic process. Following the recruitment of dynamin-related pro-fission GTPase, Drp1, and the cleavage of the inner membrane dynamin-like fusion GTPase, OPA1, the damaged mitochondria are removed from the healthy network. Outer membrane mitochondrial proteins are then ubiquitinated in a Parkin-dependent manner for proteasomal degradation. Finally, the autophagy receptor proteins bind to the mitochondria, engulfing it in a forming autophagosome which, after completion, fuse with a lysosome for degradation.

DJ-1/PARK7 is a member of the ThiJ/Pfp1 family of molecular chaperones. It is induced by oxidative stress and translocated from the cytoplasm to the outer mitochondrial membrane, regulating the clearance of endogenous ROS through modulation of the scavenging system (Canet-Avilés et al., 2004; Blackinton et al., 2005; Lev et al., 2008; Junn et al., 2009a; Im et al., 2010). *In vitro* and in living cells, DJ-1 acts like a chaperone and directly interacts with α -syn monomers and oligomers. DJ-1 overexpression attenuates α -syn dimerization while

mutant DJ-1 aggravates this process (Zondler et al., 2014). A recent study using SH-SY5Y cells showed that DJ-1 deficiency downregulated lysosomal Hsc70 and accelerated LAMP-2A degradation in the lysosomes, thus increasing α -syn aggregation (Xu et al., 2017).

2.2 Environmental factors

As mentioned in the section above, mutations in at least 23 loci and 19 disease-causing genes were identified in PD cases (Deng et al., 2018; Hewitt and Whitworth, 2017). However, mutants in single genes only account for a few percent of PD cases (15% of PD patients have family history and 5–10% of PD patients suffer from a monogenic form of the disease with Mendelian inheritance (Deng et al., 2018), which implies that ~85% of patients have no family history for the disease. Similar PD concordance in monozygotic and dizygotic twins in large studies pointed out the importance of environmental contribution to PD etiology (Elbaz et al., 1999).

2.2.1 MPTP

The first hints of the environmental toxin hypothesis were reported in 1979 and 1983 when young individuals aged 26-42 years unexpectedly developed marked PD-like motor symptoms after being injected with an opioid drug accidentally contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These manifest PD symptoms were dramatically improved by L-DOPA treatment, which implied neuronal damage from this compound in the striatum (Davis et al., 1979; Langston et al., 1983).

The decipherment of MPTP toxicity mechanisms markedly improved our understanding of environmental impacts in PD aetiology. MPTP crosses the blood-brain barrier to be converted into MPP⁺(1-methyl-4-phenylpyridinium) by MAO-B in astrocytes and released by the organic cation transporter-3 (OCT3) (Dauer and Przedborski, 2003). Uptaken by DAT into DANs, MPP⁺ escapes sequestration into vesicles through VMAT2, leaving it free to inhibit mitochondria Complex I activity and impair oxidative phosphorylation. This disruption leads to reduced ATP synthesis, increased ROS generation and malfunction of the protein degradation system.

The discovery of MPTP prompted a search for functional analogy in other environmental factors shedding light on the effect of pesticides. Afterwards, the meta-analysis of 46

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epidemiological studies revealed the link between pesticides exposure and increased risk of developing PD (van der Mark et al., 2012).

2.2.2 Paraquat

Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridinium dichloride) is a quaternary nitrogen herbicide, structurally similar to MPP⁺, the converted pathogenic form of MPTP. This compound is commercially available since 1962 and was widely used for broadleaf weed control. It has been banned since July 2007 in the European Union, and is now under restricted use in the United States (Goldman et al., 2014).

PQ can cross the brain blood barrier (BBB) via the neutral amino acid transporter (Shimizu et al., 2001; McCormack and Di Monte, 2003). Unlike MPP⁺ or the pesticide rotenone, PQ showed a low affinity for mitochondrial complex I (Nicklas et al., 1985), but it can still lead to indirect mitochondrial toxicity. The cellular toxicity of PQ is essentially due to its redox cycle including a well-known cascade of reactions leading to nicotinamide adenine dinucleotide phosphate (NADPH) consumption, generation of ROS and impairment of glutathione restoration (Bus and Gibson, 1984). It has been shown that PQ-induced neuronal loss results from a Bak-dependent mechanism, facilitating cytochrome *c* release from mitochondria and activating caspase-3, thus triggering apoptosis (Fei et al., 2008; Huang et al., 2016).

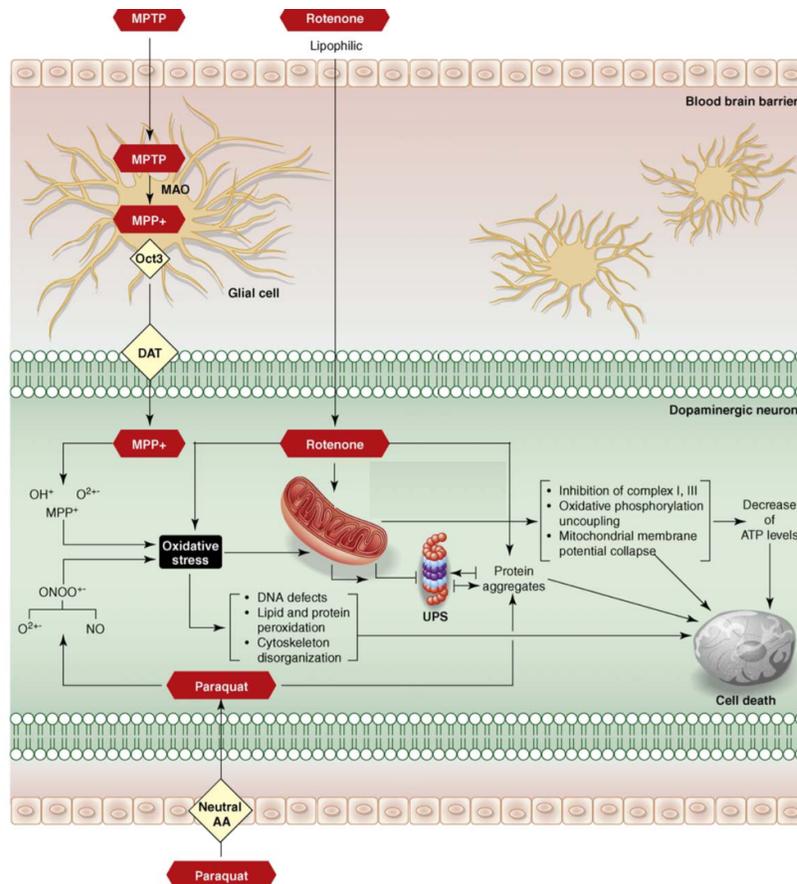


Figure 16 Mechanisms underlying various environmental factors for PD

Paraquat (PQ), rotenone and MPTP enter into the DANs via different mechanisms. Rotenone is highly lipophilic and directly cross the BBB. PQ is transported through the neutral amino acid transporter and was suggested to be taken up into DANs by DAT. MPTP is transported across the BBB, converted into MPP⁺ in glial cells, exported through the plasma membrane transporter Oct3, and finally transported into DANs by DAT. Rotenone and MPTP target the mitochondrial complex. Oxidative stress caused by those factors lead to disrupted mitochondrial function, oxidized cellular components and malfunction of proteasome activity, thereby causing neuronal dysfunction, degeneration and ultimately death. Source: Cicchetti et al., 2009.

Animals treated with PQ mimic many features of PD syndromes. In mice, PQ selectively caused a loss of DANs in the SNpc and induced motor deficits (Brooks et al., 1999; McCormack et al., 2002). Selective DAN targeting could be attributed both to PQ uptake by the DAT (Rappold et al., 2011) and their already elevated oxidative level due to high metabolic demands, pacemaking-related calcium influx, DA oxidation and presence of neuromelanin (Goldman, 2014). In addition, by inactivating autophagy and proteasomal activity (Garcia-Garcia et al., 2013; Wills et al., 2012; Navarro-Yepes et al., 2016), PQ treatment markedly accelerates formation of α -syn aggregates reminiscent of Lewy bodies in

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PD (Manning-Bog et al., 2002; Fernagut et al., 2007; Mak et al., 2010). Besides, other study in cell model showed that histone hyperacetylation caused by PQ via suppression of HDAC activity contributes to neurotoxicity as well (Song et al., 2011).

2.2.3 Rotenone

Rotenone is a chemical that belongs to the family of isoflavones naturally found in the roots and stems of several plants, used as a broad-spectrum pesticide. As shown in Figure 16, due to its highly lipophilic structure, rotenone crosses the BBB easily, independent of the membrane transporter (Higgins and Greenamyre, 1996). Once inside the cell, rotenone targets and inhibits proteasome activity. Moreover, rotenone induced dysfunction of the mitochondrial complex I, resulting in increased ROS production (Martinez and Greenamyre, 2012). Like the other environmental toxins mentioned above, rotenone-induced oxidative stress leads to the malfunction of mitochondria, with the inhibition of complex I, perturbation of oxidative phosphorylation, and mitochondrial membrane potential collapse (Cicchetti et al., 2009). The combination of damaged mitochondria unable to produce enough ATP to cope with stress condition and the accumulation of misfolded protein aggregates, trigger cell death in the end.

2.3 Ageing effect

PD affects over 1% of the population over the age of 60, while this prevalence reaches 5% among people over 85 (Reeve et al., 2014). Prevalence of most diseases usually slightly increases with ageing. In comparison, the prevalence of PD is increased more than 400 times between mid-aged people (45-54) and the elderly (>85), highlighting ageing as the key risk factor for PD.

2.3.1 ROS theory of ageing

Half of century ago, Denham Harman presented the “free-radical theory of ageing” suggesting that ageing is driven by the accumulation of oxidative damage to cellular and connective structures over time (Harman, 1956). The free radical compounds named “reactive oxygen species” refer to the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\bullet$), which are formed by the partial reduction of oxygen shown in Figure 17. The electron transport chain, a component of mitochondria is the main source of cellular ROS. Another major source of ROS is the membrane-bound protein NADPH oxidase,

which generates $O_2^{\cdot-}$ by NADPH consumption. This produced ROS have been shown to act as anti-microbial molecules (Nathan and Cunningham-Bussel, 2013)

The control of ROS level is exerted through the regulation of mitochondrial functions and metabolic pathways. Besides, the generation of ROS is tightly regulated by the ROS-scavenging systems via antioxidant enzymes. Glutathione (GSH) is one of the most abundant antioxidant synthesized by the cell. Oxidized proteins and H_2O_2 are reduced through the glutaredoxin (Grx)/GSH reductase and thioredoxin (Trx)/thioredoxin reductase systems (Figure 17C).

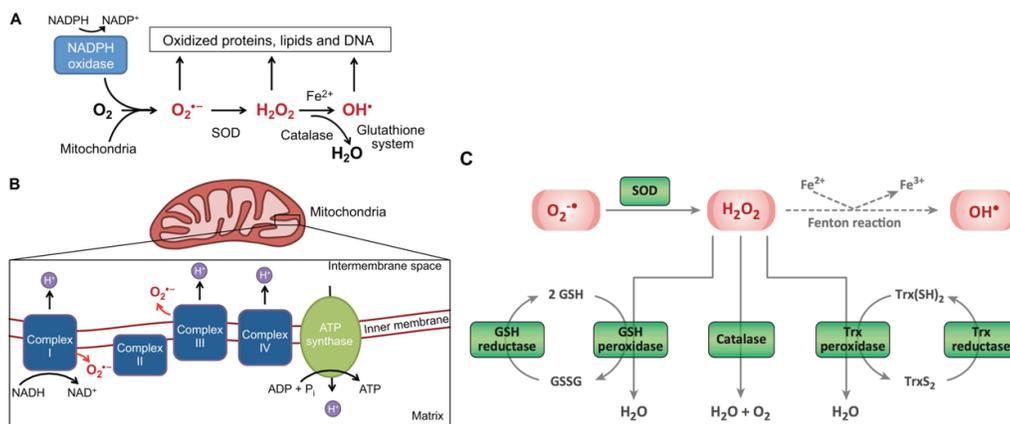


Figure 17 ROS generation and clearance system

(A) ROS refers mainly to $O_2^{\cdot-}$, H_2O_2 and OH^{\cdot} , which can cause oxidative modifications to lipids, DNA and proteins. $O_2^{\cdot-}$ derives from the one electron reduction of molecular oxygen through NADPH oxidase or the mitochondrial complex I or III. Subsequently catalysed by superoxide dismutase (SOD), $O_2^{\cdot-}$ is reduced to H_2O_2 , which can be further reduced to H_2O by catalase or can spontaneously oxidize iron to form the highly reactive OH^{\cdot} . (B) The electron transport chain complex I-IV are coupled to pumping H^+ into the mitochondrial intermembrane. The proton force drives the ATP synthase. Aberrant reduction of O_2 can happen at complex I and III with the generation of $O_2^{\cdot-}$. (C) $O_2^{\cdot-}$ can be converted to H_2O_2 by the enzyme superoxide dismutase (SOD). And H_2O_2 by the Fenton reaction can be converted into OH^{\cdot} spontaneously. H_2O_2 can be detoxified via glutathione (GSH) peroxidase, catalase or thioredoxin (Trx) to H_2O and O_2 . Source: Bigarella et al., 2014 (AB) and Li et al., 2013a (C)

Mitochondria have their own DNA, named mtDNA. Under oxidative stress, the absence of histones and relative inefficiency of DNA repair mechanisms in these organelles place them at high risk for mutations. mtDNA deletions in PD patients are a little higher than those observed in healthy aged subjects (Bender et al., 2006). Moreover, mitochondrial complex I is particularly sensitive to ROS. The reaction with free radical intermediates causes complex I protein damage and inactivation by shifting its native non-covalent polypeptide interactions

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to covalent cross-linkings (Navarro and Boveris, 2010). This inactivation of complex I in the brain has been observed in PD context as well as during ageing (Navarro et al., 2009; Schapira, 2008; Boveris and Navarro, 2008). Brain mitochondria of old animals with decreased rates of electron transfer in complex I and complex IV showed decreased membrane potential, increased content of the oxidation products, and increased size and fragility (Navarro and Boveris, 2010). Mitochondria are the main source of ROS within the cell and, importantly, the major site of ATP production, and they are also involved in apoptosis regulation. Therefore, the subtle control of mitochondrial quality, regulated in particular by Parkin and PINK1, is crucial (Gautier et al., 2008; Mouton-Liger et al., 2017).

2.3.2 Decline of proteolytic systems with ageing

One of the most obvious feature of ageing is the loss of quality control in protein turnover with the concomitant build-up of oxidatively modified proteins (Vernace et al., 2007b). Upon ageing, an accumulation of ROS leads to the oxidation of multiple cellular components, including proteins, lipids and nucleotides (Schieber and Chandel, 2014). Those modified or misfolded proteins rely on the protein clearance system (UPS and ALP) to maintain their homeostasis. However, a decrease in proteins involved in UPS and ALP was observed in aged animals compared to young ones. With regard to UPS, the expression of several genes coding for the 26S proteasome subunits declined with age (Ly et al., 2000; Carrard et al., 2002). For the ALP, autophagy-related genes (*Atg5*, *Atg7* and *Beclin-1*) are down-regulated in the ageing brain (Lipinski et al., 2010). ATP concentration is tightly correlated to 26S proteasome activity, for ATP is essential to the functional assembly of UPS. So ATP production decline with ageing lead to susceptibility to proteotoxic stress attributed to the deficit in proteasome activity (Vernace et al., 2007a). CMA activity decreases with ageing due to a reduction in LAMP-2A receptor, causing the decline of substrate binding to the lysosomal membrane and transporting into the organelle (Cuervo and Dice, 2000; Kiffin et al., 2007). Normal ageing is reported to result in a progressive decline in the activity of a lysosomal enzyme, GBA (Rocha et al., 2015). GBA mutations are the most common genetic risk factor for PD, coupled to reduced activity of the ALP (Poewe et al., 2017).

In PD patients, demise of the degradation systems with ageing lead to the failure to clear modified proteins upon oxidative stress, with the consequence of α -syn aggregation, which, in turns, blocks further proteins degradation systems. The malfunction of UPS and ALP then disturbs mitochondrial quality control processes and triggers more ROS. This “vicious cycle” ends up with apoptosis activation and neuronal death.

2.3.3 Epigenetic modification in ageing

Epigenetics by definition refers to the reversible heritable alterations in gene expression that occur without changes in DNA sequences. It modifies genes expression through DNA methylation, histone modifications (*e.g.* acetylation, methylation, phosphorylation, ubiquitination) and small non-coding RNA intervention. Epigenetic modifications provide plasticity for cells to react to changes in the environment and are essential for normal differentiation and development.

As shown in Figure 18, various changes affect the chromatin structure during the ageing process. First, transcriptional factors and histone modifications may be relocalized to different areas of the genome. For example, reduction in H3K9me3 lead to decreased heterochromatin domains (condensed regions of chromatin containing poorly-expressed genes). Second, a decline in the structure and integrity of the nucleus fails to maintain the heterochromatic level. Third, transposable elements (TEs) become active with age due to low heterochromatic condition, malfunction of the RNAi-induced silencing complex and higher chromatin accessibility (Wood and Helfand, 2013). Altogether, age-induced epigenetic abnormalities lead to misregulation of genes expression as well as the activation of TEs with deleterious effects.

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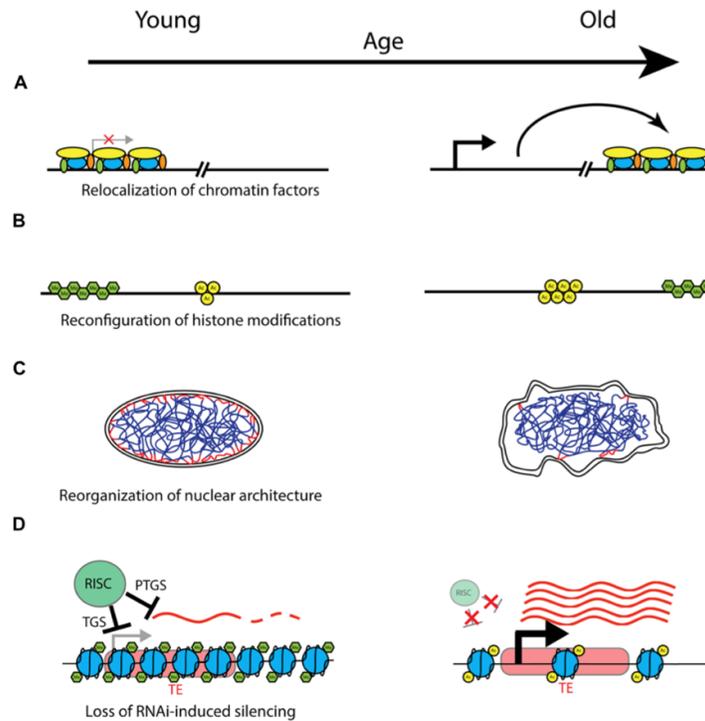


Figure 18 Chromatin-related mechanisms of ageing

(A) Chromatin factors localize to different genomic sites with age, leading to alteration in gene expression. (B) With age, aberrant histone acetylation (in yellow) and methylation (in green) affect gene expression. This could be due to loss of heterochromatin maintenance and changes in chromatin accessibility. (C) The structure and integrity and the nucleus decline with age, which affect the spatial chromatin organization. Heterochromatic LADs (lamin-associated domains) are marked in red, where they attached to the nuclear lamina on the inner nuclear membrane. With age, decline of attachment with age fails to maintain silent heterochromatin. (D) TEs activation in old stage. Activity of TEs (in red) is repressed by the RNA-induced silencing complex (RISC in green) via transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). Malfunction or inefficiency of the RISC leads to the robust TE transcription, which can undergo transposition and lead to DNA damage or insertion in the other areas of the genome. Source: Wood and Helfand, 2013.

A recent investigation proposed that epigenetic marks could act as a new source of biomarkers for PD, showing that ageing blood cells exhibited an increased profile of DNA methylation based biomarkers (Horvath and Ritz, 2015). This is consistent with previous studies showing that α -syn gene methylation is decreased in PD patients' brains (Jowaed et al., 2010; Desplats et al., 2011), highlighting that epigenetic modifications may underlie PD pathology.

2.3.4 Ageing and immune response

The other feature of ageing is the activation of astrocytes and microglia in the brain (Kanaan et al., 2010). A non-human primate study comparing ageing and PD, showed no difference in astrocyte activation in the midbrain but a more severe microglial activation in PD compared to normal ageing condition (Collier et al., 2011).

Microglial-associated central inflammation acts as a pathological hallmark of PD (Hirsch and Hunot, 2009; Tansey and Goldberg, 2012) initially demonstrated by a post-mortem study showing activated microglial in the SNpc of PD patients (McGeer et al., 1988). Activated microglia exert their neurotoxic effects by releasing proinflammatory cytokines (tumor necrosis factor (TNF)- α , Interleukin (IL)-1 β , IL-6 and (Interferon) IFN γ) and ROS leading on the long term to cell damage and death. In rats, short-term lipopolysaccharide (LPS) injection on the dopaminergic system caused microglial activation whereas long-term injection (one year) led to a progressive DANs degeneration (Castaño et al., 1998). In PD models, both wild-type and mutant form of α -syn activate microglial Toll-like receptors (TLR), triggering the release of proinflammatory cytokines and ROS, ultimately leading to neuronal loss (Zhang et al., 2005; Glass et al., 2010; Fellner et al., 2013).

2.4 Mechanisms of PD Propagation

2.4.1 Braak's model

A large number of studies have made it possible to understand the molecular and cellular mechanisms underlying PD pathologies involving the different factors described above. However, questions remained about where PD initiates, particularly the sporadic form. To address this question, Braak and his colleagues proposed a six-stage model of sporadic PD propagation based on Lewy body distribution across brain regions (Braak et al., 2003b; Braak and Del Tredici, 2017). According to this model shown in Figure 19, the disease would start in the olfactory bulb and enteric nervous system (ENS) and then affects the peripheral nervous system (PNS), finally reaching the CNS. In the CNS, the disease would affect the lower brainstem in the dorsal motor nucleus of the vagus nerve, then goes through susceptible regions of the medulla, pontine, tegmentum, midbrain and basal forebrain, to eventually reach the cerebral cortex (Braak et al., 2003b; Visanji et al., 2013). This hypothesized model of non-random Lewy pathology is consistent with the progressive clinical observation of non-motor symptoms related to the PNS in PD patients and preceding the motor symptoms. In

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accordance to the broadly accepted theory that ageing and environmental factors contribute together to the onset of sporadic PD, Braak and his colleagues proposed that potential unidentified environmental pathogens might be first taken in the body by ingestion (Braak et al., 2003a). The causative pathogen from the ENS could then enter the CNS via retrograde axonal and transneuronal transport, along the preganglionic fibres through the visceromotor cholinergic projection neurons of the vagus nerve (Braak et al., 2003b; Houser and Tansey 2017). However, this model is still under debate, because only approximately half of the cases fit with patterns, and some clinical patients have no Lewy bodies at all (Jellinger, 2008; Jellinger, 2009; Halliday et al., 2012; Surmeier et al., 2017b).

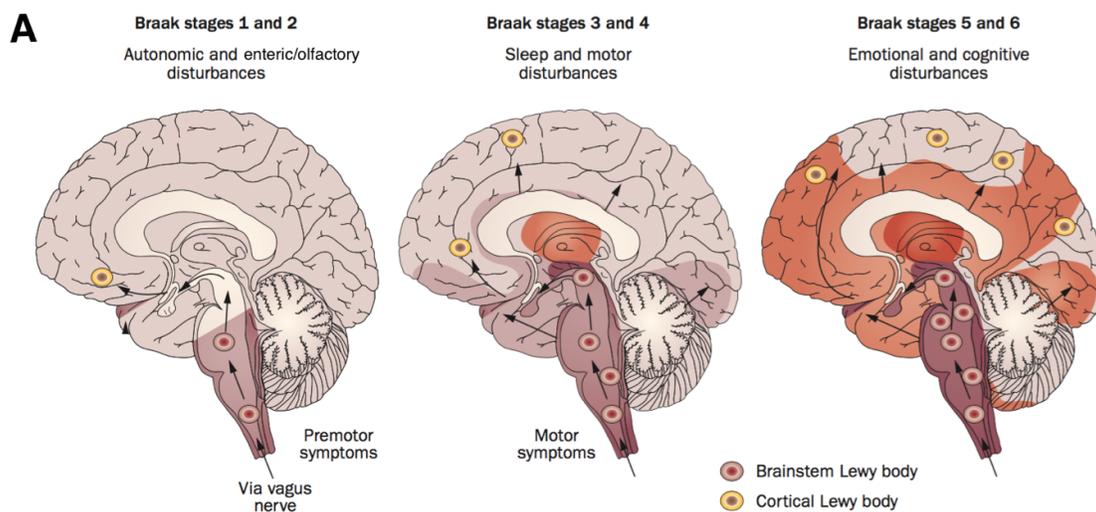


Figure 19 PD propagation model

The Braak's model of PD progression proposed that the pathology initiates in the enteric nervous system (ENS) or olfactory bulb, and then transfers via the vagus nerve to reach the CNS. Symptoms in each stage match with spreading of the Lewy pathology across the brain. Source: Adapted from Doty et al, 2012.

In recent years, PD was suggested to progress in a prion-like fashion, spreading by the seeding of misfolded proteins. This theory derives from the discovery of Lewy bodies in transplanted fetal DANs in post-mortem analysis of PD patients brain (Kordower et al., 2008a; Kordower et al., 2008b; Kurowska et al., 2011). This finding prompted a number of studies to elucidate how α -syn could be transmitted between neurons. *In vitro* studies showed that exogenous monomeric, oligomeric or fibrillar α -syn is able to infiltrate surrounding cells (Desplats et al., 2009; Hansen et al., 2011). Moreover, fibrillar α -syn can be transported via axonal transport (Freundt et al., 2012). Monomeric and aggregated forms of α -syn in

cytosolic vesicles can be released via classical ER-Golgi-dependent or non-classical exocytosis (Lee et al., 2005; Lee et al., 2008), or after their incorporation into exosomes (Soria et al., 2017), and then uptaken by surrounding neurons via conventional endocytosis, to be degraded by the lysosomes or released in the cytosol (Grozdanov and Danzer, 2018). Fibrillar α -syn can also be directly transferred from neuron to neuron through tunneling nanotubes inside lysosomal vesicles (Abounit et al., 2016; Valdinocci et al., 2017) (Figure 20).

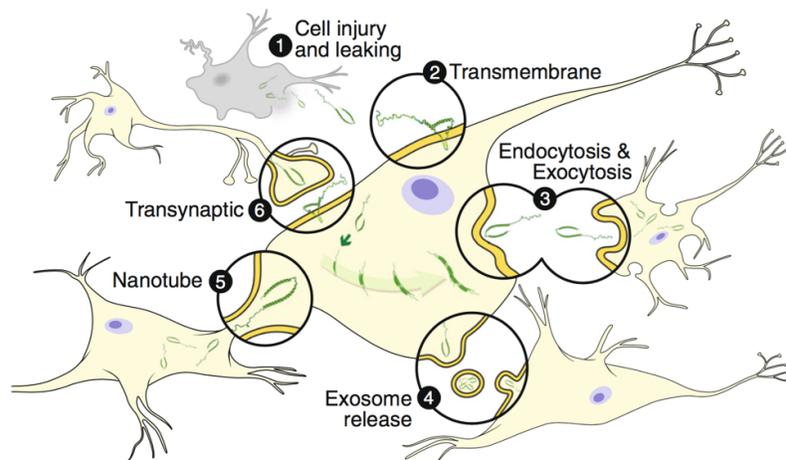


Figure 20 α -syn transmission between neurons

1-2) Extracellular α -syn released from the injured cells can translocate to the neighbour neurons by direct targeting to the membrane. 3) α -syn can be transferred between cells via exocytosis and endocytosis. 4) α -syn can be taken up via exosomes by neighbour cells. 5) α -syn transfers freely through nanotubes. 6) α -syn transmission could occur directly at the synaptic contact. From Visanji et al., 2013.

2.4.2 Role of gut microbiota in PD initiation

In line with the previously described Braak's propagation theory, research groups investigated the brain-gut axis on the hypothesis that PD could initiate in the gut (Klingelhoefer and Reichmann, 2015). First, 87% of PD patients suffer from chronic constipation preceding the motor symptoms by over a decade. The second evidence supporting this theory is the discovery of aggregated and phosphorylated α -syn in the ENS (Braak et al., 2006; Shannon et al., 2012). This accumulation could be attributed to inflammation and oxidative stress in the gastrointestinal tract caused by translocated bacteria and proinflammatory factors (Forsyth et al., 2011; Glass et al., 2010). Recent evidence

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suggest that PD could be triggered by unbalanced intestinal microbiota instead of a potential external pathogen and/or environmental toxins (Perez-Pardo et al., 2017; Sun and Shen, 2018). The gut microbiota was found to be required for α -syn-mediated motor deficits and brain pathology in mice (Sampson et al., 2016). Microbiota induced- α -syn aggregates could be retrogradely transported from the ENS to the more susceptible SNpc region, exerting its influence on PD progression. Moreover, gut-derived lipopolysaccharides (LPS) can promote disruption of the blood brain barrier, thus facilitating neuroinflammation and injury in the SNpc (Banks et al., 2008; Banks and Erickson, 2010).

2.4.3 Specific vulnerability of SNpc DANs

Apart from the presence of Lewy bodies in PD neurons, the other remarkable feature that distinguishes PD from other α -syn-related pathologies is the predominant loss of DANs in the SNpc (Spillantini and Goedert, 2000). But why are DANs of the SNpc especially vulnerable? First, DANs in SNpc have a long axon that arborizes to form a large number of synapses, loaded with a high density of mitochondria generating a higher basal rate of oxidative phosphorylation compared to other DANs (Pacelli et al., 2015). Removal of damaged mitochondria and transport of vesicles or autophagic vacuoles require ATP production to support the energetic cost of axonal transport along the microtubules (Sulzer., 2007). The vast number of mitochondria required to respond to such a high energy demand also results in a heavy ROS production.

Second, DA metabolism is considered to be a critical part of the exacerbated susceptibility of the SNpc to outside insults. Under normal condition, most DA is contained in low pH condition inside synaptic vesicles preventing its auto-oxidation. But in case of increased cytosolic levels, DA can be metabolized via MAO or go through auto-oxidation generating DA adducts, which are a source of ROS. In the presence of relatively higher iron deposit in these neurons, the H_2O_2 produced can be converted into the highly toxic radical peroxynitrite, prone to oxidize cellular components (Sian-Hülsmann et al., 2011). Elevated mitochondrial oxidative stress could trigger a DA-dependent toxic cascade. Furthermore, DA adduct formation has been shown to modify the catalytic site of GBA, a lysosomal enzyme whose mutations result in Gaucher's disease, a lysosomal storage disorder, and to lower its activity, leading to the lysosome dysfunction (Burbulla et al., 2017). Note that GBA mutations are also the major genetic risk factor for developing PD (Gegg and Schapira, 2018).

Third, DANs utilize calcium for pacemaking activity and particularly high calcium load renders SNpc DANs at risk. DANs in the SNpc are spontaneously active, and their tonic action potential firing is associated with tonic baseline DA release (Sulzer, 2007). During pacemaking, an oscillating calcium burden induces mitochondrial stress (Mosharov et al., 2009; Surmeier et al., 2010; Surmeier and Schumacker, 2013; Surmeier et al., 2017a, b). Besides, SNpc DANs expressing the calcium-binding protein calbindin to buffer calcium, located mainly in the dorsal tier of the SN, are relatively spared in PD, while the melanized ventral DANs with low calbindin are more vulnerable (Yamada et al., 1990; Collier et al., 2011; Dragicevic et al., 2015). Therefore, the cytosolic Ca^{2+} oscillation and low intrinsic Ca^{2+} buffer place DANs with more challenging calcium increase, and render them more vulnerable to PD triggers (Rcom-H'cheo-Gauthier et al., 2016).

Fourth, α -syn was proposed to regulate key stages of DA homeostasis (Venda et al., 2010; Benskey et al., 2016; Bridi and Hirth, 2018), as described in **section 2.1.3**. Under the intense oxidative stress mentioned above, any perturbation or organelle inefficiency to cope with ROS clearance would allow oxidized α -syn to rapidly impede DA homeostasis, resulting in an increased cytosolic DA level, followed by accelerated oxidative stress and mitochondria dysfunction and impaired protein degradation, here again trapping the DANs in a vicious cycle.

3. *Drosophila* models of PD

Drosophila serves as a valuable model organism for studying mechanisms of PD. Two hallmarks of PD, the loss of DANs and the locomotor defects, have been well-recapitulated in transgenic model and toxin-induced models of PD (Feany and Bender 2000; Coulom and Birman, 2004; Chaudhuri et al., 2007). Although *Drosophila* possess less genes and neurons compared to humans, the majority of genes implicated in PD pathologies are well conserved and have one homolog in flies, with the notorious exception of *SNCA* (Hirth, 2010; Vanhanwaert and Verstreken, 2015; Hewitt and Whitworth, 2017). Figure 21 shows the timeline of identification of PD genes and the loss or gain of function of the corresponding gene, or transgenic expression of the human gene, in the fly model (Vanhanwaert and Verstreken, 2015).

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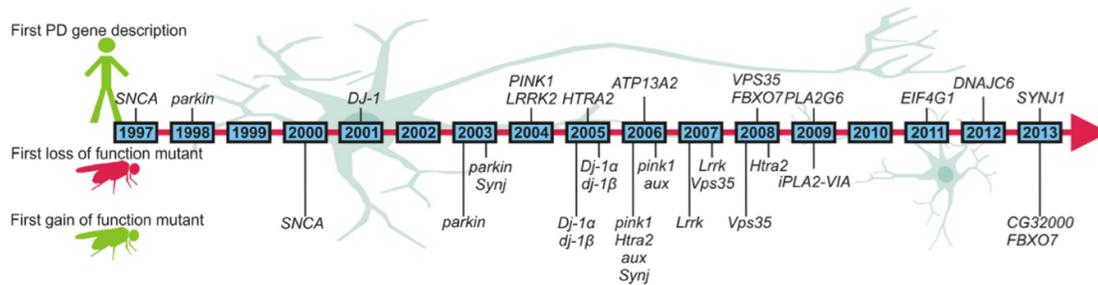


Figure 21 *Drosophila* models of PD

This scheme indicates the years when the PD genes were identified in human (top) and when *Drosophila* transgenic or loss/gain of function mutants were generated (bottom). Source: Vanhanwaert and Verstreken, 2015

3.1 Transgenic models of PD

Various *Drosophila* models have been established with different approaches to mimic mutations of PD-genes discovered in patients in order to study their role in pathogenesis. Studies of PD genes gain-of-function, even the ones that do not exist in flies, can be carried out by ectopic expression of the human mutant form or by overexpression of the fly homologs. Alternatively, when the human gene has an ortholog in the fly genome, loss-of-function or knockdown of the gene can be achieved by different genetic approaches, like RNAi or null mutants (Muqit and Feany, 2002; Şentürk and Bellen, 2017).

3.1.1 α -syn model of PD

Human *SNCA* has been the first gene used to establish a fly model of PD by Mel Feany and Welcome Bender (Feany and Bender, 2000). Due to the absence of homolog in flies, human wild-type and mutant forms of α -syn have been expressed in all neurons, driven by *elav-Gal4* (pan-neuronal driver), to assess the induced defects and its pathogenic pathway. As shown in Figure 22, flies expressing α -syn recapitulate major features of human PD, including DAN loss (PPM1/2) and age-related locomotor decline, as well as Lewy body-like inclusions.

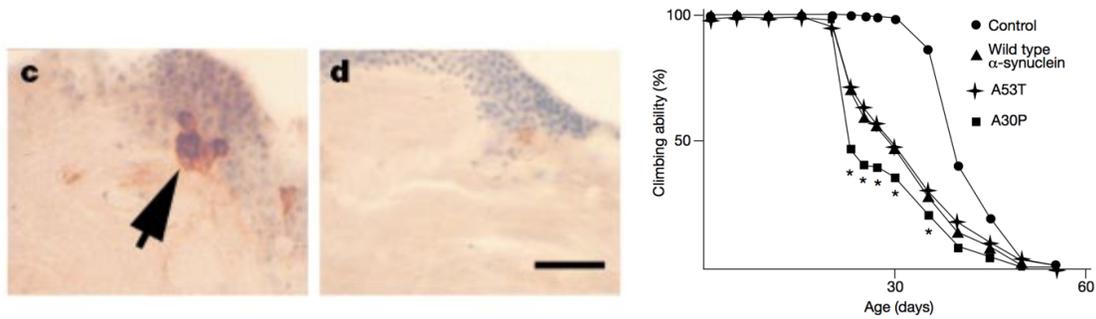


Figure 22 Two PD symptoms in the fly model

Left panels shows DAN loss in *elav>α-syn* flies (in d) compared to the control (in c). Right panel showed accelerated age-related locomotor decline (SING test) in flies expressing wild-type or mutant forms of α -syn in all neurons. Source: Feany and Bender 2000.

Interestingly, it was shown that co-expressing human Hsp70 protected the fly DANs from α -syn toxicity and that the fly Lewy bodies were positively stained both for α -syn and with an antibody against *Drosophila* Hsp70 (Auluck et al., 2002). This indicated an interaction between the two chaperone proteins that was subsequently confirmed by finding positive immunostaining for Hsp70 and Hsp40 chaperones in the Lewy bodies of human patients (Auluck et al., 2002). These observations highlight the conservation of the α -syn-induced pathology between human and fly, albeit α -syn is normally absent from the fly genome.

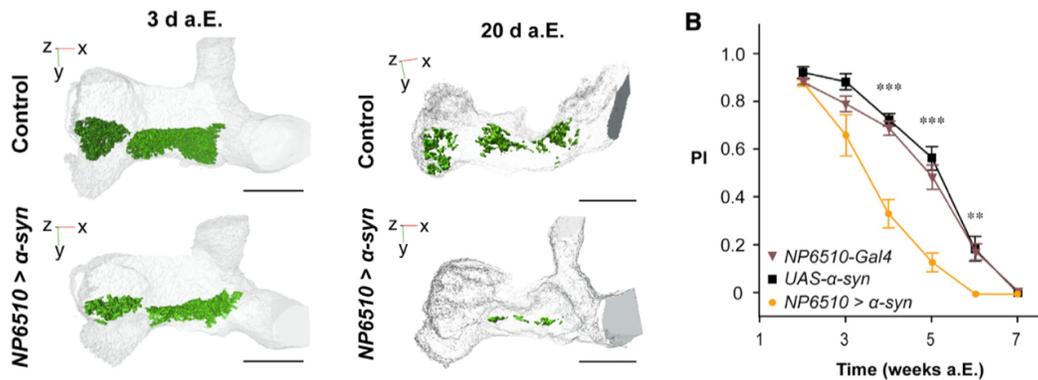


Figure 23 PAM- α -syn model of PD

Left: Compared to control, α -syn expression driven by *NP6510-Gal4* in the PAM cluster reduced connectivity from PAM DANs onto MB Kenyon cells in both day 3 and day 20 (visualized by 3D reconstruction of the dopaminergic innervation). Right: *NP6510>α-synA30P* flies showed age-related locomotor decline. Source: Riemensperger et al., 2013.

Thirteen years later, it was shown that overexpression of mutant human α -syn (the A30P form) in only 15 DANs of the PAM cluster led to age-related locomotor defects preceded by

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a rapid degeneration of descending projections from the PAM cluster onto the MB (Riemensperger et al., 2013) (Figure 23). This indicated that degeneration of the PAM DANs is for a large part responsible for the locomotor deficits induced by mutant α -syn expression in *Drosophila* neurons.

The α -syn *Drosophila* model has been widely used to study the molecular mechanisms underlying PD and other synucleopathies (Mizuno et al., 2011). It was reported that overexpressing the fly homologs of other PD-associated genes, Parkin and Pink1, could rescue α -syn-induced DAN degeneration and motor defects (Haywood and Staveley 2004, 2006; Yang et al., 2003). Over the years, many efficient neuroprotective genes or drugs have been identified using the fly α -syn-expression model that act by anti-oxidative or other mechanisms (Auluck and Bonini, 2002; Auluck et al., 2005; Outeiro et al., 2007; Trinh et al., 2008; Botella et al., 2008; Jia et al., 2008; Du et al., 2010; Barone et al., 2011; Butler et al., 2012; Shaltiel-Karyo et al., 2013; Büttner et al., 2014; Knight et al., 2014; Siddique et al., 2014; Breda et al., 2015; Wang et al., 2015), as well as candidate effectors of α -syn pathology (Büttner et al., 2013; Roy and Jackson, 2014; Suzuki et al., 2015; Jansen et al., 2017; Song et al., 2017; Ordonez et al., 2018).

3.1.2 Other fly genetic models and their contribution.

Great contributions to the understanding of mitochondrial dysfunction in PD were made from genetic models (DJ-1, PINK1 and Parkin) established in *Drosophila*. The observation that *Pink1* and *Parkin* double mutants exhibit the same muscle phenotype than the respective single mutants (Greene et al., 2003; Pesah et al., 2004; Clark et al., 2006) suggested that Pink and Parkin act in a common pathway for mitochondrial quality control. Parkin overexpression rescued the *Pink1* mutant phenotype while Pink1 overexpression had no effect on the *parkin* mutant, revealing that Parkin acts downstream of Pink1 (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). These discoveries from the PINK1/Parkin models underscore the importance of mitochondrial dysfunction as a central mechanism of PD pathogenesis.

The fly genetic models also provided important knowledge about the trafficking defects in PD, as well as about the connections between different PD-associated genes (LRRK2, Synaptojanin). Indeed, α -syn, LRRK2, Vps35, Synaptojanin, Parkin and DNAJC6/auxillin are all implicated in and connected to synaptic trafficking (Vanhouwaert and Verstreken, 2015; Penney et al., 2016). Flies overexpressing the mutated gene LRRK2-G2019S showed

several PD symptoms such as retinal degeneration, selective loss of DANs, and locomotor decline (Liu et al., 2008; West et al., 2015). LRRK2 appears to phosphorylate Endophilin A, a central component of synaptic remodelling properties. Interestingly, Endophilin A has a close functional association with Synaptojanin (Verstreken et al., 2003), which was later confirmed in PD patients (Krebs et al., 2013; Quadri et al., 2013).

3.2 Pesticide-induced models of PD

Familial PD cases are rare, which suggests that environmental insults play a primary role for the onset of sporadic PD. In *Drosophila* several pharmacological compound have been used to model sporadic PD. As shown in Figure 24, chronic exposure to the pesticide rotenone, a mitochondrial complex I inhibitor, triggered symptoms similar to the ones of PD patients like DAN degeneration and locomotor defects. Flies co-treated with rotenone and L-DOPA or rotenone and melatonin showed ameliorated locomotor performance compared to the groups with rotenone only (Coulom and Birman, 2004). The fly rotenone model has been widely used since then to study PD-related mechanisms and search for neuroprotectants (Hosamani et al., 2010; Lawal et al., 2010; Bayersdorfer et al., 2010; Girish and Muralidhara, 2012; Islam et al., 2012; Sudati et al., 2013; St. Laurent et al., 2013; Hwang et al., 2014; Araujo et al., 2015).

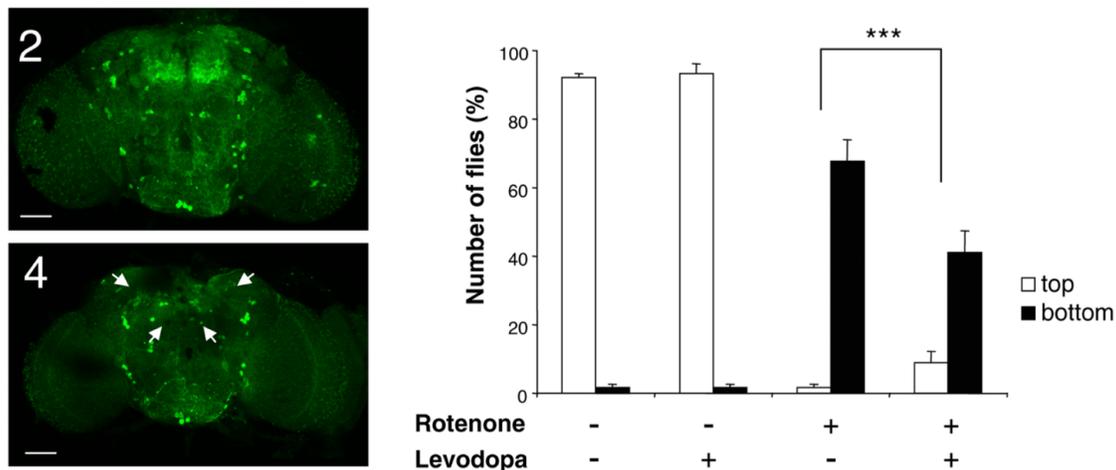


Figure 24 Rotenone model of PD

Left: TH immunostaining in the brain showing apparent lack of PAM and PAL DANs (as indicated by the white arrows in 4) in the fly brain following 7 days of exposure to 500 μM rotenone, compared to control flies (in 2). Right: Negative geotaxis test of adult flies with 7 days of exposure to

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125 μ M rotenone, 1 μ M L-DOPA or both. L-DOPA improved the locomotor defects induced by rotenone toxicity. Source: Coulom and Birman, 2004.

Flies exposed to the herbicide and pro-oxidant PQ show decreased locomotion, selective DAN loss, and die in a few days (Chaudhuri et al., 2007; Shukla et al., 2014b; Cassar et al., 2015; Shukla et al., 2016). Consistent with epidemiological studies of PD, male flies exposed to PQ exhibited PD-like symptoms earlier than females, as shown in Figure 25. PQ treated flies showed mitochondrial dysfunction and decreased superoxide dismutase (SOD) activity (Hosamani and Muralidhara, 2013; Mehdi and Qamar, 2013). Interestingly, PQ was reported to exert its neurotoxicity in *Drosophila* via overstimulation of the DA receptor Dop1R2/Damb in glutamatergic neurons (Cassar et al., 2015).

