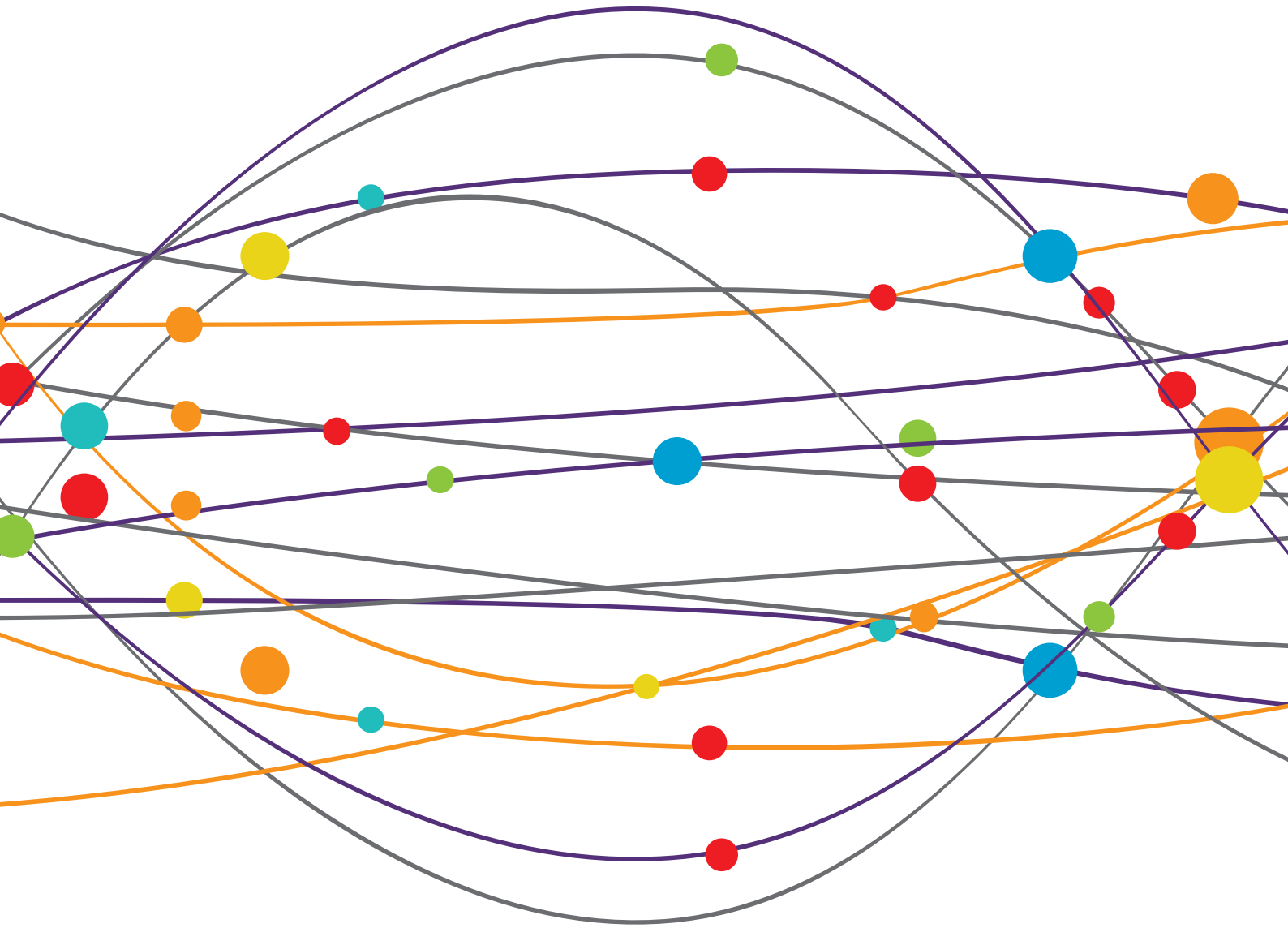
MODELING PARKINSON'S DISEASE: BRIDGING THE TRANSLATIONAL GAP

EDITED BY: Graziella Madeo, Antonello Bonci and Xiaobo Mao
PUBLISHED IN: Frontiers in Neurology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-189-6
DOI 10.3389/978-2-88963-189-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MODELING PARKINSON'S DISEASE: BRIDGING THE TRANSLATIONAL GAP