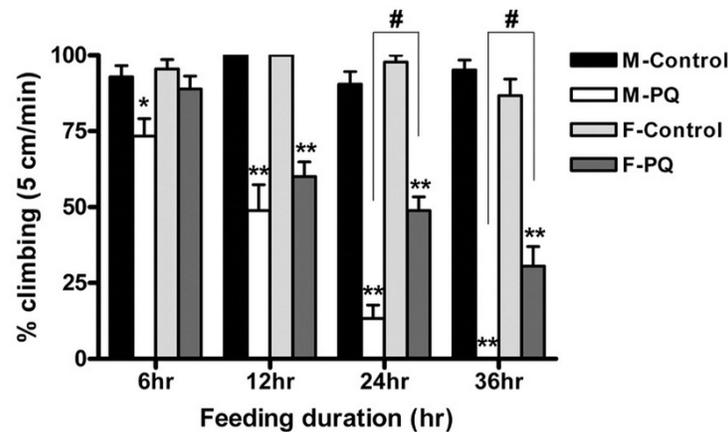


Figure 25 Males are more sensitive than females upon PQ treatment

Effect of 20 mM PQ on locomotion of wild type female and male *Drosophila*. Male flies showed total immobility after 36 h exposure to PQ. Source: Chaudhuri et al., 2007

Because of its strong redox capacity yielding stable ROS via NADPH consumption, PQ has been useful in the *Drosophila* model to evaluate neuroprotective compounds able to alleviate oxidative stress (Park et al., 2012; Minois et al., 2012; Medina-Leendertz et al., 2014; Filograna et al., 2016). Combined with other genetic approaches, PQ has been extensively used to test the influence of different genes on the sensitivity of flies to oxidative stress (Song et al., 2017; Quintero-Espinosa et al., 2017; Müller et al., 2017; Shukla et al., 2014a and b). Furthermore, a recent extensive metabolomic study has found remarkably similar alterations in metabolic pathways between the *Drosophila* PQ model and human PD patients (Shukla et al., 2016).

Chapter 3 Transposons and Argonaute proteins

1 Transposons

In the 1940s, mobile elements of the genome, called transposons or transposable elements (TEs) were identified in maize (McClintock, 1950). These “selfish” genetic elements are able to multiply themselves, mobilize, and insert into new genomic position. They take up nearly half of the human genome and 20% of that of the fly (Lander et al., 2001; Hoskins et al., 2002). TEs can be categorized into two classes according to their structure and their mobility manners: DNA transposon and DNA retrotransposon. DNA transposons go through “cut and paste” mechanisms to mobilize into an alternative position, while DNA retrotransposons mobilize through a “copy and paste” mechanism (Goodier and Kazazian, 2008). During this “copy and paste” process, an RNA intermediate transcribed from the transposon functions as a template for DNA synthesis. The synthesized DNA can be integrated back into the genome. Based on the presence or absence of long terminal repeats (LTR), retrotransposons are divided into LTRs and non-LTRs (Waddell et al., 2014). The non-LTR retrotransposon including LINE-1 (long interspersed element 1), Alu and SVA elements, are currently active in the human genome. In a large scale, retrotransposons impact on human genome evolution, local gene instability, and genomic rearrangements (Erwin et al., 2014). The epigenetic silencing of retrotransposon activity through DNA methylation and histone modification is an important defence mechanism for the cell.

Drosophila harbours distinct types of LTR elements, for example, *gypsy*, and non-LTR, LINE-like elements, such as *F-element*, *HeT-A*, and *R2-elements* that are expressed in the brain (Li et al., 2013b; Perrat et al., 2013). *HeT-A* belongs to an interesting class of elements that have their insertion sites specifically in telomeric DNA (Pardue et al., 2005; Pardue and DeBaryshe, 2011).

1.1 Highly repressed transposition in the germline

TE repression is crucial in the germline. During development in mice, a subset of cells is set aside as germ cell precursors and migrate for future gonad formation. During migration, those cells begin to undergo epigenetic changes, for example, loss of DNA methylation and histone methylation. This reprogramming process allows germline cells to reacquire a pluripotent

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state and to establish a blank genome for the sex-specific imprints of the embryo (Zamudio and Bourc'his 2010). However, this extensive epigenetic reprogramming leads to highly active transposition during this process. Accumulation of TE transcripts results in transposon-induced DNA damage, defects in homologous chromosome synapsis, meiotic arrest and sterility (Castañeda et al., 2011). To defend the genome integrity, small RNA guided pathways including small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA) are implemented to target the active sites and enhance histone or DNA methylation, thereby repressing TE activity.

1.2 Beneficial and deleterious effects of transposons in the CNS

Evidence showed that engineered human LINE-1 can retrotranspose in rat neuronal precursors (Muotri et al., 2005). Accordingly, next-generation sequencing approaches have demonstrated endogenous somatic insertions in the post-mortem human brain (Baillie et al., 2011; Evrony et al., 2012). Somatic retrotransposition increases as neural stem cells differentiate into neurons, contributing to genomic mosaicism in the brain (Erwin et al., 2014). Increased LINE-1 retrotransposition was demonstrated to occur in the neuronal genome of schizophrenic patients (Bundo et al., 2014). Those discoveries suggest a somatic genome mosaicism mechanism in the CNS by retrotransposition, which may reshape the genetic circuitry underlying normal and abnormal neurobiological processes.

1.2.1 Beneficial effect of TEs

Evolutionary conservation of neuronal somatic retrotransposition suggests that it provides benefits to the brain. By affecting genes proximal to their insertion sites, TE retrotransposition can modify the transcriptome contributing to brain cellular diversity (Cordaux and Batzer, 2009). Moreover, in some individuals, the rate of retrotransposition appears higher in the hippocampus than in the frontal cortex (Coufal et al., 2009; Baillie et al., 2011). Consistent with this, active transposition was observed in the memory-relevant $\alpha\beta$ MB neurons of adult *Drosophila* (Perrat et al., 2013). Retrotransposition events were observed in genes involved in neural-specific functions like DA receptors and neurotransmitter-related enzymes in human and *Drosophila* (Perrat et al., 2013; Baillie et al., 2011). As proposed by Erwin et al., (2014), at a single neuron level, depending on where the somatic retrotranspositions occurred, synaptic activity and neuronal response to stimuli or neuronal connections could be affected. Stochastic mobile elements insertions would bring

about the potential “variance” to enable the individual to cope with unforeseen environmental alterations.

However, in a recent critical report, Treiber and Waddell (2017) re-evaluated the frequency of somatic transposition in *Drosophila* neurons and found it to be, on the contrary, a very rare event. They observed that *de novo* transposon insertions are actually not prevalent in $\alpha\beta$ MBs KCs, and they also did not find any evidence to support the model that transposon insertions accumulate with age in the fly brain. The mistake in their previous studies (Perrat et al., 2013) was attributed to a methodological problem, i.e. chimeric DNA molecule artefacts that arose during genomic DNA amplification. These authors also suggested that previous studies in flies, rodents, humans and plants, that use similar deep-sequencing data, are subject to the same amplification artefacts. Consequently, the frequency of genuine somatic transposition in neurons, and its possible role in brain ageing, remain to this date an open question.

1.2.2 Role of TEs in neurological disorders

Increased TEs level has been shown to impact on neuronal function. Rett syndrome was the first discovered neurodevelopmental disorder related to an increased somatic retrotransposon insertion (Muotri et al., 2010). Due to the mutation in *MECP2* (methyl-CpG-binding protein 2), which repressed LINE-1 transcription in neural progenitor cells, Rett syndrome patients exhibit increased levels of somatic LINE-1 (Muotri et al., 2010). Schizophrenic patients also have a higher copy number of somatic LINE-1 retrotransposons in neurons (Bundo et al., 2014). In *Drosophila*, aged flies apparently have an increased level of TEs (LINE-like and LTR retrotransposons) in the brain. Since siRNAs are involved in TE silencing mechanism. Flies mutant for Argonaute 2 (Ago2), which is required for siRNA activity, showed elevated TE level associated with age-related memory defects and shortened lifespan (Li et al., 2013b). Conversely, overexpressing Dcr-2 (the double-stranded RNase responsible for siRNA formation) to inhibit TE activity in neurons prolonged fly lifespan (Wood et al., 2016).

Fragile X syndrome is a genetic disease caused by an expansion of a CGG triplet repeat (more than 200) within the *FMRI* gene, leading to mental retardation and other symptoms in childhood. Between 55 and 200 CGG repeats, patients are called "premutation" carriers and, although they do not have Fragile X syndrome as children, they can develop after 50 years a late-onset neurodegenerative disorder named Fragile X-associated tremor/ataxia syndrome (FXTAS). In a FXTAS *Drosophila* model, it has been shown that rCGG repeat transcripts can promote neurodegeneration by activating retrotransposition. Downregulating the

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retrotransposon gypsy was sufficient to suppress the neuronal toxicity caused by rCGG repeats (Tan et al., 2012). Furthermore, Fmr1, the *Drosophila* orthologue of Fragile mental retardation protein (FMRP), the protein coded by FMR1, was shown to genetically interact with an Argonaute protein, Aubergine (Aub), involved in the piRNA pathway (see below) and potentially TE silencing (Bozzetti et al., 2015).

In association with different Argonaute proteins, siRNAs and piRNAs can target the active TEs. However, the piRNA pathway is much more effective than the siRNA pathway, due to the large genomic “library” system of piRNA, and the capacity of PIWI proteins (piRNA binding protein) for packing TEs back into heterochromatin (Sturm et al., 2017).

2. Argonaute proteins

The gene *P-element induced wimpy testis (piwi)* was the first identified Argonaute gene in a screen for mutants that affect the asymmetric division of germline stem cells in the *Drosophila* ovary (Lin and Spradling, 1997). Then, a study in *Arabidopsis* showed that Argonaute proteins are involved in embryogenesis and leaf development (Moussian et al., 1998; Bohmert et al., 1998). These proteins are highly conserved, and exist in diverse eukaryotes, except *Saccharomyces cerevisiae*, which has lost the small RNA machinery (Drinnenberg et al., 2009). Argonaute proteins play essential roles in gene regulation mechanisms, chromosome maintenance and heterochromatin formation, by association with different classes of small non-coding RNAs (Hutvagner and Simard, 2008; Meister, 2013; Luteijn and Ketting, 2013).

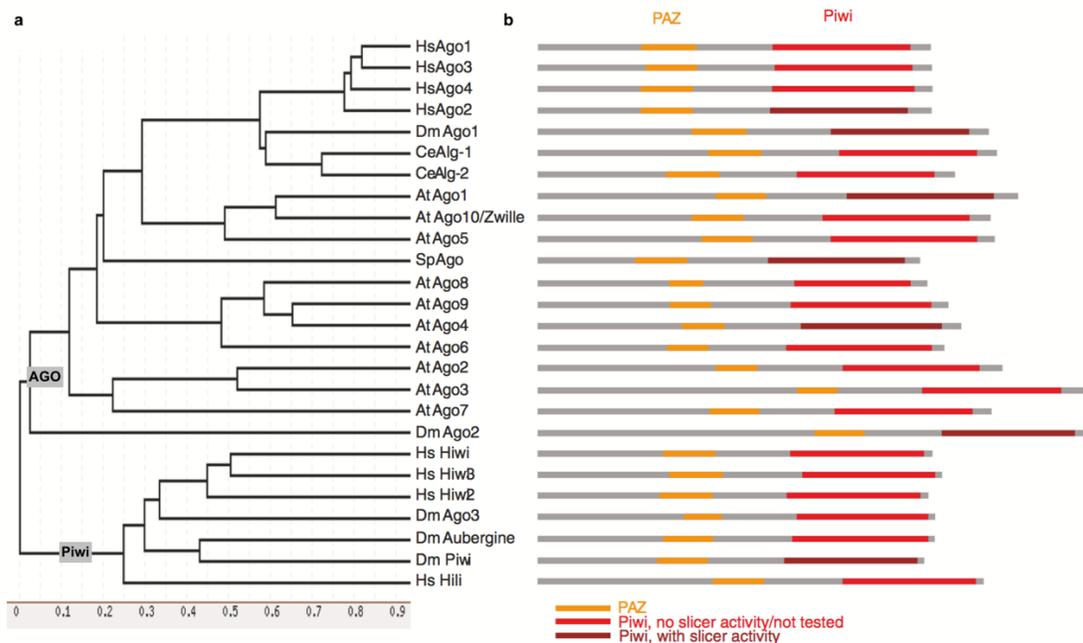


Figure 26 Phylogenetic conservation of Argonaute proteins

(a) Molecular phylogenetic analysis of Argonaute proteins was made with ClustalW and the tree was built with Treetop. Protein sequences applied for the alignment are from *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm), *Shizosaccharomyces pombe* (Sp) and *Caenorhabditis elegans* (Ce). Two subfamilies AGO and PIWI are indicated. (b) The PAZ and Piwi domains are shown in orange and red, respectively. Piwi domain of slicer-active Ago proteins are in dark red; Piwi domain without slicer activity or not tested is presented in light red. Source: Höck and Meister, 2008

According to the phylogenetic analysis shown in Figure 26, Argonaute proteins can be categorised into two subclades, the AGO subfamily and the PIWI subfamily. All Argonaute proteins share two major protein motifs: the PAZ (Piwi-Argonaute-Zwille) and Piwi domains. PAZ domain anchors the 3' end of the small RNA by loading it into a specific binding pocket (Jinek and Doudna, 2009), while the Piwi domain is structurally similar to RNase H (non-sequence-specific endonuclease enzymes that catalyse cleavage of the RNA moiety in an hybridized RNA/DNA substrate). Given these specific structures, small non-coding RNA could be loaded onto an Argonaute protein to target mRNAs. AGO subfamily proteins are ubiquitously expressed and can interact with microRNAs (miRNAs) and siRNAs, whereas PIWI subfamily proteins are expressed mainly in germline cells and exert their function via piRNAs (Iwasaki et al., 2015).

In *Drosophila*, there are five Argonaute proteins. The two AGO subfamily proteins Ago1 and Ago2 are involved in the siRNA/miRNA pathways, whereas the PIWI subfamily proteins,

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Piwi, Ago3 and aubergine (Aub) are responsible for piRNA biogenesis and piRNA-guided TE silencing mechanism (Höck and Meister, 2008). Piwi is predominately nuclear while Aub and Ago3 are cytoplasmic (Brennecke et al., 2007). The PIWI-piRNA pathway will be presented in more details below.

3. PIWI-piRNA pathway

3.1 Study of the PIWI-piRNA pathway in ovary and testes

PIWI proteins are abundant in ovary and testis in *Drosophila*, where they guard the genome integrity against TEs (Aravin et al., 2007; Senti and Brennecke, 2010). As shown in Figure 27, the three PIWI proteins Piwi, Aub and Ago3 have diverse subcellular distribution in the ovariole. Piwi localizes mainly in the nucleus of somatic follicle cells and germline cells while Aubergine and Ago3 are expressed in the cytoplasm and nuage (a perinuclear electron dense structure) (Brennecke et al., 2007). Briefly, Ago3 and Aub slice TE transcripts in the cytoplasm through post-transcriptional gene silencing (PTGS) in germline cells, whereas Piwi acts through transcriptional gene silencing (TGS) to silence TE activity, further promoting histone methylation in both somatic cells and germline cells.

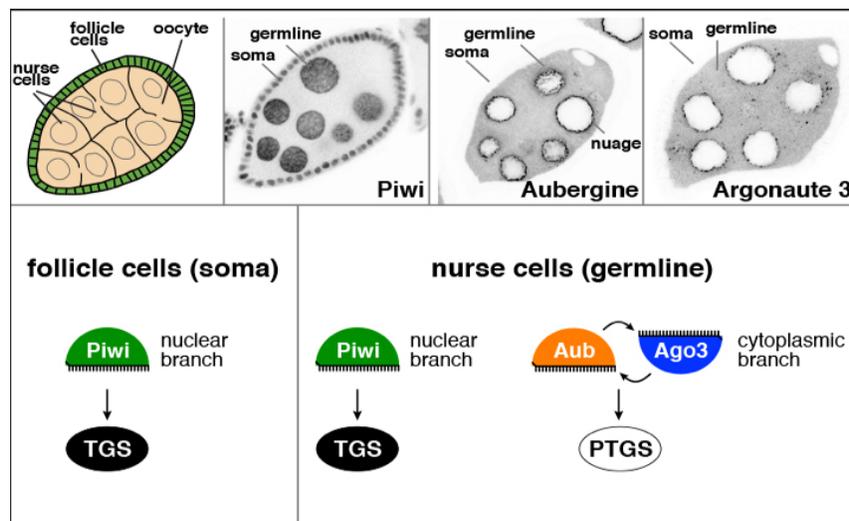


Figure 27 PIWI protein function in *Drosophila* ovary

Piwi localizes in the nucleus of germline and follicle cells (soma). Aubergine and Argonaute 3 label the cytoplasm of the germline cells and showed strong labelling in the nuage. In the germline cells, Aub and Ago3 help cleave the TE transcripts via posttranscriptional gene silencing (PTGS), whereas piwi targets and represses the TEs activity through transcriptional gene silencing (TGS). Source: <https://www.imba.oeaw.ac.at/research/julius-brennecke/projects/>

piRNAs are slightly longer (24-31 nt) than miRNAs and siRNAs and harbour 2'-O-methyl modification sites at the 3' terminal. Generated from TE-rich genomic regions called piRNA clusters (*flamenco*, *traffic jam* and other loci), piRNAs are mainly produced through primary piRNA biogenesis and amplified through a “ping-pong” pathway process (Senti and Brennecke, 2010).

In *Drosophila* ovaries, as shown in Figure 28, primary piRNA precursors derive from dual-stranded piRNA clusters or single-stranded transcripts in germline and surrounding somatic cells. The long single-stranded primary piRNA templates are exported to the cytoplasm and processed by subsequent maturation steps by Zucchini and Yb in Yb bodies (Huang et al., 2017). The Aub/Ago3-mediated ping-pong pathway is responsible for amplifying secondary piRNAs which have 10-nt complementarity with primary piRNAs at their 5' ends and possess a sense bias with adenosine at the tenth nucleotide (Brennecke et al., 2007). During the ping-pong process, the new secondary piRNAs are generated from the complementary cleavage of sense and antisense transposon transcripts by Ago3 and Aub to consume transposon transcripts in the cytoplasm. (Iwasaki et al., 2015).

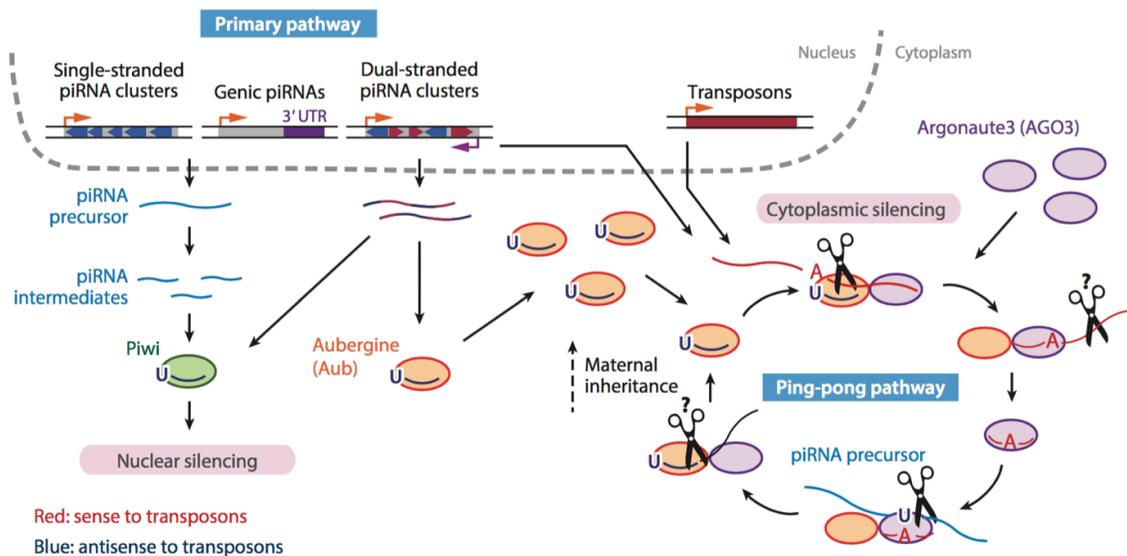


Figure 28 piRNA biogenesis

In the primary pathway, piRNA precursors are transcribed from piRNA clusters, processed and loaded on Piwi or Aub. With Ago3, Aub-piRNA complex serves as a trigger to initiate the ping-pong amplification pathway by the consumption of TE transcripts. Guided by piRNA, Piwi-piRNA complex can enter into the nucleus to target the active TEs. Source: Iwasaki et al., 2015.

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piRNAs are necessary for Piwi function in the nucleus where *piwi* deletion causes the loss of repressive histone H3 lysine 9 trimethylation (H3K9me3) marks at the TE loci, increases distribution of RNA polymerase II (RNA Pol II), followed by elevated level of TE transcripts (Huang et al., 2013; Le Thomas et al., 2013; Wang and Elgin, 2011; Sienski et al., 2012; Rozhkov et al., 2013). Besides, guided by piRNAs, Piwi enters the nucleus and is also involved in epigenetic regulation in somatic cells of the ovary by TGS and PTGS pathways as illustrated in Figure 29.

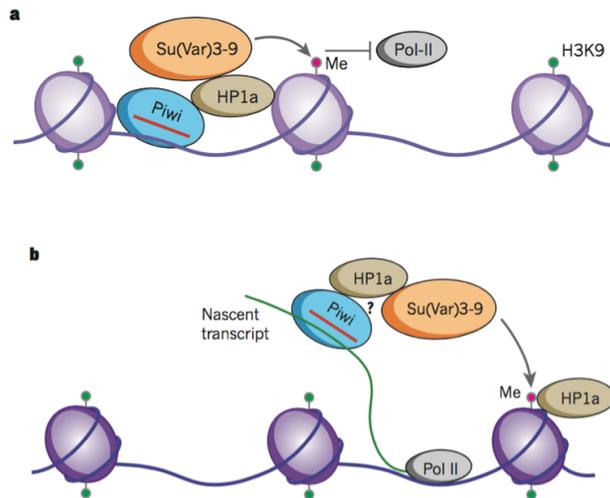


Figure 29 Piwi represses TE activity

(a) Transcriptional gene silencing mechanisms. Guided by piRNAs, Piwi can bind to the TEs that are complementary to these piRNAs. There Piwi can recruit Heterochromatin protein 1a (HP1a) and the methyltransferase Su(Var)3-9 to generate *de novo* methylation and inhibit RNA Pol II, thus silencing TEs. (b) At the active TE locus, Piwi-piRNA can recruit HP1a and Su(Var) 3-9 to increase methylation and promote heterochromatin formation. Source: Ross et al., 2014.

In the *Drosophila* heterochromatin, Piwi directly targets genomic DNA. At piRNA target sites, Piwi associates with Heterochromatin protein 1a (HP1a) to recruit the major histone methyltransferase Su(var)3-9 and locally promotes H3K9 methylation (Brower-Toland et al., 2007; Yin and Lin, 2007). These repressive chromatin marks inhibit RNA Pol II activity, thereby silencing the TE locus. In euchromatin, guided by the piRNA which is complementary to the nascent transcripts from the TEs, Piwi directly recruits HP1a and Su(var)3-9 to increase H3K9me3 of euchromatic transposon and decrease RNA Pol II occupancy (Rozhkov et al., 2013; Sienski et al., 2012; Le Thomas et al., 2013). With HP1a binding to the formed H3K9me3, Piwi can promote heterochromatin formation and maintain proper heterochromatin at Piwi targets (Luteijn and Ketting, 2013).

PIWI function in germline cell development is conserved among species. The Piwi ortholog in mice, *Miwi2*, localizes to the nucleus and possesses TGS mechanism to establish *de novo* DNA methylation of the TE loci in testes (Carmell et al., 2007; Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). So does the zebrafish Piwi protein, *Zili*, showing dynamic nucleocytoplasmic distribution (Houwing et al., 2008). Cytoplasmic nuage localization of PIWI proteins and related PTGS mechanism is well conserved among species as well. Mutations in murine *Mili* showed impaired secondary piRNA production and de-repressed TEs (De Fazio et al., 2011). A loss of *Ziwi*, the other Piwi protein in zebrafish, caused apoptosis in germline cells. Work in mice also showed that pachytene piRNA caused mRNA destabilization during spermatogenesis (Gou et al., 2014).

3.2 Evidence of PIWI expression and function beyond the gonad

PIWI proteins are expressed in all animals from sponges to humans (Lim and Kai, 2015). Apart from the gonads, PIWI/piRNA is presumed to function in non-ageing cells to maintain their proliferative potential but at a lower expression level than those detected in the germline. As shown in Figure 30, PIWI protein expression ranges from naïve pluripotent stem cells to differentiated somatic cells (Ross et al., 2014). In the stem cells of metazoans from diverse phyla, PIWI proteins are essential in maintaining pluripotency as well as their capacity for partial or whole body regeneration (Alié et al., 2011; Funayama et al., 2010). Consistent with this, murine induced pluripotent stem cells derived from various cell types showed increased *Mili* expression during reprogramming. In the intestinal stem cells (ISCs) of the *Drosophila* posterior midgut epithelium, exposure to regenerative pressure induced *Piwi* expression via STAT dependent signal pathway for the regenerative capacity and the ISCs maintenance (Sousa-Victor et al., 2017). In addition, *piwi* knockdown in the ISCs showed elevated TEs activation, loss of heterochromatin maintenance and ISC apoptosis. Mouse mesenchymal stem cells expressed *Mili*, and *Mili* knockdown *in vitro* resulted in higher cell division rate, suggesting the broad and diverse roles for Piwi in the stem cells (Wu et al., 2010).

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Phylum	Common name	Species	Known Piwi genes	PIWI protein expression
Porifera	Sponge	<i>Ephydatia fluviatilis</i>	<i>EfPiwiA</i> and <i>EfPiwiB</i>	Archeocytes (stem cells that differentiate into both somatic and germ cells)
Cnidaria	Jellyfish	<i>Clytia hemisphaerica</i>	<i>Piwi</i>	Somatic stem cells of the tentacle bulb (produce stinging cells characteristic of the cnidarians)
	Jellyfish	<i>Podocoryne carnea</i>	<i>Cniwi</i>	Somatic stem cells of the tentacle bulb (see above); striated muscle cells capable of transdifferentiation
Ctenophora	Comb jellyfish	<i>Pleurobrachia pileus</i>	<i>PpiPiwi1</i> and <i>PpiPiwi2</i>	Actively dividing adult somatic cells; germ line
Platyhelminthes	Planaria	<i>Schmidtea mediterranea</i>	<i>smedwi-1</i> , <i>smedwi-2</i> and <i>smedwi-3</i>	Neoblasts (totipotent stem cells that can repopulate all somatic and germline lineages)
	Saltwater flatworm	<i>Macrostomum lignano</i>	<i>macpiwi</i>	Neoblasts (see above)
Mollusca	Sea slug	<i>Aplysia californica</i>	<i>Piwi</i>	Nervous system, heart and germ line
Arthropoda	Fruitfly	<i>Drosophila melanogaster</i>	<i>piwi</i> , <i>aub</i> and <i>AGO3</i>	Gonad, brain, salivary gland
Chordata	Sea squirt (ascidian)	<i>Botrylloides leachii</i> and <i>Botryllus schlosseri</i>	<i>Piwi</i>	Stem cell population (capable of whole-body regeneration)
	Mouse	<i>Mus musculus</i>	<i>Miwi</i> , <i>Mili</i> and <i>Miwi2</i>	Diverse cancers (breast cancer, rhabdomyosarcoma, medulloblastoma); male germ line
	Human	<i>Homo sapiens</i>	<i>HIWI</i> , <i>HILI</i> , <i>HIWI2</i> and <i>HIWI3</i>	Diverse cancers (breast cancer, cervical cancer, endometrial carcinoma, seminomas, hepatocellular carcinoma, gastric cancer, pancreatic adenocarcinoma, gastrointestinal stromal tumours, colon cancer, renal cell carcinoma); haematopoietic stem cells; and male germ line

Figure 30 PIWI function across metazoans

Source: Ross et al., 2014

In addition, PIWI proteins are expressed in adult cancer stem cells. Ectopic PIWI expressions in various tumorous tissues could be intrinsically connected to cancer development and modulation of tumour cell proliferation (See the reviews by Suzuki et al., 2012; Tan et al., 2015; Han et al., 2017). *Drosophila* brain tumours showed upregulation of *piwi* and *aub*, and inactivation of *piwi* and *aub* prevented tumour growth and metastasis (Janic et al., 2010).

Landry et al. (2013) proposed a potential role for piRNAs in epigenetic control of memory, based on the discovery of their function in learning and memory in *Aplysia*. Upon treatment with serotonin, an important modulatory transmitter for memory, there was unexpectedly an abundant expression of piRNAs. Furthermore, *piwi* depletion or overexpression affects learning-related synaptic plasticity by a piRNA-dependent mechanism (Rajasethupathy et al., 2012). A review by Zuo et al (2016) summarized potential expression and function of PIWI/piRNA in the brain of different species. In mice, piRNA-like species were identified in the mammalian cortex (Yan et al., 2011) and murine MIWI, a homolog of *Drosophila* Ago3, was detected in hippocampal neurons (Lee et al., 2011a). In mice, the PIWI-piRNA complex can modulate dendritic spine development, postsynaptic density and is involved in neuronal migration (Adams et al., 2002; Lee et al., 2011a).

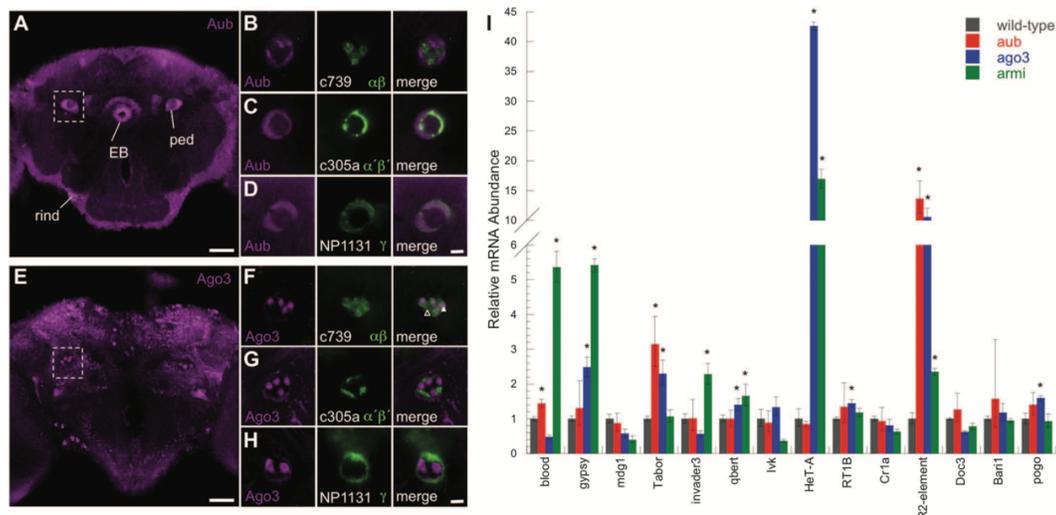


Figure 31 PIWI proteins are differentially labelled in the brain

(A-H) Immunostaining for Aub and Ago3 showed that these two PIWI protein are differentially expressed in the brain. A: Aub staining showed clear labelling in ellipsoid body (eb), peduncles (ped) and cortical cell body layer (rind). By using the driver *c305a-Gal4* $\alpha'\beta'$ -B), *c739-Gal4* ($\alpha\beta$ -C) and *NP1131-Gal4* (γ -D), Aub showed converged signalling with GFP expressed in the MB subdivisions with more in $\alpha'\beta'$ than in $\alpha\beta$ lobes. E: Ago3 labelling had a more complex pattern with labelling in β core (F), no $\alpha'\beta'$ -G) and overlapping with γ neurons (H). (I) Ago3, Aub and Arm mutant flies showed dramatically elevated TE levels in the brain. Source: Perrat et al., 2013

Perrat et al (2013) have reported that the PIWI proteins Ago3 and Aub are expressed in the *Drosophila* brain. More precisely, neurons of the MBs, the integrated centre for memory formation, showed different Ago3 and Aub levels, with less labelling in $\alpha\beta$ than in $\alpha'\beta'$ and γ neurons. *Ago3* and *aub* inactivation elicited an increase in TE level in the brain (Figure 31). As mentioned above in Chapter 3 - 1.2.1, however, a recent work from the same laboratory demonstrated that genuine somatic transposition is less prevalent in *Drosophila* than previously envisaged (Treiber and Waddell, 2017). Consistent with this, in an unpublished recent work, van den Beek et al (2018) detected a small amount of piRNA-sized, “ping-pong” negative molecules in *Drosophila* heads. The identification of piRNA-like non-coding RNA in heads confirms potential expression of PIWI proteins in the fly brain.

Finally, in a *Drosophila* model of Alzheimer’s disease, Tau-induced neurodegeneration was found to be linked to heterochromatin loss, as attested by decreased H3K9me2 level and downregulation of HP1a in neurons (Frost et al., 2014). Tau toxicity reduced H3K9me2 binding to the *Ago3* gene, ending up with an increased level of Ago3 protein. Interestingly, *Ago3* knockdown in all neurons could rescue the locomotor defects induced by Tau toxicity.

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One possible hypothesis is that Tau-induced Ago3-piRNA pathway would switch on proliferative activity in post-mitotic neurons, thus causing neuronal apoptosis.

PhD objectives

PD is a neurodegenerative movement disorder that is still incurable and for which transgenic and pharmacological *in vivo* models are available in *Drosophila*. These Parkinsonian flies are generally characterized by progressive locomotor defects or shortened survival due to increased brain oxidative stress. The general goal of my PhD project has been to use these models to understand better how α -syn accumulation in DANs can induce locomotor defects and to find novel proteins or pathways that may confer neuroprotection in such pathological conditions.

My PhD objectives have therefore focused on three topics:

- **A. To identify DA-related neuronal circuits that regulate startle-induced locomotion in the fly brain**
- **B. To understand how α -syn expression in a few DANs can induce dramatic locomotor deficits in ageing flies**
- **C. To identify novel endogenous proteins or pathways that can protect neurons against PD-like acute oxidative stress.**

A. *Drosophila* PD models are characterized by a progressively impaired locomotor reactivity that leads to reduced performance in startle-induced locomotion (SING behaviour), while spontaneous locomotion is not as strongly affected. This suggests that components of the brain circuits controlling the SING reflex are specifically targeted in PD models. Because these circuits had not been thoroughly studied, my first objective was to identify some of the neuronal components involved. This work led us to highlight the role of the MBs, associated DANs and output neurons in SING modulation.

B. Previous studies from my host laboratory showed that expressing mutant human α -syn in as few as 15 neurons of the PAM DANs caused a progressive loss of their projections to the MB neurons in adult flies, followed by strongly accelerated SING decline. Here I will show evidence that mutant α -syn triggers mitochondrial dysfunction in these DANs that propagate by a non-cell autonomous mechanism, to their non-dopaminergic MB target cells, causing the characteristic locomotor deficits in this fly PD model.

PhD objectives

C. An essential aim is to discover novel mechanisms that could delay neuronal ageing and disease progression, which would be helpful for the development of effective therapeutic strategies. During my PhD, I have contributed to the identification and/or characterization of different protective proteins that act by restoring mitochondrial homeostasis or enhancing the autophagy-lysosomal pathway in neurons. In this dissertation, I will focus on the results showing that the Argonaute protein Piwi is a potent neuroprotectant that is induced by oxidative stress in the *Drosophila* brain.

Materials and Methods

1. *Drosophila* and culture

Fly stocks were raised and crossed at 25°C on standard corn meal-yeast-agar *Drosophila* medium supplemented with methyl-4-hydroxybenzoate as a mold protector, under a 12h-12h light-dark cycle. The driver (*Gal4* or *LexA*) were crossed with the effector (*UAS* or *LexAop*) strains to express genes ectopically in specific tissues. The w^{118} line was used as wild-type for control crosses.

Table 3 Driver strain information

Driver	Expression pattern
<i>elav-Gal4</i>	All neurons
<i>elav-Gal80</i>	Inhibits Gal4 activity in all neurons
<i>TH-Gal4</i>	All TH-expressing cells including central DA neurons except a major part of the brain PAM cluster
<i>TH-LexA</i>	Similar to <i>TH-Gal4</i>
<i>R58E02-Gal4</i>	Major part of PAM DANs
<i>R58E02-LexA</i>	Same as <i>R58E02-Gal4</i>
<i>NP6510-Gal4</i>	15 DANs including MB-MVP1 plus 3 non-DANs
<i>NP5272-Gal4</i>	3 PAM DANs (MB-M3) that are also labelled by <i>TH-Gal4</i>
<i>Mz840-Gal4</i>	PPL1 MB-V1 plus other neurons and glial cells
<i>NP2758-Gal4</i>	PPL1 MB-MP1
<i>TH-D'-Gal4</i>	PAL, PPM2, PPM3 and PPL1
<i>TH-C'-Gal4</i>	PAL, PPM2 and PPL2ab
<i>TH-C1-Gal4</i>	PAM, PAL, PPM2 and PPL2ab
<i>TH-LexA</i>	Similar to <i>TH-Gal4</i>
<i>Tdc2-Gal4</i>	Octopaminergic neurons
<i>VGlut-Gal4</i>	Glutamatergic neurons
<i>OK371-Gal4</i>	Glutamatergic neurons
<i>Gad1-Gal4</i>	GABAergic neurons

Materials and methods

<i>C316-Gal4</i>	Dorsal paired medial (DPM) neurons
<i>238Y-Gal4</i>	All MB lobes (mushroom body neurons)
<i>VT30559-Gal4</i>	All MB Kenyon cells
<i>c305a-Gal4</i>	MB α' β' lobes and broad expression out of the MB
<i>G0050-Gal4</i>	MB $\alpha'\beta'$ lobes
<i>4-59-Gal4</i>	MB $\alpha'\beta'$ lobes
<i>R35B12-Gal4</i>	MB $\alpha'\beta'$ lobes
<i>H24-Gal4</i>	MB γ lobes
<i>R16A06-Gal4</i>	MB γ lobes
<i>mb247-Gal4</i>	MB $\alpha\beta$ and γ lobes
<i>R14C08-LexA</i>	MBON-M4/M6
<i>NP2492-Gal4</i>	MBON-V2
<i>R71D08-Gal4</i>	MBON-V2
<i>G0239-Gal4</i>	MBON-V3
<i>c41-Gal4</i>	EB neurons (ellipsoid body neurons)
<i>c105-Gal4</i>	EB ring neurons R1
<i>EB1-Gal4</i>	EB ring neurons R2
<i>c232-Gal4</i>	EB ring neurons R3/R4d
<i>R52G12-Gal4</i>	FSB columnar neurons (fan-shaped body neurons)
<i>R89F06-Gal4</i>	FSB columnar neurons
<i>R94F05-Gal4</i>	FSB layer neurons
<i>R84C10-Gal4</i>	FSB layer neurons

Table 4 Effector strain information

UAS/LexAop strain	Effector function
<i>UAS-nSyb::GFP</i>	GFP fused to neuronal Synaptobrevin (vesicle marker)
<i>UAS-mCD8::GFP</i>	GFP fused to mouse CD8 (plasma membrane marker)
<i>UAS-msGFP</i>	<i>UAS-mCD8::GFP</i> , <i>UAS-nSyb::GFP</i>
<i>UAS-CD4::spGFP₁₁</i>	splitGFP ₁₁ fused to mouse CD4
<i>LexAop-CD4::spGFP₁₁</i>	Same as <i>UAS-CD4::spGFP₁₁</i>
<i>UAS-nSyb::spGFP₁₋₁₀</i>	<i>spGFP₁₋₁₀</i> fused to neuronal Synaptobrevin
<i>LexAop-nsyb::spGFP₁₋₁₀</i>	Same as <i>UAS-nSyb::spGFP₁₋₁₀</i>
<i>UAS-dTrpA1</i>	Heat-activated cation channel for thermogenetic neuronal activation
<i>LexAop-dTrpA1</i>	Same as <i>UAS-dTrpA1</i>
<i>UAS-shi^{Δs1}</i>	Temperature-sensitive Shibire (dynamin homolog)
<i>UAS-ChR2-XXL</i>	Modified Channelrhodopsin 2 for optogenetic neuronal activation
<i>UAS-damb^{RNAi}</i>	<i>Dopamine 1-like receptor 2 (Dop1R2)</i> RNAi inactivation
<i>UAS-dumb^{RNAi}</i>	<i>Dopamine 1-like receptor 1 (Dop1R1)</i> RNAi inactivation
<i>UAS-piwi^{RNAi-31}</i>	<i>piwi</i> RNAi inactivation (<i>TRiP HMS00606</i>)
<i>UAS-piwi^{RNAi-33}</i>	<i>piwi</i> RNAi inactivation (<i>TRiP JF01394</i>)
<i>piwi^{EP1024}</i>	<i>piwi</i> loss-of-function mutant and overexpression (<i>UAS-piwi</i>)
<i>UAS-α-synA30P</i>	Expresses mutant human <i>α-synuclein A30P</i>
<i>LexAop-α-synA30P</i>	Same as <i>UAS-α-synA30P</i>
<i>UAS-α-synWT</i>	Expresses wild-type human <i>α-synuclein</i>
<i>UAS-mitoGFP</i>	GFP addressed to the mitochondrial matrix
<i>UAS-MitoTimer</i>	Fluorescent timer DsRed1-E5 addressed to the mitochondrial matrix
<i>UAS-mito-roGFP2-Grx1</i>	Redox-sensitive GFP fused to glutaredoxin-1 and addressed to the mitochondrial matrix
<i>UAS-hUCP2</i>	Expresses human Uncoupling Protein 2
<i>UAS-DmUCP5</i>	Expresses <i>Drosophila</i> Uncoupling Protein 5 (Bmcp)

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2. Binary expression systems

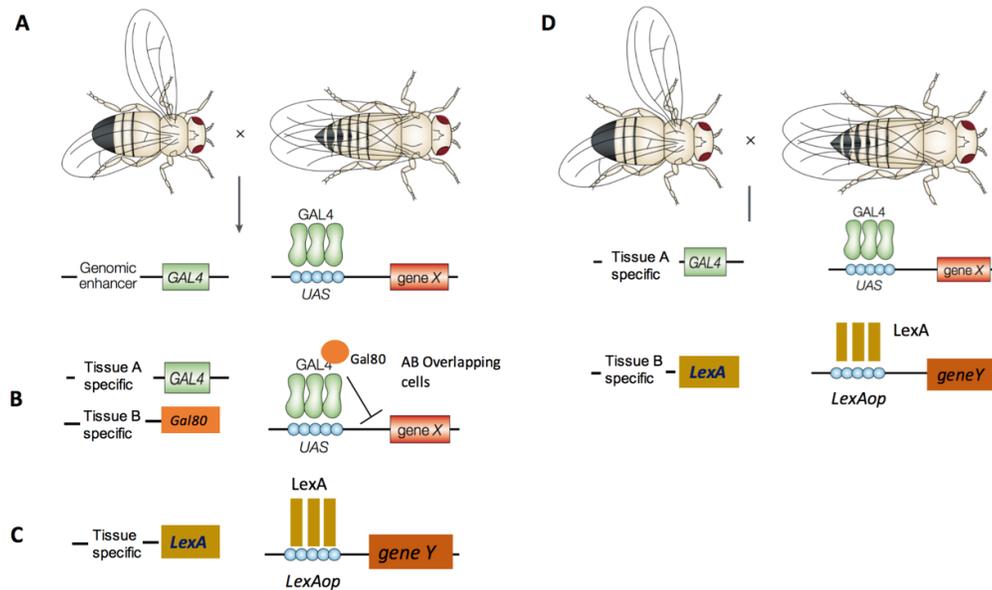


Figure 32 Use of binary expression systems in *Drosophila*

Adapted from St Johnston, 2002

The advantage of the binary expression systems *Gal4/UAS* and *LexA/LexAop* has been presented in **Chapter I, section 3.1**. The yeast *Gal4* gene can be fused to enhancer sequences of specific *Drosophila* genes and then inserted either at random (by transposase activity) or in targeted positions (via PhiC31 integrase-mediated cassette exchange) in the *Drosophila* genome to generate transformed lines expressing *Gal4* in a tissue-specific manner. These *Gal4*-transformed flies are crossed to flies carrying the gene of interest (*gene X*) fused downstream of yeast upstream activating sequences (*UAS*) (*UAS-gene X* strain). In the progeny of this cross, *Gal4* will bind to the *UAS* and switch on *gene X* expression in a tissue-specific manner (Figure 32 A). The yeast Gal80 protein can bind to Gal4 and repress its activity. By combining tissue-specific *Gal4*- and *Gal80*, as well as *UAS-gene X*, transgenes in the same fly, *gene X* will be expressed only in the cells that express *Gal4* and not *Gal80* (Figure 32 B). Similarly, in the bacterial *LexA/LexAop* system, LexA binds to *LexAop* sequence and can induce expression of the *gene Y* located downstream of *LexAop* (Figure 32 C). Since expression systems in bacteria and yeast are independent, combining both systems in the same fly allows two different genes, named here *X* and *Y*, to be expressed in different tissues at the same time (Figure 32 D).

3. Startle-induced locomotion assay

Startle-induced negative geotaxis (SING) test is commonly used in *Drosophila* to monitor performance in locomotor reactivity and climbing ability. The flies are kept inside a narrow column to prevent flight and the assay is initiated by a gentle mechanical shock that makes them fall to the bottom. As an innate response to this startle, flies climb rapidly upwards.