Topic Editors:
Graziella Madeo, National Institutes of Health (NIH), United States
Antonello Bonci, National Institute on Drug Abuse (NIDA), United States
Xiaobo Mao, Johns Hopkins University, United States

Citation: Madeo, G., Bonci, A., Mao, X., eds. (2019). Modeling Parkinson's Disease: Bridging the Translational Gap. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88963-189-6

Table of Contents

frontiers in Neurology

MINI REVIEW
published: 04 October 2017
doi: 10.3389/fneur.2017.00527

Mitophagy in Parkinson's Disease: Pathogenic and Therapeutic Implications

Fei Gao, Jia Yang, Dongdong Wang, Chao Li, Yi Fu, Huaishan Wang, Wei He and Jianmin Zhang**

Department of Immunology, Research Center on Pediatric Development and Diseases, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College, State Key Laboratory of Medical Molecular Biology, Beijing, China

Neurons affected in Parkinson's disease (PD) experience mitochondrial dysfunction and bioenergetic deficits that occur early and promote the disease-related α-synucleinopathy. Emerging findings suggest that the autophagy-lysosome pathway, which removes damaged mitochondria (mitophagy), is also compromised in PD and results in the accumulation of dysfunctional mitochondria. Studies using genetic-modulated or toxin-induced animal and cellular models as well as postmortem human tissue indicate that impaired mitophagy might be a critical factor in the pathogenesis of synaptic dysfunction and the aggregation of misfolded proteins, which in turn impairs mitochondrial homeostasis. Interventions that stimulate mitophagy to maintain mitochondrial health might, therefore, be used as an approach to delay the neurodegenerative processes in PD.

Keywords: mitophagy, Parkinson disease, mitochondria, synuclein, autophagosome

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Paola Bonsi, Fondazione Santa Lucia (IRCCS), Italy
Ludivine S. Breger, Lund University, Sweden

****Correspondence:***
Wei He
heweingd@126.com;
Jianmin Zhang
jzhang@ibms.pumc.edu.cn

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *16 June 2017*
Accepted: *21 September 2017*
Published: *04 October 2017*

Citation:
Gao F, Yang J, Wang D, Li C, Fu Y, Wang H, He W and Zhang J (2017) Mitophagy in Parkinson's Disease: Pathogenic and Therapeutic Implications. Front. Neurol. 8:527. doi: 10.3389/fneur.2017.00527

INTRODUCTION

Parkinson's disease (PD) is an incurable chronic progressive disease affecting nearly 2% of the "over 50" population with an approximately estimate of more than 6 million cases worldwide (1). The cause of PD is generally unknown, but it is believed to involve both genetic and environmental factors (2). Epidemiological studies have revealed that fewer than 10% of PD cases are inherited from family, whereas the majority of cases are sporadic (3). Discoveries of genes linked to rare familial forms of PD have confirmed the critical role of genes in the development of PD and made great contributions in understanding the molecular pathogenesis behind this common but complex illness. Autophagy is a conserved pathway that degrades damaged organelles and misfolded proteins (4). Here, we consider the roles of autophagy in neuronal health and the pathological mechanisms leading to disease progression to help us seek for potential targets for neuroprotective interventions, which may revolutionize the treatment of this incurable disease.

PROTEIN AGGREGATION AND MITOCHONDRIAL DYSFUNCTION IN PD

Parkinson's disease is a neurodegenerative movement disorder characterized by the preferential loss of dopaminergic neurons in the substantia nigra, which results in progressive motor system malfunction (5). Primary motor signs that characterize PD include rigidity, bradykinesia, postural instability, and tremor (6). The pathology of PD remains unknown, but almost all cases show the

presence of intraneuronal misfolded protein aggregates forming Lewy bodies, the primary component of which is α-synuclein (7). Protein homeostasis is crucial to sustain cellular health and viability in neurons (8). The process of α-synuclein accumulation resulting in the generation of highly diffusible small oligomers and fibrils, which abnormally aggregate and can be visualized as eosinophilic cytoplasmic inclusion in neurons (9). Evidence indicates that the accumulation of [α-synuclein, possibly oligomers, without insoluble aggregates, may lead to oxidative stress and give rise to deleterious effects in dopamine (DA) neurons (10–13)].