➤ SING assay conditions

Groups of 10 flies of the same genotype prepared one night before the test were placed in a vertical column (25 cm long, 1.5 cm diameter) with a conic bottom end. SING assays were carried out as previously described (Coulom and Birman, 2004; Riemensperger et al., 2013) at 23°C. After 1 min, flies having reached the top of the column (above 22 cm) and flies remaining at the bottom end (below 4 cm) were separately counted. Three rounds of test were performed three times in a row per column. Results are the mean \pm SEM of the scores obtained with ten groups of flies per genotype. The performance index (PI) is defined as $\frac{1}{2}[(n_{\text{tot}} + n_{\text{top}} - n_{\text{bot}})/n_{\text{tot}}]$, where n_{tot} , n_{top} , and n_{bot} are the total number of flies, the number of flies at the top, and the number of flies at the bottom, respectively.

➤ Monitoring age-related SING decline

Over 75 male flies of the same genotype were collected for testing the effect of ageing on locomotor performance. The SING test was performed as described above. The first experiment was carried out with 10 day-old adult flies, then the flies were kept at 25°C for ageing and tested again once a week until they did not climb anymore. Dead flies were replaced by substitutes of the same age. Two-way ANOVA with Tukey's *post-hoc* tests for multiple comparisons was used to compare locomotor ageing between different genotypes at different time point.

➤ Thermogenetic modulation of neuronal activity

The *Drosophila* gene *shibire* (*shi*) codes for the homolog of Dynamin, a GTPase that is implicated in synaptic vesicle recycling at nerve terminals (Chen et al., 1991; Bliok and Meyerowitz, 1991). The temperature-sensitive mutant Shi^{ts1} has a single amino acid substitution in the GTPase domain that impairs its activity at restrictive temperature ($>29^\circ\text{C}$), which results in depletion of synaptic vesicles and suppression of neurotransmission, as shown in Figure 33A. The *Gal4/UAS-shi^{ts1}* strategy was introduced in 2001 (Kitamoto, 2001); since then it has been widely used to analyse the behavioural consequences of neurotransmission blockade in specific neurons (Kasuya et al., 2009).

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Membrane proteins of the Transient Receptor Potential (TRP) family, which were initially discovered in *Drosophila*, are ion channels that can be activated by a wide variety of stimuli (light, warm or cold temperature, diverse mechanical and chemical stimuli). *Drosophila* dTrpA1 encodes a heat-activated channel involved in temperature sensation. When the temperature increases above 26°C, the ion channel opens and can generate an action potential in neurons, thus promoting neurotransmitter release (Hamada et al., 2008) (Figure 33B).

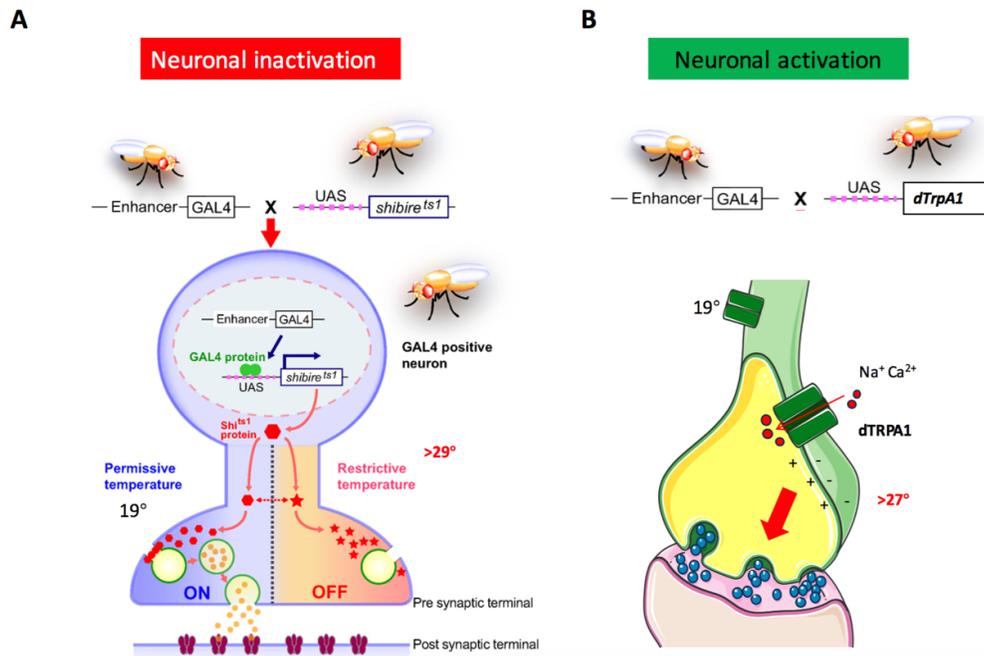


Figure 33 Genetic tools used for thermogenetic modulation of neuronal activity

Adapted from Kasuya et al., 2009

To perform SING assay following thermogenetic inhibition or activation of neuronal activity, around 100 7 to 10-day old adult flies expressing either *Shi^{ts1}*, *dTrpA1* or *msGFP* (*mCD8::GFP + nSyb::GFP*) as control, respectively, in neuronal subsets, were kept at 19°C overnight. The next day, groups of 10 flies of the same genotype were placed in the SING test columns and left for about 20 min at 19°C for habituation. Thermomodulation was performed by incubating each column for 10 min at 32°C, or at 23°C for control of heat effect, just before the locomotor assay. SING assays were carried out as described above. Two-way ANOVA with Bonferroni's or Tukey's tests for multiple comparisons was used to analyse the effects of thermogenetic activation or inhibition on locomotor performance in flies of various genotypes.

➤ **Optogenetic photostimulation of neuronal activity**

To perform SING assay during optogenetic photostimulation, 7 to 10-day old flies expressing Channelrhodopsin-2-XXL (ChR2-XXL) (Dawydow et al., 2014) were kept in constant darkness, and all manipulation were done under dim red light. Groups of 10 flies per column were prepared one night before the test. The transparent column with 10 flies inside were introduced in a dark box and illuminated during the locomotion test with either blue-light diode (peak wavelength 468 nm) from two sides (intensity range 6-11x 10³ Lux), or red light as control (see Figure S2 in Sun et al. (2018)). Six rounds of tests were performed in a row per column, 3 under red light and 3 under blue light. One minute after startle, the number of flies was counted as described above for PI calculation. Two-way ANOVA with Bonferroni's *post-hoc* tests for multiple comparisons was applied to analyse the effect of optogenetic photostimulation on fly locomotion.

4. Immunohistochemistry

Adult brains or ventral nerve cords (VNCs) were quickly dissected in ice-cold *Drosophila* Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 0.3 mM Na₂HPO₄, 0.35 mM KH₂PO₄, pH 7.5). Then brains were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM KH₂PO₄) with agitation at room temperature for 1 h, followed by three 20-min washes in PBS. After 2 h of preincubation in PBS containing 0.5% Triton X-100 (PBST) plus 2% (wt/vol) bovine serum albumin (BSA), brains were incubated with the primary antibodies at proper dilution in the same solution overnight at 4 °C. The next day, after three 20-min washes in PBST, brains were incubated with the secondary antibody at room temperature for 2 hours. After two 20-min washes in PBST and one 20-min wash in PBS, the brains were mounted in ProLong Gold Antifade reagent (ThermoFisher Scientific). Images were acquired with a Nikon A1R confocal microscope and processed using the Fiji software (Schindelin et al., 2012).

The primary antibodies used were: mouse anti-GFP (Invitrogen Molecular Probes 1:500 for msGFP detection or Sigma-Aldrich #G6539 1:200 for reconstituted splitGFP (rsGFP) detection), rabbit anti-TH (Novus Biologicals #NB300-109, 1:1000), mouse anti-TH (Immunostar #22941 1:1000), rabbit anti-H3K9me3 (Abcam #ab8898, 1:1000), mouse anti-Fas II (DSHB #1D4, 1:50), and rabbit anti- α -synuclein (Abcam #ab138501, 1:1000). The

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secondary antibodies were goat anti-mouse and anti-rabbit conjugated to Alexa fluor 488 or 555 (Invitrogen Molecular Probes, 1:1000)

5. SplitGFP reconstitution

The GFP-reconstitution across synaptic partners (GRASP) method was used for the visualization of proximity and potential synaptic connectivity between neurons (Feinberg et al., 2008; Gordon and Scott, 2009; Pech et al., 2013a; Macpherson et al., 2015). The *Drosophila* line *LexAop-n-syb::spGFP₁₋₁₀, UAS-CD4::spGFP₁₁* was crossed to the recombinant driver line *NP2492-Gal4; TH-LexA* (MBON-V2 and DANs), and the line *UAS-n-syb::spGFP_{1-10, LexAop-CD4::spGFP₁₁}* was crossed to the recombinant driver lines *R14C08-LexA; R58E02-Gal4* (MBON-M4/M6 and PAM DANs) and *NP2492-Gal4; R14C08-LexA* (MBON-V2 and MBON-M4/M6). 7-10 day-old female flies were collected for brain dissection followed by whole-mount brain immunostaining as described in the previous paragraph.

6. Fluorescent probes for monitoring mitochondrial dynamics.

The green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea Victoria* (Shimomura et al., 1962). This soluble protein and its variants are resistant to common proteases and stable under physiological pH conditions, thus providing an excellent scaffold for the development of biosensors (Day and Davidson, 2009). MitoGFP targets GFP to mitochondria using a localization signal and three amino acids of cytochrome c oxidase VIII, and has proved to be as a valuable tool for *in situ* observation of organelle distribution, morphology and dynamics in *Drosophila* (Verstreken et al., 2005; Yang et al., 2008; Liu and Lu, 2010).

Fluorescent Timer is a mutant (DsRed1-E5) of the red fluorescent protein DsRed (Terskikh et al., 2002) that was cloned from the coral *Discosoma coral*. The two substitutions, V105A and S197T are responsible for enhanced fluorescent intensity and fast maturation. During protein maturation, its fluorescence starts from green for the newly synthesised form (excitation 483 nm/emission 500 nm) to red for the mature form (excitation 558 nm/emission 583 nm). This colour shift is independent from pH, ion and protein concentration, but is affected by temperature, oxygen, and light exposure (Terskikh et al., 2000; Gross et al., 2000). Timer was

fused to the mitochondrial targeting sequence to make the MitoTimer construction (Hernandez et al., 2013). The mitochondrial targeting sequence is derived from cytochrome c oxidase subunit VIII, which is used to deliver fusion proteins across the mitochondrial inner membrane. *In vivo*, a shift in the abundance of newly expressed vs. mature MitoTimer can be used to reveal changes in the rate of mitochondrial turnover (the balance between biogenesis and mitophagy) (Ferree et al., 2013; Gottlieb and Stotland, 2015). In *Drosophila*, PQ and rotenone-treated flies showed increased red/green ratio of MitoTimer expressed in the heart tube compared to the control group (Laker et al., 2014).

Redox homeostasis (i.e. the balance between oxidants and antioxidants in cells) is closely related to mitochondrial function. Redox-sensitive GFP (roGFP) was generated to measure redox changes in cells (Meyer and Dick, 2010). The biosensor applied in our work, roGFP2, is derived from enhanced GFP with an engineered dithiol/disulfide switch on their surface. Two excitation maxima characteristic of GFP (400 nm for the A-band and 475-490 nm for the B-band) are altered in different redox context. Oxidation results in an increase in the A-band and a decrease in the B-band whereas under reducing conditions, the reverse occurs as shown in Figure 34. Because the midpoint redox potential of roGFP2 is more negative than that of glutathione at physiological concentrations, roGFP2 exhibit sensitivity to glutathione redox potential (E_{GSH}).

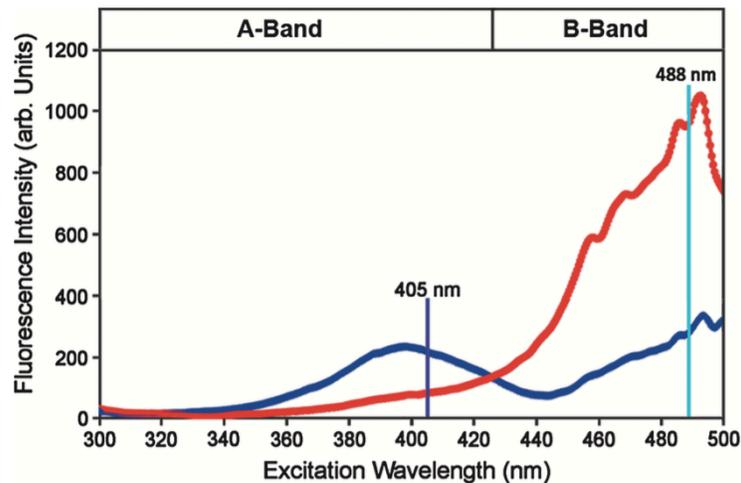


Figure 34 Redox-dependent changes in the excitation spectrum of roGFP2

Source: Meyer and Dick, 2010

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The intracellular response of roGFP2 is not capable of interacting with the thioredoxin (Trx) system and is restricted by the endogenous concentration of glutaredoxins (Grx) as well. To solve this problem, roGFP2 was fused to Grx to enforce continuous equilibration between the two redox pairs of roGFP-reduced/roGFP2-oxidized and two glutathione/glutathion disulfide (GSH/GSSG), which ensured rapid and efficient equilibration under all circumstances (Meyer and Dick, 2010). In flies, targeting roGFP2-Grx1 in mitochondria, using *UAS-mito-roGFP2-Grx1*, enables *in vivo* observation of redox alteration inside the mitochondria (Albrecht et al., 2011; 2014).

Fly expressing MitoTimer or mitoGFP in brain were quickly dissected in ice-cold Ca^{2+} -free *Drosophila* Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.8 mM MgCl_2 , 0.74 mM KH_2PO_4 , 0.35 mM Na_2HPO_4 , pH 7.5), fixed for 20 min in 4% PFA in PBS and washed 3 x 20 min in PBS with agitation at room temperature. For mito-roGFP2-Grx1, brains were freshly dissected in PBS and transferred immediately for confocal scanning one by one. All the brains were mounted in ProLong Gold Antifade reagent for confocal visualisation. Excitation/emission (488/515-530nm) was set for mitoGFP fluorescent detection, while green (excitation/emission 488/515-530 nm) and red (excitation/emission 560/570-620 nm) were used for MitoTimer detection. For mito-roGFP2-Grx1, biosensor was excited sequentially at 405nm and 488 nm (line by line) and detected at 500-530 nm (Albrecht et al., 2011). Settings for different probes were kept constant across samples during time course studies.

7. DHE staining

ROS are very reactive and unstable molecules, so it is difficult to detect them directly in cells. Dihydroethidium (DHE) reacts with ROS to form 2-hydroxyethidium (2-OH-E^+) and ethidium, which intercalate with DNA and stain the whole nucleus with a bright red fluorescence (Zhao et al., 2005; Dikalov et al., 2007; Zielonka and Kalyanaraman, 2010) (Figure 35). DHE is able to permeate cell membranes freely and it has been used extensively to monitor ROS production in *Drosophila* tissues (Chang and Min, 2005; Owusu-Ansah et al., 2008; Bahadorani et al., 2010; Rera et al., 2011; Wang et al., 2011b; Icreverzi et al., 2015; Vaccaro et al., 2017).

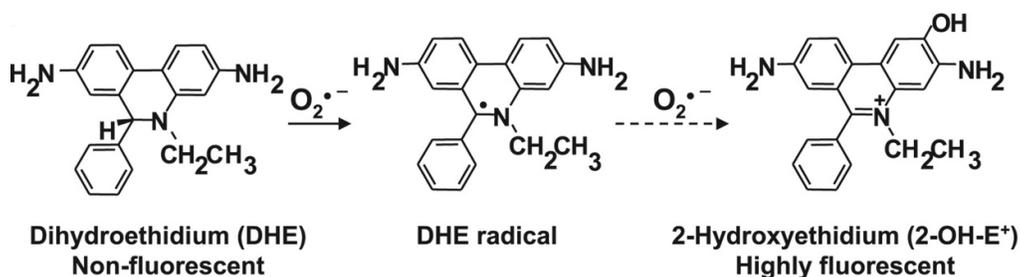


Figure 35 The reaction of $O_2^{\bullet-}$ with DHE produces 2-hydroxyethidium (2-HE⁺)

Source: Dikalov et al., 2007

5-10 fly brains per genotypes were quickly dissected in Schneider's insect medium (Sigma Aldrich, #S0146), and then incubated in 30 μ M DHE (Thermo Fisher Scientific, #D11347) diluted in Schneider's medium for 5-7 mins. After three 5-min washes in Schneider's medium, brains were slightly fixed for 7 minutes in 7% formaldehyde in PBS, followed by 5 min rinsing in PBS, then processed for mounting in Vectashield Antifade Mounting Medium (Vector Laboratories). Images were immediately captured on a Nikon A1R confocal microscope at constant gain setting. The average fluorescence intensity of Z-projected stacks were measured using the Fiji software.

8. Western blotting

20 fly heads were homogenized in 100 μ l RIPA buffer (Sigma-Aldrich) containing protease inhibitors (cOmplete Protease Inhibitor Cocktail, Roche Diagnostics) using a Minilys apparatus (Bertin Instruments), or 30 dissected brains were homogenized in 50 μ L RIPA buffer containing protease inhibitors. The lysates were incubated on ice for 30 min and centrifuged at 8,000 g for 10 min at 4°C. Protein samples were prepared by diluting the supernatant in 1 x final NuPAGE LDS Sample Buffer and Reducing Agent, followed by 10 min denaturation at 70°C and 5 s centrifugation at 1,000 g. Proteins were separated in 4-12% Novex NuPAGE Bis-Tris precast polyacrylamide gels (Life Technologies), using PageRuler Plus Prestained Protein Ladder (ThermoFisher Scientific) as migration marker, and electrotransferred to Amersham Hybond P 0.45 PVDF membranes for 90 mins (GE Healthcare Life Sciences).

Membranes were blocked after transfer for 2 h at room temperature in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 (TBS-T) supplemented with 5% skimmed milk.

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Membranes were then incubated overnight at 4°C in TBS-T containing 5% (w/v) skimmed milk in 0.05% TBS-T with the following primary antibodies: rabbit polyclonal anti-Piwi (1:1000 kindly provided by Gregory J. Hannon), mouse monoclonal anti-Piwi (1:500, Santa Cruz Biotechnology #sc-390946) and mouse monoclonal anti- α -tubulin (DHSB 1:500, #12G10). Membranes were washed six times for 6 min in TBS-T and then incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (Invitrogen Molecular Probes) or anti-mouse (Sigma-Aldrich) secondary antibodies at 1:3,000. Immunolabeled bands were revealed by using ECL RevelBIOT Intense (Ozyme, France) as chemiluminescent HRP substrate and digitally acquired at different exposure times using ImageQuant TL software (GE Healthcare Life Science). Densitometry measures were made with the Fiji software and normalized to the tubulin measures as internal controls.

9. RT-PCR and quantitative PCR

Reverse transcription PCR (RT-PCR) was performed to examine *piwi* and *HeT-A* expression in dissected tissues. The primers for RT were: for *piwi*, sense primer 5'-ACGCATTCGCGACCACAATCAG, antisense primer 5'-TGTACTIONTCTGTGACGTTCTTGTCCAGT; for *HeT-A*, sense primer 5'-CGCAAAGACATCTGGAGGACTACC, antisense primer 5'-TGCCGACCTGCTTGGTATTG; *rp49* (reference), sense primer 5'-GACGCTTCAAGGGACAGTATC, antisense primer 5'-AAACGCGGTTCTGCATGAG). RT-PCR analysis was carried out and analysed as previously described (Cassar et al., 2015). Quantitative PCR was conducted on a Roche LightCycler 480. System with the Roche SYBR Green Master mix using the following primer sets: for *piwi*, sense primer 5'-GACGTTGCTCACACAATCCG, antisense primer 5'-CATGGCACGCATAAGCTGAAA; for *rp49*: sense primer 5'-GACGCTTCAAGGGACAGTATC, antisense primer 5'-AAACGCGGTTCTGCATGAG.

10. Statistics

All statistical analyses were performed with the GraphPad Prism 6 software. Data from locomotor assays were analysed using two-way ANOVA with Bonferroni's or Tukey's *post-hoc* tests for multiple comparisons. Data for PQ test were analysed using one-way ANOVA

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with Bonferroni's for multiple comparisons, data for fluorescence intensity with one-way or two-way ANOVA with Bonferroni's for multiple comparisons. Data comparing mitochondrial size were analysed by Frequency Distribution followed by Gaussian distribution analysis. All data are presented as mean \pm SEM. Significant values in all figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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Results

Results

Results A - Neural network controlling locomotor reactivity

Summary

Locomotor reactivity to startling stimuli is commonly used in *Drosophila* research to monitor performance in various behaviours such as climbing, flight initiation, phototaxis and optomotor responses. A widely used paradigm is called startle-induced negative geotaxis (SING), in which flies entrapped in a narrow column respond to a gentle mechanical shock by climbing rapidly upward. SING progressively declines with age or under various neuropathological conditions, making this test valuable for various types of studies.

Here we used thermogenetic and optogenetic tools to activate precise neuronal subsets in the *Drosophila* brain, or block their synaptic output, in order to identify components of the circuits underlying the modulation of this behaviour. We demonstrate that manipulating the activity of different clusters of DANs afferent to the MBs causes diverse effects on SING, and that DAN-mediated SING regulation requires expression of the DA receptor Dop1R1 (Dumb) in the MBs. We confirm our previous observation that activating MB $\alpha'\beta'$, but not $\alpha\beta$, KCs decreased the SING response, and we identify further MB neurons implicated in SING control, including KCs of the γ lobe and two subtypes of MB output neurons (MBONs).

Strikingly, we observed that co-activating the $\alpha\beta$ KCs potently antagonizes $\alpha'\beta'$ and γ KC-mediated SING modulation, suggesting the occurrence of fine regulatory mechanisms between different MB compartments in locomotion control. We also observed that the fan-shaped body (FSB) appears to be involved in this neural network, but not the ellipsoid body (EB), suggesting that the FSB could serve as a relay between the MB control and the descending neurons involved in SING execution. Finally, splitGFP reconstitution was used to identify potential synaptic connections between identified neural components of the SING modulation network.

Altogether, this study contributes to an emerging picture of the brain circuits controlling locomotor reactivity in *Drosophila* that appears both to overlap and differ from those underlying associative learning and memory, sleep/wake state and stress-induced hyperactivity.

Personal contribution: I have carried out most of the experiments in this chapter, except for initial studies that were performed in collaboration with two undergraduate interns (An Qi Xu

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and Julia Giraud), and a collaboration with André Fiala's laboratory (Thomas Riemensperger and Haiko Poppinga) for the analysis of MB driver patterns and the optogenetic photostimulation setup.

Part I

Neural Control of Startle-Induced Locomotion by the Mushroom Bodies and Associated Neurons in *Drosophila*

Jun Sun, An Qi Xu, Julia Giraud, Haiko Poppinga, Thomas Riemensperger, André Fiala and Serge Birman

Article published on 28 March 2018 in *Frontiers in Systems Neuroscience*

Part II

Complementary data on the role of neurotransmitters and the fan-shaped body in SING control

Result A - Part I

Neural Control of Startle-Induced Locomotion by the Mushroom Bodies and Associated Neurons in *Drosophila*

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Results



Neural Control of Startle-Induced Locomotion by the Mushroom Bodies and Associated Neurons in *Drosophila*

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Startle-induced locomotion is commonly used in *Drosophila* research to monitor locomotor reactivity and its progressive decline with age or under various neuropathological conditions. A widely used paradigm is startle-induced negative geotaxis (SING), in which flies entrapped in a narrow column react to a gentle mechanical shock by climbing rapidly upwards. Here we combined *in vivo* manipulation of neuronal activity and splitGFP reconstitution across cells to search for brain neurons and putative circuits that regulate this behavior. We show that the activity of specific clusters of dopaminergic neurons (DANs) afferent to the mushroom bodies (MBs) modulates SING, and that DAN-mediated SING regulation requires expression of the DA receptor Dop1R1/Dumb, but not Dop1R2/Damb, in intrinsic MB Kenyon cells (KCs). We confirmed our previous observation that activating the MB $\alpha'\beta'$, but not $\alpha\beta$, KCs decreased the SING response, and we identified further MB neurons implicated in SING control, including KCs of the γ lobe and two subtypes of MB output neurons (MBONs). We also observed that co-activating the $\alpha\beta$ KCs antagonizes $\alpha'\beta'$ and γ KC-mediated SING modulation, suggesting the existence of subtle regulation mechanisms between the different MB lobes in locomotion control. Overall, this study contributes to an emerging picture of the brain circuits modulating locomotor reactivity in *Drosophila* that appear both to overlap and differ from those underlying associative learning and memory, sleep/wake state and stress-induced hyperactivity.

Keywords: dopamine, mushroom bodies, startle-induced negative geotaxis, neural circuits, *Drosophila melanogaster*

INTRODUCTION

The identification of neural circuits that modulate innate or reflex behaviors is essential to better understand how the brain functions and adapts to a changing environment (LeBeau et al., 2005; Dickinson, 2006; Marder, 2012; Su and Wang, 2014). *Drosophila* is an advantageous organism for studying the neural basis of behavior using genetically-encoded probes that enable *in vivo* control of neuronal activity (White and Peabody, 2009; Griffith, 2012; Yoshihara and Ito, 2012; Kazama, 2015; Oswald et al., 2015b; Riemensperger et al., 2016; Martín and Alcorta, 2017). In this

organism, spontaneous locomotor activity and locomotor reactivity have been described as two separate behavioral systems that are regulated differently (Connolly, 1967; Meehan and Wilson, 1987; O'Dell and Burnet, 1988; Martin et al., 1999a). A sudden external stimulus (startle) usually triggers inhibition or arrest of spontaneous locomotion followed by an appropriate behavioral response, which may itself be a locomotor reaction. Startle-induced reactivity has long been used in *Drosophila* to monitor various behavioral performances, such as phototaxis (Benzer, 1967) or negative geotaxis (Miquel et al., 1972). A widely used paradigm relies on the fast climbing reaction initiated by a gentle mechanical shock of flies entrapped in a vial or a narrow column, an innate reflex called startle-induced negative geotaxis (SING). SING performance progressively declines with age (Ganetzky and Flanagan, 1978; Le Bourg and Lints, 1992; Grotewiel et al., 2005; White et al., 2010; Jones and Grotewiel, 2011; Vaccaro et al., 2017), in contrast to spontaneous locomotion that does not vary during the adult life and even increases in old flies (White et al., 2010). The SING reflex is also progressively altered in various mutant or under neuropathological conditions, as is the case in *Drosophila* models of Parkinson disease (Feany and Bender, 2000; Coulom and Birman, 2004; Chaudhuri et al., 2007; Riemensperger et al., 2013; Bou Dib et al., 2014). It is therefore of particular interest to identify precise neural components underlying the modulation of startle-induced locomotion, in *Drosophila* as in other species (Hale et al., 2016).

The mushroom body (MB) is a paired structure of the insect brain that has important behavioral functions, including the formation of olfactory memory (Heisenberg, 2003; Fiala, 2007; Davis, 2011; Kahsai and Zars, 2011; Waddell, 2013) and the control of sleep (Bushey and Cirelli, 2011; Tomita et al., 2017). The *Drosophila* MB is composed of intrinsic neurons known as Kenyon cells (KCs) and it is innervated by afferent modulatory neurons, in particular subsets of dopaminergic neurons (DANs), as well as efferent MB output neurons (MBONs) (Tanaka et al., 2008; Pech et al., 2013a; Aso et al., 2014a,b). The cell bodies of the KCs form a large cluster in the dorsal posterior brain; their dendritic branches make up the calyx and their axons bundle up in the peduncles. The KCs are named according to the lobes in which they send axonal projections: $\alpha\beta$, $\alpha'\beta'$; and γ (Lee et al., 1999; Tanaka et al., 2008). At the distal end of the peduncles, the axons of the $\alpha\beta$ and $\alpha'\beta'$ KCs bifurcate dorsally and medially to form the vertical (α and α') and horizontal (β and β') lobes, while the γ KCs form only the γ horizontal lobes.

Around 60 years ago, experiments carried out on crickets provided the first evidence that the insect MB contains neurons inhibiting locomotion (Huber, 1960, 1967; Howse, 1975). In

Drosophila, both the *mushroom body miniature* mutation or chemical ablation of the MB increased walking activity when measured over long time intervals, confirming that the MB normally suppresses locomotor behavior (Heisenberg et al., 1985; Martin et al., 1998; Helfrich-Förster et al., 2002), while similar experiments suggested that, by contrast, the MB stimulates initial stages of walking activity (Serway et al., 2009). Neuroanatomical defects in the MB lobes were observed in a set of mutants giving rise to changes in startle-induced locomotion behavior, but without a clear correlation between the two phenotypes (Yamamoto et al., 2008). Furthermore, we previously reported that SING is controlled by the activity of the $\alpha'\beta'$ KCs (Riemensperger et al., 2013). Determining the precise contributions of the various subtypes of MB neurons to startle-induced locomotion required, therefore, further investigations.

Dopamine (DA) is an important neurotransmitter that, in flies, was implicated in the modulation of diverse behaviors including appetitive or aversive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005, 2011; Schroll et al., 2006; Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010; Waddell, 2010; Berry et al., 2012; Burke et al., 2012; Plaçais et al., 2012; Cohn et al., 2015; Musso et al., 2015; Aso and Rubin, 2016; Yamagata et al., 2016) and sleep-wake mechanisms (Van Swinderen and Andretic, 2011; Liu et al., 2012b; Ueno et al., 2012; Berry et al., 2015; Sitaraman et al., 2015b; Pimentel et al., 2016). It is also well established that DA prominently controls locomotor activity in *Drosophila* (Yellman et al., 1997; Bainton et al., 2000; Friggi-Grelin et al., 2003; Kume et al., 2005; Lima and Miesenböck, 2005; Wu et al., 2008; Lebestky et al., 2009; Kong et al., 2010; Riemensperger et al., 2011; Van Swinderen and Andretic, 2011) as it does in vertebrates (Beninger, 1983; Zhou and Palmiter, 1995; Giros et al., 1996; Blum et al., 2014). We have recently reported that the degeneration of DANs of either the protocerebral anterior medial (PAM) or protocerebral posterior lateral 1 (PPL1) clusters afferent to the MBs was associated with an accelerated decline of SING performance in aging flies (Riemensperger et al., 2013; Vaccaro et al., 2017). Further recent studies support a function for the PAM and PPL1 clusters in climbing or flight control (Bou Dib et al., 2014; Agrawal and Hasan, 2015; Pathak et al., 2015). However, the role of these and other DANs in SING modulation has not yet been precisely investigated.

Here we used activation or silencing of synaptic transmission in neuronal subsets targeted with selective drivers in order to identify the MB-associated neurons (KCs, DANs, and MBONs) that control startle-induced locomotion in *Drosophila*. Neuronal activation revealed that several classes of DANs projecting to the MBs have diverse roles in modulatory mechanisms. We show that DANs in the PPL1 cluster act as inhibitory neurons in the SING-modulating circuits, while the PAM cluster appears to contain both inhibitory and excitatory DAN subsets. We also confirm that MB $\alpha'\beta'$ KCs are implicated in SING control and demonstrate that γ KCs are involved in this modulation as well. Interestingly, we find that $\alpha'\beta'$ and γ neuron-mediated SING modulation is antagonized by co-activating the $\alpha\beta$ KCs. Finally, we show that the MBONs M4/M6 and V2 are part of the network, suggesting that they convey SING modulatory information to downstream motor circuits. Overall,

Abbreviations: CRE, crepine; DA, dopamine; DAN, dopaminergic neuron; Dop1R1, Dopamine 1-like receptor 1; Dop1R2, Dopamine 1-like receptor 2; EB, ellipsoid body; dFSB, dorsal fan-shaped body; GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; KC, Kenyon cell; MB, mushroom body; MBON, MB output neuron; msGFP, mCD8::GFP, n-syb::GFP; PAL, protocerebral anterior lateral; PAM, protocerebral anterior medial; PI, performance index; PPL, protocerebral posterior lateral; PPM, protocerebral posterior medial; RNAi, RNA interference; rsGFP, reconstituted splitGFP; SING, startle-induced negative geotaxis; SIP, superior intermediate protocerebrum; SLP, superior lateral protocerebrum; SMP, superior medial protocerebrum; TH, tyrosine hydroxylase.

this work provides a first picture of the brain network and modulatory mechanisms controlling startle-induced locomotion in *Drosophila* that centrally involve a subset of MB-associated neurons.

MATERIALS AND METHODS

Drosophila Culture and Strains

Fly stocks were raised and crossed at 25°C on the standard corn meal/yeast/agar medium supplemented with methyl-4-hydroxybenzoate as a mold protector, under a 12 h/12 h light-dark cycle. The following effector lines were used: *UAS-mCD8::GFP*, *UAS-n-syb::GFP* (here named *UAS-msGFP*) (Riemensperger et al., 2013), *UAS-shi^{ts1}* (Kitamoto, 2001), *UAS-dTrpA1* (Hamada et al., 2008), *UAS-ChR2-XXL* (Dawydow et al., 2014), *LexAop-dTrpA1* (Burke et al., 2012), *UAS-Dumb-RNAi* (Bloomington *Drosophila* Stock Center, line 62193), *UAS-Damb-RNAi* (Vienna *Drosophila* RNAi center, line v3391) (Cassar et al., 2015), *UAS-n-syb::spGFP₁₋₁₀*, *LexAop-CD4::spGFP₁₁/CyO* and *LexAop-n-syb::spGFP₁₋₁₀*, *UAS-CD4::spGFP₁₁* (Bloomington *Drosophila* Stock Center, lines 64314 and 64315) (Macpherson et al., 2015). The driver lines used and their brain expression patterns are described in Table S1. Except for those that were generated in our laboratories, these lines were either obtained from the Bloomington *Drosophila* Stock Center or kindly provided by: Ronald L. Davis (*TH-LexA*, Berry et al., 2015), Thomas Preat and Pierre-Yves Plaçais (*4-59-Gal4*, *238Y-Gal4*, *G0050-Gal4*, *NP2758-Gal4*, *R71D08-Gal4*, *NP2492-Gal4*, *R27G01-Gal4*, *R14C08-LexA*), Hiromu Tanimoto (*R58E02-Gal4*, Liu et al., 2012a) and Mark Wu (*TH-C1-Gal4*, *TH-C'-Gal4*, and *TH-D'-Gal4*) (Liu et al., 2012b).

Locomotion Assay Coupled With Genetic Manipulation of Neuronal Activity

SING assays were generally carried out following thermogenetic inhibition or activation of neuronal activity. Seven- to ten-day-old flies expressing *Shi^{ts1}* or *dTrpA1*, respectively, or *msGFP* as a control, in neuronal subsets, were kept at 19°C overnight. The next day, groups of 10 flies of the same genotype were placed in a vertical column (25 cm long, 1.5 cm diameter) with a conic bottom end, and left for about 20 min at 19°C for habituation. Thermogenetic activation or silencing of neurons was performed by incubating each column for 10 min at 32°C, or at 23°C for control of a potential temperature effect. SING assays were carried out immediately afterwards at 23°C as previously described (Coulom and Birman, 2004; Riemensperger et al., 2013). Briefly, flies were suddenly startled by gently tapping them down. After 1 min, flies having reached the top of the column (above 22 cm) and flies remaining at the bottom end (below 4 cm) were separately counted. Three rounds of test were performed in a row per column. Results are the mean \pm SEM of the scores obtained with ten groups of flies per genotype. The performance index (PI) is defined as $\frac{1}{2}[(n_{\text{tot}} + n_{\text{top}} - n_{\text{bot}})/n_{\text{tot}}]$, where n_{tot} is the total number of flies, and n_{top} and n_{bot} the number of flies at the top and at the bottom, respectively.

In some experiments, optogenetic photostimulation was performed instead on 7 to 10-day-old flies expressing the

channelrhodopsins *ChR2-XXL* (Dawydow et al., 2014) in neuronal subsets. In this case, flies were kept in constant darkness, and all manipulations before the SING assay were done under dim red light. The transparent columns were introduced in a dark box and illuminated during locomotion testing with either blue-light diodes (peak wavelength 468 nm) from two sides (intensity range $6\text{--}11 \times 10^3$ Lux), or red light as a control. Six rounds of tests were performed in a row per column, 3 under red light and 3 under blue light. Further details on the SING assay procedure under optogenetic photostimulation are provided in the legends to Figures S2A,B.

Immunohistochemistry

Adult brains were dissected in ice-cold *Drosophila* Ringer's solution and processed for whole mount immunostaining as previously described (Riemensperger et al., 2011). The primary antibodies were mouse anti-GFP (ThermoFisher Scientific 33-2600, 1:500 for *msGFP* detection or Sigma-Aldrich G6539, 1:200 for reconstituted splitGFP (rsGFP) detection) and rabbit anti-TH (Novus Biologicals NB300-109, 1:1,000). The secondary antibodies were goat anti-mouse and anti-rabbit conjugated to Alexa fluor 488 or 555 (Invitrogen Molecular Probes, 1:1,000). The brains were mounted in ProLong Gold Antifade reagent (ThermoFisher Scientific). Images were acquired with a Nikon A1R confocal microscope and processed using the Fiji software (Schindelin et al., 2012).

For the quantification of Gal4 expression patterns in KC subpopulations, the brains of 5–7 day-old female flies expressing *mCD8::GFP* under the control of different Gal4 drivers were dissected in ice cold Ringer's solution, fixed for 2 h on ice in 4% paraformaldehyde and washed 3×20 min in phosphate-buffered saline + 0.6 % Triton X-100 (PBSTx). After a 2 h pre-incubation in PBSTx + 2% bovine serum albumin, brains were incubated overnight at 4°C in the same buffer with mouse monoclonal anti-Bruchpilot antibody (1:10, nc82, Developmental Studies Hybridoma Bank) to visualize synaptic neuropils. After 3×20 min washes in PBSTx, samples were incubated for 2 h with Cy3-conjugated anti-mouse secondary antibody (1:300, Jackson ImmunoResearch), then washed 3×20 min in PBSTx and additionally overnight in PBS. Brains were mounted in Vectashield (Vector Laboratories) and scanned using a Leica SP8 confocal laser scanning microscope equipped with hybrid detectors. Quantification of Gal4-expressing KC somata was conducted by monitoring GFP autofluorescence with the Fiji Cell Counter plugin across the focal planes.

Split-GFP Reconstitution

For the visualization of potential synaptic connectivity with the GRASP method (Feinberg et al., 2008; Gordon and Scott, 2009; Pech et al., 2013a; Macpherson et al., 2015), the *Drosophila* line *LexAop-n-syb::spGFP₁₋₁₀*, *UAS-CD4::spGFP₁₁* was crossed to the recombined driver line *NP2492-Gal4*; *TH-LexA* (MBON-V2 and DANs), and the line *UAS-n-syb::spGFP₁₋₁₀*, *LexAop-CD4::spGFP₁₁* was crossed to the recombined driver lines *R14C08-LexA*; *R58E02-Gal4* (MBON-M4/M6 and PAM DANs) and *NP2492-Gal4*; *R14C08-LexA* (MBON-V2 and MBON-M4/M6). 7–10 day-old female flies were collected for brain

dissection followed by whole-mount brain immunostaining as described in the previous paragraph.

Statistics

All statistical analyses were performed with the GraphPad Prism 6 software. Data from locomotor assays were analyzed using two-way ANOVA with Bonferroni's or Tukey's *post-hoc* tests for multiple comparisons. All data are presented as mean \pm SEM. Significant values in all figures: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RESULTS

Activation of *TH-Gal4*-Targeted DANs Inhibits Fly Locomotor Reactivity to Startle

To determine the effect of DAN inhibition or activation on SING response, we first used *TH-Gal4*, a driver that expresses selectively in brain DANs, except in the PAM cluster where it only labels 12 DANs out of ~ 90 in total (Friggi-Grelin et al., 2003; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010; White et al., 2010; Pech et al., 2013a). We crossed *TH-Gal4* with *UAS-shi^{ts1}* flies to express the thermosensitive variant of *Drosophila* Dynamin *Shi^{ts1}* (Kitamoto, 2001) that blocks neurotransmitter release above 30°C (Kitamoto, 2001), in DANs of the progeny. After a 10-min incubation, these *TH>shi^{ts1}* flies showed no difference in SING performance between the permissive (23°C) and restrictive (32°C) temperatures, indicating that *TH-Gal4*-targeted DANs are not required for the execution of this locomotor response (Figure 1A). We checked that the *UAS-shi^{ts1}* transgene was active by expressing *Shi^{ts1}* in all neurons with *elav-Gal4*, which led to fly paralysis at the restrictive temperature (data not shown). Flies expressing a membrane-associated form of GFP (msGFP, described in section Materials and Methods) in *TH-Gal4* DANs neither showed any difference in SING performance between the two temperatures. This indicates that temperature by itself had no significant effect on the test (Figure 1A). In contrast, expressing the heat-inducible cation channel *dTrpA1* (Hamada et al., 2008) in *TH-Gal4*-targeted DANs (*TH>dTrpA1* flies) led to altered SING performance after activation at 32°C, which was decreased to $\sim 20\%$ of the 23°C control value (Figure 1A). After 10 min of neuronal thermoactivation, *TH>dTrpA1* flies were in fact very active without any inhibition of their spontaneous locomotion (Movie S1). After the startle, most of these thermoactivated flies stayed at the bottom of the column and a few climbed up to the middle and stopped (Movie S2), while in the absence of neuronal thermoactivation, these same flies generally climbed to the top of the column quickly like wild-type flies (Movie S3). This indicates that DANs labeled by *TH-Gal4* inhibit the SING response i.e., locomotor reactivity, but not spontaneous locomotion, when they are stimulated.

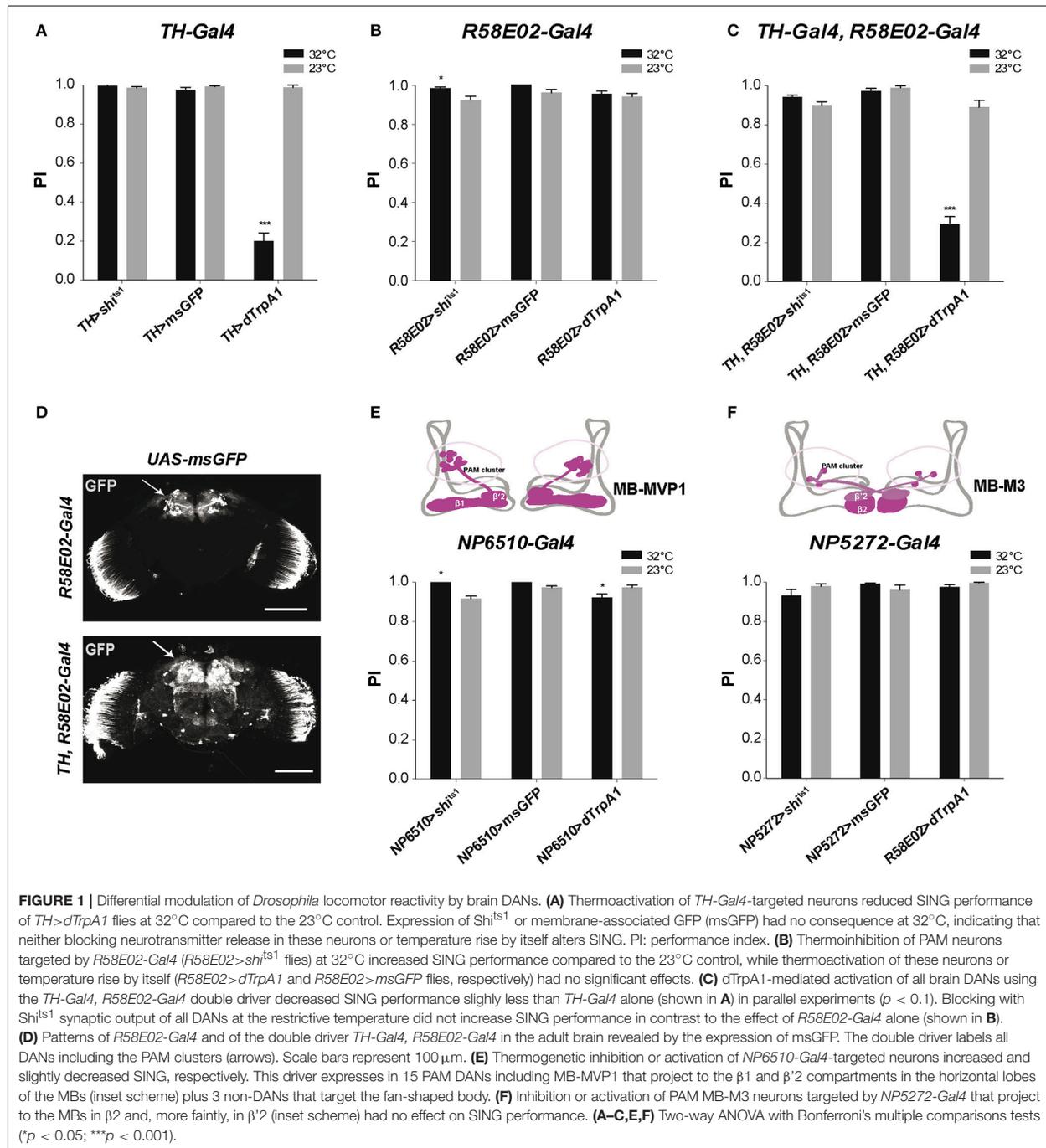
In order to better characterize this behavioral modulation, we have monitored the SING performance of *TH>dTrpA1* flies after various times of incubation at 32°C (Figure S1A). We observed that 2 min were required for the temperature inside the column to reach above 30°C. Nevertheless, a decrease in SING performance could be observed after only 1 min of incubation, indicating that this modulation is actually rapid. SING performance continued to decrease until ~ 5 min of

DAN thermoactivation, after what it remained stable at a low value (Figure S1A). Next, we checked whether DAN activation triggered during the climbing test could modulate as well SING behavior. We used for that optogenetic photostimulation in order to activate neurons instantly without the latency of thermoactivation, by expressing in DANs the channelrhodopsin *ChR2-XXL* (Dawydow et al., 2014; Riemensperger et al., 2016; Figures S2A,B). We first tested the efficiency of the system by expressing these optogenetic effectors in all GABAergic neurons with *Gad-Gal4*. As expected, blue light but not red light illumination after startle prevented *Gad>ChR2-XXL* flies from climbing (data not shown). Next we tested optogenetic stimulation of the DANs. We found that illuminating *TH>ChR2-XXL* flies with blue light, but not red light, during the test, i.e., within less than 1 min, was sufficient to reduce significantly their SING performance by $\sim 22\%$ (Figure S2C). These results indicate that DAN-mediated SING modulation is a fast and physiologically-relevant process.

DANs in the PAM Cluster Are Also Involved in SING Modulation

Because the *TH-Gal4* pattern excludes a large part of the PAM clusters, we used the *R58E02-Gal4* driver that labels $\sim 80\%$ of the PAM DANs (Liu et al., 2012a; Pech et al., 2013a) to investigate the role of this cluster in SING modulation. Again, no effect of temperature was detected in control *R58E02>msGFP* flies expressing msGFP in the PAM neurons (Figure 1B). Stimulating PAM DANs activity by *dTrpA1* caused no inhibitory effect on fly locomotion, whereas blocking output from these neurons with *Shi^{ts1}* led to a small but statistically significant increase in SING performance at 32°C compared to 23°C (Figure 1B). This result suggests that the PAM clusters contain neurons that inhibit locomotor reactivity. These neurons appear spontaneously active during the test because their blockade by *Shi^{ts1}* increased SING while their stimulation by *dTrpA1* did not lead to any effect. Indeed, it has recently been shown that some PAM DANs are spontaneously active (Yamagata et al., 2016).

We then constructed a double-driver strain containing both *TH-Gal4* and *R58E02-Gal4*. We checked that this double driver labeled all brain DANs, including the PAM clusters, by expressing msGFP and comparing to the pattern of the *R58E02-Gal4* strain (Figure 1D). Like with *TH-Gal4* alone, *TH, R58E02>shi^{ts1}* and *TH, R58E02>msGFP* flies showed similar SING performance at low and high temperatures. In contrast, *TH, R58E02>dTrpA1* flies showed at 32°C a climbing performance that was reduced to $\sim 33\%$ of the 23°C control value (Figure 1C), an effect that was slightly but significantly lower compared with the decrease observed in a parallel experiment with *TH-Gal4* alone ($19.7 \pm 4.5\%$ vs. $33.1 \pm 4.0\%$ of the 23°C control for *TH>dTrpA1* and *TH, R58E02>dTrpA1* flies, respectively). This result suggests that PAM neuron co-stimulation somewhat offsets the inhibition of locomotor reactivity induced by *TH-Gal4*. It therefore seems that the PAM clusters contain not only neurons that constitutively inhibit locomotor reactivity, but also neurons that, on the other hand, increase SING when stimulated. The PAM clusters are known, indeed, to include functionally heterogeneous subsets of DANs (Liu et al., 2012a; Waddell, 2013).



The driver *NP6510-Gal4* expresses in 15 PAM DANs that are not labeled by *TH-Gal4* and that project to the MB horizontal lobe $\beta 1$ and $\beta 2$ compartments (Figure 1E; Aso et al., 2010; Riemensperger et al., 2013). We previously showed that the degeneration of these 15 DANs induced by mutant α -synuclein accumulation led to progressive SING defects that were as

strong as those observed by expressing mutant α -synuclein in all neurons of the fly (Riemensperger et al., 2013). This suggested that *NP6510-Gal4* DANs could be involved in SING modulation. *NP6510>sh^{ts1}* flies showed indeed a slight increase in SING performance at the restrictive temperature, similar to the effect observed with *R58E02-Gal4*, whereas dTrpA1-induced

thermostimulation of these neurons by contrast led to decreased SING response (Figure 1E). No such effects were observed with *NP5272-Gal4* that expresses in three PAM cells involved in aversive odor memory, the MB-M3 neurons, which innervate the tip of the MB horizontal lobes (β_2 and β'_2 compartments) and are labeled by *TH-Gal4* (Aso et al., 2010; Figure 1F). Neither did a *NP6510-Gal4*, *R58E02-Gal80* recombinant driver that expresses only in three *NP6510*-targeted non-DANs have any effect on SING (data not shown). Our results suggest, therefore, that the PAM neurons that inhibit SING correspond to the *NP6510*-targeted DANs or a subset of these cells.

MB-Afferent DANs of the PPL1 Clusters Inhibit the SING Response

We recently reported that the progressive degeneration of DANs in the PPL1 clusters induced by a mutation of the circadian gene *Clock* severely accelerates age-related SING decline (Vaccaro et al., 2017). To identify whether PPL1 plays a direct role in SING modulation, we employed two drivers that label specific neurons in this cluster: *Mz840-Gal4* labeling the MB-V1 neuron that projects to MB dorsal lobes α_2 , α'_2 compartments and *NP2758-Gal4* that expresses in the MB-MP1 neuron sending projection to the γ_1 peduncle (Figures 2A,B; Aso et al., 2010, 2012). Whereas, the inhibition of the neurons targeted by each of these drivers had no effect on SING, their thermoactivation significantly decreased performance of the flies to around 41 and 78% of the 23°C control value for *Mz840-Gal4* and *NP2758-Gal4*, respectively (Figures 2A,B). We then used the driver *TH-D'-Gal4* (Liu et al., 2012b) that expresses strongly in the PPL1 cluster (Figure 2C). SING performance of *TH-D'>dTrpA1* flies at 32°C was markedly reduced to ~16% of the 23°C control (Figure 2C), an effect comparable to that of *TH-Gal4* itself (see Figure 1A). However, *TH-D'-Gal4* expresses in other DAN clusters than the PPL1 such as PPM2 and PPM3 (Liu et al., 2012b) that could contribute as well to SING modulation.

DANs Localized in Other Clusters Are Also Implicated in SING Regulation

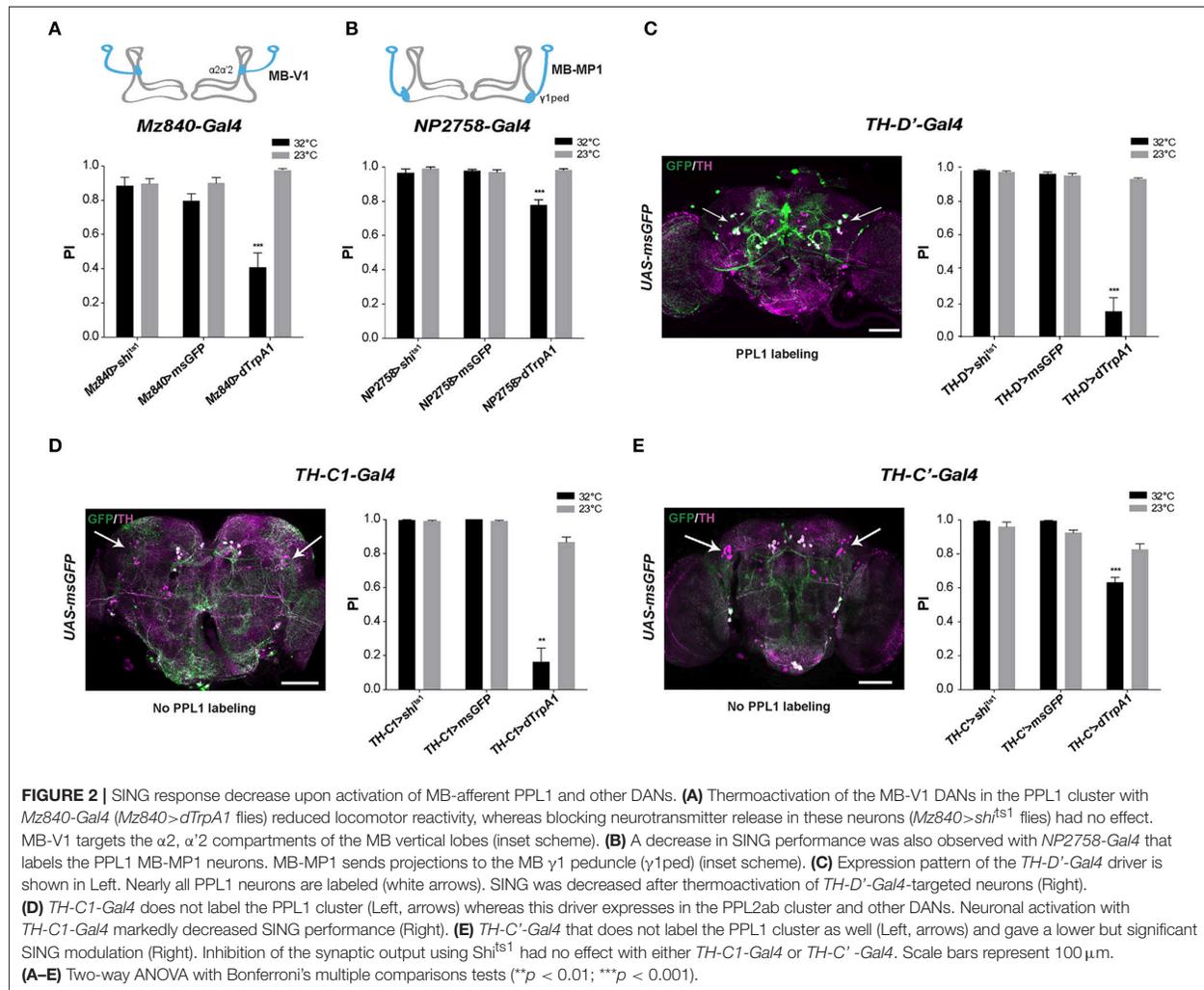
To determine whether other DANs modulate the SING response, we selected two drivers, *TH-C1-Gal4* and *TH-C'-Gal4*, both of which do not express in the PPL1 (Liu et al., 2012b). We first verified that the PPL1 clusters were not labeled by these drivers (Figures 2D,E, left). The use of these drivers did not cause any effect on SING upon synaptic blockade with *Shi^{ts1}* but induced down-regulation of SING upon neuronal thermoactivation, which was strong with *TH-C1-Gal4* (18% of the 23°C control) (Figure 2D, right) and lower, but still significant, with *TH-C'-Gal4* (77% of the control) (Figure 2E, right). Both drivers express similarly in the protocerebral anterior medial (PAL), PPL2 and PPM2 DAN clusters, indicating that some of these clusters, and possibly the PPL2ab neurons that project to the MB calyx (Mao and Davis, 2009), could also be involved in SING modulation. Overall, our results suggest that several brain DAN subsets have the ability to hinder SING behavior when activated or inhibited, indicating that DA-mediated modulation of locomotor reactivity is an important and complex process in the insect brain.

Activation of MB $\alpha'\beta'$ and γ Neurons Decreases SING Performance

We previously reported that SING performance was decreased when synaptic activity in the MB prime ($\alpha'\beta'$) lobes, targeted with *c305a-Gal4*, was either thermogenetically inhibited or stimulated, and that the defect was stronger in the latter case (Riemensperger et al., 2013). We confirmed those results in the present work using either *c305a-Gal4* or *G0050-Gal4*: both drivers did induce SING inhibition at 32°C either with *Shi^{ts1}* or with *dTrpA1* (Figures 3A,B). *c305a-Gal4* labels the entire MB $\alpha'\beta'$ lobes and the γ lobes faintly, as well as the antennal lobes, the central complex and other neuropils (Krashes et al., 2007; Pech et al., 2013b), while *G0050-Gal4* selectively labels the $\alpha'\beta'$ lobes in the MB, and also the ellipsoid body and brain glial cells (Lin et al., 2007; Chen et al., 2012). To ascertain the role of the $\alpha'\beta'$ lobes in SING modulation, we used two other drivers, *4-59-Gal4* and *R35B12-Gal4*, that restrictedly express in the MB prime lobes (Figures 3C,D, insets). Neuronal activation within *4-59-Gal4*- and *R35B12-Gal4*-labeled KCs decreased SING to around 21 and 54% of the 23°C control value (Figures 3C,D), compared to around 12.5% with *c305a-Gal4* and 4% with *G0050-Gal4* (Figures 3A,B). In contrast, *Shi^{ts1}* expression with *4-59-Gal4* and *R35B12-Gal4* did not cause any decrease in SING behavior at the restrictive temperature (Figures 3C,D). This suggests that the $\alpha'\beta'$ KCs are rather involved in SING inhibition than activation, and that another, still unidentified, targeted neuropile must be responsible for the *Shi^{ts1}*-induced decrease observed with *c305a-Gal4* and *G0050-Gal4* (Figures 3A,B).

As mentioned, *c305a-Gal4* expresses in the $\alpha'\beta'$ lobes and in the γ lobes faintly. To further investigate the role of γ lobe KCs, we used the drivers *R16A06-Gal4* and *H24-Gal4* that target selectively γ neurons in the MB. Their expression patterns are shown in Figure 3G. We obtained discrepant results. Expressing *dTrpA1* with *R16A06-Gal4* nearly abolished fly locomotor reactivity at 32°C to around 4% of the 23°C control (Figure 3E), while the same experiment performed with *H24-Gal4* had no effect on SING (Figure 3F). Such a difference prompted us to analyze more precisely the expression patterns of these γ lobe drivers. First, *H24-Gal4* also labels the $\alpha\beta$ lobes slightly in contrast to *R16A06-Gal4* that appears selective for the γ lobes. Second, by counting the labeled MB neurons using two-photon microscopy, we found that *R16A06-Gal4* expresses in around 500 γ lobe KCs per hemisphere while *H24-Gal4* labels around 300 γ neurons only (Figure 3G). It is quite possible that *H24-Gal4* does not express in a specific subset of γ KCs involved in SING control that would be in contrast targeted by *R16A06-Gal4*.

The γ lobe driver *R16A06-Gal4* had such a strong effect that we looked more closely at SING modulation in *R16A06>dTrpA1* flies. Kinetics studies showed that the inhibition was fast with this driver indeed, decreasing SING performance to ~10% of controls after only 3 min of thermoactivation (Figure S1B). Optogenetic photostimulation of *R16A06>ChR2-XXL* flies during the climbing test was also able to reduce efficiently SING performance by ~30% (Figure S2D). Remarkably, at the end of a 10-min thermoactivation period, *R16A06>dTrpA1* flies



were not paralyzed but in contrast very active in the column (Movie S4). After being tapped down, they did not start climbing, possibly because the startle stopped spontaneous locomotion while thermoactivation of the γ lobe prevented their locomotor reactivity (Movie S5). These experiments confirmed that γ lobe activation has a stronger effect on SING than DAN activation.

$\alpha\beta$ Lobe Co-activation Antagonizes SING Modulation by $\alpha\beta\beta'$ and γ KCs

In our previous work, we considered that the $\alpha\beta$ lobe neurons were not involved in SING modulation, because no effect could be seen after synaptic blockade or activation with *mb247-Gal4* that strongly targets the $\alpha\beta$ and γ KCs (Riemensperger et al., 2013). Again, the result with *mb247-Gal4* could be confirmed here (Figure 3H). Similarly, the use of an $\alpha\beta$ -specific driver, *c708a-Gal4*, did not induce any effect on SING (data not shown). Neuronal thermoactivation with *H24-Gal4* did not show any

difference compared to the control, while that of *R16A06-Gal4*-targeted neurons led to a strong SING decrease (Figures 3E–G). Remarkably, both *mb247-Gal4* and *H24-Gal4*, which induce no effect on SING, express both in the $\alpha\beta$ and γ KCs, whereas *R16A06-Gal4* that induce strong effect on SING targets the γ KCs selectively. This led us to the hypothesis that co-activation of $\alpha\beta$ neurons could potentially antagonize SING modulation caused by γ lobe activation.

To test this possibility, a recombined *R16A06-Gal4*, *mb247-Gal4* double driver line was constructed. The pattern of this driver, as characterized by msGFP expression, showed even and strong α , β and γ lobe labeling (Figure 3I). Expressing *dTrpA1* with *R16A06-Gal4* confirmed the decreased fly locomotor reactivity at 32°C (15% of the 23°C control, Figure 3J), while neuronal activation of KCs targeted by the *R16A06-Gal4*, *mb247-Gal4* double driver in a parallel experiment showed remarkably rescued SING response that rose up to 53% of the control in the first round of test (Figure 3J). The response of these flies

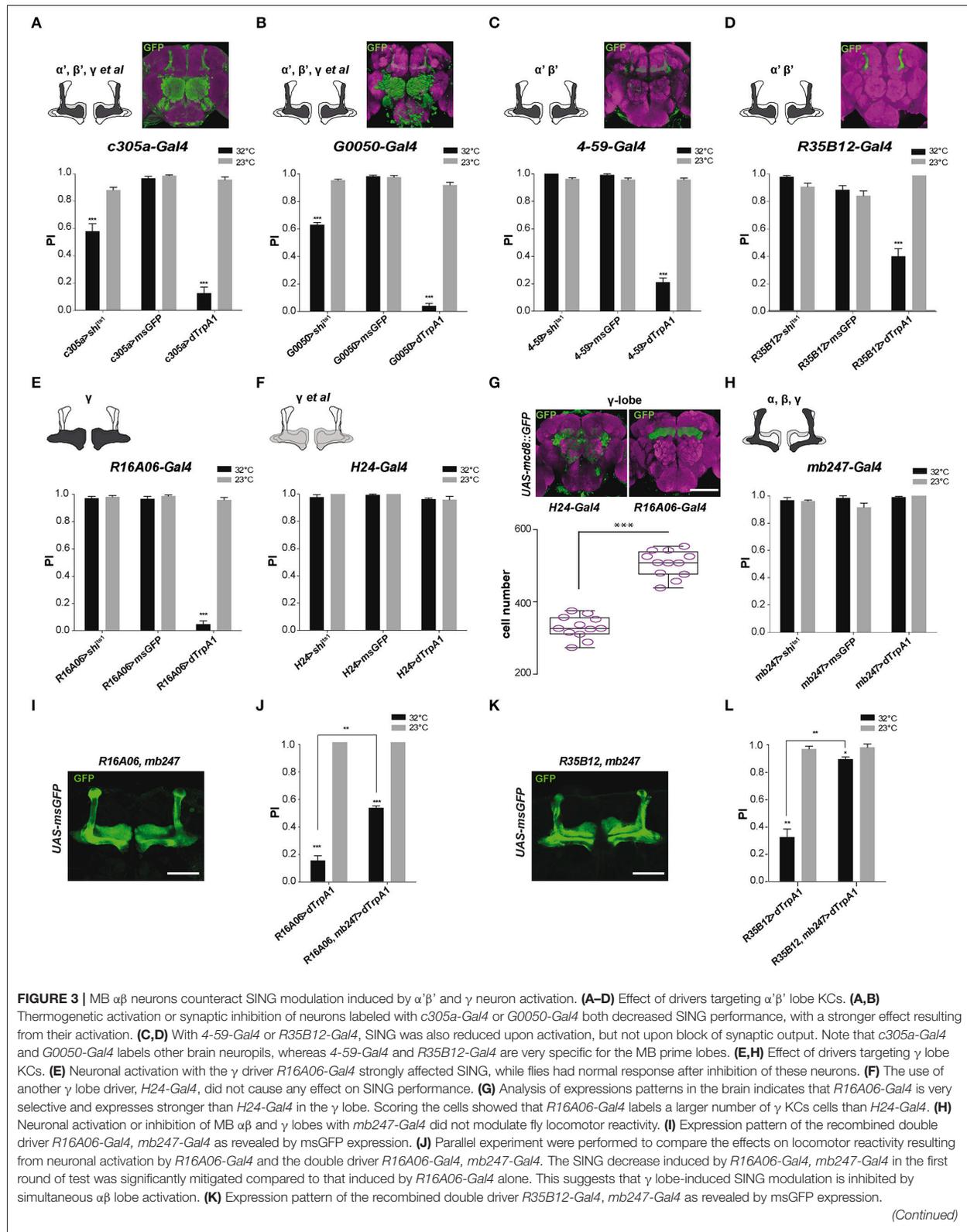


FIGURE 3 | (L) Parallel experiment was performed to compare the effects on locomotor reactivity resulting from neuronal activation by *R35B12-Gal4* and the double driver *R35B12-Gal4, mb247-Gal4*. Flies with neuronal activation in both $\alpha\beta\gamma$ and $\alpha'\beta'$ with *R35B12-Gal4, mb247-Gal4* showed normal SING performance compared to reduced performance in *R35B12>dTrpA1* flies. This suggests that activation of $\alpha\beta\gamma$ KCs blocked SING modulation induced by $\alpha'\beta'$ KCs. Scale bars represent 100 μm . **(A–F,H)** Two-way ANOVA with Bonferroni's multiple comparisons tests ($***p < 0.001$). **(G)** One-way ANOVA with Tukey's multiple comparisons test ($***p < 0.001$). **(J,L)** Two-way ANOVA with Tukey's multiple comparisons tests ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

then declined in the two subsequent tests, possibly related to a dominant effect of *R16A06-Gal4*-induced neuronal activation. This result indicates that co-activating the $\alpha\beta$ lobes can at least transiently inhibit SING blockade induced by activation of the γ lobe intrinsic neurons.

We then checked if activation of the $\alpha\beta$ KCs could similarly interfere with SING modulation induced by $\alpha'\beta'$ KC activation. A recombined *R35B12-Gal4, mb247-Gal4* double driver line was constructed that strongly expresses in the $\alpha\beta$, γ and $\alpha'\beta'$ KCs, i.e., in all the MB lobes (Figure 3K). Strikingly, the significant effect of $\alpha'\beta'$ neuron thermoactivation by *R35B12-Gal4* on SING modulation (reduction of the response to 33% of the 23°C control) was nearly abolished when the double-driver *R35B12-Gal4, mb247-Gal4* was used in a parallel experiment (reduction to 90.5% of the control only) (Figure 3L). Therefore, co-activation of the $\alpha\beta$ and γ neurons blocked the inhibitory effect induced by $\alpha'\beta'$ neuron activation. Accordingly, we observed that thermoactivation or synaptic blockade with a driver that expresses specifically in all MB lobes, *VT30559-Gal4*, only had little effects on SING modulation (data not shown). Overall, these results indicate that activity of the $\alpha\beta$ KCs potentially counteracts by an unknown mechanism the behavioral modulation induced by the $\alpha'\beta'$ and γ KCs.

Regulation of Locomotor Reactivity Requires DA Receptor Signaling in the MB

We next investigated whether down-regulation of DA receptor expression in the MB could prevent the decrease in SING caused by thermoactivation of DANs. Two DA receptors, D₁-like *Dumb/Dop1R1* and D_{1/5}-like *Damb/Dop1R2*, are abundant in the MB lobes where they play key roles in olfactory memory (Kim et al., 2007; Seugnet et al., 2008; Selcho et al., 2009; Berry et al., 2012; Musso et al., 2015; Plaçais et al., 2017). *Dumb* has also been implicated in arousal and grooming (Andretic et al., 2008; Lebestky et al., 2009; Pitmon et al., 2016) and *Damb* in paraquat- and DA-induced neurotoxicity (Cassar et al., 2015). Taking advantage of the *LexA-LexAop* and *Gal4-UAS* expression systems, we expressed *dTrpA1* in DANs using *LexAop-dTrpA1* and the *TH-LexA* driver, whose expression pattern is similar to that of *TH-Gal4* (Berry et al., 2015), while inactivating by targeted RNA interference (RNAi) the genes encoding *Dumb* or *Damb* in all MB lobes with the *238Y-Gal4* driver. As shown in Figure 4A, *TH-LexA*-controlled *dTrpA1* expression in the presence of *238Y-Gal4* alone induced a significant decrease in SING performance at 32°C (~48% of the 23°C control value). We observed that adding the *UAS-Dumb-RNAi* construct to allow *Dumb* inactivation in the MB fully restored SING performance to control level despite DAN thermoactivation (Figure 4A). In contrast, selective *Damb* inactivation had no such effect

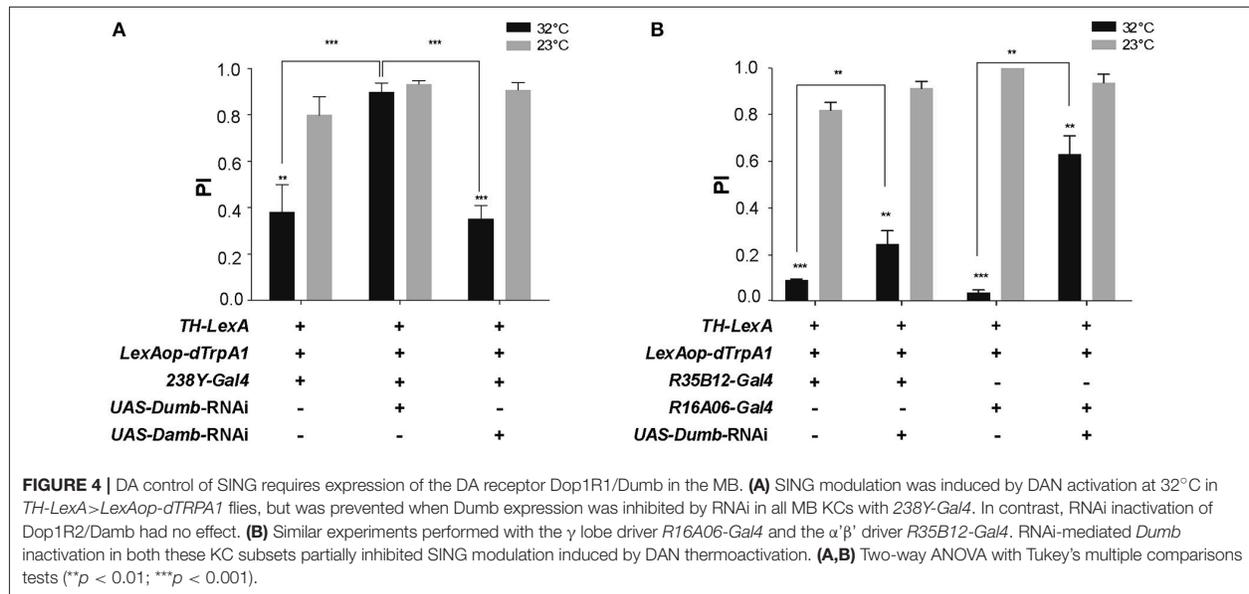
(Figure 4A). This experiment suggests that DA modulation of SING requires DA receptor expression in the MB KCs and that this regulation specifically depends on signaling through the *Dumb* receptor.

Next we investigated whether RNAi-mediated inactivation of *Dumb* expression in specific MB lobes could have a similar antagonistic effect on DA modulation of SING. We found that targeting *Dumb* RNAi selectively in the $\alpha'\beta'$ or γ lobes using *R35B12-Gal4* and *R16A06-Gal4*, respectively, in both cases significantly rescued the SING response, in spite of *TH-LexA*-mediated DAN activation (Figure 4B). This effect was most prominent with the strong and specific γ driver *R16A06-Gal4* (Figure 4B). This indicates a requirement for the DA receptor *Dumb* in the $\alpha'\beta'$ and γ lobes for DAN-mediated SING modulation.

MBON-M4/M6 and MBON-V2 Relay SING Modulation

We then attempted to identify specific MB-output neurons (MBONs) that could transfer MB modulatory information to downstream motor circuits. Since the intrinsic KCs in the MB $\alpha'\beta'$ and γ lobes appear to play a role in SING control, we studied the role of MBONs whose dendrites arborize on these lobes. The glutamatergic MBON-M4 β , M4 β' and M6 (also named MBON- $\beta 2\beta'2$ and MBON- $\beta'2\text{mp}$ for M4, and MBON- $\gamma 5\beta'2a$ for M6) arborize on the tip of the β , β' , and γ lobes, respectively (Tanaka et al., 2008; Aso et al., 2014b; Oswald et al., 2015a) (Figure 5A). These neurons are known to be involved in sleep regulation and the expression of appetitive and aversive memory performance (Aso et al., 2014b; Bouzaiane et al., 2015; Oswald et al., 2015a; Sitaraman et al., 2015a). Using *NP3212-Gal4* and *R27G01-Gal4* that both target the MBON-M4 and M6 neurons (Tanaka et al., 2008; Bouzaiane et al., 2015), we observed that thermogenetic activation of these MB efferent neurons significantly reduced locomotor reactivity, while inhibiting their synaptic output with *Shi^{ts1}* had no effect (Figures 5B,C).

The cholinergic MBON-V2 α and V2 α' (also named MBON- $\alpha 2\text{sc}$ and MBON- $\alpha'3$, respectively) have their dendrites in the MB vertical lobes ($\alpha 2$, $\alpha'3$) and are required for retrieval of aversive olfactory memory from the $\alpha\beta$ lobe (Tanaka et al., 2008; Séjourné et al., 2011; Aso et al., 2014b; Bouzaiane et al., 2015; Figure 5D). Two specific drivers, *NP2492-Gal4* and *R71D08-Gal4* (Tanaka et al., 2008; Séjourné et al., 2011) were used to test whether V2 neurons are implicated in SING modulation. Activating these neurons with either of these drivers greatly reduced SING performance to around 33 and 21% of the 23°C control value, respectively, and again inhibition of synaptic output had no effect (Figures 5E,F). Finally, neither activation nor blocking of the MBON-V3 (alias MBON- $\alpha 3$) output, targeted



by *G0239-Gal4*, had any effect on the SING response (data not shown), indicating that specific MBONs are involved in SING control. Hence, we propose that both MBON-M4/M6 and MBON-V2 participate in the transmission of MB regulatory information to the downstream SING reflex motor circuits.

The Ellipsoid Body Does Not Play a Role in the Modulation of Startle-Induced Locomotion

The *Drosophila* ellipsoid body (EB) is a region of the central complex in the brain that controls locomotor patterns (Strauss and Heisenberg, 1993; Martin et al., 1999b, 2001; Strauss, 2002), as well as spatial orientation and visual pattern memories (Neuser et al., 2008; Pan et al., 2009). Subsets of DANs labeled by *TH-Gal4* heavily innervate the EB (Mao and Davis, 2009; White et al., 2010; Ueno et al., 2012; Riemensperger et al., 2013). Due to the complex structure of the EB, different driver lines have been used which express in various areas of the EB: *c41-Gal4* (all EB neurons), *c105-Gal4* (R1 neurons), *EB1-Gal4* (R2/R4d neurons), and *c232-Gal4* (R3/R4 neurons). Neuronal activation or synaptic inhibition with any of these drivers had no significant effect on the fly's locomotor reactivity, as tested by SING (Figure 6). This suggests that the EB is not involved in the neuronal circuits modulating startle-induced locomotion in *Drosophila*.

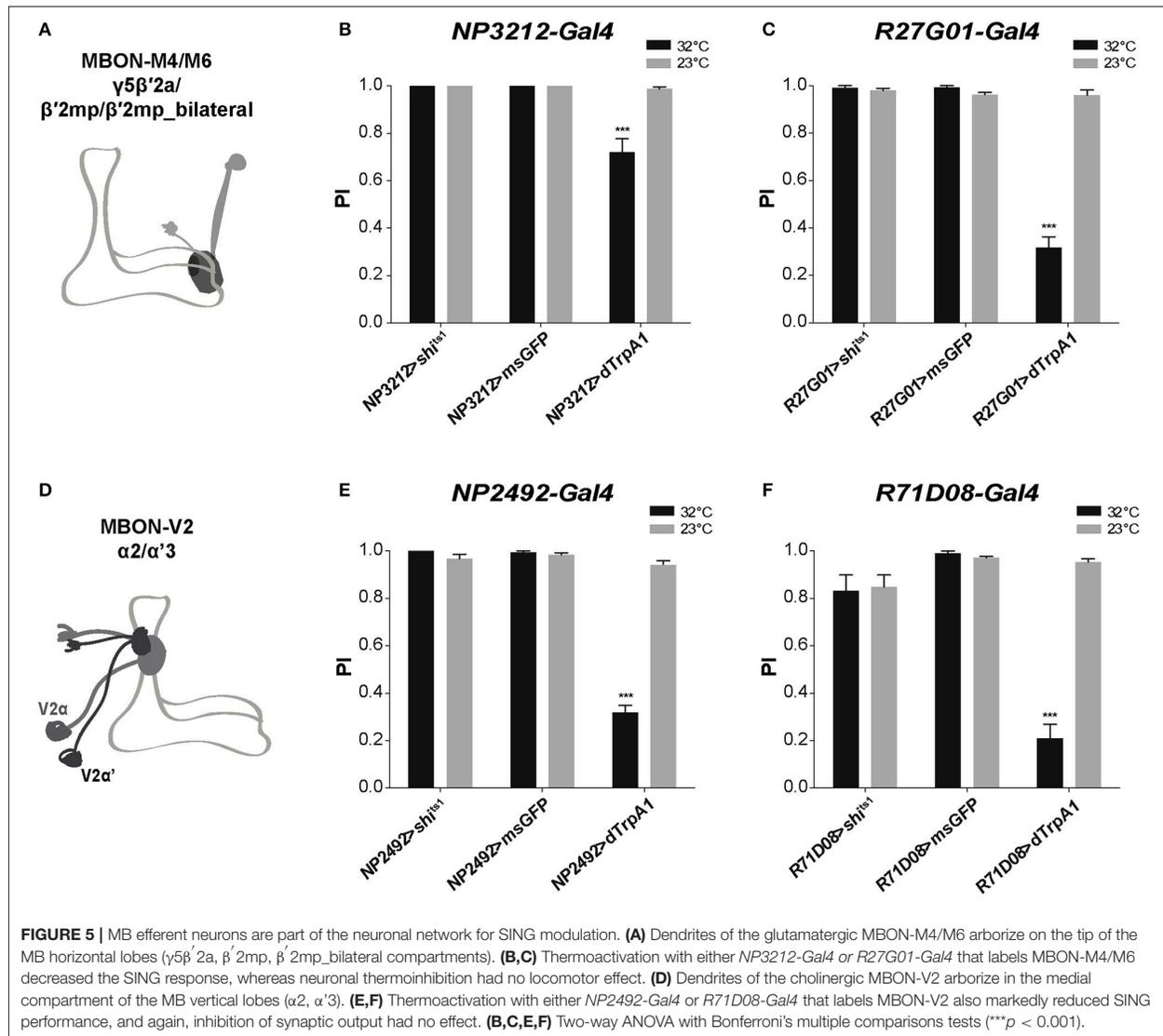
Potential Synaptic Convergence Between DANs and MBONs Controlling SING

According to the MB neuronal architecture reported by Aso et al. (2014a), dendrites from the PAM DANs mainly reside in the crepine (CRE) and superior medial protocerebrum (SMP) brain regions, and slightly also in the superior intermediate protocerebrum (SIP) and superior lateral protocerebrum (SLP). The PPL1 DANs have a large part of their dendrites in the SMP,

which is also where the MBON-M4/M6 and MBON-V2 send axonal projections. In order to detect zones of potential synaptic connections between the afferent and efferent MB neurons, we used the technique of splitGFP reconstitution (also named GFP reconstitution across synaptic partners, GRASP) coupled with the *LexA-LexAop* and *Gal4-UAS* systems (Feinberg et al., 2008; Gordon and Scott, 2009; Pech et al., 2013a; Macpherson et al., 2015).

The PAM DAN projections mainly tile the MB horizontal lobes where the MBON-M4/M6 dendrites arborize (Pech et al., 2013b; Riemensperger et al., 2013; Aso et al., 2014a). Results of splitGFP experiments indicated a potential synaptic convergence between these two groups of neurons in the tips of the MB horizontal lobes ($\gamma 5$, $\beta 2$, and $\beta 2'$ compartments) (Figure 7A1–3) and also in the CRE and SMP neuropiles (Figure 7A2–4). This suggests, in agreement with a previous report (Owald et al., 2015a), that the zones of convergence between PAM and M4/M6 neurons not only localize in the MB horizontal lobes but also in the superior protocerebrum where the M4/M6 neurons appear to project onto the PAM DAN dendrites.

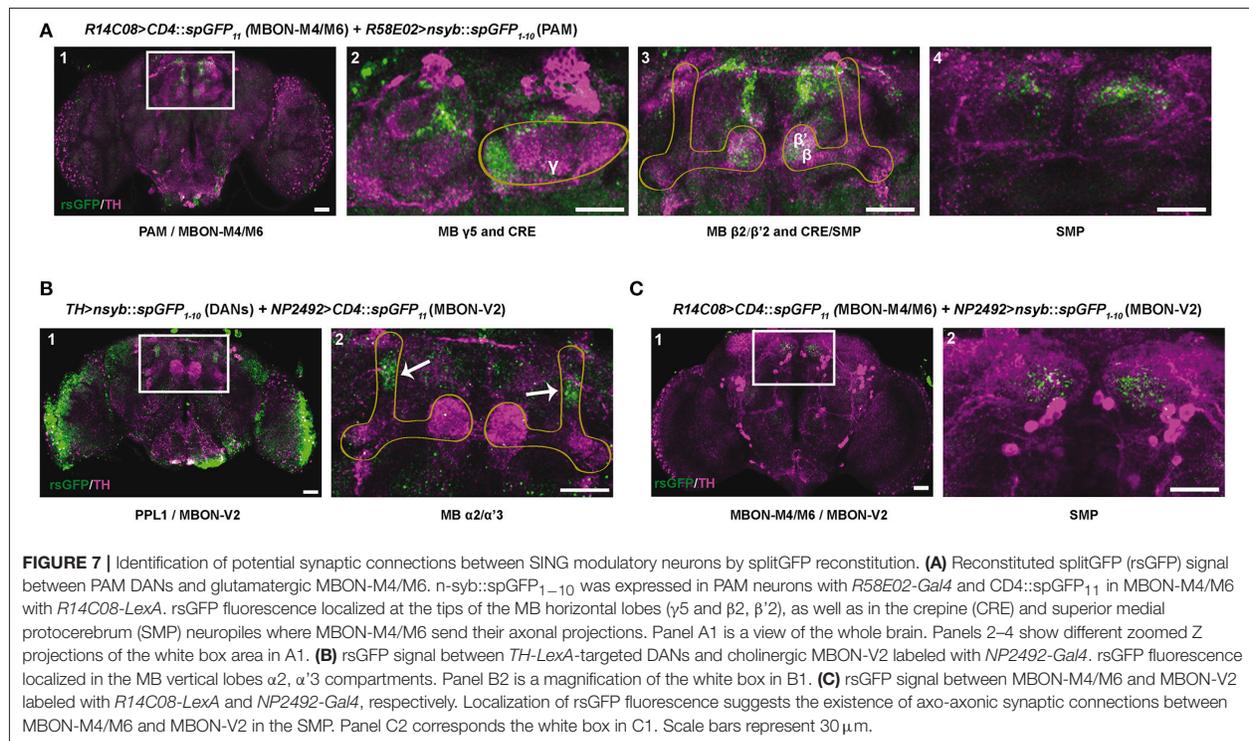
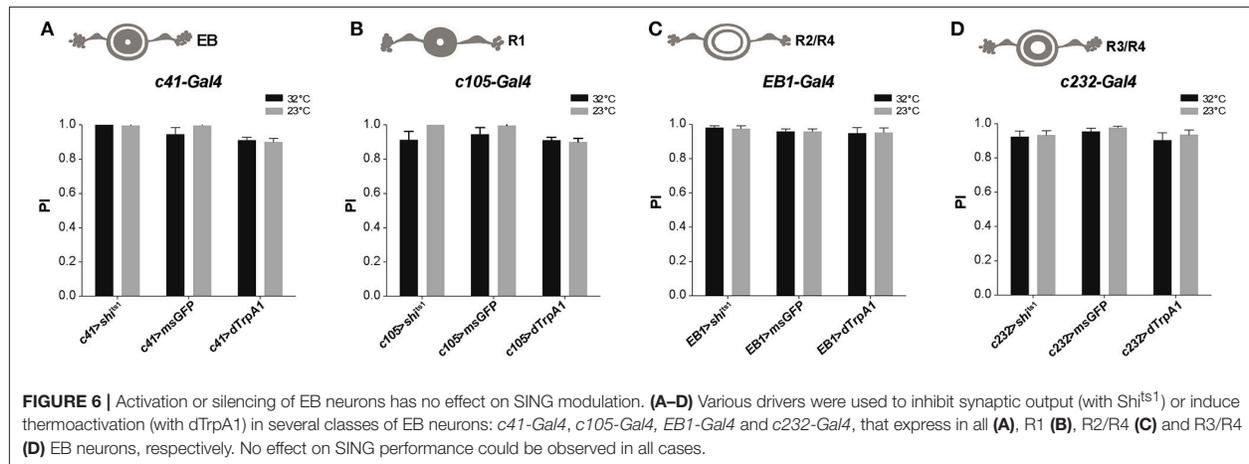
MBON-V2 arborizes on the MB vertical lobes (Tanaka et al., 2008; Séjourné et al., 2011; Aso et al., 2014b). Reconstituted split GFP (rsGFP) signals between MBON-V2 and DANs targeted by *TH-LexA* could be detected in the MB α and α' medial compartments, where the PPL1 MB-V1 neurons send projections (Aso et al., 2010, 2014b), indicating a close proximity between these neurons (Figure 7B1,2). A strong rsGFP signal was only observed when the presynaptic marker *nsyb::spGFP_{1–10}* was driven with *TH-LexA* and *CD4::spGFP₁₁* by the MBON-V2 driver *NP2492-Gal4* (Figures 7B1,2) and not the opposite (not shown), suggesting that DANs project to the MBON-V2 in the MB vertical lobe compartments. The occurrence of DAN > MBON synapses in the MB has recently



been demonstrated in a comprehensive electron microscopy study (Takemura et al., 2017). Furthermore, rsGFP signals were visible between MBON-V2 and MBON-M4/M6 in the SMP region, which suggests that these MBONs may form axo-axonic reciprocal synapses (Figures 7C1,2). It seems that MBON-V2 could be presynaptic and MBON-M4/M6 postsynaptic in these contacts because a rsGFP signal in the SMP was only observed when the V2 driver *NP2492-Gal4* expressed the presynaptic marker *nsyb::spGFP₁₋₁₀* and the M4/M6 driver *CD4::spGFP₁₁* (Figures 7C1,2) and not the opposite (not shown). Therefore, there might be feedback signals from the MBON-V2 to MBON-M4/M6 and DANs that could optimize SING modulation, possibly in relation to learning and memory processes, and thus coordinate locomotor behavior with the environment.

DISCUSSION

In this study, we have identified MB afferent, intrinsic and efferent neurons that underlie modulation of startle-induced locomotion in the *Drosophila* brain. Using *in vivo* activation or silencing of synaptic transmission in neuronal subsets, we showed that specific compartments of the MBs are central to this modulation. Implicated neurons include $\alpha' \beta'$ and γ KCs, subsets of PAM and PPL1 DANs, and the MBONs V2 and M4/M6. We have also characterized some of the potential synaptic connections between these elements using splitGFP reconstitution across cells. Although the picture is not complete, these results led us to propose a first scheme of the neuronal circuits underlying the control of locomotor reactivity in an insect brain.



DANs Show Diverse Functions in the Control of Locomotor Reactivity

We previously reported that the degeneration of DANs afferent to the MBs in the PAM and PPL1 clusters is associated with accelerated decline of SING performance in aging flies (Riemensperger et al., 2013; Vaccaro et al., 2017). Here we have specifically addressed the role of these and other DANs in SING modulation. Our initial observation was that thermoactivation of *TH-Gal4*-targeted DANs consistently led to decreased locomotor reactivity, while silencing synaptic output from these neurons

had no effect. This result was verified by rapid optogenetic photostimulation, indicating that indeed DAN activation affects locomotor reactivity during the execution of the behavior. In contrast, blocking selectively synaptic output of the PAM DANs neurons resulted in a slight increase in SING performance, suggesting that a subset of spontaneously active neurons in the PAM inhibits SING. It should be noted, however, that this effect appeared small probably in part because SING performance was already very high for the control flies in our assay condition. This issue may have prevented us from detecting other modulatory

neurons in the course of this study. Interestingly, our data suggest that those PAM neurons that inhibit SING are targeted by *NP6510-Gal4*, a driver that expresses in 15 PAM DANs that project to the MB $\beta 1$ and $\beta 2$ compartments. The degeneration of these neurons also appears to be largely responsible for α -synuclein-induced decline in SING performance in a Parkinson disease model (Riemensperger et al., 2013). Moreover, we provided one observation in this study, using DAN co-activation with *TH-Gal4* and *R58E02-Gal4*, suggesting that other subsets of the PAM cluster may modulate locomotor reactivity with opposite effects, i.e., increase SING when they are stimulated.

Our study further indicated that thermoactivation of two DANs of the PPL1 cluster, either MB-MP1 that projects to the $\gamma 1$ peduncle in the MB horizontal lobes or MB-V1 that projects to the $\alpha 2$ and $\alpha' 2$ compartments of the MB vertical lobes, was sufficient to significantly decrease SING performance. This suggests that the MB-afferent DANs of the PPL1 cluster are also implicated in SING modulation. Other DAN subsets could play a role and are still to be identified. However, inactivation of a DA receptor, Dop1R1/Dumb, in MB KCs precluded DAN-mediated SING modulation, strongly suggesting that DANs afferent to the MBs plays a prominent role in the neuronal network controlling fly's locomotor reactivity. In contrast, inactivating Dop1R2/Damb in KCs did not show any effect on DA-induced SING control.