Recent evidence suggests that α-synuclein is a lipophilic protein, localized to mitochondria and connected to endoplasmic reticulum (ER) through mitochondrial-associated ER membrane (MAM) (14, 15). Overexpression of α-synuclein inhibits the normal function of inner-mitochondrial membrane-anchored respiratory chain complexes in whole brain of PD patients, but mostly in nigrostriatal neurons. Increased levels of reactive oxygen species (ROS) might be the cause of neuronal death (16). A study has also demonstrated that α-synuclein overexpression in mitochondria increases the number of fragmented mitochondria in vitro (17). In addition, intermediate α-synuclein accumulation (pre-fibrillar forms) reduces mitochondrial Ca^{2+} retention (18). Ca^{2+} is required by mitochondria for the generation of ATP *via* the tricarboxylic acid cycle (19). Perturbed neuronal Ca^{2+} levels caused by soluble pre-fibrillar α-synuclein lead to altered mitochondrial membrane potential and NADH oxidation, which indicate the dysfunction of complex I (20). The effect of complex I inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite 1-methyl-4-phenylpyridinium (MPP+) on dopaminergic cell death is inhibited in mouse models lacking α-synuclein, which is mainly due to the inactivation of nitric oxide synthase (NOS) (21). In addition, siRNA-mediated knockdown of α-synuclein also protects cells from NOS activation in cellular models, rescuing cells from MPP^+-induced apoptosis (22).

Posttranslational modification of α-synuclein is also a crucial factor in the pathological mechanisms of PD. Many PD-associated mutations in α-synuclein also induce mitochondrial dysfunction. The H50Q mutation is proved to induce aggregation of α-synuclein oligomers in SH-SY5Y cells and increase the number of fragmented mitochondria in hippocampal neurons *in vivo* (23, 24). Ser129-induced α-synuclein aggregation is involved in the formation of Lewy bodies and plays a critical role in the neurodegenerative process (25). SH-SY5Y cells expressing A53T α-synuclein exhibit depolarized mitochondrial and increased ROS levels when exposed to rotenone (26). Studies in transgenic mice overexpressing the A53T-mutant human α-synuclein revealed that intracerebral inoculation of aggregated α-synuclein or preformed recombinant α-synuclein fibrils induces a progressive and ultimately lethal α-synucleinopathy in inoculated animals (27, 28).

Damaged cellular function and decreased ATP levels induced by α-synuclein are detrimental to dopaminergic neurons and provide implications for disease pathogenesis in PD. Impaired mitochondrial function may lead to a reduction in cellular energy levels and excessive ROS production in neurons, which in turn exacerbate mitochondrial damage (29). As a result, measures to enhance the degradation of abnormally aggregated proteins and the clearance of damaged mitochondria seem to be the most promising strategies in rescuing neurodegeneration in PD patients.

PD-RELATED GENES AND THEIR ROLES IN MITOPHAGY AND MITOCHONDRIAL DYSFUNCTION

Autophagy is an evolutionarily conserved process in which cytoplasmic substrates are engulfed in autophagic vesicles and fused to lysosomes for degradation and recycling (30). The specific autophagic elimination of mitochondria is defined as mitophagy (31). Autophagy is classified into various subgroups based on the mechanism of substrate delivery to the lysosome, including macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy (4). The process of mitophagy is directed mainly by macroautophagy. Genome-wide association studies implicate that PD-related genes and their products are responsible for mitochondrial homeostasis and mitophagy (32, 33).