Therefore, these results suggest that DA input to the MBs can inhibit or increase the reflexive locomotor response to a mechanical startle, allowing the animal to react to an instant, sudden stimulus. In accordance with this interpretation, previous reports have shown that the MB is not only a site for associative olfactory learning, but that it can also regulate innate behaviors (Hige et al., 2015; Lewis et al., 2015; Oswald et al., 2015a). By combining synaptic imaging and electrophysiology, Cohn et al. (2015) have demonstrated that dopaminergic inputs to the MB intrinsic KCs play a central role in this function by exquisitely modulating the synapses that control MB output activity, thereby enabling the activation of different behavioral circuits according to contextual cues.

Interactions Between MB Compartments Contribute to SING Modulation

We previously reported a decrease in SING performance when KCs in the $\alpha'\beta'$ lobes, but not in the $\alpha\beta$ and γ lobes, were thermogenetically stimulated or their synaptic output silenced (Riemensperger et al., 2013). Here, using a set of specific drivers, we have more precisely studied the contribution of the various MB lobes in the modulation of this innate reflex. We confirmed that the $\alpha'\beta'$ KCs down-regulate SING when they are activated but not when their output is inhibited. Other unidentified neurons, which are targeted by the rather non-selective *c305a-Gal4* and *G0050-Gal4* drivers, trigger a decrease in SING performance when they are inhibited by Shi^{ts1}, and are therefore potential SING-activating neurons. We further found that the MB γ lobes contain KCs that strongly inhibit SING when activated, both by thermogenetic and optogenetic

stimulation, as shown with the γ -lobe specific driver *R16A06-Gal4*. However, thermoactivation of γ neurons with other drivers, like *mb247-Gal4*, which express both in the $\alpha\beta$ and γ lobe, did not decrease SING (Riemensperger et al., 2013 and this study). This could result from an inhibitory effect of $\alpha\beta$ neuron activation on SING modulation by γ neurons. To test this hypothesis, we have generated a double-driver by recombining *mb247-Gal4* with *R16A06-Gal4*. Because both drivers express in the γ lobes, one would expect a stronger effect on SING modulation after thermoactivation with the double-driver than with *R16A06-Gal4* alone. We observed strikingly the opposite, i.e., that SING was decreased to a less extent with the double-driver than with *R16A06-Gal4* alone. Activation of *mb247-Gal4* $\alpha\beta$ neurons therefore likely counterbalanced the effect of γ neuron activation with *R16A06-Gal4* on SING modulation. A similar and even more obvious results was obtained when *mb247-Gal4* was recombined with the $\alpha'\beta'$ driver *R35B12-Gal4*: co-activation of the neurons targeted by these two drivers prevented the strong SING modulation normally induced by *R35B12-Gal4* alone. These results suggest the existence of an inter-compartmental communication process for locomotor reactivity control in the *Drosophila* MB. Comparably, it was recently suggested, in the case of memory retrieval, that MB output channels are ultimately pooled such that blockade (or activation) of all the outputs from a given population of KCs may have no apparent effect on odor-driven behavior, while such behavior can be changed by blocking a single output (Owald et al., 2015a). Such a transfer of information could occur, as was previously reported, through connections involving the MBONs within the lobes or outside the MB (Aso et al., 2014b; Oswald et al., 2015a).

Role of Specific MBONs in Innate Reflex Suppression

Finally, the activation of two sets of MB efferent neurons, cholinergic MBON-V2 and glutamatergic MBON-M4/M6, consistently decreased SING performance of the flies. In contrast, silencing these neurons had no effect on locomotor behavior, as was previously observed (Aso et al., 2014b). The dendrites of these MBONs arborize in the medial part of the vertical lobes ($\alpha 2$, $\alpha' 3$) and the tips of the horizontal lobes ($\beta 2$ and $\gamma 5$), respectively, as a further evidence that the prime and γ lobes, and DANs efferent to these compartments, are involved in SING modulation. We also show results from GRASP observations suggesting that the PAM DANs lay very close or make potential synaptic connections with the MBON-M4/M6 neurons in their MB compartments, as well as the M4/M6 with the PAM in the SMP, in agreement with recent evidence from other laboratories (Lewis et al., 2015; Oswald et al., 2015a; Takemura et al., 2017). Our results also provide evidence that the PPL1 DANs and MBON-V2 contact each other in the vertical lobes and that axo-axonic synaptic contacts may occur between the MBON-V2 and M4/M6 neurons in their common projection region in the SMP.

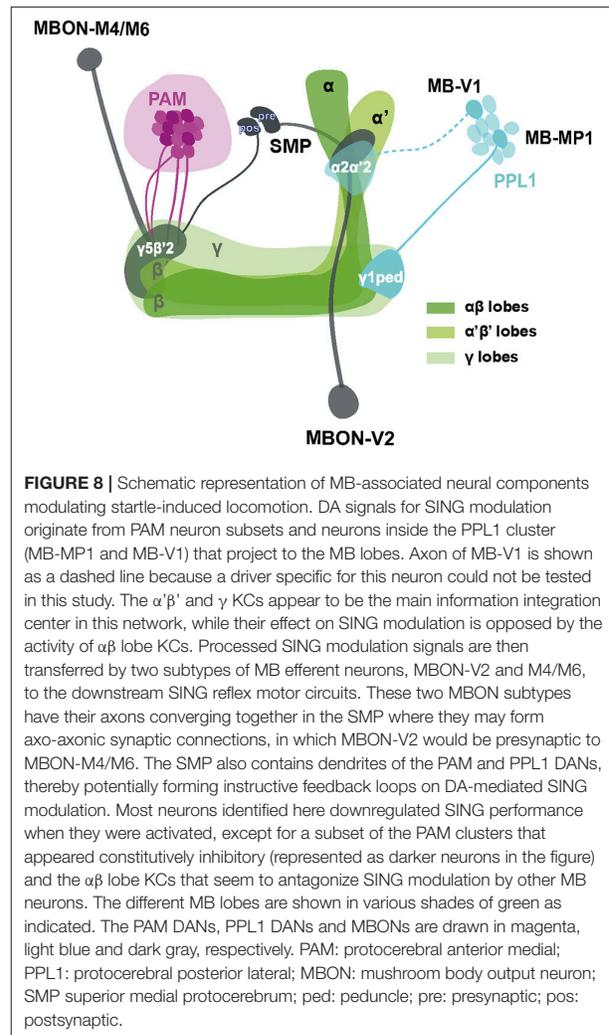
These MBONs are known to be involved in opposite ways in olfactory memory: DAN-induced synaptic repression

of cholinergic or glutamatergic MBONs would result in the expression of aversive or attractive memory, respectively (Aso et al., 2014b). Here we find, in contrast, that the activation of these two sets of MBONs had similar depressing effects on SING behavior. Interestingly, it has been recently reported that the glutamatergic MBONs and PAM neurons that project to the MB β '2 compartment are also required for modulation of another innate reflex, CO₂ avoidance (Lewis et al., 2015). CO₂ exposure, like mechanical startle, represents a potential danger for the flies, thus triggering an avoidance behavior that can be suppressed by silencing these MBONs in specific environmental conditions. However, it is the activation of glutamatergic MBONs that inhibits SING. This apparent discrepancy might be explained if the downstream circuits were different for these two escape behaviors (CO₂ avoidance and fast climbing). Overall, our results further support the hypothesis of a primary role of the MB as a higher brain center for adapting innate sensory-driven reflex to a specific behavioral context (Cohn et al., 2015; Lewis et al., 2015).

Different Neuronal Circuits Control Locomotor Reactivity, Sleep/Wake State and Hyperactivity

Even though the model remains to be confirmed and elaborated, a proposed scheme summarizing our current working hypothesis of the neural components underlying SING control is presented in **Figure 8**. Sensory information from mechanical stimulation triggers an innate climbing reflex (negative geotaxis) that can be regulated by signals transmitted from MB-afferent DANs (in the PAM and PPL1 cluster) to select KCs and two sets of MBONs (V2 and M4/M6) in specific MB compartments. Processing of this information could occur through synergistic or antagonistic interactions between the MB compartments and, finally, the MBON neurons would convey the resulting modulatory signal to downstream motor circuits controlling the climbing reflex. We observed that the axonal projections of these MBONs make synaptic contacts with each other and converge together to the SMP where the dendrites of DANs lie (Aso et al., 2014a), suggesting that they might form feedback loops to control DA signaling in the circuits.

SING performance can be affected by a collection of factors including the arousal threshold of the fly, the ability to sense gravity and also climbing ability. "Arousal" is defined as a state characterized by increased motor activity, sensitivity to sensory stimuli, and certain patterns of brain activity (Coull, 1998; Pfaff and Banavar, 2007). A distinction can be made between endogenous arousal (i.e., wakefulness as opposed to sleep) and exogenous arousal (i.e., behavioral responsiveness) (Van Swinderen and Andretic, 2011). In *Drosophila*, DA level and signaling control all known forms of arousal (Friggi-Grelin et al., 2003; Birman, 2005; Kume et al., 2005; Lebestky et al., 2009; Van Swinderen and Andretic, 2011; Kumar et al., 2012; Liu et al., 2012b; Ueno et al., 2012; Nall et al., 2016). Because the MB plays an important role in sleep regulation (Sitaraman et al., 2015a; Artushin and Sehgal, 2017; Tomita et al., 2017), sleep- or wake-promoting networks might indeed in part interact or overlap with



those controlling locomotor reactivity. However, we observed that thermoactivation with various drivers had in a number of cases opposite effects on sleep/wake state and SING. First, neuronal thermoactivation with *TH-Gal4* suppresses sleep (Liu et al., 2012b) but decreases the SING response. Second, extensive thermogenetic activation screen revealed that α ' β ' and γ KCs are wake-promoting and γ d KCs are sleep-promoting (Sitaraman et al., 2015a). In our experiments, neuronal activation of α ' β ' or γ KCs both led to strongly decreased locomotor reactivity. Third, stimulating MBON-M4 and M6, which are wake-promoting (Sitaraman et al., 2015a), decreased SING performance.

Another brain structure, the EB, plays important roles in the control of locomotor patterns (Strauss, 2002) and is also sleep-promoting (Liu et al., 2016). Furthermore, the EB is involved in the dopaminergic control of stress- or ethanol-induced hyperactivity (Lebestky et al., 2009; Kong et al., 2010), which can be considered as forms of exogenously-generated arousal. We used several drivers labeling diverse EB neuronal layers

and found no noticeable effects of thermoactivation of these neurons on the SING response. We conclude that the circuits responsible for SING modulation, although they apparently share some similarities, are globally different from those controlling sleep/wake state and environmentally-induced hyperactivity.

Overall, this work identified elements of the neuronal networks controlling startle-induced locomotion in *Drosophila* and confirmed the central role of the MBs in this important function. Future studies are required to complete this scheme and explore the intriguing interactions between the different MB compartments in SING neuromodulation.

ETHICS STATEMENT

Experiments on *Drosophila* are not subject to the approval of ethics committee. All experiments were nevertheless performed in accordance with ethic procedures and by minimizing the number of animals required for data gathering.

AUTHOR CONTRIBUTIONS

JS, TR, AF, and SB: Conceived and designed the experiments; JS, AX, JG, HP, and TR: Performed the experiments; JS, AX, JG, HP,

TR, AF, and SB: Analyzed data; JS, AX, TR, AF, and SB: Wrote the paper; SB: Designed and supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnsys.2018.00006/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Neural control of startle-induced locomotion by the mushroom
bodies and associated neurons in *Drosophila***

**Jun Sun, An Qi Xu, Julia Giraud, Haiko Poppinga, Thomas Riemensperger, André Fiala and
Serge Birman ***

SUPPLEMENTARY MATERIAL

- Figures S1 and S2**
- Movie Captions**
- Table S1**
- Supplementary References**

Supplementary Figures

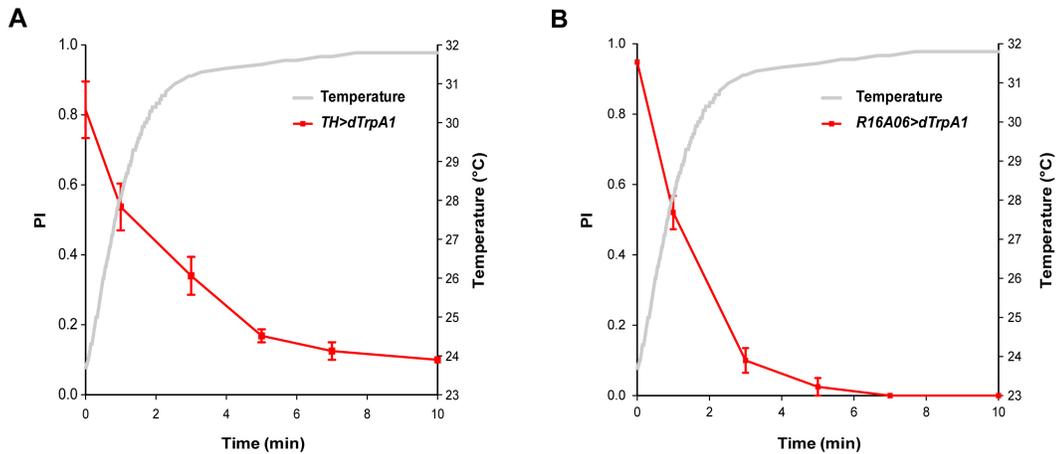


FIGURE S1 | Time course of SING behavior modulation during thermogenetic neuronal activation. **(A)** Effect of *TH-Gal4*-targeted DANs. The SING performance of *TH>dTrpA1* flies was determined after various times of incubation at 32°C (red curve). The grey curve shows the kinetics of the temperature increase within the column measured with a thermometer during a 10-min incubation period. A decrease in SING PI could be observed at 1 min compared to the control (time 0) when the temperature of the column reached about 28°C. SING performance did not decrease significantly and stayed stable after 5 min of DAN thermoactivation. **(B)** Effect of MB γ neurons. *R16A06>dTrpA1* flies in which γ lobe KCs were selectively thermoactivated showed a more rapid decrease in SING that was ~90% inhibited in 3 min.

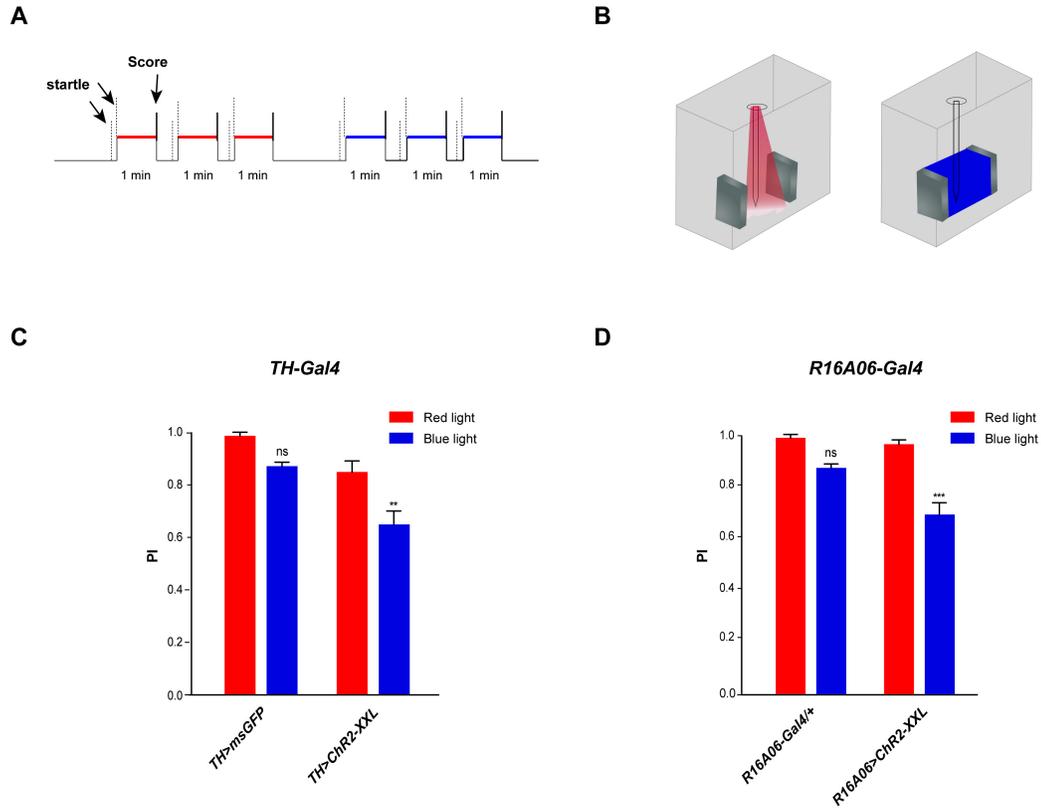


FIGURE S2 | SING modulation by optogenetic photostimulation. **(A)** Protocol used to assay startle-induced locomotion during optogenetic photoactivation. Mechanical startle was applied just before the column was introduced in the illuminated box and a second time in the box previous to the start of the test (dotted vertical lines). Flies were counted to score PI after 1 min (black vertical lines) using video monitoring. The test was repeated 3 times in red light (control, red horizontal lines) and 3 times in blue light (blue horizontal lines). **(B)** Schematic representation of the experimental setup showing the illuminated dark box with the column inside. Note that the red light covered 100% of the column length and the blue light only 45%. **(C)** Expression of channelrhodopsin in DANs. Photostimulation of *TH>ChR2-XXL* flies with blue light during the test significantly decreased SING performance by ~22% compared to controls illuminated with red light. No significant effect of blue light was observed with control *TH>msGFP* flies. **(D)** Expression of channelrhodopsin in MB γ lobes. Photostimulation of *R16A06>ChR2-XXL* flies with blue light during the test decreased SING performance by ~30% compared to controls illuminated with red light. No effect of blue light was observed with control *R16A06-Gal4/+* flies. These experiments indicate that activation of DANs or γ KCs had significant effects on startle-induced locomotion within less than 1 min. **(C,D)** Two-way ANOVA with Bonferroni's multiple comparisons tests (ns: not significant; ** $p < 0.01$; *** $p < 0.001$).

Movie Captions

MOVIE S1 | Spontaneous locomotion of *TH>dTrpA1* flies after neuronal thermoactivation. Following prolonged thermoactivation of the DANs (10 min at 32°C), *TH>dTrpA1* flies were very active with apparently normal spontaneous locomotion. Note that each column usually contained 10 flies.

MOVIE S2 | SING performance of *TH>dTrpA1* flies after neuronal thermoactivation. Suite of Movie S1. After 10-min thermoactivation, flies were gently tapped down (startle), which stops spontaneous locomotion and normally triggers climbing as a locomotor reaction (see Movie S3). Thermoactivated *TH>dTrpA1* flies were still active and showed no signs of paralysis after startle. However, most of them stayed at the bottom of the column instead of climbing up quickly like control flies. A few started to climb but generally stopped in the middle and sometimes fell. This suggests that DAN thermoactivation inhibited startle-induced locomotion.

MOVIE S3 | SING performance of control *TH>dTrpA1* flies maintained at 23°C. In the absence of DAN thermoactivation, most of these flies climbed rapidly to the top of the column similarly to wild-type flies.

MOVIE S4 | Spontaneous locomotion of *R16A06>dTrpA1* flies after neuronal thermoactivation. After prolonged thermogenetic activation of MB γ neurons (10 min at 32°C), *R16A06>dTrpA1* flies were very active and spontaneously walked rapidly along the column. Note that the two columns shown contain flies of the same genotype.

MOVIE S5 | SING performance of *R16A06>dTrpA1* flies after neuronal thermoactivation. Suite of Movie S3. Upon startle which stops spontaneous locomotion, *R16A06>dTrpA1* flies were still active but their locomotor reactivity appeared to be inhibited as they did not climb efficiently. 10-min thermoactivation of MB γ neurons therefore fully prevented startle-induced locomotion.

Table S1. Driver strains used in this study and their brain expression patterns.

Drivers	Expression patterns	References
<i>TH-Gal4</i>	All DAN clusters except a major part of the PAM	Friggi-Grelin et al., 2003; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010
<i>R58E02-Gal4</i>	~80% of PAM DANs	Liu et al., 2012a; Pech et al., 2013b
<i>NP6510-Gal4</i>	15 PAM DANs including MB-MVP1 plus 3 non-DANs in the PAM	Liu et al., 2006; Tanaka et al., 2008; Aso et al., 2010; Riemensperger et al., 2013
<i>NP5272-Gal4</i>	3 PAM DANs (MB-M3) that are also labelled by TH-Gal4	Tanaka et al., 2008; Aso et al., 2010; Aso et al., 2012
<i>Mz840-Gal4</i>	PPL1 MB-V1	Aso et al., 2010; Aso et al., 2012
<i>NP2758-Gal4</i>	PPL1 MB-MP1	Tanaka et al., 2008; Krashes et al., 2009; Aso et al., 2010
<i>TH-D'-Gal4</i>	PAL, PPM2, PPM3 and PPL1	Liu et al., 2012b
<i>TH-C'-Gal4</i>	PAL, PPM2 and PPL2ab	Liu et al., 2012b; Pathak et al., 2015
<i>TH-C1-Gal4</i>	PAM, PAL, PPM2 and PPL2ab	Liu et al., 2012b, Kayser et al., 2014
<i>TH-LexA</i>	Similar to <i>TH-Gal4</i>	Berry et al., 2015
<i>238Y-Gal4</i>	All MB lobes	Armstrong et al., 1998; Aso et al., 2009
<i>c305a-Gal4</i>	MB $\alpha\beta'$ lobes and broad expression out of the MB	Krashes et al., 2007; Aso et al., 2009; Chen et al., 2012; Riemensperger et al., 2013
<i>G0050-Gal4</i>	MB $\alpha\beta'$ lobes	Lin et al., 2007; Pech et al., 2013b
<i>4-59-Gal4</i>	MB $\alpha\beta'$ lobes	Kaun et al., 2011

<i>R35B12-Gal4</i>	MB α' β' lobes	Jenett et al., 2012; Lin et al., 2014; Cohn et al., 2015
<i>H24-Gal4</i>	MB γ lobes	Martin et al., 1998; Zars et al., 2000; Aso et al., 2009; Christiansen et al., 2011
<i>R16A06-Gal4</i>	MB γ lobes	Jenett et al., 2012; Issman-Zecharya and Schuldiner, 2014; Cohn et al., 2015
<i>mb247-Gal4</i>	MB $\alpha\beta$ and γ lobes	Zars et al., 2000; McGuire et al., 2001; Aso et al., 2009
<i>NP3212-Gal4</i>	MBON-M4/M6	Tanaka et al., 2008; Bouzaiane et al., 2015
<i>R27G01-Gal4</i>	MBON-M4/M6	Jenett et al., 2012; Bouzaiane et al., 2015
<i>R14C08-LexA</i>	MBON-M4/M6	Jenett et al., 2012; Lewis et al., 2015
<i>NP2492-Gal4</i>	MBON-V2	Tanaka et al., 2008; Séjourné et al., 2011
<i>R71D08-Gal4</i>	MBON-V2	Jenett et al., 2012; Séjourné et al., 2011
<i>c41-Gal4</i>	EB neurons	Sakai and Kitamoto, 2006
<i>c105-Gal4</i>	EB ring neurons R1	Renn et al., 1999; Baker et al., 2007; Martín-Peña et al., 2014
<i>EB1-Gal4</i>	EB ring neurons R2	Young and Armstrong, 2010; Thran et al., 2013
<i>c232-Gal4</i>	EB ring neurons R3/R4d	Renn et al., 1999; Young and Armstrong, 2010

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Result A - Part II

Complementary data on the role of neurotransmitters and the fan-shaped body in SING control

1. Role of different neurotransmitters in SING control

According to previous studies, not only DANs project to the MBs, but axons of neurons utilizing other neurotransmitters like serotonin or octopamine reach the MB as well (Pech et al., 2013a; Yu et al., 2005; Keene et al., 2006; Han et al., 1998; Sinakevitch and Strausfeld, 2006; Blenau and Thamm, 2011; Giang et al., 2011; Lee et al., 2011c; Kim et al., 2013; Wu et al., 2013; Niens et al., 2017; Ries et al., 2017). To explore how SING modulation is affected by the different neurotransmitters, we used selective drivers that target each neuronal subtypes: *Trh-Gal4* carries regulatory sequences of tryptophan hydroxylase that synthesizes serotonin (Cassar et al., 2015), *Tdc2-Gal4* uses the promoter of the *Tyrosine decarboxylase 2* (*Tdc2*) gene and expresses in tyraminergetic neurons (Cole et al., 2005), both *OK371-Gal4* inserted near the *Drosophila Vesicular glutamate transporter* (*VGlut*) gene (Mahr and Aberle, 2006) and *VGlut-Gal4*, containing regulatory sequences of *VGlut* (Cassar et al., 2015) target glutamatergic neurons, and *Gad1-Gal4* uses regulating sequence of *Glutamic acid decarboxylase 1* (*Gad1*) to express in GABAergic neurons (Ng et al., 2002).

c316-Gal4 labels the dorsal paired medial (DPM) neurons, which are critical to memory consolidation. This pair of serotoninergetic/peptidergic neurons innervate all the MB lobes (Waddell et al., 2000; Yu et al., 2005; Keene et al., 2006; Haynes et al., 2015). Activation of *c316-Gal4*-targeted neurons fully inhibited the SING reflex (PI = 0,001) (**Figure 1A**), while flies were not paralysed (data not shown). However, this driver expresses in many other neuropils in the brain, including some of the MB KCs (Wu et al., 2011), so more specific drivers should be used to evaluate the role of the DPM in SING modulation. Unlike deficits in learning and memory resulting from neuronal inhibition with *c316-Gal4*, silencing with this driver had apparently no significant effect on the locomotor response (**Figure 1A**).

Increasing octopamine and tyramine release by expressing dTrpA1 with *Tdc2-Gal4* caused decreased startle-induced locomotion (**Figure 1B**). Because octopaminergic neurons innervate some of the MB-associated DANs (Burke et al., 2012), the observed decrease in SING performance observed could be explained either by direct modulation of the MBs or

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indirect effects of octopaminergic or tyraminergetic neurons on MB-associated DANs or other potential neuropils. *Trh>dTrpA1* flies showed a decrease of SING performance at 32 °C (**Figure 1C**), suggesting that serotonin modulates the locomotor response of fly to stimuli as well.

Glutamatergic neurons and GABAergic neurons are widely distributed in the nervous system, and the motor neurons are glutamatergic in *Drosophila*. Apart from *VGlut>dTrpA1* flies showed slight response to startle. Neuronal activation or silencing driven by *VGlut-Gal4* or *OK371-Gal4* (glutamate), and by *Gad1-Gal4* (GABA) caused, as expected, fly paralysis even without startle (**Figure 1D-F**).

2. The fan-shaped body is involved in SING modulation

The FSB, EB, protocerebral bridge, and noduli constitute the central complex (CC), which was proposed to correspond to the vertebrate basal ganglia (Strausfeld and Hirth, 2013; Fiore et al., 2015). CC is suggested to have a role in locomotion control (Strauss, 2002; Martin et al., 1999; Kahsai et al., 2010). In part I, we have ruled out the role of EB in the SING modulation. Therefore, we continued to test whether FSB would be implicated in neural network of SING control. The FSB is composed of horizontal layers and vertical columns (Wolff et al., 2015; Weir et al., 2015). We used *R52G12-Gal4* and *R89C06-Gal4* targeting horizontal layers and *R94C05-Gal4* and *R84C10-Gal4* labelling vertical columns. Neuronal activation driven by *R52G12-Gal4* or *R94C05-Gal4* caused a similar ~25% decrease in SING PI (**Figure 2A, 2C**), while *R89C06-Gal4* and *R84C10-Gal4* had no effect (**Figure 2B, 2D**). *R52G12-Gal4* labels more posterior and anterior neurons than *R89F06-Gal4* (Weir et al., 2015). Indicated by the pattern below, *R52G12-Gal4* labels the FSB more selectively and stronger than *R89F06-Gal4*, while *R89F06-Gal4*, not *R84C10-Gal4* labels the ventral FSB. This suggests that the ventral FSB may play a role in SING, as a decrease in SING performance was observed for *R52G12>dTrpA1* and *R94C05-Gal4>dTrpA1* flies. Altogether, these data indicate that the FSB might be part of neural circuits and act as pre-motor center coordinating SING modulation. Because the FSB is innervated by neurons using a variety of neurotransmitters and neuropeptides (Kahsai and Winther, 2011), whether FSB involves in DA-mediated neural network need to be elucidated.

3. Further data on SING control using MB split drivers

Split-Gal4 drivers were generated by the combination of an enhancer with p65ADZp and another enhancer with ZpGAL4DBD to drive expression in more specific brain regions that are targeted by both enhancers. The reconstitution of an active GAL4 transcription factor requires heterodimerization of p65ADZp and ZpGAL4DBD, which only occurs in cells expressing both proteins (Luan et al., 2006; Pfeiffer et al., 2010). We selected some of the split-Gal4 drivers that were generated previously (Aso et al., 2014a) and used them in our SING modulation test.

We used *Mb011B-Gal4* that labels the MBON-M4/M6. Neuronal activation with this split driver caused a small but significant effect on startle-induced locomotion, in agreement with our previous results (**Figure 3A**). Neuronal activation or inactivation with *Mb077C-Gal4* that targets the MBON- γ 2 α '1 showed no effects (**Figure 3B**).

We have shown previously that thermoactivation of MBON-V2 (MBON- α '3ap, MBON- α 2sc) decreased SING performance. However, neuronal activation with *Mb594C-Gal4* that labels MBON-V2 did not elicit any change in SING performance, possibly due to the low expression of this driver (**Figure 3C**). In contrast, and interestingly, neuronal activation with *Mb082C-Gal4*, which labels MBON-V3 (MBON- α '2 and MBON- α 3) (Shyu et al., 2017), decreased SING performance (**Figure 3D**). We showed before that activation with *G0239-Gal4* (MBON-V3 α 3) had no effect. So neuronal activation of MBON- α '2 appears selectively responsible for the effect on SING performance, suggesting that MBON- α '2 may be downstream to the PPL1 DAN MB-V1 (α '2, α 2), conveying the information from DAN>KC to the SMP neuropil.

Mb022B-Gal4 labels the octopamine-ventral paired medial 3 (OA-VPM3) and OA-VPM4 which utilizes octopamine as neurotransmitter. The cell bodies of these neurons lie in the SEZ, but they send large projections covering the PAM cluster, crepine, FSB, MB peduncle, SMP, SIP, SLP and then reach to the MB calyx (according to Flylight). Unlike the slight effect obtained from the other split drivers, at 32°C, *Mb022B>dTrpA1* flies showed nearly 40% decrease in SING PI compared to the control, demonstrating that octopamine is implicated in SING modulation (**Figure 3E**). On the contrary, the neuropeptide SIFamide may be not involved, for there is no effect resulting from neuronal activation with *Mb013B-Gal4* (**Figure 3F**).

Results

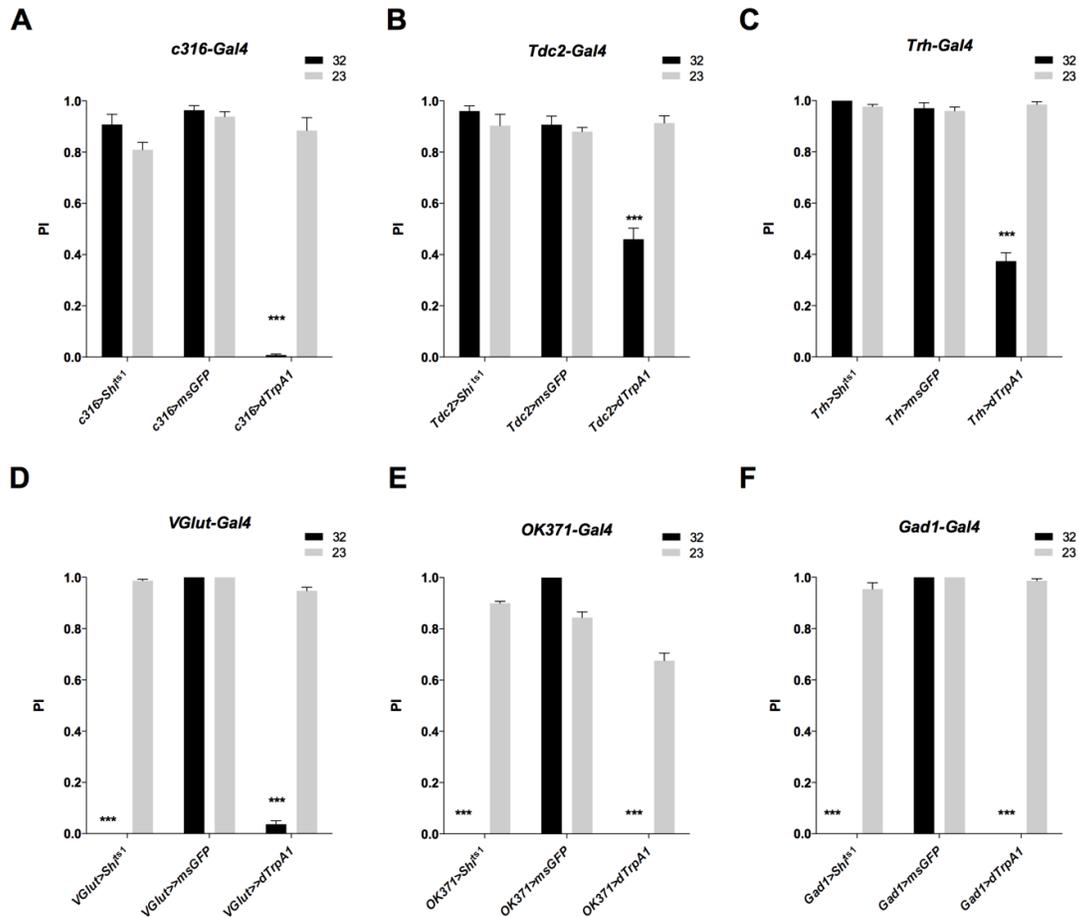


Figure 1. Effect of neurotransmitter-specific drivers on SING modulation

A. *c316-Gal4* expresses in the DPM neurons that project to all MB lobes and in many other brain cells. Flies with neuronal activation in DPM were not paralysis, but did not respond to startle. **B.** *Tdc2-Gal4* (octopamine and tyramine) targets the calyx of the MBs and slightly the MB lobes. Flies with thermoactivation driven by *Tdc2-Gal4* had decreased SING performance. **C.** *Trh-Gal4* (serotonin) targets the MB lobes and accessory calyx. Neuronal activation of serotonergic neurons decreased SING performance. **D-F.** *VGlut-Gal4* (glutamate), *OK371-Gal4* (glutamate) and *Gad1-Gal4* (GABA). Without startle, flies with neuronal activation or inhibition driven by those drivers were paralysed, except *VGlut>dTrpA1* flies, which showed slight locomotor reactivity upon startle.

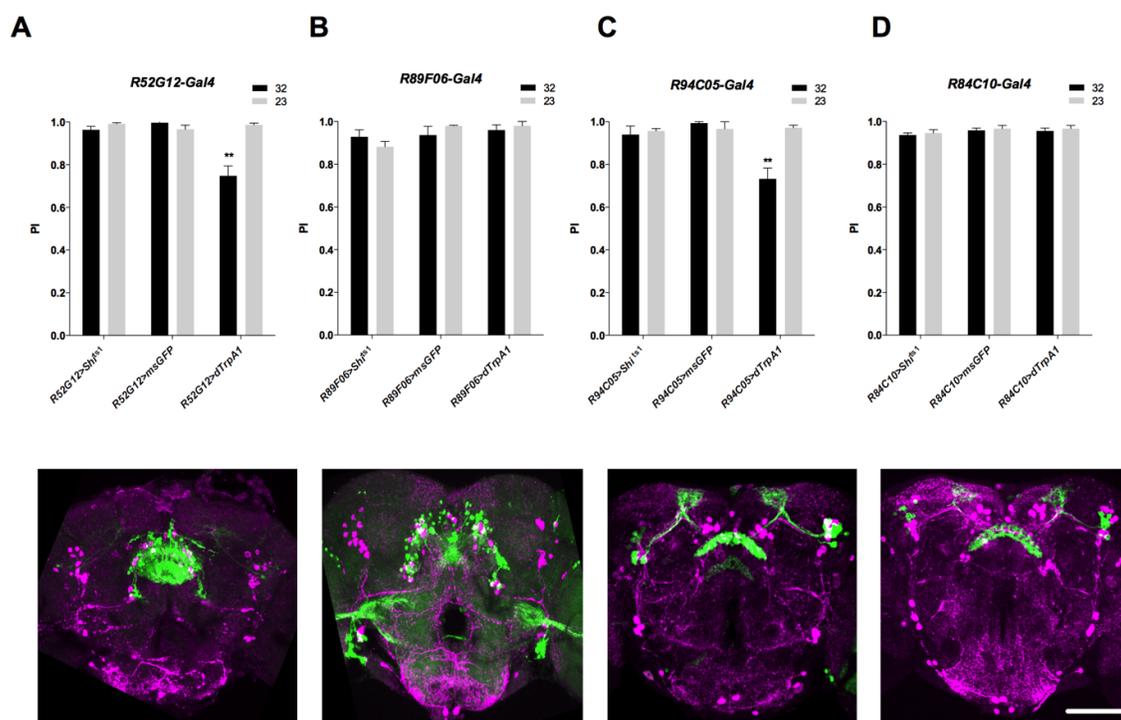


Figure 2 Fan-shaped body may play a role in SING modulation.

A-B. Neurons targeted by *R52G12-Gal4* and *R89F06-Gal4* send projections that constitute of the vertical columns of the FSB. *R52G12-Gal4* labels 262 posterior and 38 anterior FSB neurons (Weir et al., 2015), strongly labelling the ventral FSB. Neuronal activation driven by *R52G12-Gal4* led to SING inhibition. Thermoactivation or inhibition by *R89F06-Gal4* caused no effect on fly locomotion. **C-D.** Projections from the neurons targeted by *R84C10-Gal4* or *R94C05-Gal4* form the horizontal layers of the FSB. *R84C10-Gal4* only labels 31 posterior neurons, localized near the cell bodies of PPL1 DANs. Axons of those neurons, whose dendrites arborized in SMP region, form the horizontal layers of the FSB. Thermoactivation of *R94C05-Gal4*-labelled neurons reduced the SING response. The corresponding pattern of each driver is displayed below the graphs. Scale bar: 100µM

Results

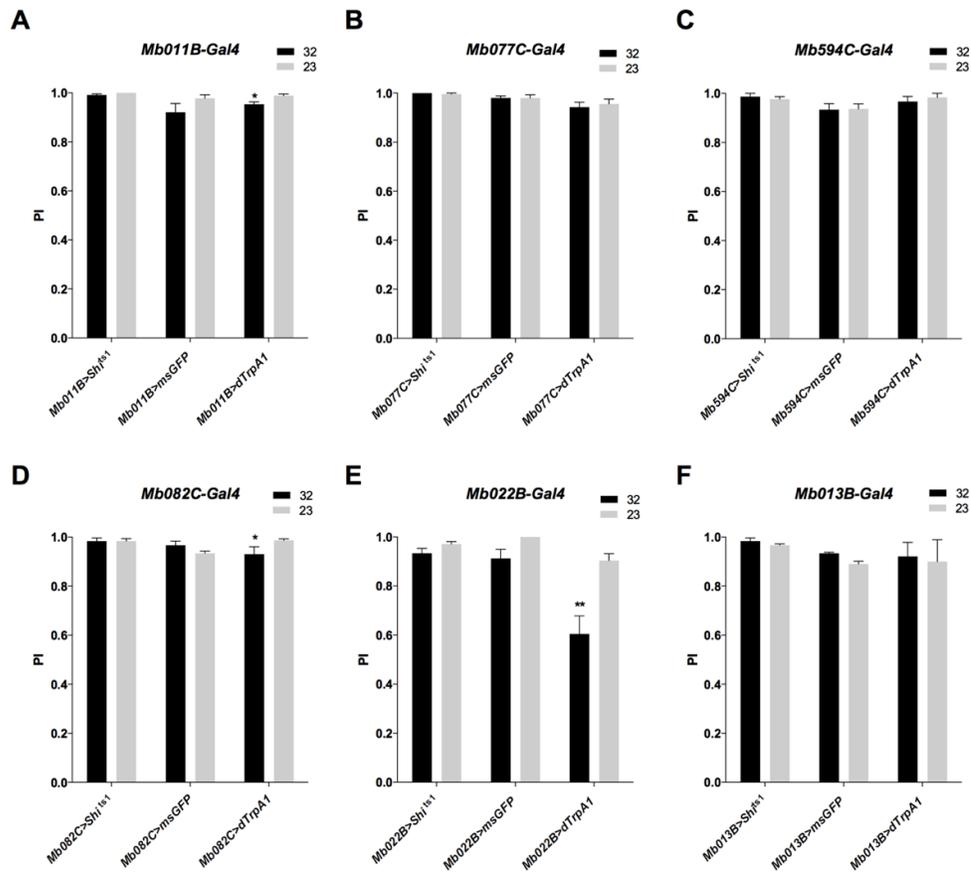


Figure 3 Assay of various split-Gal4 drivers for SING modulation

A. *Mb011B-Gal4* targets MBON-M4/M6. Activating with this driver induced a slight effect on locomotion. **B.** *Mb077C-Gal4* labels the MBON-MV1, neuronal activation and inactivation had no effect. **C.** *Mb594-Gal4* labels the MBON-V2, thermoactivation or silencing in those neurons did not change SING performance. **D.** *Mb082C* targets the MBON-V3 (MBON- α 2 and MBON- α 3). Neuronal activation with this driver caused a slight effect on the SING response. **E.** Neuronal activation by *Mb011B-Gal4* that targets octopaminergic neurons caused a significant decrease in locomotor reactivity. **F.** *MB013B-Gal4* labels the SIFamide neurons and had no effects on SING.

Results B – Non-cell autonomous mechanism of α -synuclein toxicity

Summary

The PD pathology has been suggested to first develop in anterior olfactory structures and the enteric nervous system, then spread to the brainstem and other parts of the CNS in a stereotypical manner along interconnected anatomical pathways. Neuronal degeneration in PD could therefore result from a combination of cell autonomous (CA) and non-cell autonomous (NCA) mechanisms. Previous work done in our laboratory showed that expressing human α -synuclein (α -syn) in only 15 PAM DANs in the *Drosophila* brain led to accelerated age-related locomotor decline (ARLD), as monitored with the SING assay, associated with the loss of their projections to the horizontal lobes of the MB (Riemensperger et al., 2013). Blocking synaptic output of the PAM DANs did not inhibit the SING reflex but, on the opposite, instantly led to a small increase in SING performance, as show in the previous Chapter (Sun et al., 2018). This suggests that the accelerated locomotor defects induced by α -syn did not result from decreased synaptic activity but were an indirect consequence of the slow and progressive degeneration of the DANs. Here we investigated the mechanisms by which α -syn expression in the PAM DANs can progressively decrease fly locomotor reactivity. Because we knew that the MBs are involved in SING control (Riemensperger et al., 2013; Sun et al., 2018), we hypothesized that degeneration of the PAM DANs could disturb the function of their downstream target cells by a non-cell autonomous process and from there progressively impair the whole brain neuronal network that underlies the SING response. Strong evidence from the literature suggests that oxidative stress and mitochondrial dysfunction play a role in α -syn toxicity and PD pathogenesis. Here, we monitored ROS level alterations and mitochondrial dynamics in the PAM cluster and MB neurons in the course of the disease. We show that rescuing the mitochondria by uncoupling protein (UCP) expression either in the PAM DANs or their target MB cells is sufficient to prevent α -syn-induced acceleration of ARLD in the fly model.

Personal contribution: Abdul-Raouf Issa has shown the protective effect of human and *Drosophila* UCP expression against α -syn in the PAM DANs during his PhD. The experiments to visualise mitochondrial dynamics and the effect of non-cell autonomous mechanisms on SING were started by Eva Islimye and Denisa Rusu as a Master 2 and summer internship project, respectively, under the supervision of Serge and myself. I have

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confirmed their initial results and conducted all the other experiments that are described in the following manuscript.

α -synuclein induces locomotor deficits through a non-cell autonomous mechanism in *Drosophila*

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Abstract

Accumulating evidence support the hypothesis that Parkinson disease (PD) is a prion-like pathology that can spread from peripheral structures to the brainstem and then reach other parts of the CNS in a stereotypical manner along interconnected neuroanatomical pathways. Neuronal degeneration and the motor symptoms of PD could therefore result from a combination of cell autonomous and non-cell autonomous mechanisms. In *Drosophila melanogaster*, pan-neuronal expression of human α -synuclein (α -syn) induces progressive locomotor deficits, characterized by accelerated age-related locomotor decline (ARLD). We have shown that expressing mutant α -synA30P in a subset of brain dopaminergic neurons (DANs) of the protocerebral anterior medial (PAM) cluster in *Drosophila* leads to similarly accelerated ARLD associated with the loss of projections from the PAM DANs to the mushroom bodies (MBs). However, inhibition of activity of these PAM neurons failed to induce major locomotor defects, which suggests that the locomotor deficits are not just caused by a dysregulation in DA transmission. Here, we have used genetically-encoded fluorescent probe to monitor mitochondrial dynamics and ROS level alterations in the PAM and MB neurons in the course of the disease. Quite remarkably, we observed that redox defects can transmit non-cell autonomously from the degenerating PAM DANs to their target MB cells in the PAM- α -synA30P model. We show that expressing uncoupling proteins (UCPs), which decrease mitochondrial ROS production, either in the PAM DANs or in their target MB cells is sufficient to prevent α -synA30P-induced locomotor defects and fly

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lethality. Taken together, these findings reveal a novel non-cell autonomous mechanism that couple defects initiated in the PAM cluster to target MBs and underlie locomotor defects induced by α -synA30P expression in DANs in the *Drosophila* PD model.

Key words

α -synuclein - mitochondria - age-related locomotor decline - non-cell autonomous mechanisms - intercellular propagation – Parkinson disease - *Drosophila*

Introduction

Parkinson disease (PD) is a slowly progressive neurodegenerative disorder affecting more than 5% of the population over 85 (Reeve et al., 2014; Rodriguez et al., 2015). It is characterised by the progressive loss of midbrain dopaminergic neurons (DANs) in the substantia nigra *pars compacta* (SNpc) accompanied by the accumulation of cytoplasmic inclusions composed of α -synuclein (α -syn) termed Lewy bodies in neuronal somata or Lewy neurites in dendrites and axons (Poewe et al., 2017). At the clinical level, PD has typically been considered as a motor disorder as patients show a slowness in movement (bradykinesia), resting tremor, muscle rigidity and postural instability. However, non-motor symptoms such as impaired olfaction or disordered sleep can appear prior to the clinical diagnosis of PD, whereas psychosis emerges in later stages (Kalia and Lang, 2015; Postuma et al., 2016; Schapira et al., 2017). Because the aetiology of this disease is complex and multifactorial, and because there are still grey areas in our understanding of the molecular pathways underlying its pathogenesis, neuroprotective treatments are lacking and current therapies are always palliative (Oertel and Schulz, 2016).

In 2003, Braak and colleagues introduced the concept that α -syn could be transferred from the enteric nervous system to the central nervous system (CNS) (Braak et al., 2003a; Braak et al., 2003b; Rietdijk et al., 2017). However, while the prion-like property of α -syn largely supports the Braak hypothesis of PD propagation (Brundin and Melki, 2017), the pertinence of this model for the various forms of PD is still a matter of controversy (Surmeier et al., 2017a). Neuron-to-neuron transmission of α -syn could take place via exocytosis and endocytosis, by exosomes or directly through synaptic contact (Visanji et al., 2013; Dehay et

al., 2016; Grozdano and Danzer, 2017; Wong and Krainc, 2017). In addition, evidence from recent studies suggest that extracellular α -syn can activate surrounding microglia and cause severe reactive oxygen species (ROS) production resulting in the activation of NADPH oxidase and the release of pro-inflammatory factors (Zhang et al., 2005; Glass et al., 2010; McCann et al., 2016), providing a clue that reactive oxygen and nitric oxide species would accelerate PD propagation via a neuroinflammatory mechanism.

The specific loss of DANs is a key feature that distinguishes PD from other α -synucleinopathies (Beyer and Arize et al., 2007). It has been suggested that DANs of the SNpc are particularly vulnerable to cell death in PD due to their unique and elaborate physiology with their thin unmyelinated axons and numerous synaptic terminals whose activity requires a high energy level supplied by local mitochondria (Lotharius and Brundin et al., 2002; Matsuda et al., 2009; Surmeier and Schumacker, 2013; Surmeier 2017b). Highly active mitochondria typically generate large amounts of free radicals that can in turn build up in DANs and lead to oxidative stress. The sustained calcium entry into DAN cytoplasm and the ability of dopamine (DA) to auto-oxidise results in elevated levels of ROS (Surmeier et al., 2010). Interestingly, the disease-causing PD protein, α -syn is susceptible to oxidative modification and is implicated in DA metabolism and release (Venda et al., 2010; Benskey et al., 2016; Bridi and Hirth, 2018). Therefore, modified or aggregated α -syn could selectively impair DA homeostasis. Additional perturbations in mitochondrial and proteolytic systems in these DANs could in turn aggravate α -syn aggregation and impaired DA homeostasis, further elevating ROS levels.

Over the past decades, many lines of evidence have shown that mitochondrial dysfunction plays a central role in the pathogenesis of PD (Exner et al., 2012; Mullin and Schapira, 2013; Bose and Beal, 2016). Interestingly, while α -syn also localizes to mitochondria and its mitochondrial accumulation can disrupt the organelle's dynamics - fusion and fission (Kamp et al., 2010; Nakamura et al., 2011). Mitochondria are the primary source of ROS that are produced in the process of oxidative phosphorylation, but also the main targets of ROS as oxidative damage of mitochondrial proteins or DNA can result in mitochondrial defects (Lin and Beal, 2006). To protect themselves against high ROS levels, mitochondria are however equipped with antioxidant defence including the presence of uncoupling proteins (UCPs) in the inner mitochondrial membrane that allow protons to leak into the matrix and lower ROS generation (Krauss et al., 2005; Wolkow and Iser, 2006). Indeed, both *in vitro* and *in vivo* studies showed that human UCP2 (hUCP2) upregulation can provide neuroprotection against

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neuronal stress (Diano et al., 2003; Mattiasson et al., 2003), and mice that overexpress hUCP2 are resistant to 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-induced nigral DAN degeneration (Andrews et al., 2005a; Andrews et al., 2005b). In *Drosophila melanogaster*, neuronal expression of hUCP2 in adult stage extended lifespan (Fridell et al., 2005) and protected DANs in a rotenone-induced model of PD (Islam et al., 2012; Hwang et al., 2014). The *Drosophila* uncoupling protein UCP5/Bmcp (DmUCP5) is predominantly expressed in the brain (Fridell et al., 2004; Sánchez-Blanco et al., 2006) and its overexpression rescued locomotor defects in a fly glial model of Huntington disease (Besson et al., 2010).

In *Drosophila*, the neuronal expression of human α -syn accelerates age-related locomotor decline (ARLD), which was generally monitored by a startle-induced negative geotaxis (SING) climbing assay (Feany and Bender, 2000; Barone et al., 2011; Butler et al., 2012; Riemensperger et al., 2013; Breda et al., 2015; Ordonez et al., 2018). However, the cellular mechanisms behind this progressive locomotor defect have not been well explained to date. We have shown that α -syn expression (particularly its mutant pathogenic form α -synA30P) in a small number of DANs of the protocerebral anterior medial (PAM) cluster in the fly brain recapitulates key PD characteristics including premature ARLD accompanied with the loss of projections from the DANs onto the mushroom body (MB) (Riemensperger et al., 2013). The MB is a paired brain structure in insect involved in the control of essential brain functions such as memory, sleep and locomotion. DANs innervate all the lobes of the MBs (Tanaka et al., 2008; Mao and Davis, 2009; Pech et al., 2013; Aso et al., 2014) that are made up of projections from specific neurons, the Kenyon Cells (KC), known to be peptidergic and cholinergic (Knapek et al., 2013; Barnstedt et al., 2016). Recent studies from our and other laboratories provided evidence that synaptic projections of the DANs to the MBs, and the MB KCs themselves, are involved in the control of locomotion (startle-induced climbing and flight) (Riemensperger et al., 2013; Bou Dib et al., 2014; Agrawal and Hasan, 2015; Pathak et al., 2015; Vaccaro et al., 2017; Sun et al., 2018). However, we have also observed that activity of these DAN>MB synapses is not necessary for expression of the SING reflex (Sun et al., 2018). This led us to hypothesize that α -syn-induced degeneration of the PAM DANs could disturb the function of their downstream target cells by a non-cell autonomous (NCA) process that would propagate and progressively disrupt the SING neuronal network.

One of the consequences of α -syn accumulation in neurons is an increase in oxidative stress, as was shown both in mammalian cells and *Drosophila* (Junn and Mouradiah, 2002; Parihar

et al., 2008; Barone et al., 2011; Wang et al., 2015). Here, we monitored ROS levels and mitochondrial dynamics in the PAM DA and MB neurons in the *Drosophila* α -syn PD model. Using genetically-encoded fluorescent probes to monitor mitochondrial dynamics and redox status *in situ*, we observed that α -synA30P expression in the PAM cluster disrupted mitochondrial turnover and increased ROS levels both in the DANs and in their MB target. We show that increasing mitochondrial uncoupling by co-expressing hUCP2 or DmUCP5 together with α -synA30P in the PAM DANs fully rescued α -syn-induced locomotor defects. Remarkably, UCP expression selectively in the MBs also rescued premature ARLD and the lethality induced by α -synA30P expression in the PAM DANs. These findings support a model in which impaired mitochondrial function in DANs would non-cell autonomously induce mitochondrial perturbations in interconnected neighbouring neurons to trigger PD-like symptoms in *Drosophila*.

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ROS accumulation is implicated in α -synA30P-induced locomotor deficits

The prolonged pan-neuronal expression of wild-type and mutant forms (A30P and A53T) of human α -syn in *Drosophila* established the first transgenic PD model (Feany and Bender, 2000). It recapitulated, defining features of PD including adult-onset loss of selective DN clusters, Lewy body-like α -syn-positive neuronal inclusions and accelerated locomotor dysfunction. Interestingly, flies with α -synA30P expression exhibited more severe ARLD compared to flies overexpressing wild-type (WT) α -syn (Feany and Bender, 2000; Riemensperger et al., 2013). Here we monitored ROS levels in whole brains of flies expressing either α -synWT or α -synA30P in all neurons with the *elav-Gal4* driver using dihydroethidium (DHE) staining. ROS and oxidative damage are known to accumulate in the mammalian and *Drosophila* brain during aging (Lu et al., 2004; Murali and Panneerselvam, 2007; Hirano et al., 2012; Oka et al., 2015; Vaccaro et al., 2017). Here we confirmed that ROS levels increases with age as flies aged 30 days after adult eclosion (d a.E.) exhibited higher brain ROS signals than 2-day old flies independently of the genotype (*elav>w*¹¹¹⁸ (control), *elav> α -synWT* and *elav> α -synA30P*) (Figure 1A-B). Interestingly, *elav> α -synA30P* brains showed higher oxidised DHE fluorescence compared to *elav>w* and *elav> α -synWT* brains, both in flies aged 2 and 30 d a.E. (Figure 1A-B).

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In order to determine if the locomotor defects caused by α -synA30P expression were related to ROS accumulation in DANs, we used a previously established PD model in which α -synA30P is selectively expressed in a subset of PAM DANs with the *NP6510-Gal4* driver (Riemensperger et al., 2013). We co-expressed with this driver human or *Drosophila* uncoupling proteins, (hUCP2 or DmUCP5, respectively), together with α -synA30P in the PAM DANs. Strikingly, increased mitochondrial uncoupling largely improved fly locomotion compared to flies expressing α -synA30P alone (Figure 1C). The ARLD profile of the UCP-rescued α -syn30P-expressing flies was actually similar to that of wild-type flies (Riemensperger et al., 2013). UCP also reduced the lower locomotor deficits of *NP6510-Gal4*> *α -syn^{WT}* flies but this effect was only significant at week 4 (Figure 1D). This indicates that increasing UCP activity, and therefore decreasing ROS production by mitochondria, in the PAM DANs can efficiently rescue the fly locomotor deficits induced by α -synA30P expression in these cells.

α -synA30P expression modifies mitochondrial structure in the PAM neurons

We then attempted to look at mitochondrial structure when human α -synA30P is expressed in the fly DANs. PAM is the largest DAN cluster in the *Drosophila* brain that possesses around 100 neurons mainly projecting to the horizontal lobes of the MB. We used the *R58E02-Gal4* driver, whose regulatory sequence are from the first intron of the *Drosophila* DA transporter (*DAT*) gene (Liu et al., 2012), to express α -synA30P in all PAM neurons with mitoGFP. mitoGFP is a mitochondria-targeted GFP transgene that allowed to monitor mitochondrial structure and dynamics in the course of the disease. mitoGFP fluorescence was found to increase dramatically with age between 2 and 10 d a.E. and it appeared that α -synA30P expression caused a further fluorescence increase that was, interestingly, more prominent at day 10 than at day 30 (Figure 2A and Figure S1).

Next we selected two regions to visualize mitochondria in the PAM DANs at higher magnification: the crepine (CRE) that contains the axons and the tip of the horizontal lobes where the dopaminergic projections arborize. In the CRE region, axonal mitochondria showed a rather fragmented and blob-like structure in the *R58E02*>*mitoGFP*, *α -synA30P* flies, whereas mitochondria in controls without α -synA30P appeared more fibrillar in shape with a bit less strong fluorescence intensity (Figure 2B, top panels). Mitochondria at the tip of the horizontal lobes had a round morphology at day 30 a.E. with or without α -synA30P (Figure 2B, bottom panels). Quantification of the blob-shaped mitochondria in the PAM

neurons on all confocal scans showed that the presence of α -synA30P generally widened their size distribution profile at day 30, with a shift toward larger sizes (Figure 2C). Therefore, α -synA30P expression in the PAM DANs modifies mitochondrial morphology from a tubular shape to round and more swollen structures as visualised by mitoGFP.

α -synA30P alters the redox status of PAM DAN mitochondria

MitoTimer is another fluorescent probe derived from a mutant of the protein DsRed (Terskikh et al., 2002) that is used to monitor mitochondrial dynamics (Hernandez et al., 2013; Ferree et al., 2013; Laker et al., 2014; Gottlieb and Stotland, 2015). MitoTimer's fluorescence shifts from green for the newly synthesised form to red of the mature oxidized form. This colour shift is independent from pH, ion and protein concentration, but is affected by temperature, oxygen, and light exposure (Terskikh et al., 2000; Gross et al., 2000). Interestingly, MitoTimer was spontaneously more oxidized in the PAM DANs than in other brain cells as early as 2 d a.E: indeed, MitoTimer expression driven by *R58E02-Gal4* exhibited intense red fluorescence and weak green signal, indicative of higher redox levels in these cells (Figure S2). Using this probe, we also observed that there was nevertheless a significant increase in the red/green ratio induced by α -synA30P expression in the PAM DANs at day 10 compared to the control (see Figure S6A). This suggests that MitoTimer is more rapidly oxidized in DANs in the presence of α -synA30P.

To further validate this observation, we took advantage of a redox sensitive GFP (roGFP) fused to Grx1 (mito-roGFP2-Grx1, named here mito-roGFP2) (Albrecht et al., 2014). roGFP2 was engineered such that two excitation maxima characteristics of GFP are altered according to the redox context (Gutscher et al., 2008; Lukyanov and Belousov, 2014; Meyer and Dick, 2010): reduced roGFP2 exhibits a predominant excitation at around 488 nm, while the oxidized form gains excitability at 405 nm and is no more excitable at 488nm. At the 405 nm excitation wavelength, we could detect oxidized roGFP2 signal in the brain but also some background endogenous fluorescence in the trachea (Figure 3A). We then compared the redox levels at different ages by computing the 405/488 nm oxidized/reduced signal ratio in a region of interest including the PAM clusters in *R58E02>mito-roGFP2* and *R58E02>mito-roGFP2, α -synA30P* flies. The presence of α -synA30P led to a 20% and 35% increase in the oxidized/reduced ratio of mito-roGFP2 at day 10 and day 30, respectively (Figure 3B). Together, these observations suggest that α -synA30P accumulation progressively increases mitochondrial ROS level in the PAM DANs.

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PAM expression of α -synA30P induces mitochondrial dysfunction in the MBs

Recent work from our laboratory demonstrated that the MBs are required for DA modulation of the SING reflex (Sun et al., 2018). Because the PAM DANs project to the MBs, we hypothesised that α -synA30P-induced mitochondrial defects in the PAM neurons could transfer to the MB lobes, leading to accelerated ARLD. We therefore monitored the mitochondrial status by expression fluorescent probes selectively in the MBs with the *VT30559-Gal4* driver, while simultaneously expressing α -synA30P in the PAM cluster with *R58E02-LexA* (Figure 4A). At day 10, the expression of α -synA30P in the PAM cluster caused a significant 30% increase in the ratio of oxidized/reduced mito-roGFP2 fluorescence in the MB lobes compared to controls (Figure 4B-C), whereas no difference with controls was observed at day 2 and day 30 (Figure 4B and Figure S4). In contrast, mitochondrial morphology of the MB lobes probed by mitoGFP did not change significantly at any age following α -synA30P expression in the PAM cluster compared to the control genotypes (Figure S3). This shows, interestingly, that the expression of α -synA30P in the PAM DANs non-cell autonomously induces an increase in mitochondrial ROS in the MB neurons, at a ‘pre-symptomatic’ time when locomotion is still not significantly altered in the PD-mimic flies.

Due to its prion-like property of α -syn in mammals, we suspected that α -synA30P expressed in the PAM could be released from the DANs and enters the MB KCs, thus inducing elevated ROS and dysfunction of these cells. However, α -syn immunostaining failed to detect obvious MB accumulation of α -syn in the PAM- α -synA30P flies at day 30 (Figure S5A), though it cannot be excluded that a low amount of transfer occurred that would not be detectable. Then we assessed the fly locomotor performance after α -synA30P was directly expressed in the MBs. This appeared innocuous as *VT30559> α -synA30P* flies had a quite similar ARLD profile as the controls (Figure S5B). Finally, flies were constructed that express MitoTimer or mito-roGFP2 together with α -synA30P in the MBs. As shown in Figure S6B and D, in 10-day old flies, there was no effect of α -synA30P on mitochondrial redox level in MB KCs, compared to its cell-autonomous effect in DANs (Figure S6A and C). This suggests that α -synA30P transmission from the PAM to the MB could not by itself trigger mitochondrial perturbation in the KCs.

UCP expression in the MB neurons rescues locomotor deficits of the PAM- α -synA30P flies

Finally, we tested if scavenging ROS accumulation by UCP overexpression in the MBs (*VT30559>DmUCP5* or *VT30559>hUCP2* flies) could rescue locomotor dysfunction induced by α -synA30P expression in the PAM. Indeed, not only did expressing DmUCP5 or hUCP2 in the MBs dramatically ameliorate α -synA30P-accelerated ARLD (Figure 5A and B), but it also rescued the lethality of *R58E02> α -synA30P* adult flies: 1-5% mortality rate at week 5 for flies expressing DmUCP5 or hUCP2 in the MBs and α -synA30P in the PAM, which was similar to the control without α -synA30P, compared to 30% mortality in flies expressing α -synA30P in the PAM only (Figure 5C). These results provide strong evidence that α -synA30P-mediated defects in the PAM DANs impairs mitochondrial redox status and ROS levels in the target MB neurons to affect fly locomotion and, ultimately, survival.

Discussion

In this study, we aimed at investigating the hypothesis that the PD pathology propagates from one cell to another in the nervous system by performing studies in the *Drosophila* PAM- α -synA30P model (Riemensperger et al., 2013). Advantages of this model is that the circuits formed by the PAM DANs and their target MB cells have been extensively characterized because they play a central role in the neuromodulation of many brain functions, including memory, arousal and locomotion. We used different fluorescent reporter probes to examine the consequences of α -synA30P expression on mitochondrial structure and health in the PAM DANs and searched for evidence of a non-cell autonomous (NCA) effect in the MBs. We did observe NCA propagation of mitochondrial toxicity in the MB lobes early in the pathogenesis. Importantly, we were able to rescue ARLD and induced lethality in PAM- α -synA30P flies by expressing human of *Drosophila* UCPs in the MBs, which suggests that a mechanism of propagation from the degenerating DANs to the MBs and other brain cells underlies the locomotor deficits in the *Drosophila* PD model.

Comparison of different fluorescent probes

In this study, we investigated the toxic effects of a mutant form of human α -syn on mitochondrial functions in *Drosophila* DANs and the MBs using three independent fluorescent reporter probes: mitoGFP, MitoTimer and mito-roGFP2-Grx1.

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Several authors previously used mitoGFP to monitor mitochondrial morphology and dynamics under oxidative stress in *Drosophila*. Liao et al. (2017) reported a decrease in mitochondrial length upon H₂O₂ treatment. Ordonez et al (2018) showed mitochondrial enlargement in neurons of the central brain of transgenic flies expressing wild-type α -syn, demonstrating the failure of fission attributed to α -syn-induced lack of translocation of Drp1 to the mitochondrial outer membrane. In another work, rotenone treatment induced a change in mitochondrial morphology from tubular networks and fibrillary to blob-like round mitochondria with strong fluorescent intensity (Ahmad et al., 2013). In the present study, we visualized mitochondrial morphology with mitoGFP and observed that α -synA30P expression in DANs of the PAM cluster led to a higher frequency of large mitochondria at a late stage of the pathology. Given that oxidative stress triggers Parkin/PINK1 pathway-dependent mitophagy (Wang et al., 2012; Xiao et al., 2017), one would expect that α -synA30P induces a decrease in mitoGFP fluorescence intensity. But, unlike other mutant and wild-type forms of α -syn that promote macroautophagy, mutant α -synA30P inhibits the autophagic flux (Koch et al., 2015). Therefore, the increase in mitoGFP fluorescence with α -synA30P expression we observed in the PAM, could be caused by a blockade of the autophagy-lysosomal pathway by α -synA30P preventing mitochondrial degradation.

In the PAM DANs, interestingly, MitoTimer showed a higher red/green ratio compared to other brain cells, indicating that the probe matures faster in these cells and suggesting that they have a naturally higher level of oxidative stress (Figure S2). In addition, α -synA30P expression in the PAM cluster resulted in an even faster maturation of the probe and/or disturbed mitochondrial turnover, as shown by the higher red/green ratio for MitoTimer compared to control without α -synA30P at day 10 (Figure S6A). As another independent way to monitor mitochondrial dysfunction, we used mito-roGFP2-Grx1, with which the ratio of oxidized/reduced form is an index of the redox state of mitochondria (Meyer and Dick, 2010). The results obtained with mito-roGFP2 to monitor the cell-autonomous effect of α -synA30P either in the PAM-DANs or the MBs were consistent with those with MitoTimer (Figure S6). Together, these findings, in agreement with those recently published by Ordonez et al (2018), demonstrated that mitoGFP, MitoTimer, and mito-roGFP2 are useful tools to investigate the mitochondrial turnover and redox level in fly neurons under stress.

α -synA30P induces mitochondrial dysfunction cell autonomously in the PAM DANs but not in the MB cells

Previously we reported that α -synA30P expression in PAM DANs led to locomotor defects and degeneration of their synaptic projections to the MB lobes (Riemensperger et al., 2013). We have now found that α -synA30P also disrupted mitochondrial turnover and redox status in the PAM DANs (Figure 2). This is consistent with a recent study showing that wild-type α -syn expressed pan-neuronally at a high level triggers mitochondrial dysfunction through altered localization of the essential fission protein Drp1 (Ordonez et al., 2018). We observed that the PAM DANs are more oxidised than many other brain cells, which could be due to DA auto-oxidation (Figure S2). This highly oxidative environment could favour α -syn aggregation and contribute to its cell-autonomous toxicity. Aggregated α -synA30P could then impair the homeostasis of DA metabolism and release (Venda et al., 2007; Benskey et al., 2016; Bridi and Hirth, 2018), thereby resulting in further elevated ROS levels. Interestingly, α -synA30P expression selectively in the MBs showed no effect on ARLD, suggesting that α -synA30P is not toxic to KCs (Figure S5B). In addition, at day 10, flies expressing α -synA30P in the MB KCs exhibited no disturbance of mitochondrial redox status compared to controls (Figure S6B, D). The lack of α -synA30P-mediated neurotoxicity in cholinergic/peptidergic neurons of the MBs is therefore likely due to the inability of α -synA30P to aggregate and generate pathological ROS in these cells.

 α -syn-induced mitochondrial defects propagate from the PAM DANs to their MB target by a non-cell autonomous mechanism

Here, using mito-roGFP2-Grx1, we detected alterations in mitochondrial redox status in MB neurons at day 10 resulting from α -synA30P overexpression in the upstream PAM DANs (Figure 4C). How exactly did the toxicity propagated from the DANs to their target neurons to induce mitochondrial perturbations in MB neurons remains to be elucidated. We could not detect convincing evidence that α -synA30P itself is transferred from one neuron to the other in this model (Figure S5A). The propagation of ROS by a NCA mechanisms has previously been reported in a mammalian cell PD model, in which α -syn induced $O_2^{\bullet-}$ production in activated microglia that acted then on DANs (Zhang et al., 2005). Interestingly, the trans-synaptic effect of α -synA30P on MB mitochondria was transient as no difference with controls could be detected at day 30 (Figures 3B and S4). As we showed previously, the number of PAM DANs>MBs synaptic projections are already strongly reduced at day 20

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when α -synA30P is expressed in the PAM (Riemensperger et al., 2013). These synapses are therefore likely to be absent at day 30, which may explain why the mitochondrial perturbations do not transfer between the two cell types anymore at this age. We propose therefore that ROS accumulation induced by α -synA30P expression in the DANs could trans-synaptically transfer to their target MN neurons to affect the latter's cellular redox state.

α -syn-induced locomotor deficits result from a non-cell autonomous mechanism

Our study demonstrates that co-expressing mitochondrial UCP proteins in DANs can significantly ameliorate ARLD when α -synA30P is expressed in the PAM neurons (Figure 1C). Surprisingly, UCP expression in the MB neurons also much improved ARLD and fly survival in the PAM- α -synA30P model (Figure 5). It appears, therefore; that in the PAM- α -synA30P flies, degeneration of the DANs can disrupt the function of their downstream MB target cells by a NCA process. Our hypothesis is that mitochondrial dysfunction could propagate from there to other cells, thus progressively impairing the whole brain network that underlies the SING response and compromising in the end fly survival.

Overall, our work provides evidence for a NCA process that links dysfunction in DANs to downstream defects in target MB neurons. Future studies will aim at identifying the nature of the toxic species that transfer from one cell type to another and the mechanisms involved.

Material and methods

***Drosophila* culture and strains**

Fly stocks were raised and crossed at 25°C on the standard *Drosophila* corn meal-yeast-agar medium supplemented with methyl-4-hydroxybenzoate as a mold protector, under a 12h-12hr light-dark cycle. The following effector lines were used: *UAS-mitoGFP* (Pilling et al., 2006), *UAS-MitoTimer* (Laker et al., 2014), *UAS-mito-roGFP2-Grx1* (Albrecht et al., 2011), *UAS- α -synA30P* and *UAS- α -syn WT* (Feany and Bender, 2000), *UAS-hUCP2* and *UAS-DmUCP5* (Fridell et al., 2005; Besson et al., 2010), and *LexAop- α -synA30P* (this study). The driver lines used are: *R58E02-Gal4* and *R58E02-LexA* (Liu et al., 2012), *NP6510-Gal4* (Liu et al., 2006), *elav-Gal4* (*elav*^{C155}, #458), *VT30559-Gal4* (gift from Thomas Preat).

Startle-induced negative geotaxis (SING) assay

Over 75 male flies were collected to assay for ARLD by the SING test as previously described (Riemensperger et al., 2013; Vaccaro et al., 2017). Ten-day old flies were used for the first experimental day. Groups of 10 flies of the same genotype were collected one night before the test and placed in a vertical column (25 cm long, 1.5 cm diameter) with a conic bottom end. After startle for 1 min, flies having reached the top of the column (above 22 cm) and flies remaining at the bottom end (below 4 cm) were separately counted. Three rounds of test were performed three times in a row per column. Results are the mean \pm SEM of the scores obtained with ten groups of flies per genotype. The performance index (PI) is defined as $\frac{1}{2}[(n_{tot} + n_{top} - n_{bot})/n_{tot}]$, where n_{tot} , n_{top} , and n_{bot} are the total number of flies, the number of flies at the top, and the number of flies at the bottom, respectively. To test the ageing effect, the flies were flipped back to keep ageing for the subsequent evaluation every week to detect the locomotor performance with age. Dead flies were replaced by flies of the same genotype and age. Two-way ANOVA and Tukey's *post-hoc* test for multiple comparisons was used to compare ARLD across genotypes at different time points.

Use of genetically-encoded mitochondrial fluorescent probes

Brains of flies expressing MitoTimer or mitoGFP were quickly dissected in ice-cold Ca^{2+} -free *Drosophila* Ringer's solution, fixed for 20 min in 4% PFA in PBS and washed 3x 20 min in PBS on an agitator at room temperature. For mito-roGFP2-Grx1, brains were freshly dissected in PBS and transferred immediately for confocal scanning. All the brains were mounted in ProLong Gold Antifade Reagent for confocal visualisation. excitation/emission (488/515-530 nm) was set for mitoGFP fluorescent detection, while green (excitation/emission 488/515-530 nm) and red (excitation/emission 560/570-620 nm) were used for MitoTimer detection. As to mito-roGFP2-Grx1, this biosensor was excited sequentially at 405 nm and 488 nm (line by line) and detected at 500-530 nm (Albrecht et al., 2014). Settings for different probes were kept constant across samples for the comparison for time course studies. The image quantification (fluorescence intensity and mitochondrial perimeter) was done using Fiji software (Schindelin et al., 2012).

To quantify mitochondrial morphology, and in particular “blob”-like mitochondria, the whole PAM cluster, including the DAN cell bodies and their projections, was imaged by confocal microscopy. We set the circularity of the analysed particles making sure that our analysis only included round mitoGFP-positive structures but not fibrillary mitochondria. The size of mitochondria was determined by the perimeter indicated by the round particles. Data

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generated in Fiji were then used in Graphpad Prism to compute the Gaussian distribution of mitochondrial perimeters.

To exhibit the redox status of mitochondria *in situ*, oxidized roGFP2 stack was directly divided by the reduced roGFP2 stack by the image J → process → Image Calculator → divide. The calculated data were processed by the Image J plugin 3D surface plot (kindly provided by Diana Zala in Centre de Psychiatrie et Neuroscience) to get final images shown in Figure 4C and Figure S4.

Immunostaining

Adult brains were dissected in ice-cold *Drosophila* Ringer's solution and processed for whole-mount immunostaining as previously described (Riemensperger et al., 2011). Primary antibodies were mouse anti-FasII (DSHB, 1:50) and rabbit anti- α -syn (Abcam, 1:1000). Secondary antibodies were goat anti-mouse and anti-rabbit conjugated to Alexa fluor 488 or 555 (Invitrogen Molecular Probes, 1:1000). The brains were mounted in ProLong Gold Anti-fade reagent (Thermo Fisher Scientific) with the anterior side facing upward. Images were acquired on a Nikon AIR confocal microscope and processed using the Fiji software.

ROS detection and quantification

ROS levels in brains were detected with the fluorescent dye dihydroethidium (DHE) (Life Technologies) following a published protocol adapted to whole-mount *Drosophila* brains (Owusu-Ansah et al., 2008; Vacarro et al., 2017). Briefly, brains of 5-10 flies from different genotypes were quickly dissected in Schneider's insect medium, and incubated on an orbital shaker in 30 μ M DHE diluted in Schneider's medium for 5-7 mins. After 3 5-min washes with Schneider's medium, brains were fixed quickly for 7 mins in 7% formaldehyde in PBS, rinsed 5 mins in PBS, then mounted in Vectashield (Vector Laboratories). Images were captured immediately after staining on a Nikon AIR confocal microscope. The average intensity of the stacks of Z-projections was processed by Fiji software for the whole brain.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 6. Data were analysed using one-way ANOVA or two-way ANOVA with Bonferroni's or Tukey's *post-hoc* tests for multiple comparisons.

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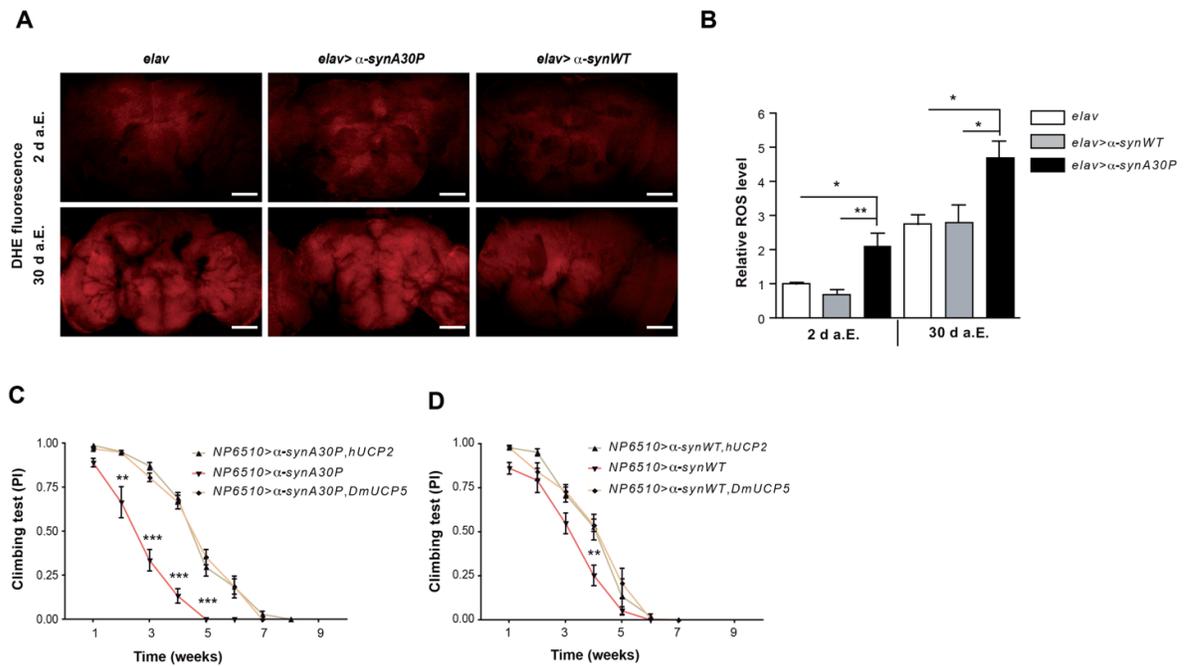


Figure 1 Mitochondrial UCPs prevents locomotor defects induced by α -synA30P expression in PAM DANs. (A) Representative images of DHE staining of adult flies aged 2-day or 30-day after eclosion (d a.E.) expressing no or mutant (A30P) or wild type (WT) α -syn in all neurons. Scale bars: 50 μ m. (B) Quantification of relative ROS levels in whole brains of different genotypes at day 2 or day 30. Compared to controls, brains with pan-neuronal α -synA30P overexpression driven by *elav-Gal4* showed increased ROS levels at day 2 and day 30 while α -synWT expression showed no effect on brain ROS levels. Scale bar: 100 μ m. (C) Flies co-expressing hUCP2 or DmUCP5 with α -synA30P driven by *NP6510-Gal4* showed delayed α -synA30P-induced locomotor defects compared to its respective control *NP6510> α -synA30P*. (D) Compared to control *NP6510> α -synWT* flies, co-expression of hUCP2 or DmUCP5 proteins with α -syn WT significantly improved fly locomotion at week 4 only.

Results

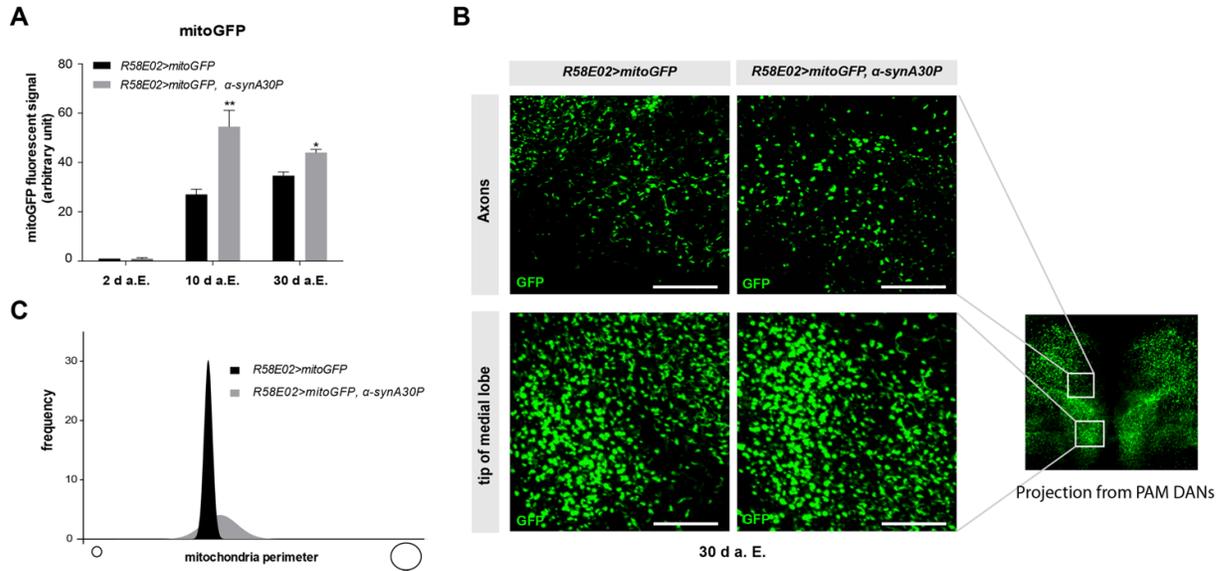


Figure 2 α -synA30P accelerates age-related mitochondrial alterations in the PAM cluster. (A) *R58E02>mitoGFP* flies showed increase in fluorescence intensity with age in the PAM cluster. α -synA30P expression in these DANs caused a significant increase in mitoGFP fluorescence intensity at day 10 and day 30. (B) Distribution of mitochondrial size for *R58E02>mitoGFP* and *R58E02>mitoGFP, α -synA30P* flies at day 30. Expression of α -synA30P in the PAM cluster induced significant difference in mitochondrial morphology compared to controls, with more numerous enlarged mitochondria. (C) High magnification images of mitochondrial morphology. Mitochondria from axons lying in the crepine neuropil and at the tip of the MB medial lobes are shown. Mitochondria in PAM neurons have a fibrillary shape in *R58E02>mitoGFP* flies, whereas *R58E02>mitoGFP, α -synA30P* flies showed increased number of round mitochondria. Scale bars: 10 μ M.

Results

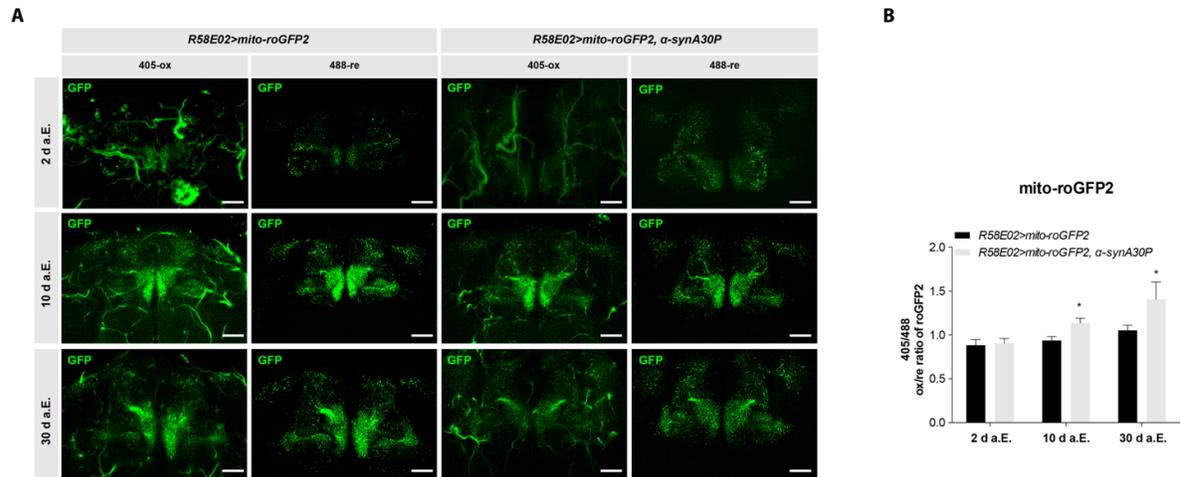


Figure 3 α -synA30P progressively impairs mitochondrial redox status in PAM neurons
(A): Representative images of oxidized roGFP2 (405 nm) and reduced roGFP2 (488 nm) patterns in the fly brain with or without α -synA30P expression in PAM DANs at different age. Scale bars: 30 μ M. (B): Quantification of mitochondrial redox status. 10-day-old flies co-expressing mito-roGFP2-Grx1 (denoted as mito-roGFP2) with α -synA30P in PAM showed an increased ratio of oxidized/reduced mito-roGFP2 compared to control flies without α -synA30P expression.

Results

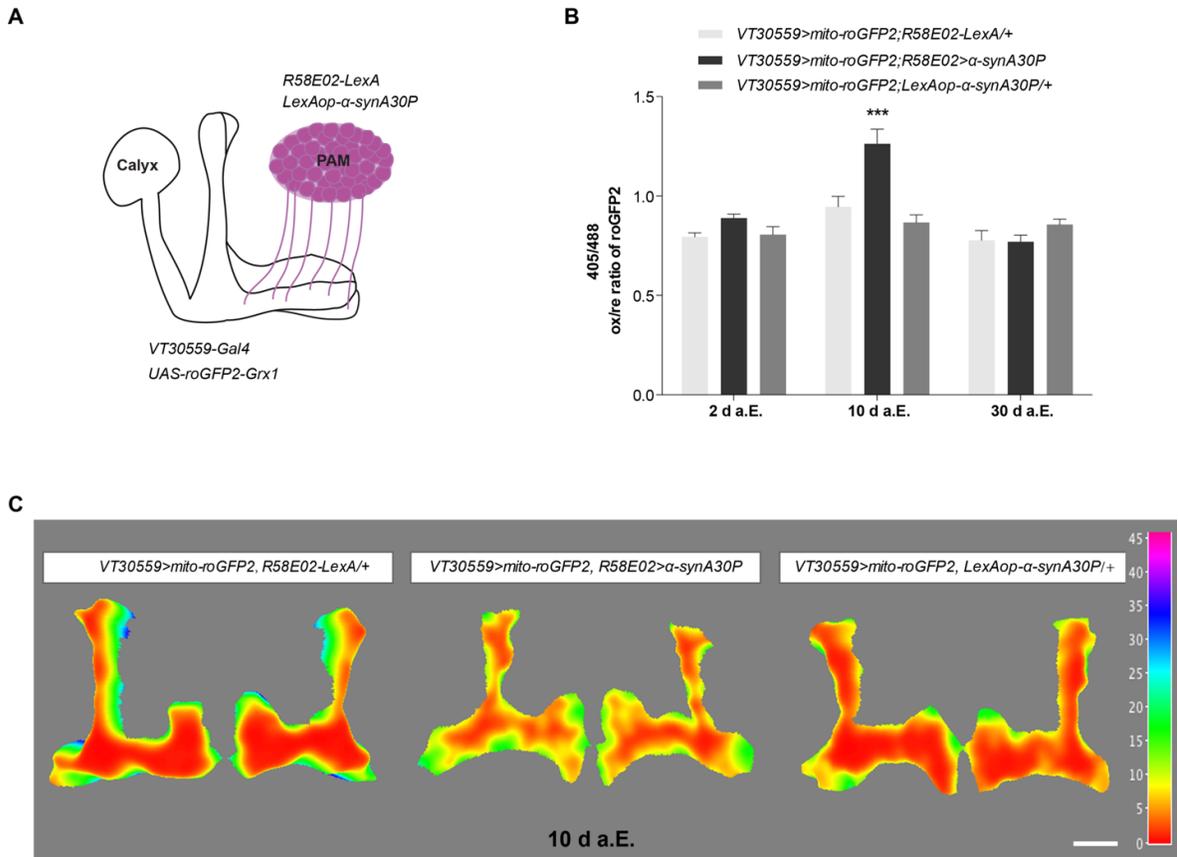


Figure 4 ROS levels increase in target MB neurons when α -synA30P is expressed in the PAM. (A) Schematic representation of the dual *in vivo* expression system that allows independent expression of α -synA30P in PAM DANs with *R58E02-LexA*, while simultaneously monitoring the mitochondrial redox status in the MB by expressing mito-roGFP2 with *VT30559-Gal4*. (B) At day 10, flies with α -synA30P in the PAM DANs showed increased oxidized/reduced (ox/red) ratio of mito-roGFP2 in the MB lobes. There was no difference in MB mito-roGFP2 ox/red ratio at day 30 compared to control. (C) 3D-reconstruction of mito-roGFP2 ox/re ratio in the MB lobes at day 10. False color representation. Scale bar: 30 μ m.

Results

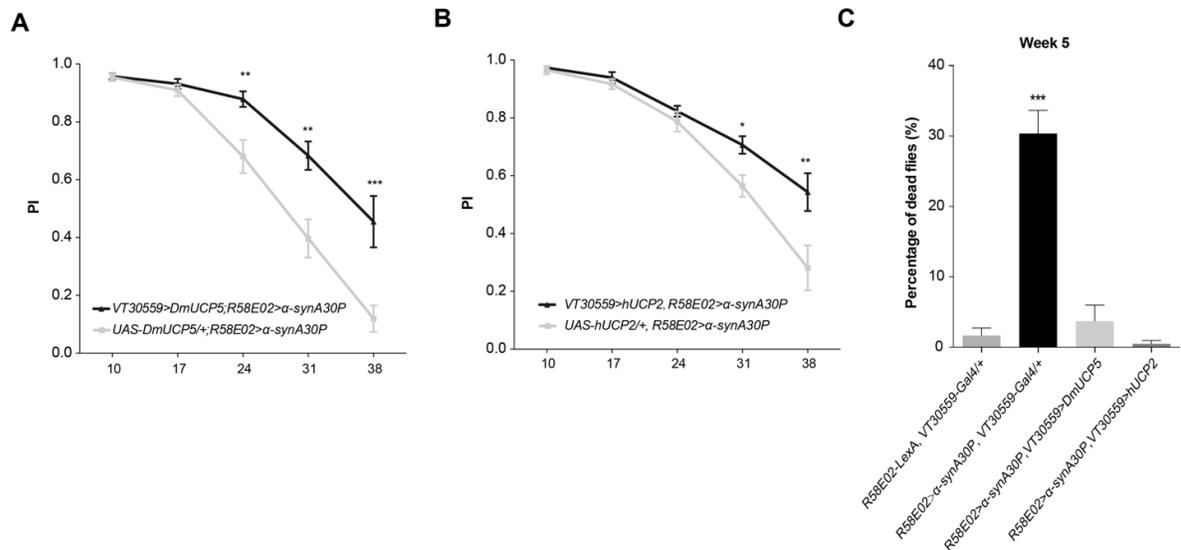


Figure 5 Expression of mitochondrial UCPs in the MBs prevents locomotor deficits and lethality induced by α -synA30P expression in PAM DANs. (A) MB expression of DmUCP5 (driven by *VT30559-Gal4*) significantly ameliorated the SING response in the PAM- α -synA30P flies. (B) hUCP2 overexpression in the MB contributed to the same extent to the alleviation of PAM- α -synA30P-induced locomotor deficits. (C) α -synA30P expression in the PAM cluster increased the fly lethality rate that was ~30% at week 5 compared to ~3% in controls without α -synA30P. Overexpressing DmUCP5 or hUCP2 in the MBs rescued the lethality of the PAM- α -synA30P flies.