PINK1 and Parkin are the most well-known proteins related to PD. PINK1, encoded by PARK6 gene, is a mitochondrial-targeted serine/threonine kinase, while Parkin, encoded by the PARK2 gene, is a 465-amino acid E3 ubiquitin ligase (34, 35). "Loss-of-function" mutations in either PINK1 or Parkin lead to autosomal recessive forms of PD (35, 36). PINK1-dependent activation of Parkin is recognized as a major pathway of mitophagy (37). When mitochondria become depolarized, PINK1 accumulates on the surface of the outer membrane of mitochondria, where it phosphorylates both ubiquitin and Parkin and activates Parkin's ubiquitin E3-ligase activity. Moreover, it was recently shown that wild-type PINK1 recruits Parkin to damaged mitochondria during mitophagy rather than the PD-linked PINK1-mutant forms (38). The subsequent recruitment of ubiquitin-binding mitophagy receptors lead to the formation of LC3-positive phagophores, which sequester damaged mitochondria from the cytosol and eventually degrade by lysosomal hydrolases (39). PINK1 and Parkin are also important for sustaining mitochondrial homeostasis through the regulation of mitochondrial fission and fusion. A study has shown that the ubiquitination process of mitochondrial fusion protein mitofusin (Mfn) is mediated by both PINK1 and Parkin. Loss of PINK1 or Parkin causes damaged mitophagy process and elongated mitochondria in *Drosophila* (40). Genetic loss of Mfn1 and Mfn2 leads to the dissipation of membrane potential in a subset of mitochondria, preventing Parkin's recruitment process through the translocase of the inner membrane complex (41). Parkin-mutant or PINK1-mutant *Drosophila* display a severe defect in flight muscle, leading to behavioral locomotive problems and greater susceptibility to oxidative stress (42, 43). Indirect flight muscles and DA neurons in this model are filled with swollen mitochondria (44, 45). Mutant-Parkin displays degeneration of a subset of DA neurons, exhibiting shrinkage in morphology and decreased DA level in *Drosophila* brains (42, 46, 47). PINK1 knockout fibroblasts and neurons exhibit reduced membrane potential, overloaded Ca^{2+} levels and increased ROS production in mitochondria (48, 49). Meanwhile, mitochondria

isolated from the brain of PINK1 knockout mice show defects in Ca^{2+}-buffering capacity and increased vulnerability of neurons in oxidative stress caused by inflammation (50). DA neuronal death is also observed in a conditional Parkin ablation mouse model after lentivirus delivers the Cre recombinase to the mouse brain, which suggests that Parkin plays an important role in neuronal survival (51).

Mutations in the PARK7 gene, which encode DJ-1, cause a rare autosomal recessive form of PD (52, 53). DJ-1, a transcriptional regulator, is often known as a redox sensor/reductase which influences mitochondrial homeostasis and mitophagy (54). It is long believed that DJ-1 is a neuroprotective factor (55). Mitochondria localized DJ-1 is a component of thioredoxin/apoptosis signal-regulating kinase 1 (Trx/Ask1) complex, which regulates the clearance of endogenous ROS through the modulation of scavenging systems (56). DJ-1 deficiency decreases brain mitochondria consumption of H_2O_2, leading to the increased level of oxidative stress, and eventually causes cell death in DA neurons (54, 57). In addition, DJ-1 directly interacts with α-synuclein. The mutant form of DJ-1 in PD causes misfolded α-synuclein aggregate in DA neurons, while the overexpression of DJ-1 reduces the dimerization of α-synuclein (55).

LRRK2 is a member of the leucine-rich repeat kinase family that is encoded by the PARK8 gene (58). Mutations in LRRK2 are associated with autosomal-dominant PD (33). Expression of mutant LRRK2 may have a variety of negative effects on mitochondrial and cellular health (59, 60). Endogenous LRRK2 directly interacts with the mitochondrial fission and fusion regulators dynamin-related protein 1, Mfn, and optic atrophy 1 (OPA1) to maintain the balance among mitochondrial biogenesis, intracellular material trafficking, metabolic demands, and mitochondrial morphology (61–63). G2019S mutant LRRK2 in sporadic PD patients showed decreased levels of OPA1, indicating that LRRK2 kinase activity is also an important factor in mitochondrial dynamics (64). The overexpression of G2019S mutant LRRK2 in mouse brains showed mitochondrial uncoupling accompanying with an increased basal oxygen consumption in both fibroblast and neuroblastoma cells, resulting in decreased ATP level and compromised cellular function (65). Fibroblasts with G2019S mutant LRRK2 from PD patients showed increased susceptibility to MPP^+ induced cell death (66). Meanwhile, the depletion of LRRK2 or mutant LRRK2 impair the autophagy/lysosomal pathway, leading to the accumulation of autophagosomes (67, 68). The degradation of LRRK2 in lysosomes is mediated by CMA in nervous system, while the mutant forms of LRRK2 and also high concentrations of wild-type LRRK2 interfere with the CMA translocation complex, resulting in defective CMA (67, 69). Inhibition of CMA in neurons induces the accumulation of both soluble and insoluble α-synuclein, which in turn could compromise the degradation of α-synuclein and initiate protein aggregation in PD (70, 71).