Results

Supplemental Figure Legend

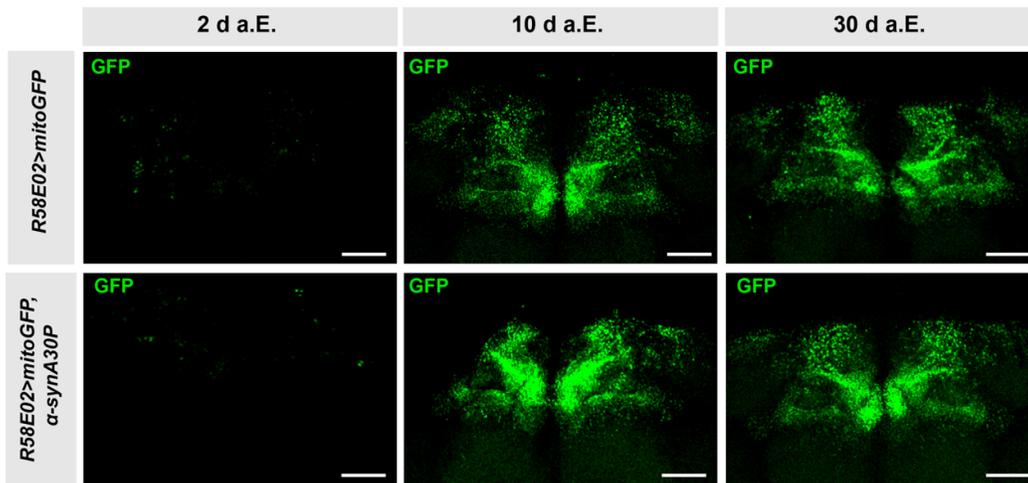


Figure S1 Representative pictures of mitoGFP fluorescence in the PAM cluster. Flies expressing α -synA30P in PAM DANs showed higher intensity of mitoGFP fluorescence at day 10 and 30 compared to controls. Scale bar : 30 μ M.

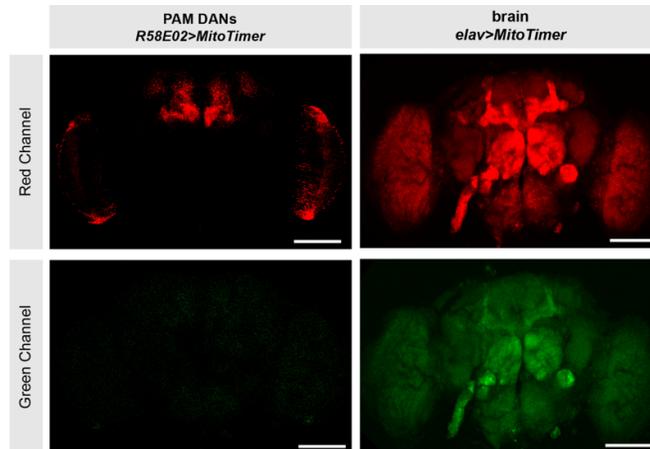


Figure S2 MitoTimer exhibited a rapid maturation in PAM DANs specifically. MitoTimer showed intense red and very faint green fluorescence at day 2 of adult flies in the PAM cluster (*left pictures*). Expressing MitoTimer in all neurons with the *elav-Gal4* driver led to intense red MitoTimer and weak but visible green signals throughout the brain (*right pictures*). Scale bar: 100 μ M.

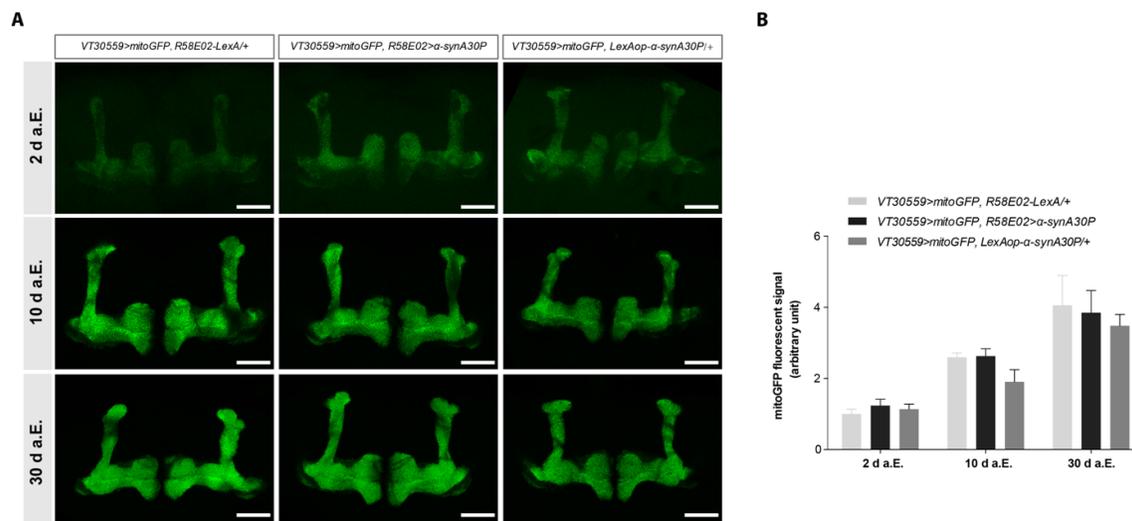


Figure S3 α -synA30P expression in PAM neurons did not change mitoGFP fluorescence in the MBs. (A) Representative images of mitoGFP fluorescence in the MB lobes of flies expressing or not α -synA30P in the PAM cluster at day 2, 10 and 30. (B) mitoGFP quantification. There was no significant difference between the three genotypes. Scale bar: 30 μ M.

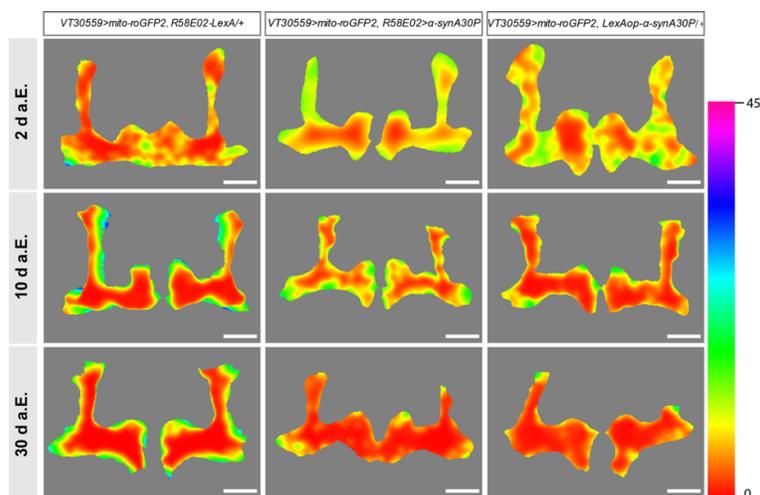


Figure S4 PAM- α -synA30P impacts on the redox ratio of mito-roGFP2 in the MBs. Representative images of mito-roGFP2 redox ratio in MB lobes of flies expressing or not α -synA30P in the PAM cluster at day 2, 10 and 30. False color representation. Scale bar: 30 μ M.

Results

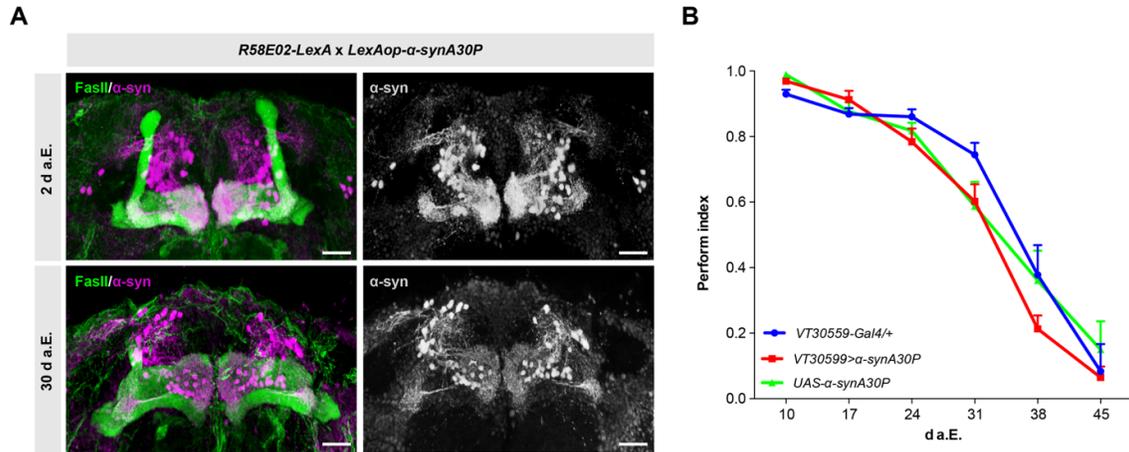


Figure S5 Lack of evidence for PAM to MB α -syn propagation (A) α -syn immunostaining in the brain of *R58E02> α -synA30P* flies labelled the PAM DANs but failed to detect evidence of α -synA30P spreading into the MB lobes at day 30. Scale bar: 20 μ M (B) α -synA30P expression in the MBs (driven by *VT30559-Gal4*) had no prominent effect on ARLD.

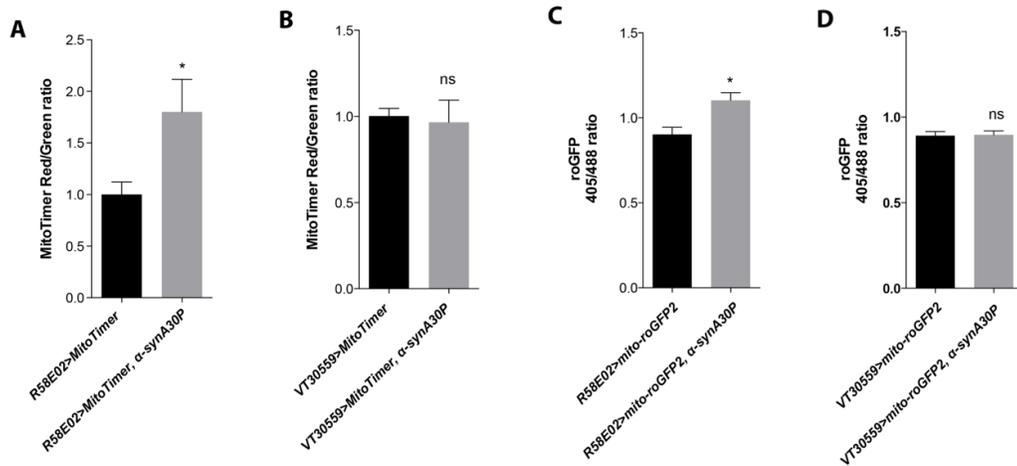


Figure S6 α -synA30P induces mitochondrial redox alteration cell-autonomously in PAM but not in MB neurons. (A) 10-day old flies co-expressing α -synA30P and MitoTimer in the PAM cluster showed increased MitoTimer red/green ratio in the PAM compared to controls without α -synA30P. (B) There was no difference in red/green ratio at day 10 in the MBs of flies co-expressing α -synA30P and MitoTimer in all KCs compared to controls without α -synA30P. (C) Same as (A) but using mito-roGFP2 instead of MitoTimer showing increased ratio of oxidized/reduced (405/488) mito-roGFP2 in the PAM at day 10. (D) Same as in (B) but using mito-roGFP2 instead of MitoTimer, showing lack of alteration in ox/red mito-roGFP2 ratio in the MBs.

Results C – Neuroprotective role of Piwi in the *Drosophila* brain

Summary

Epigenetic remodelling of chromatin has been involved in several neurodegenerative disorders and in PD mouse models. Decreased genomic DNA methylation levels associated with heterochromatin alterations increase TE transcription and mobility, inducing progressive DNA damages that contribute to aging and, possibly, to the onset of neurodegenerative diseases. The small RNA machinery, including the siRNA and piRNA pathways, prevents TE transcription and expression. The proteins involved in RNA-mediated TE silencing pathways belong to the Argonaute family. In *Drosophila*, there are five proteins of this family: Ago1 is responsible for miRNA function, Ago2 participates in the siRNA pathway, and the PIWI proteins (Ago3, Aub and Piwi) are involved in piRNA biogenesis and TE-silencing activity.

Piwi is the only PIWI protein that enters the nucleus where it can represses transposons through transcriptional gene silencing (TGS). Previous reports suggested that *Drosophila* Piwi plays a role in the prevention of TE reactivation and transposition both in the germline and in somatic cells, including the adult fat bodies and intestinal stem cells. Initial experiments performed in our team suggested that inhibiting Piwi expression by RNAi in dopaminergic neurons decreased fly resistance to paraquat, a pro-oxidant environmental PD factor. Here I show that Piwi functions as an inducible neuroprotector against oxidative stress and other aging-related processes, and promotes histone H3K9 methylation and TE inactivation in adult *Drosophila* neurons.

Personal contribution: Initial experiments on this project were performed in our group, following invaluable suggestions from Alain Prochiantz, by Marlène Cassar, Abdul-Raouf Issa and three undergraduate interns before I started my PhD. They found that Piwi inactivation in brain DA neurons increased PQ resistance in *Drosophila*. I then repeated and extended their observations by showing that Piwi is indeed expressed at very low level in the fly CNS, and that it is markedly induced in this tissue under oxidative stress and aging conditions, and by studying systematically the effect of Piwi inactivation and overexpression on oxidative stress resistance, ROS accumulation, transposon transcription and H3K9 methylation in the brain.

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Piwi is a stress-inducible protein that protects against Parkinson disease and aging-related processes in the *Drosophila* brain

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Abstract

Epigenetic remodelling of chromatin has been involved in several neurodegenerative disorders and in Parkinson disease (PD) mouse models. Decreased genomic DNA methylation levels associated with heterochromatin alterations increases transposable element (TE) transcription and mobility, inducing progressive DNA damages that contribute to aging and, possibly, to the onset of neurodegenerative diseases. The PIWI/piRNA pathway was demonstrated to target specifically the active TEs and pack them in heterochromatin. *Drosophila* possess three PIWI proteins, Ago3, Aub and Piwi. Previous studies indicated that *Drosophila* Piwi plays a role in the prevention of TE reactivation and transposition both in the germline and in somatic cells, including the adult fat bodies and intestinal stem cells. However, Piwi was not detected to date in the fly brain. Here we find that *piwi* is normally expressed at very low levels in the adult *Drosophila* brain and ventral nerve cord (VNC), while it is upregulated in this nervous tissue either by exposure to paraquat (PQ), a pro-oxidant and PD-causing toxin, or during normal aging. We observed that neuronal *piwi* expression increases fly resistance to oxidative stress and reduces marks of aging in the brain. Our observations further provide evidence that the protective role of Piwi against aging and

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PD-like conditions involves TE repression and the maintenance of heterochromatin state in *Drosophila* neurons. This suggests that Piwi is an essential protein in the nervous system that could be considered as a valuable target for aging-related neurodegenerative disorders.

Key words

Argonaute proteins - Piwi - piRNAs - transposable elements - H3K9me3 - paraquat - oxidative stress- aging - nervous system – Parkinson disease - *Drosophila melanogaster*

Introduction

Over generations, during meiosis and embryonic development, the small RNA-induced gene silencing complex (RISC) maintains genomic integrity against transposable elements (TEs) through transcriptional and post-transcriptional gene silencing processes (Aravin et al., 2007). In diverse organisms, these mechanisms are universally employed to silence TEs, in particular in germline and stem cells, where they serve as the main line of defence against TE transposition allowing the permanence of genome stability (Juliano et al., 2011). RISC assembly requires binding between small non-coding RNAs and the Argonaute proteins, that belong to the PIWI protein family in the case of the PIWI-interacting RNA (piRNA) pathway. *Drosophila* encodes three PIWI proteins: Piwi, Aubergine (Aub) and Ago3. The function of piRNAs in genome protection has been demonstrated in germline cells (Senti and Brennecke, 2010). Piwi represses transcription of transposons that are complementary to associated piRNAs in the nucleus by recruiting Heterochromatin protein 1a (HP1a, also named Suppressor of variegation 205 (Su(var)205) in *Drosophila*) and increasing Histone 3 lysine 9 trimethylation (H3K9me3) marks. This induces a repressive heterochromatin state in the target TE loci (Le Thomas et al., 2013; Brower-Toland et al., 2007).

In the nervous system, it was suggested that TE mobility could contribute to cellular diversity during neurogenesis, and that TE misregulation is associated with certain neurological disorders (Erwin et al., 2014). The Piwi/piRNA complex can also have other targets than the TEs and participate in cell-specific gene regulations for higher brain functions such as memory. This was demonstrated in *Aplysia* (Rajasethupathy et al., 2012) and piRNAs were also detected in the nervous tissue, including in mouse hippocampal neurons and *Drosophila* mushroom body (MB) neurons that are essential for memory formation (Lee et al., 2011,

Perrat et al., 2013; Ross et al. 2014; Bozzetti et al., 2015). Clinical data demonstrate that transcription and retrotransposition of long interspersed nuclear elements-1 (LINE-1), which constitute ~20% of the human genome, increase in certain neurological disorders, including Rett syndrome (Muotri et al., 2010), autism (Shpyleva et al., 2018) and schizophrenia (Doyle et al., 2017).

TE activation has been observed during aging in mice and *Drosophila*, which could be attributed to chromatin relaxation (De Cecco et al., 2013; Li et al., 2013; Chen et al., 2016; Wood and Helfand, 2013). Conversely, TE transposition would trigger aging acceleration as a consequence of DNA damage and mutation accumulation (Lenart et al., 2018). Promoting the heterochromatin state was shown to mitigate age-related increase in TE expression (Wood et al., 2016). Since aging is the primary factor for the onset of neurodegenerative disorders like Alzheimer disease and PD, the function of the Piwi/piRNA pathway in heterochromatin maintenance and TE silencing could be beneficial to protect against neurodegeneration. In line with this hypothesis, *Ago2* mutant fly showed progressive and age-dependent memory impairments, as well as shortened lifespan, that were attributed to high TE activity in brain (Li et al., 2013). Moreover, the protein Tau, involved in AD, could induced loss of heterochromatin and led to aberrant expression of *Ago3* in flies (Frost et al., 2014), highlighting the role of Argonaute proteins in neurodegenerative processes.

Previous works suggested that *Drosophila* Piwi plays a role in the prevention of TE reactivation and transposition both in the germline and in somatic cells, including the adult fat bodies and intestinal stem cells (Brennecke et al., 2007; Le Thomas et al., 2013; Jones et al., 2016; Klein et al., 2016; Sousa-Victor et al., 2017; Akkouche et al., 2017). Here we show that Piwi functions as an inducible neuroprotector against oxidative stress and other aging-related processes in the *Drosophila* nervous system. We also provide evidence that Piwi exerts its neuroprotection by targeting induced TEs and maintaining heterochromatin status in adult brain neurons.

Results

piwi is induced by oxidative stress in the *Drosophila* CNS

The Piwi protein was not detected in the *Drosophila* brain in a previous study (Perrat et al., 2013), suggesting that it is expressed at a very low level or not expressed at all in this tissue. We performed RT-PCR and western blots on dissected brains and VNCs to avoid potential

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contamination by the eyes, fat bodies and other non-nervous tissues. To detect *piwi* by RT-PCR, we designed primers that bridge the fourth intron to avoid contamination by products from genomic DNA. RT-PCR showed that *piwi* is very slightly expressed in the brain and VNC compared to its regular expression in the ovary (Figure 1A). On western blots, the brain and VNC samples showed weak bands, at around 100 kDa. Those bands co-migrate with the Piwi band of the ovary sample (Figure 1B). Quantified data from those these experiments are shown in Figure S1A and B.

The herbicide paraquat (PQ) is a superoxide generator that has been used extensively to increase oxidative stress and model PD in *Drosophila*. Flies exposed to PQ show decreased locomotion, selective DA neuron loss, and they die in a few days (Chaudhuri et al., 2007; Shukla et al., 2014; Cassar et al., 2015; Shukla et al., 2016). Because TE transposition and chromatin alterations can be increased by oxidative stress in other systems (Stoycheva et al., 2010; Giorgi et al., 2011; Song et al., 2011), we examined Piwi expression in the CNS after exposing Canton-S flies to a relatively low dose of PQ (10 mM). Strikingly, we found that *piwi* mRNA level was remarkably elevated in the CNS after 24 h showing a 2-fold and 10-fold increase in the brain and VNC, respectively. This effect was observed both by RT-PCR (Figure 1C) and confirmed by qPCR (Figure S1C). Then we determined Piwi protein level in the CNS by western blot after a similar PQ exposure. We observed that Piwi increased 3 folds in the brain and 2 folds in the VNC (Figure 1D). This suggests that *piwi* expression is regulated both at the transcriptional and translational levels in the *Drosophila* nervous system.

Neuronal expression of Piwi protects against PQ toxicity

Because PQ ingestion induced Piwi expression in the CNS, it was quite possible that neuronal expression of Piwi could have an influence on PQ susceptibility. We first investigated the effect of a loss-of-function of Piwi in all neurons by monitoring the survival rate of flies exposed to 20 mM PQ as in usual assays (Cassar et al., 2015). After 24 h, the survival of flies with pan-neuronal *piwi* inactivation (*elav>piwi RNAi*) was 30-40% lower than controls (Figure 2A). Previous work showed that PQ primarily triggers DA neuron degeneration in the *Drosophila* CNS (Chaudhuri et al., 2007; Shukla et al., 2014; Cassar et al., 2015). We therefore silenced *piwi* selectively in DA neurons with *TH-Gal4*. After 24h PQ treatment, we observed that the survival rate of *TH>piwi RNAi* flies was ~60% lower than the controls (Figure 2B). *TH-Gal4* also targets DA-producing cells in the hypoderm and other non-nervous tissues (Friggi-Grelin et al., 2003). In order to eliminate the effect from these non-neuronal cells, we used *elav-Gal80* together with *TH-Gal4* to block *piwi* inactivation in

DA neurons (Figure 2B). We observed that the *elav-Gal80, TH>piwi RNAi* flies had a PQ susceptibility comparable to controls, which showed that *piwi* expression is required specifically in neurons to increase PQ resistance. Another *piwi* RNAi also decreased fly survival under PQ when expressed in DA neurons (Figure S1D), indicating that the effect comes from *piwi* inactivation and not from another gene.

To verify the role of Piwi, we tested the PQ resistance of flies in which Piwi was, conversely, overexpressed. Remarkably, *piwi* overexpression in all neurons resulted in higher tolerance of flies to PQ with 80% survival rate after 30 h PQ ingestion, compared to around 50% for controls. In contrast, *piwi* overexpression with *TH-Gal4* did not induce significant protection, which could come from the fact that DA neurons represent less than 1% of the total number of neurons in the *Drosophila* brain, or suggest that Piwi expression is tightly controlled at the translation level in these neurons. Altogether, these observations suggest that Piwi is induced by oxidative stress in the CNS and functions as a potent neuroprotective protein since its expression can significantly prolong the survival of flies exposed to a dangerous pro-oxidant neurotoxin.

Piwi alleviates age-related ROS accumulation in the *Drosophila* brain.

Aging is a major risk factor for the onset of PD (Malkus et al. 2009; Rodriguez et al., 2015). ROS and oxidative damage were shown to accumulate in the *Drosophila* brain during aging (Hirano et al., 2012; Oka et al., 2015; Vaccaro et al., 2017). To determine if neuronal Piwi can protect against aging, we quantified ROS levels in the brain by DHE staining at different ages and examined the effect of *piwi* inactivation or overexpression in all neurons (Figure 2E and F). At day 2 after adult eclosion, ROS levels were low in brain and *piwi* silencing or overexpression made no difference with the driver and effector controls. At day 30, brain ROS levels were strongly increased in all groups. Interestingly, ROS level was found to be 25% higher in the *piwi* RNAi flies (Figure 2E) and 40% lower in the *piwi* overexpressing flies (Figure 2F) compared to the two controls. These data suggest that Piwi reduces age-induced ROS accumulation in the fly neurons.

Neuronal Piwi represses TE activity and increases heterochromatin formation

Piwi belongs to the Argonaute PIWI family whose main function is to target TEs. piRNAs and other PIWI proteins were shown to be expressed in the *Drosophila* brain (Perrat et al., 2013). We therefore looked at the effect of PQ and Piwi on transposon activity in the fly brain. Like LINE-1 in mammals, *HeT-A* is non-long terminal repeat (LTR) retrotransposon that is predominantly inserted in the chromosome telomeres (Pardue et al., 2005). RT-PCR

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results demonstrated a 2-fold increase in *Het-A* mRNA level after 24h PQ treatment (Figure 3A). We observed that Piwi overexpression in all neurons decreased *HeT-A* level compared to controls in PQ ingestion conditions (Figure 3B).

Previous studies revealed that epigenetic modifications are implicated in PD pathogenesis (Urduingio et al., 2009; Desplats et al., 2011; Rekaik et al., 2015). Piwi can directly target the genome inside the nucleus to promote heterochromatin formation, thereby silencing active TE loci (Le Thomas et al., 2013; Brower-Toland et al., 2007). We examined heterochromatin H3K9me3 marks in the fly brain by immunostaining. First, we observed decreased H3K9me3 levels after 24 h exposure to 10 mM PQ compared to controls without PQ treatment (Figure 3C). In *elav>piwi* flies, this effect was significantly mitigated compared to controls, indicating that Piwi can promote heterochromatin formation under oxidative stress in *Drosophila* neurons (Figure 3C). Overall, these results suggest that Piwi ensures better fly survival and neuroprotection under PQ-induced acute oxidative stress owing to its action against TE mobilization and H3K9 demethylation.

Piwi impacts on TEs during aging in the fly brain

Several TEs were reported to be highly active in the *Drosophila* brain during normal ageing (Li et al., 2013), suggesting the transposon activation may contribute to age-dependent neuronal dysfunction and degeneration. First, we examined the effect of aging on Piwi expression level in the brain. Both *piwi* mRNA and protein levels were found to be increased in old wild-type Canton-S flies (>30 days) (Figure 4A and B). Wild-type flies aged for 30 days showed a one-fold increase in *HeT-A* (and R2, not shown) retrotransposons compared to that from 2-day old flies (Figure 4C). Similar to the effect of Piwi on *HeT-A* mRNA level in PQ condition (Figure 3B), pan-neuronal piwi overexpression decreases *HeT-A* mRNAs in 30-day old flies (Figure 4D). The potential role of piwi on maintaining H3K9 methylation state under PQ (Figure 3C) suggested that Piwi may have a similar role during normal aging. As shown in Figure 4E, flies with *piwi* silencing in all neurons showed a slight decrease in H3K9me3 immunostaining in 30 day-old flies compared to the controls without neuronal RNAi expression. In contrast, no difference in H3K9me3 staining was observed in old flies when Piwi was overexpressed in all neurons, suggesting possibly that the age-related increase in Piwi level was enough to ensure H3K9 methylation at piRNA-complementary loci.

Discussion

The mechanisms for maintenance of chromatin integrity by the PIWI/piRNA pathway has been well-conserved across species. Aging is a major risk factor in PD. Although no specific TE clusters were reported to relate to the PD pathologies, age-induced alterations in the epigenetic landscape, such as histone modification and active TE transposition, causing DNA damage or ROS accumulation, may be a potential driving force for PD onset in the aged individuals. Therefore, the PIWI/piRNA pathway could serve as an endogenous self-defense mechanism to delay PD pathologies. Here we provide evidence that Piwi is indeed induced by increased oxidative stress in the *Drosophila* CNS. Piwi overexpression in all neurons decreased fly susceptibility to PQ toxicity associated with lower TE mobility and higher H3K9me3 level, while *piwi* inactivation in neurons had opposite effects. Together, these data suggest that *piwi* function as an inducible protective protein against PD-like oxidative stress and marks of aging in the fly nervous system.

In *Drosophila*, Piwi has been detected in the gonad tissue in the first place and then revealed to play a role in early embryogenesis (Mani et al., 2014). Piwi was then localized in the stem cells of the gut, in the nuclei of abdominal fat bodies, and in follicle and germline cells in gonad tissue by studies later on (Brennecke et al., 2007; Jones et al., 2016; Sousa-Victor et al., 2017). Previous works did not report a neural localization and function of Piwi. The study by Perrat et al. (2013) showed immunostaining patterns for Ago3 and Aub in the *Drosophila* MB lobes and stated that Piwi was not detected in the brain. Moreover, Jones et al., (2016) showed by western blots that Piwi is less expressed in the fat body than in the ovary, and no signal was obtained in head and thorax samples. Here we provide evidence that Piwi is expressed, but normally at hardly detectable levels, in the *Drosophila* brain and VNC, while it is strongly induced in those tissues by PQ-induced oxidative stress and present at a significantly higher level in the brain of old flies. We ascertained the presence of Piwi in young flies by using two different antibodies, a rabbit polyclonal from G. Hannon and a commercially available mouse monoclonal antibody. We obtained identical bands in our brain samples with these two antibodies that co-migrated with the ovary signal, highly suggesting that Piwi is expressed in the fly CNS. Furthermore, the effect of *piwi* inactivation on fly resistance to PQ toxicity using neuronal drivers and two different RNAi constructs provided more evidence that Piwi is expressed and may function as a neuroprotective protein in the fly CNS. Neurons are usually considered as postmitotic cells, and in the fly brain, there are few adult stem cells (von Trotha et al., 2009; Herrup and Yang, 2007). Because the

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PIWI/piRNA pathway normally functions in non-aging cells to maintain their proliferative activity (Sturm et al., 2017), Piwi could be expressed in those few adult neuronal stem cells, or, by an unconventional mechanism, Piwi may also be expressed ubiquitously and at a low level in the fly CNS.

Our work indicates that Piwi in the CNS and in the gonad tissue share similar mechanisms of action. Overexpressing Piwi in all neurons could mitigate the elevated TE level induced by PQ toxicity, and maintain heterochromatin status in this context. In a fly model of Alzheimer disease (Frost et al., 2014), *Drosophila* Ago3 was found to be upregulated by Tau-toxicity, which is comparable to the induced Piwi level we observed upon PQ ingestion. The toxicity of Tau and PQ shares the similarity that both of them can induce the epigenetic abnormalities (Frost et al., 2014; Song et al., 2011). However, a divergence between the AD model and our PD model is that downregulation of Ago3 could alleviate the toxic effect from Tau whereas we find that *piwi* inactivation in contrast decreased fly resistance to PQ toxicity. Ago3 and Piwi are categorized as PIWI subfamily proteins, responsible for piRNA biogenesis and function (Brennecke et al., 2007; Lutejin and Ketting, 2013). Ago3/piRNAs target TE transcripts in the cytoplasm and induce their cleavage to generate new piRNAs, whereas, guided by piRNA, Piwi enters the nucleus, where the Piwi/piRNA complex targets TE transcripts and active TE locus (Iwasaki et al., 2015). Furthermore, by recruiting HP1a/Su(var)205, Piwi can increase H3K9me3 level, thus packing the TE site in the heterochromatin status (Lutejin and Ketting, 2013). Due to the diverse role of Piwi in the nuclei and in histone modifications, it is reasonable that *piwi* and *ago3* inactivation elicited different consequences upon the toxicity induced by Tau and PQ. Tau transgenic flies exhibited significant loss of H3K9me2 in the Ago3 gene, leading to an increase of Ago3 expression (Frost et al., 2014). The authors considered the consequence of increased Ago3 expression as a loss of control of neuronal proliferation that would re-enter into an inappropriate cell cycle inducing subsequently apoptosis. This hypothesis could not explain the neuroprotective effect of Piwi overexpression, given that the PIWI/piRNA pathway is implicated in proliferation (Yakushev et al., 2016; Ponnusamy et al., 2017). Since we could not determine the exact localization of *piwi* or even the localization of induced Piwi upon PQ treatment in the brain, we cannot rule out an unconventional mechanism for Piwi function in differentiated neurons, in which overexpressed Piwi would target the aberrant TEs in all cells and maintain the heterochromatin without initiating cell cycle re-entry.

Exposure to environmental insults has been implicated in the pathogenesis of PD. PQ-treated animals exhibited loss of DA neurons with increase in ROS generation and caspase-3 activation (Bus and Gibson, 1984; McComack et al., 2002; Chaudhuri et al., 2007; Fei et al., 2008; Hosamani and Muralidhara, 2013; Shukla et al., 2014; Cassar et al., 2015). In a cell culture model of PD, PQ induced epigenetic change by promoting histone acetylation (Song et al., 2011). Applying a DNA methyltransferase inhibitor increased cell sensitivity to PQ toxicity (Kong et al., 2012). However, the effects of PQ on heterochromatic status *in vivo* was not characterized yet. Here we observed a decrease in H3K9me3 in the brain of flies treated 10 mM PQ compared to those without treatment. This loss of H3K9me3 heterochromatin marks is probably related to the TE activation that is also induced by PQ exposure.

Heterochromatin decreases with aging, leading to increased TE transcription (Wood and Helfand, 2013) and aging acts as a “priming” factor for the onset of PD. TE activation was suggested to be a major factor of ageing, because it better explained the exponentially increase in mortality rate during the adult life (Sturm et al., 2015). Here we showed that ROS accumulation, induced TEs and decreased H3K9me3 levels in the brain of aging flies, were all alleviated by pan-neuronal Piwi overexpression. Altogether, our work provides the first evidence that Piwi is expressed in the fly CNS and identifies it as an inducible neuroprotective protein. The neuronal function of Piwi against PQ toxicity and aging-related chromatin defects in flies suggest that targeting the Piwi/piRNA pathway, which involves heterochromatin maintenance and repressed TE activity, could be an effective way to delay or protect against PD pathogenesis.

Materials and Methods

Drosophila culture and strains

Fly stocks were raised and crossed at 25°C on standard corn meal/yeast/agar medium supplemented with methyl-4-hydroxybenzoate as a mold protector, under a 12h-12hr light/dark cycle. The following lines were used: Canton-S and w^{1118} as wild-type controls, *elav-Gal4* (Lin and Goodman, 1994), *TH-Gal4* (Friggi-Grelin et al., 2003), *elav-Gal80* (Rideout et al., 2010) (kindly provided by Sean Sweeney, University of York, UK), *piwi*^{EP1024}/*UAS-piwi* (Cox et al., 2000) (kindly provided by Haifan Lin, Yale University, New Haven, CT, USA), *UAS-piwi*^{RNAi-33} and *UAS-piwi*^{RNAi-31} (Bloomington *Drosophila* Stock

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Center TRiP lines #33724 and #31610, respectively). Except otherwise indicated, *UAS-piwi*^{RNAi-33} was generally used and named in figures *UAS-piwi RNAi*.

Oxidative stress resistance

Oxidative stress resistance was assayed by exposure to paraquat (PQ) (methyl viologen, Sigma-Aldrich) using a previously described dietary ingestion procedure (Cassar et al., 2015). ~100 non-virgin 8-day old female *Drosophila* per genotype were collected and exposed to 20 mM PQ diluted in 2% (wt/vol) sucrose (or sucrose only for controls) in 2-inch (5.2 cm) diameter Petri dishes (10 flies per dish) containing two layers of Whatman filter soaked with the PQ solution, then incubated at 25°C in saturating humidity conditions. Fly survival was scored after 24 h and 30 h. In some experiments, brains from flies exposed to 10 mM PQ for 24h were dissected and processed for PCR, Western blots and immunostaining.

ROS detection and quantification

Reactive oxygen species (ROS) level was determined with the fluorescent dye dihydroethidium (DHE) (Life Technologies) following a published protocol adapted to whole-mount *Drosophila* brains (Owusu-Ansah et al., 2008; Vacarro et al., 2017). 5-8 adult brains per genotype were dissected in Schneider's insect medium and incubated on an orbital shaker for 5-7 min at room temperature in 30 μM DHE diluted in the same medium. The brains were then washed 3 times in Schneider's medium and fixed for 7 minutes in 7% formaldehyde in PBS. After rinsing in PBS and mounting in Vectashield (Vector Laboratories), the brains were immediately scanned at constant gain setting on a Nikon A1R confocal microscope. Relative ROS levels were measured by quantification of whole brain average intensity level of the dye fluorescence using the Fiji software.

Immunostaining

7-10 adult brains were dissected in ice-cold *Drosophila* Ringer' solution and processed for whole mount immunostaining as previously described (Riemensperger et al 2011). The primary antibodies were rabbit anti-H3K9me3 (Abcam, 1:1000) and mouse anti-TH (Immunostar, 1:1000). The secondary antibodies were goat anti-mouse and anti-rabbit conjugated to Alexa Fluor 488 or 555 (Invitrogen Molecular Probes, 1:1000). The brains were mounted in ProLong Gold Antifade reagent (Thermo Fisher Scientific). Images were acquired with a Nikon A1R confocal microscope and processed using the FIJI software to quantify the fluorescent intensity for H3K9 tri-methylation in the brain.

RT-PCR and quantitative PCR

RT-PCR was performed to examine *piwi* expression in different dissected tissues as previously described (Cassar et al., 2015). The primers used in the RT reaction were: for *piwi*, sense 5'-ACGCATTCGCGACCACAATCAG, antisense 5'-TGTACTTCTGTGACGTTCTTGTCCAGT; for *Het-A*, sense 5'-CGCAAAGACATCTGGAGGACTACC, antisense 5'-TGCCGACCTGCTTGGTATTG; for *rp49*, sense 5'-GACGCTTCAAGGGACAGTATC, antisense 5'-AAACGCGGTTCTGCATGAG. Quantitative PCR was conducted on a Roche LightCycler 480. System with the Roche SYBR Green Master mix using the following primer sets: *piwi*: sense primer 5'-GACGTTGCTCACACAATCCG, antisense primer 5'-CATGGCACGCATAAGCTGAAA; *rp49*: sense primer 5'-GACGCTTCAAGGGACAGTATC, antisense primer 5'-AAACGCGGTTCTGCATGAG.

Western Blots

25-30 dissected brains or VNCs were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (cOmplete Protease Inhibitor Cocktail, Roche Diagnostics). The lysates were incubated on ice for 30 min and centrifuged at 8,000 g for 10 min at 4°C. Protein samples were prepared by diluting the supernatant in 1 x final NuPAGE LDS Sample Buffer and Reducing Agent, followed by 10 min denaturation at 70°C and 5 s centrifugation at 1,000 g. Proteins were separated in 4-12% Novex NuPAGE Bis-Tris precast polyacrylamide gels (Life Technologies), using PageRuler Plus Prestained Protein Ladder (ThermoFisher Scientific) as migration marker, and electrotransferred to Amersham Hybond P 0.45 PVDF membranes (GE Healthcare Life Sciences). Membranes were blocked after transfer for 2 h at room temperature in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 (TBS-T) supplemented with 5% skimmed milk. Membranes were then incubated overnight at 4°C in TBS-T containing 5% (w/v) skimmed milk in 0.05% TBS-T with the following primary antibodies: rabbit polyclonal anti-*Drosophila* Piwi (kindly provided by Gregory J. Hannon, University of Cambridge, UK), mouse monoclonal anti-*Drosophila* Piwi (1:500, Santa Cruz Biotechnology, #sc-390946) mouse monoclonal anti- α -tubulin (DHSB 1:500, #12G10). The horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Invitrogen Molecular Probes) were used at 1:3000. Immunolabeled bands were revealed with ECL RevelBIOT Intense (Ozyme, France) and digitally acquired at different exposure times using Image Quant TL software (GE Healthcare Life Science). Densitometry measures

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were made with the Fiji software and normalized to the α -tubulin measures as internal controls.

Statistical analysis

All statistical analyses were performed with the GraphPad Prism 6 software. Data were analysed using Mann-Whitney test or one-way ANOVA or two-way ANOVA with Bonferroni's or Tukey's post-hoc tests for multiple comparisons. All data are presented as mean \pm SEM. Significant values in all figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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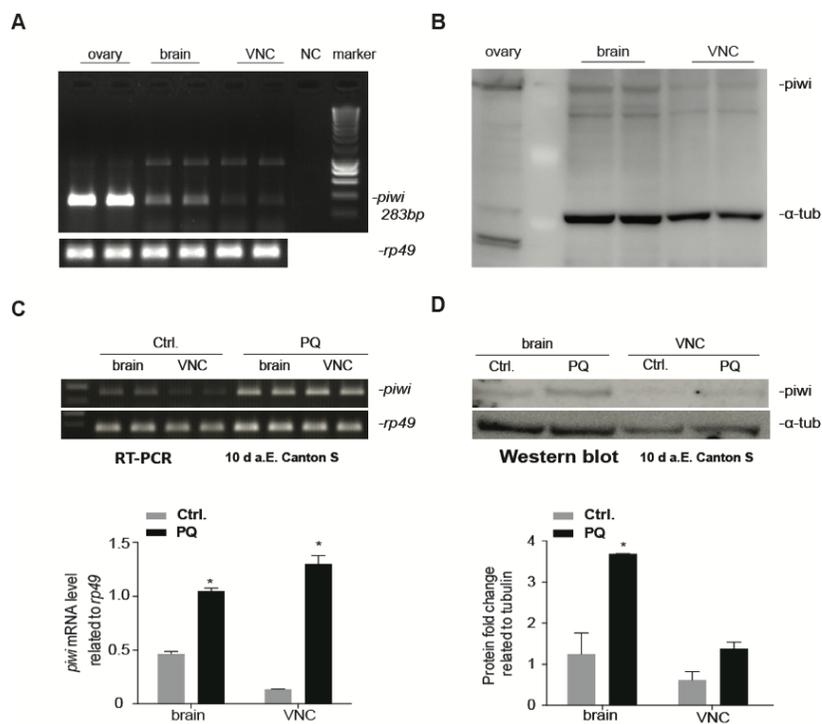


Figure 1. Piwi is induced by oxidative stress in the nervous system. (A, B) Piwi expression in normal conditions. Both *piwi* mRNA (A) and protein (B) were hardly detectable in the adult brain and ventral nerve cord (VNC) of wild-type flies in normal conditions, compared to much higher *piwi* expression in the ovary. (C, D) Piwi expression in oxidative stress conditions. Both *piwi* mRNA (C) and protein (D) levels were strongly up-regulated after 24 h exposure to 10 mM PQ. Ctrl: control, NC: negative control-PCR reaction without template, PQ: paraquat, α -tub: α -tubulin, VNC: ventral nerve cord.

Results

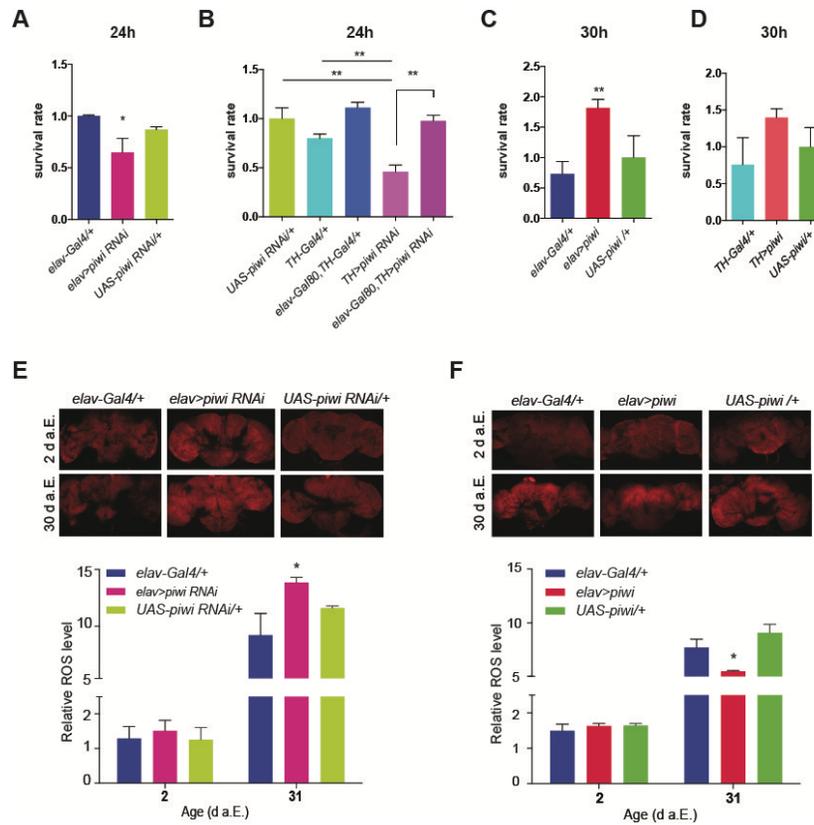


Figure 2. Effect of piwi inactivation or overexpression in neurons on PQ resistance and age-related ROS accumulation. (A-D) Effect of piwi on fly PQ resistance. (A) piwi silencing by RNAi in all neurons (*elav-Gal4* driver) decreased *Drosophila* survival after 24 h of exposure to PQ, as compared to the driver and RNAi alone controls. (B) piwi silencing in DA-synthesizing cells (*TH-Gal4* driver) also decreased PQ resistance, while blocking this inactivation selectively in DA neurons with *elav-Gal80* rescued fly survival. (C) piwi overexpression in all neurons strongly increased fly survival after 30 h exposure to PQ compared to both controls. (D) Flies with Piwi overexpressed in DA-synthesizing cells showed no significant difference in survival rate compared to controls in the same conditions as (C). (E-F) Effect of piwi on ROS accumulation in the brain. piwi inactivation or overexpression had no effect on ROS levels in the brain of young adult flies aged 2 days a.E. In contrast, in the brain of aged flies (30 days a.E.) piwi inactivation in all neurons increased (E), while piwi overexpression decreased (F), age-induced ROS accumulation in the brain. Representative pictures of fluorescence levels in whole-mount dissected brains are shown on top of the graphs. d a. E.: day after eclosion.

Results

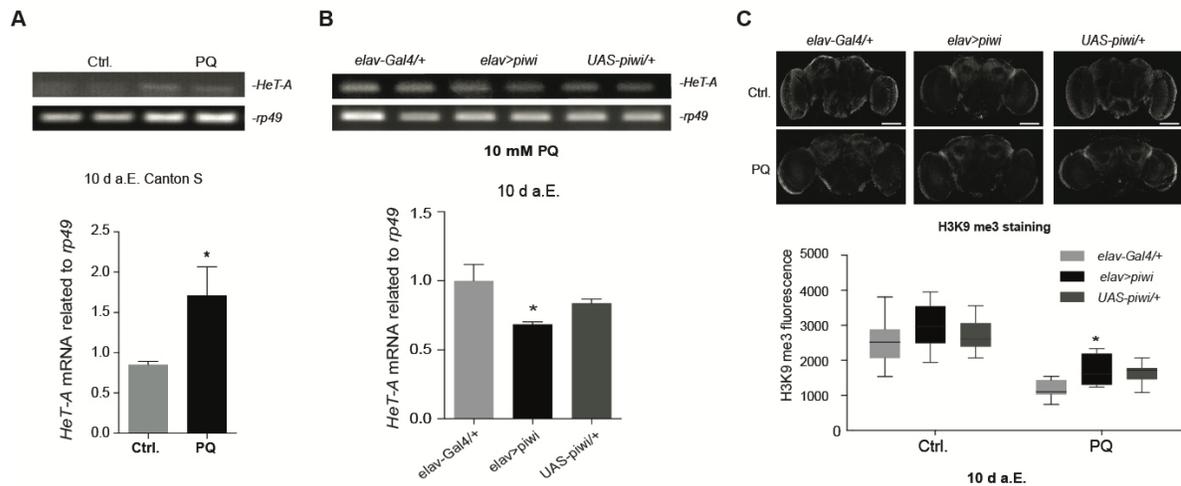


Figure 3 Neuronal Piwi overexpression decreases PQ-induced TE transcription and H3K9 demethylation. (A) Transcription of *HeT-A* retrotransposon is increased by 10 mM PQ exposure for 24h. (B) RT-PCR results showed a slight decrease in *HeT-A* transcription in *elav>piwi* flies after 24h exposure to 10 mM PQ. (C) PQ ingestion decreased H3K9me3 level in the whole brain compared to untreated controls. Pan-neuronal *piwi* overexpression partially prevented PQ-induced H3K9 demethylation.

Results

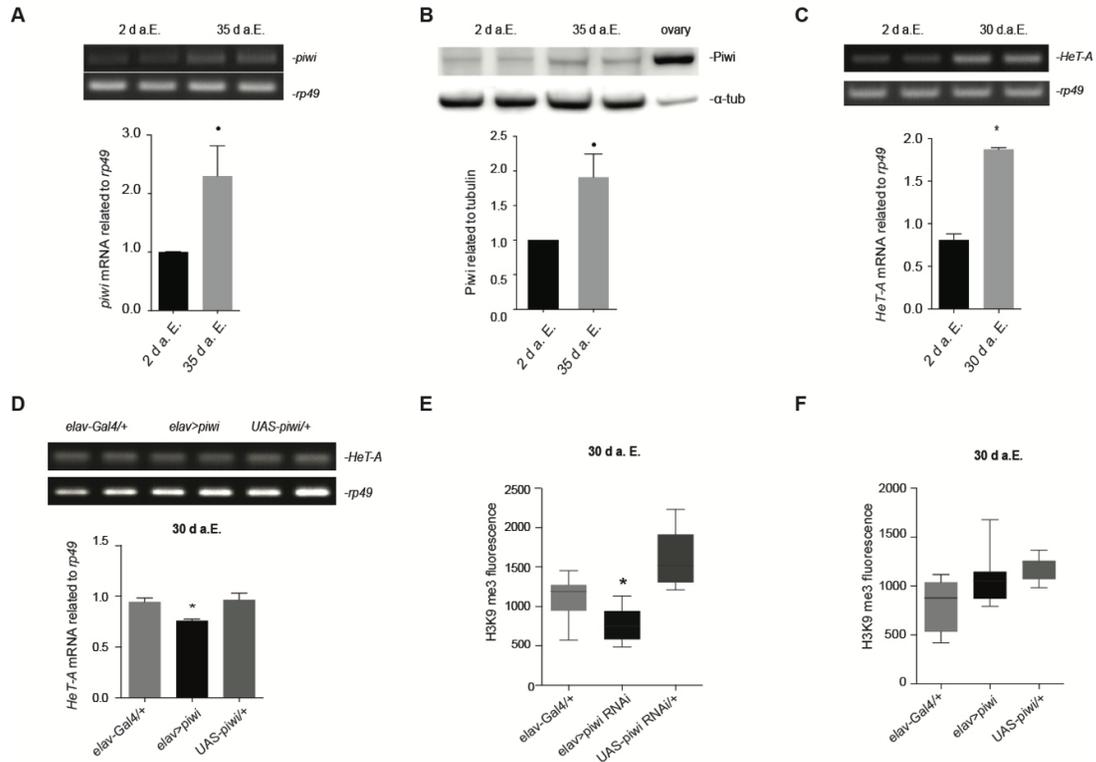


Figure 4 Neuronal Piwi expression increases with age and attenuates aging-related chromatin defects. (A,B) Effect of aging on *piwi* mRNA in the heads (A) and protein (B) levels in brain. Piwi expression increases in old flies (C) Transcription of *HeT-A* retrotransposon increases with age in adult flies, as shown by RT-PCR performed on head mRNAs. (D) Piwi overexpression in all neurons significantly mitigated the increase in *HeT-A* in heads of 30-day old adult flies. (E,F) *piwi* inactivation increased the loss of H3K9me3 marks in brains of 30-day old adult flies (E) while *piwi* overexpression had no significant effects (F).

Supplemental Figure Legend

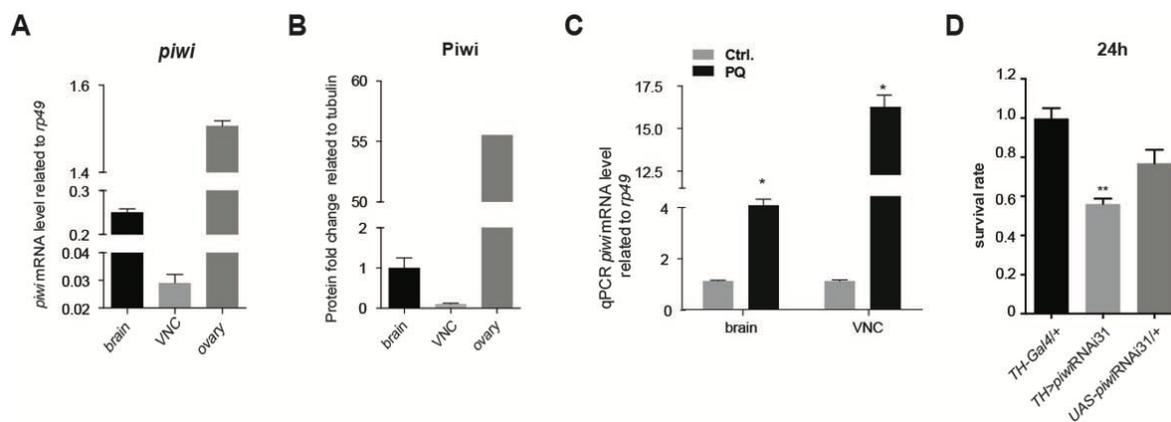


Figure S1 (A,C) Quantification of *piwi* mRNA (A) and protein (B) levels in brain, VNC and ovary based on data shown in Figure 1A,B. (C) qPCR performed on samples used for Figure 1C showed increase in *piwi* transcript after 24 h exposure to 10 mM PQ. (D) *piwi* inactivation in DA neurons with another *piwi* RNAi strains. *TH>piwi* RNAi-31 flies are more sensitive to PQ than the two controls.

Results

Discussion

Discussion

1. Neural circuits modulating locomotor reactivity

We have shown that the MBs are involved in SING modulation. The three main components of the MBs, afferent modulatory neurons (including DANs), intrinsic neurons (KCs) and efferent neurons-MBONs, participate in this neuromodulation. We have found that the DA receptor Dop1R1, not Dop1R2, is required in the MB lobes for DA control of startle-induced locomotion, particularly from the PPL1 cluster. After delineation of the role of the MBs in SING modulation, we completed this work in Part II of Results A to evaluate which other neurotransmitters could be involved in the networks controlling locomotor reactivity. We showed that both serotonin and octopamine are implicated in SING modulation, which was not surprising because serotonergic and octopaminergic neurons are afferent to the MBs (Pech et al., 2013a). Moreover, we observed an effect of neuronal activation of the FSB, but not of the EB, which are both part of the CC involved in the coordination of locomotor behaviour (Kashai et al., 2010; Wolff et al., 2015). Therefore, FSB may serve to convey sensorimotor representations between the SMP (projection region of the MBs) and the descending neurons, which carry signal from brain to the thoracic ganglia (Hsu and Bhandawat, 2016), to execute the SING reflex.

1.1 Implication of the MBs in locomotion control

The point highlighted in Result A-Part I is that the MBs not only serve as an integrated centre for learning and memory (Aso et al., 2014a, 2014b), but it also has an inhibitory action on locomotor reactivity. A role for the MBs in locomotion control has been first shown in *Drosophila* by Heisenberg et al. (1985) who reported that *mushroom body miniature* mutant flies exhibited increased locomotor activity and then it was revealed by Martin et al. that MBs suppressed locomotor activity (Heisenberg et al., 1985; Martin et al., 1998). The locomotor activity here refers to the free walking (spontaneous) activity, potentially modulated by different neural circuits from those controlling the SING (White et al., 2010; Sun et al., 2018). However, the neuronal circuits regulating these two different locomotor behaviours appear to have the MBs in common.

Based on the nice correspondence of the neural assemblies of the mammalian basal ganglia and insect central complex the MB is reminiscent of the hippocampus and amygdala in mammals (Strausfeld and Hirth et al., 2013). Interestingly, in mammals, an injury to the hippocampus region caused disrupted locomotor circuits (López Ruiz et al., 2015) and the hippocampus was implicated in the control of locomotion speed (Bender et al., 2015),

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comparably to the insect MB function in the control of locomotor behaviours. Activating DANs decreased the SING response while activating MB α' β' or γ KCs similarly exhibited a marked decrease in locomotor reactivity. This confirmed that the MBs in this network are inhibitory for startle-induced locomotion. DA binds to excitatory Dop1R1 in the MB activating subtypes of the intrinsic KCs. Therefore, it is logical that both increased DA release or the activation of MB KCs elicited a decrease in locomotor reactivity. Activation by dTrpA1 of specific MBONs induced a similar decline in SING performance, which implied that signalling input activated those MBON to convey the “stop” message to the sensorimotor neuronal network. But what could be the input signal on MBONs? Probably either direct DA release on MBONs or integrated information from DANs through MB intrinsic neurons.

1.2 Neuronal networks modulating startle-induced locomotion

A scheme in Results A, Part I (Figure 8 of Sun et al., 2018) summarized the MB-associated neuronal components controlling startle-induced locomotion that we have identified. One important question is how the integrated information in the higher brain centres (MB, SMP) is transferred through descending neurons and connects with the motor system?

The CC in the fly brain is considered as a pre-motor center control (Strauss, 2002; Martin et al., 1999; Kahsai et al., 2010; Kahsai and Winther, 2011). It comprises four neuropils: PB, FSB, EB, and noduli. Classified by Lin et al. (2013), neurons extending from the PB, FB and EB send projections to the LALs. Meanwhile, the LALs are innervated by many neurons, including dendrites of the descending neurons, so the LAL selects the activity of descending output according to received input from the CC, which coordinates the behaviour (Fiore et al., 2015; Turner-Evans and Jayaraman, 2016). Moreover, activity of EB layers was associated to the LALs, encoding premotor command (Fiore et al., 2017; Namiki and Kazaki, 2016). However, our work showed no effect of the EB in SING modulation, which is consistent with a recent demonstration from Kottler et al. (deposited in BioRxiv). In contrast, the FSB appears to be involved in SING modulation.

As experiments in Results A, Part II, suggest that the FSB could be responsible for the transmission of integrated information from the SMP region. Neuronal activation by the columnar neurons extending across the PB and FSB (Figure 2A, driver *R52G12-Gal4*) or in the neurons that release neuroactive peptides in different layers of the FSB (Figure 2C, driver *R94C05-Gal4*) caused a decrease in locomotor reactivity. We have evidence for an involvement of the FSB in SING modulation, but we cannot tell for now if the FSB acts as

the pre-locomotor command centre responding directly to DA modulation for SING modulation, as DAN terminals reach to the FSB (Mao and Davis, 2009; Kong et al., 2010, Alekseyenko et al., 2013; Niens et al., 2017; Kahsai and Winther, 2011; Kahsai et al., 2012). Work from various groups has demonstrated that DAN of the PPL1 and PPM3 clusters can project to different layers of the FSB. For examples, PPL1 neurons tile the dorsal FSB part and projection of PPM3 arborizes on the ventral FSB and noduli (Kong et al., 2010; Alekseyenko et al., 2013; Strausfeld and Hirth et al., 2013). These DA terminals correspond to Dop1R1 localization in the FSB and noduli (Kahsai et al., 2012; Alekseyenko et al., 2013). Localization of Dop2R in the PB has also been reported in the work of Alekseyenko et al (2013). This indicates that the CC, reminiscent of mammalian basal ganglion, possesses two types of DA receptors with excitatory and inhibitory function, respectively, underpinning DA-related modulation. But, because of the heavy projections from different DAN clusters onto the MBs (PPL1, PAM and PPL2ab) and the potential expression of Dop2R in the MBs (Scholz-Kornehl and Schwärzel, 2016), the MBs may also be involved in the excitatory/inhibitory mechanism for modulating locomotor reactivity.

According to the pattern of the *R94C05-Gal4* driver, projections of these neurons go through the SMP region where the dendrites of DANs and terminals of MBONs lie. In the flesh fly *Neobellieria bullata*, dendrites of neurons reaching to the FSB largely lie in the SMP (Phillips-Portillo and Strausfeld, 2012), suggesting that input to the FSB may come from the MBONs terminals after DAN>KC>MBON integration. Further work is needed to characterize the synaptic interactions between the DANs, MBONs and FSB in this interesting convergence region, the SMP, to complete an overall view of DA-mediated SING control.

Discussion

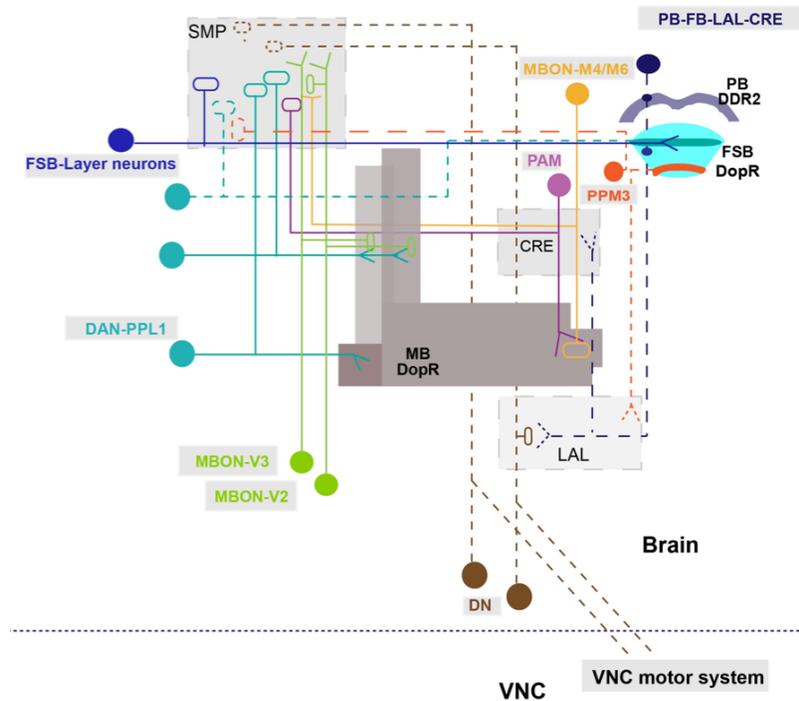


Figure 36 Startle-induced locomotion modulatory network

Different neuronal clusters are presented in different colour, with round solid circles cell body, rectangles dendrites and “V” axonal projections. The dashed line indicates neuronal connections based on previous studies but not verified by our work. Distribution of DA receptors is indicated in each neuronal compartment with Dop1R1 (DopR) in the MBs and two segments of the FSB and Dop2R (DDR2) in the FSB. The SMP region contains dendrites of DANs from the PPL1 (light blue), PAM (purple) and PPM3 (orange) clusters. FSB-layer neurons (blue), axons of MBON-M4/M6 (yellow) and MBON-V2/V3 (green) also lay in the SMP region, as well as potential dendrites and axons from descending neurons (DNs, brown). CRE: crepine; DN: descending neuron; DAN: dopaminergic neurons; FSB: fan-shaped body; LAL: lateral accessory lobes; MB: mushroom body; MBON: mushroom body output neuron; PAM: protocerebral anterior medial; PB: protocerebral bridge; PPL: protocerebral posterior lateral; PPM: protocerebral posterior medial; SMP: superior medial protocerebrum; VNC: ventral nerve cord.

Our work has identified three subtypes of MBONs (MBON-M4/M6, V2 and V3) conveying information processed through DANs-MBs interactions to the SMP region for SING modulation. They join there with the dendrites of PPL1/PAM DANs and intriguingly a few dendritic projections from the descending neurons (Namiki et al., 2017, BioRxiv) placing the SMP region as a potential zone of convergence of DANs, MBONs, CC and the sensorimotor system, all underpinning startle-induced locomotion. As described in this work of Namiki,

both LAL, SMP, SLP exhibited smooth processes (post-synaptic), while SMP and SLP tiny varicose processes (pre-synapse) of descending neurons. No dendrites of descending neurons were observed in the CC or MB by Namiki et al., suggesting no direct descending output from these high-order integrative regions. However, there is scarce but existing tiny varicose process of descending neurons in the calyx of the MBs, FSB and PB, implying tiny input feedback to the higher order brain.

1.3 Impaired SING in fly as a model of motor symptoms in PD patients

DA can be considered as a neuromodulator involved in locomotion control, not only for spontaneous locomotion (walking and flying) but also for locomotor reactivity (Riemensperger et al., 2011, 2013; Bou Dib et al., 2014; Agrawal and Hasan, 2015; Pathak et al., 2015; Vaccaro et al., 2017; Sun et al., 2018). Brain DA-deficient flies showed a decrease in walking speed, walking distance and strongly impaired locomotor reactivity (Riemensperger et al., 2011). In the Results-A part I, because of the inhibitory role of MBs in SING control, SING performance was normal or slightly improved when DA release onto the MB was blocked in most of the DANs (see Fig. 2A-C in Sun et al., 2018)). However, one could wonder then why the DA-deficient flies showed impaired locomotor reactivity. On the other hand, previous work from our group showed that α -synA30P expression in 15 DANs of the PAM (*NP6510-Gal4 driver*) caused degeneration of PAM projections onto the MBs and to premature locomotor decline with age (Riemensperger et al., 2013). Similar progressive locomotor defects were observed when TH was inactivated by RNAi during the whole life of the fly in these 15 PAM DANs (Riemensperger et al., 2013). Even more strikingly, TH RNAi inactivation in a single DAN of the PPL1 cluster (MB-V1) that also projects to the MBs also led to accelerated SING decline (Vaccaro et al., 2017). On the other hand, short-term or long-term blockage of DA release from the 15 PAM neurons with Shi^{ts1} (for 12 h per day during 3 weeks), elicited no impact on the locomotor reactivity (Riemensperger et al., 2013; Sun et al., 2018). Apparently then, accelerated SING decline can be caused either by a long-term lack of DA in MB-afferent DANs (as in the case of DA-deficient or TH-RNAi flies), or by long-term ROS accumulation in these MB-afferent DANs (as in the PAM- α -synA30P model described in Results B of this study, or, quite likely, in the case of *Clk*^{AR} mutant flies described in our group (Vaccaro et al., 2017). Overall, and although we don't understand all about it by far, a combination of DA deficiency and ROS accumulation appears to underpin the progressively impaired SING decline in flies, which suggests that studying further this effect is a good model to understand better what causes motor symptoms in PD patients.

Discussion

2. Parkinson disease-like pathogenesis

2.1 DANs as “greenhouse” for α -syn-induced defects

As shown in the Result B part, pan-neuronal expression of mutant α -synA30P induced ROS accumulation in the whole fly brain, whereas no obvious difference in ROS levels could be seen between α -synWT and control. This could be due to the fact that prefibrillar oligomer form may represent the toxic among all the forms, and the A30P mutant form has a reduced speed of fibrillization compared to the other mutant form or the wild-type protein (Seidel et al., 2010; Lashuel et al., 2012). Therefore, it is possible that the relatively prolonged presence of α -synA30P oligomers would enhance damage to mitochondria, lysosome, and microtubules, elevating the ROS level (Lashuel et al., 2012). Indeed, the higher ROS accumulation in the fly with α -synA30P expression correlated to the more severe locomotor defects.

DANs offer the best “hotbed” for α -syn-induced oxidative stress. When α -syn is expressed in the *NP6510-Gal4*-targeted DANs, the toxicity manifested with locomotor defects (Riemensperger et al., 2013). But, expressing α -synA30P with *VT30559-Gal4* in all MB KCs, which are in large part cholinergic, did not trigger such locomotor deficits, indicating the higher vulnerability of DANs than cholinergic neurons upon α -synA30P toxicity in the fly, as is the case in the human brain. Please note that *VT30559-Gal4* labels more than 1000 KCs, *R58E02-Gal4* around 170 PAM DANs plus cells in the optic lobes, and *NP6510-Gal4* 30 PAM DANs plus 3 non-DANs in the PAM (F neurons). *VT30559-Gal4* drives gene expression much stronger than *R58E02-Gal4* according to our observations. However, as we showed in Results B, only flies expressing α -synA30P in DANs and not in MB cells exhibited alterations in mitochondrial redox level.

As stated in a large number of reviews, the specific vulnerability of SNpc DANs to PD conditions would be due to high energy demand that requires a large quantity of mitochondria, heavy calcium loading for autonomous pacemaking activity but less calcium buffer proteins (Faustini et al., 2017; Surmeier and Schumacker, 2013; Reeve et al., 2014; Surmeier et al., 2017c). DANs that are equipped with a large quantify of mitochondria offer the best and more numerous engines for α -synA30P-mediated ROS production (Martin, 2006; Parihar et al., 2008). Mitochondria act as the powerhouse guarantying proper neuronal function, thus, mitochondrial equilibrium should be exquisitely regulated (Tatsuta and Langer, 2008), especially for DANs of the SNpc. A recent study showed that high wild-type α -syn levels in

fly neurons altered the localization of the mitochondrial fission protein Drp1 in the axons, subsequently disturbing mitochondrial quality control and enhancing α -syn toxicity. Strongly expressing α -syn in all neurons also led to enlarged mitochondria as visualized by mitoGFP, and oxidized mitochondria revealed with MitoTimer (Ordonez et al., 2018). It is known that a reduction in mitochondrial size is a requirement for mitochondrial clearance (Pozo Devoto and Falzone, 2017). What we can infer from those studies is that the enlarged mitochondria may slow down mitochondrial degradation, promoting aberrant mitochondria accumulation. On the other hand, due to the toxicity of α -synA30P, the aggregates of α -synA30P were reported to block the autophagic flux in the rat optic nerve (Koch et al., 2015), possibly undermining the autophagy-lysosomal pathway on the long term. Our own observations with mitoGFP in the PAM DANs are consistent therefore with previously published observations.

It is known that human α -syn expression increases intracellular ROS and mitochondrial stress (Junn and Mouradian, 2002; Parihar et al., 2008; Dias et al., 2013). As proton-carrier proteins located in the inner membrane of mitochondria, activated by mitochondrial ROS, UCP can effectively uncouple substrate oxidation from ATP phosphorylation, and decrease oxidative stress (Andrews et al., 2005a,b). Here our work showed that ROS clearance with UCP proteins in the PAM DANs mitigated locomotor defects induced by α -synA30P, highlighting that mitochondrial homeostasis inside these DANs is pivotal for normal startle-induced locomotion in the fly.

2.3 Non-cell autonomous mechanism underlying disease propagation

PD is now considered as a prion-disorder that involves neuron-to-neuron transmission of an α -syn-derived pathogen (Olanow and Prusiner, 2009; Costanzo and Zurzolo, 2013; Brundin and Melki, 2017), though this theory is still in part under debate (McCann et al., 2016; Surmeier et al., 2017b). ROS and reactive nitric oxide (RNS) species produced by extracellular α -syn-induced microglia activation would then accelerate the neurodegeneration around (Zhang et al., 2005). The loss of DANs in post-mortem PD brains is indeed associated with microgliosis (Przedborski, 2017). A recent study has lent further support that misfolded preformed fibrils of α -syn can be transferred from an affected neuron to a previously healthy one by binding to the surface motif lymphocyte-activation gene 3 *in vitro* and *in vivo* (Mao et al., 2016). In addition, this study also demonstrated that the accumulation of pathological α -syn can decrease levels of synaptic proteins, impair neuronal excitability, and finally lead to neuronal death. This could be the NCA mechanism caused by the preformed fibrillar form of α -syn.

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Because the effects of α -synA30P we observed in the PAM DANs were a disruption of mitochondrial structure and an increase in mitochondrial oxidation level, we searched for similar alterations in the MB target cells as an evidence of a NCA process, which was not yet demonstrated in the *Drosophila* model. Using the mito-roGFP2-Grx1 fluorescent probe, we did observe redox alterations in the MB mitochondria in 10-day old flies when α -synA30P was expressed in the PAM DANs. Strikingly, alleviating mitochondrial stress by UCP expression in the MBs improved the locomotor performance in the PAM- α -synA30P model. Not restricted to the amelioration of locomotion, UCP expression in the MB could also significantly decrease the lethality caused by PAM-expressed α -synA30P. This indicates that the NCA process we detected with the mito-roGFP2 probe is neither insignificant nor harmless, as rescuing the mitochondria in the MB target cells was sufficient to protect against both faster ARLD and lethality in this model.

Remarkably, in a proteome analysis of the α -synA30P *Drosophila* model (Xun et al., 2007), around 42% of the dysregulated proteins were found to be mitochondrion-associated at day 10, which is exactly the pre-symptomatic time at which we detected non-cell autonomously-induced redox alterations in the MB mitochondria. In contrast, we could not detect any difference with controls not expressing α -synA30P at day 30, which could be related to the fact that the PAM DAN synaptic projections to the MBs have probably all degenerated at this time (Riemensperger et al., 2013). This reinforces the hypothesis of the trans-synaptic transfer of a pathological agent between the degenerating DANs and their MB target.

What could be the nature of this transferred pathological agent? The first candidate is of course α -synA30P itself. Therefore we attempted to mimic such a transmission by expressing α -synA30P directly and selectively in the MB neurons. However, this did not lead to any obvious defects in locomotion nor alteration in the redox status of mitochondria in 10-day old flies. This might be explained if the cholinergic MB neurons were able to cope with α -synA30P toxicity by enhancing protein degradation and also maintain mitochondrial activity. Devoid of the ROS-producing context within DANs, α -synA30P expression in these MB neurons may not be sufficient to produce massive ROS accumulation and locomotor defects. Another hypothesis we could suggest is that α -synA30P needs to be generated in DANs to acquire the conformation and aggregation state of a dangerous prion protein able to induce dysfunction in target neurons. It is known indeed that DA itself can interact with α -syn to induce the formation and stabilization of toxic soluble α -syn oligomers or protofibrils

(Conway et al., 2001; Xu et al., 2002; Mor et al., 2017), which could then be transferred from the PAM DANs to the MBs as long as the contacts between these cells are maintained.

However, the prion hypothesis does not seem to hold in the fly model for several reasons. First, we could not detect the presence of α -synA30P in the MB cells by immunostaining in symptomatic state (at day 30) while this protein was readily detectable in the close by-located DANs. Second, a released α -synA30P oligomer could not induce pathological propagation after being taken up into another neuron by converting the α -syn present in this target cell into a prion, for the simple reason that there is no endogenous α -syn expressed in the fly. Third, we have shown previously that long-term TH inactivation in MB-afferent DANs can induce quite similar progressive locomotor deficits as those induced by α -synA30P expression in these DANs (Riemensperger et al., 2013; Vaccaro et al., 2017). Clearly, α -syn oligomers are not necessarily required for this NCA process. Based on that, we would like to propose that α -synA30P expression in DANs would rather facilitate pathological propagation through a ROS-associated NCA mechanism. Either ROS would be directly transferred trans-synaptically between neurons, or, perhaps, the escaping ROS would activate glia cells around, which would release pro-inflammatory factors and more ROS, promoting the degeneration of neurons in the vicinity. H_2O_2 permeation through aquaporin channels and $O_2^{\cdot-}$ penetration through anion channels are possible mechanisms for ROS translocation across the plasma membrane (Fisher, 2009). The neuronal membrane could also be damaged directly by ROS or by specific “pore-like” structure formed by the α -syn oligomers (Chen et al., 2015) that could facilitate ROS leakage through the membrane. Our idea that ROS transmission within the pathological propagation process undermined locomotion in the *Drosophila* PD model fits well with the popular microbiota-gut-brain axis theory for PD propagation. This interesting concept proposed that bacterial proteins may trigger inflammation and glial activation in the enteric system, causing oxidative stress transmission, neurodegeneration and motor deficits (Mulak and Bonaz, 2015; Sampson et al., 2016).

3. Piwi guards DANs against epigenetic modifications in PD

3.1 Neuroprotective role of Piwi in a fly model of PD

Neuronal DNA damage primarily induced by oxidative stress has been long considered as a potential cause of DAN loss in PD (Robison and Bradley, 1984; Sanders and Greenamyre, 2013; Coppedè and Migliore, 2015; Li et al., 2016). Oxidative stress can indeed trigger TE overexpression leading to chromatin alterations and DNA breaks (Stoycheva et al., 2010;

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Giorgi et al., 2010; Song et al., 2011). Maintaining chromatin integrity and preventing DNA damage could, therefore, be a trackable approach to treat or delay PD pathology. Heterochromatin-associated protein contributes to DNA repair in various conditions (Sansó et al., 2011; Dinant and Luijsterburg, 2009; Frost et al., 2014; Lemaître and Soutoglou., 2014; Rekaik et al., 2015). The Argonaute protein Piwi silences transcription of transposons that are complementary to piRNAs by increasing histone methylation and maintaining a repressive heterochromatin state at TE loci (Pal-Bhadra et al., 2004; Brower-Toland et al., 2007; Castel and Martienssen, 2013; Le Thomas et al., 2013; Tóth et al., 2016;) thereby potentially defending the genome against environmental insults.

In our study, we have shown that *piwi* inactivation in DANs markedly increased *Drosophila* lethality after PQ ingestion, suggesting that perturbation to chromatic homeostasis impaired fly anti-oxidative ability. DANs is the most vulnerable neuronal clusters in the brain. Overexpressing Piwi in DANs only was not enough to protect the flies against the PQ induced oxidative stress. But, flies with pan-neuronal Piwi overexpression exhibited higher resistance to oxidative stress induced by PQ. One possibility is that other subset of neurons might uptake PQ or be sensitive to the oxidative stress generated by PQ in DANs. Or this could be related to a tighter regulation of Piwi translation in DANs than in other neurons. As visualized with a H3K9me3 antibody, we observed that Piwi overexpression in all neurons relatively preserved histone methylation in the fly brains after PQ ingestion. This result is consistent with the increased PQ sensitivity of rat PC12 cells after pre-treatment with a DNA methyltransferase inhibitor (Kong et al., 2012). DNA methylation and histone modification are involved in establishing patterns of gene repression. Although DNA methylation and histone methylation are carried out by different chemical reactions and require different enzymes, it is apparent that these two pathways can be dependent on one another (Cedar and Bergman, 2009). Other than the effect on histone methylation, PQ toxicity was also associated with histone H3 hyperacetylation in the DANs due to reduced activity of HDAC (Song et al., 2010, 2011).

Convincing evidence of epigenetic abnormalities in PD has been reported by Desplats et al., (2011) who showed that DNA methyltransferase 1 (Dnmt1), an enzyme that maintains DNA methylation, was sequestered from the nucleus by α -syn, resulting in global DNA hypomethylation, in particular in CpG islands upstream of *SNCA* itself. Moreover, the methylation status of the gene encoding the cytokine Tumour necrosis factor α (TNF α) was significantly decreased in the SN compared to the cortex of both PD patients and healthy

individuals, implying that increased TNF α expression may trigger an inflammatory process, in this brain region specifically, rendering DANs more susceptible to environmental insults (Pieper et al., 2008). Based on a genome-wide DNA methylation analysis of PD patients' brains, Kaut et al. (2012) reported that the gene coding for cytochrome P450 2E1 (*CYP2E1*) was hypomethylated and its protein product overexpressed in the putamen and cortex of PD patients. Although *CYP2E1* is involved in the detoxification of xenobiotics, it has been associated with increased generation of ROS.

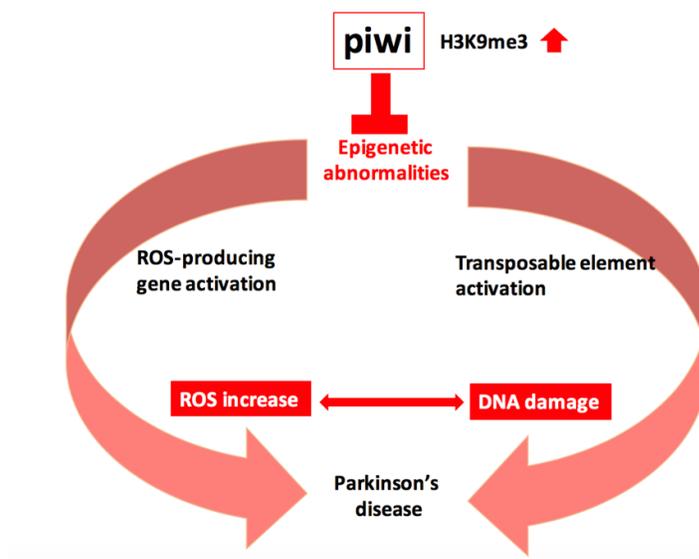


Figure 37 Hypothesis: protective role of Piwi in PD-like pathogenesis

By assessing TE levels, we have found that *HeT-A* and *R2* elements, two non-LTR retrotransposons, comparable to mammalian LINE-1, were induced during PQ treatment. *HeT-A* has a very interesting insertion site preference in telomeric DNA, and mutations in related genes mediating TE activity, showed increased telomere length (Pardue et al., 2005). In our work, the consequence of induced *HeT-A* during PQ exposure is unclear, but we considered this alteration as resulting from a general derepression of heterochromatin and activation of TEs. Similar to the maintenance of H3K9me3 marks by Piwi overexpression, PQ-induced *HeT-A* overexpression was partly prevented by overexpressed Piwi. From the data described in Results C, we proposed a potential protective role of Piwi against PD-like pathogenesis in flies (Figure 37). By maintaining histone methylation level, Piwi could preserve repressive epigenetic marks, leading to a decrease in the expression of ROS-producing genes and silencing of TE activity.

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3.2 PIWI functions outside the gonad

Piwi function is crucial to maintain the heterochromatin status, repress retrotransposon activation and prevent DNA damage (see in 3.1 above) both in germline and somatic cells in *Drosophila*. In the intestine, for example, Piwi is required for the long-term preservation of intestinal stem cells (ISC) function by maintaining heterochromatin marks and preventing apoptosis (Sousa-Victor et al., 2017). Piwi is induced in the gut by the JAK/STAT signaling pathway under infection, thus supporting the high rate of proliferative activity of ISC. In our study, we showed that Piwi is expressed at very low level in the *Drosophila* CNS, but can be induced by PQ, and that it protects neurons against acute oxidative stress. Another PIWI Argonaute *Drosophila* protein, Ago3, was shown to play a role in neurodegeneration. Frost et al. (2014) have reported a loss of H3K9me2 and HP1a in a fly model of Tau pathology. More precisely, Tau-induced decreased occupancy of H3K9me2 of the *Ago3* gene elevated Ago3 expression, which led to aberrant cell cycle activation and subsequent neuronal apoptosis. This observation was reminiscent of autopsy analyses from Alzheimer's disease patients, showing depleted H3K9me2 and increased PIWIL1 (mammalian homolog to Ago3). Surprisingly, although Ago3 and Piwi function in the same pathway to enhance piRNA function, they appear to have opposite effects in neurodegenerative context. In the AD model, elevated Ago3 induced by Tau was suggested to be associated with piRNA dysregulation and a loss of proliferative control. In our PD model, Piwi was induced by PQ, and, in contrast, Piwi overexpression protected against oxidative damage. The contrasted consequences between Ago3 and Piwi effects might be related to the nuclear localization of Piwi, which is not shared by Ago3, as increased Piwi levels could directly target the TE loci *in situ*, therefore silencing the TEs and restoring heterochromatin maintenance.

We also observed decreased level of H3K9me3 in the brain of aged *elav>piwi* RNAi flies. Decreased H3K9 methylation suggests decline of heterochromatin-enriched histone code with activation of gene expression. Until now, there is no direct evidence where Piwi localizes in the fly brain. Is it slightly expressed in the all neurons? Or it could be expressed restrictedly in few neuronal stem cells (von Trotha et al., 2009), which would be involved in proliferative function identically to Piwi in the stem and germline cells. piRNAs were previously detected in the brain, including in mouse hippocampus and *Drosophila* MBs (Lee et al., 2011, Perrat et al., 2013; Ross et al. 2014; Bozzetti et al., 2015). In our study, induced or overexpressed Piwi in the brain could maintain the heterochromatin state, suggesting that its neuroprotective function depends on its activity in the nucleus. The elaborate mechanisms

to explain how Piwi protects against PQ toxicity and ageing will require further studies to be elucidated.

3.3 Potential function of PIWI/piRNAs in PD pathology

PIWI proteins use a group of small non-coding RNA, the piRNAs, to regulate cellular activity by RNA silencing. In fly, over 60% of piRNAs map to genomic locations, with 70% targeting to transposons, the rest mapping to non-annotated regions and protein-coding genes (Senti and Brennecke, 2010). Deep sequencing of extracted RNAs from the mouse hippocampus led to characterize a subset of piRNAs; which was followed by the discovery that Miwi (mouse homolog of Ago3) is expressed in the hippocampal neurons. One type of piRNA, DQ541777, was predicted to target genes coding Cyclin-dependent protein kinases 5 regulatory subunit-associated protein 1 (*Cdk5rap1*) and microtubule affinity-regulating kinase 1/2 (*MARK1/2*) (Lee et al., 2011a).

Cdk5rap1 inhibits the activation of the kinase *Cdk5* by p25 (Ching et al., 2002). *Cdk5* has no function in cell-cycle progression, but rather in the organization of the cytoarchitecture of the CNS (Dhavan and Tsai, 2001). PIWI/piRNA binding to *Cdk5rap1* was shown by the presence of *Cdk5rap1* in MIWI-IP (immunoprecipitation done with mouse MIWI) products (Nishibu et al., 2012), suggesting that PIWI/piRNA may mediate its expression. Conceivably, aberrant regulation of *Cdk5rap1* would lead to hyperactivated *Cdk5* binding to p25. Both were identified as the components of Lewy bodies in the PD patients (Beyer and Ariza et al., 2007; Wakabayashi et al., 2013) and *Cdk5* is involved in the pathology of PD (Wilkaniec et al., 2016). The oligomers of α -syn can induce *Cdk5* hyperactivity. Then, this elevated *Cdk5* activity could impact on parkin phosphorylation and lead to its aggregation, followed by the mitochondrial dysfunction, oxidative stress and dopaminergic neurodegeneration.

As to the other piRNA binding gene, *MARK1/2*, activation of MARK proteins was observed to accumulate in the Lewy bodies-like inclusions as well as in the Lewy bodies of postmortem synucleinopathies brains, suggesting the role of MARK in α -syn pathology (Henderson et al., 2017). Besides, MARKs have been associated to the PD-associated genes, *Pink1* and *Lrrk2*. MARK2 and PINK1 have been reported to localize with mitochondria and regulate their transport. MARK2 was shown to interact and preferentially phosphorylate the cleaved form of PINK1, thus increasing PINK1 activity. Interestingly, the main phosphorylated site by MARK2 on the cleaved PINK was Thr-131, a frequent mutated site of familial PD (Matenia et al., 2012). MARK1 was validated as a substrate of LRRK2,

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potentially acting as a key player in the pathway by which LRRK2 regulates cytoskeletal dynamics (Krumova et al., 2015). In addition, MARK1 serves as the binding partner of 14-3-3 probably involving in cytoskeletal organization and cellular trafficking (Göransson et al., 2006). Potentially, via employing piRNA DQ541777 and other not-yet identified piRNAs, dysregulation of PIWI could be directly associated with PD pathology.

4. Ageing, environmental factors, ROS, and PD

Ageing is the major risk factor for the onset of PD, not only because of accumulated mutations and misfolded proteins, but also due to alterations in the genome landscape, with the consequence of ROS build-up and abnormal gene expression (Reeve et al., 2014; Wood and Helfand, 2013). This likely leads to inefficiency in mitochondrial activity and malfunction of the proteolytic systems, and ultimately failure to maintain cellular homeostasis in DANs (Reeve et al., 2014; Surmeier et al., 2017b).

Derepression of transposon activity is associated with ageing and some TEs in the brain are overexpressed upon PQ treatment as shown in our study. A causative relation between transposons and the PD pathology has not been established yet. However, some report suggested that TE activity can influence the biosynthesis of a number of neurotransmitters, contributing to neurodegenerative disorders, particularly in PD (Baillie et al., 2011; Abrusán, 2012). Interestingly, the endogenous mechanisms to silence TEs mainly take place in the gonad tissue rather than the somatic tissue. Given that TEs accumulate in the brain with ageing, the relevant mechanisms perhaps emerged to defend the integrity of the genome, which would help understand why Piwi is induced in the PQ toxicity context, as well as in the ageing brain. Piwi could prevent PQ-induced DNA damage (not tested yet) and loss of heterochromatin marks, thus compromising the subsequent ROS production and epigenetic abnormalities triggered by PQ and ageing. The observation that Piwi overexpression in all neurons efficiently protect against acute PQ toxicity, suggests that damage to the genome during the PD pathology should not be neglected.

A major difference between germline and somatic cells is that the Piwi/piRNA pathway is highly used in germline cells to target TEs activity and maintain their non-ageing phenotypes. According to Sturm et al (2015), the mortality rate rises exponentially throughout the adult life suggesting the primary factors for ageing might be TE activity rather than the decline in efficiency of cellular repair processes. In agreement with the retrotransposon theory of aging, the overexpression of genes involved in heterochromatin maintenance or the silencing of TEs

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by inhibiting reverse transcriptase increased lifespan in the fly (Wood et al., 2016). Therefore, TE-repressive mechanisms combined with enhanced ROS clearance and cellular repair process could postpone the onset of PD and other ageing-associated neurodegenerative disorders.

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder, characterized at the cellular level by the loss of midbrain dopaminergic neurons (DANs) and the presence of cytoplasmic inclusions mainly composed of α -synuclein (α -syn) named Lewy bodies. A combination of multiple factors, including ageing, genetic mutations, and environmental insults contribute to the development of PD, making it difficult to understand its neuropathological pathways. Although research on PD has remarkably advanced, we still do not have a curable treatment. A useful *Drosophila* PD model showed that mutant α -synA30P expression in a subset of DANs of the brain protocerebral anterior medial (PAM) cluster caused progressive locomotor deficits and loss of projections of these cells to the mushroom bodies (MBs). The objectives of my PD work were to characterize further this *in vivo* model to better understand how α -syn accumulation in DANs can disrupt startle-induced locomotion and to search for novel proteins or pathways that may confer neuroprotection in PD-related pathological conditions.

In this work we first identified putative networks underlying the modulation of startle-induced locomotion in the fly, which primarily involve subsets of DANs and MB-associated neurons. We then obtained evidence that α -synA30P expression in PAM DANs triggers mitochondrial dysfunction both in the DANs and, through a non-cell autonomous process, in their target MB cells. Alleviating mitochondrial stress by expressing uncoupling proteins (UCPs) either in DANs or, remarkably, in the MBs mitigated the locomotor deficits and increased fly survival in the PAM- α -synA30P model. Finally, we report that the Argonaute protein Piwi is induced by oxidative stress and neuroprotective in the *Drosophila* brain. Piwi can delay neuronal ageing and PD-like progression by enhancing H3K9 methylation and potentially silencing transposable elements. Overall, our work provides new insights on PD-related pathological and neuroprotective mechanisms in the *Drosophila* model.

Key words: Parkinson's disease, startle-induced locomotion, mitochondrial homeostasis, non-cell autonomous process, Piwi, epigenetic modifications, *Drosophila melanogaster*