Lysosomal defects in the clearance of cytosolic substrates also contribute to the progression of PD (72). PARK9 encoded lysosomal ATPase ATP13A2 is a P-type transport ATPase which protects against cellular dysfunction caused by α-synuclein (73). PD-linked mutations in ATP13A2 reduce the activity of proteolytic processing enzymes, disturbing the acid environment in lysosomes, resulting in the impaired degrading capacity of autophagosomes (74).

As we can see, these PD-related genes not only play a role in the maintenance of mitochondrial homeostasis but also are important for the clearance of aggregated proteins and damaged organelles through mitophagy. Mitochondrial deficiency is responsible for neurodegeneration in PD, but the specific mechanism between mitochondrial deficiency and α-synuclein aggregation remains to be discovered.

THERAPEUTIC IMPLICATIONS FOR PHARMACOLOGICAL TARGETING AND GENE THERAPY

Intracellular misfolded proteins contribute to cellular dysfunction and neuronal death in PD patients. Moreover, compromised clearance pathways aggravate the pathological process of this neurodegenerative disease. Since autophagy plays an important role in selectively degrading misfolded proteins and damaged organelles, it could be an interesting target for the development of efficient treatment for PD. Nowadays, up-to-date researches also give us implications on PD-related genes and their influence on mitochondrial homeostasis. The obstacles between this promising therapeutic targets and mitochondrial dynamic are still a major challenge for us to overcome.

Methods identified to enhance autophagy in several preclinical PD models are proven to be effective. The serine/threonine protein kinase mTOR is a component of the mTOR complex 1 and suppresses autophagy under nutrient-rich conditions (75). The mTOR inhibitor rapamycin, which stabilizes the association of mTOR complex and inhibits the kinase activity, is the most widely used small molecule drug which is proved effective in enhancing autophagy activity in many disease models (76–81). Rapamycin selectively suppresses the activity of mTOR through the dephosphorylation of Akt kinase, which is crucial for neuronal survival in PD models (82, 83).

Beclin 1 is encoded by autophagy-related gene 6. This protein interacts with either BCL-2 or the class III phosphatidylinositol 3-kinase (PI_3K) VPS34, playing a critical role in the localization of other autophagy-regulatory proteins to the preautophagosomal structure (84). Beclin 1 is negatively regulated by BCL-2 and BCL-X_L at ER membranes (85). Mutations in BH3-related domain in Beclin 1 disrupt the formation of Beclin 1–BCL-2 complex, leading to enhanced autophagy (86). Chronic administration of trehalose results in a reduction of the frontal cortex p62/beclin 1 level, suggesting an elevated state of autophagy (87–90). Moreover, ER stress is responsible for the activation of autophagy through the unfolded-protein response (UPR) (91). Tunicamycin Induced mild ER stress shows a promising treatment potential in protecting dopaminergic neurons from death in PD models (92). Gene therapy approaches to handle the unfolded protein load *via* the activation of UPR are designed to manipulate autophagy in a more specific manner (93). Beclin 1 gene therapy mediated by lentivirus exhibits not only positive effects in the clearance of intraneuronal α-synuclein proteins but also a proved synaptic function in PD models (94). Gene therapy also exhibits great

potential in the clearance of abnormally aggregated proteins in other neurodegenerative diseases through the activation of autophagy (95–97).

Although methods to activate autophagy are promising novel therapeutic approaches for PD, a complex scenario is emerging in which the alteration of distinct regulatory steps in autophagy may perturb the homeostasis of the cell, contributing to the disease progression as well (98). Therefore, the mere enhancement of autophagy may have detrimental consequences by provoking neurodegeneration and exacerbating disease progression. Thus, it is critical that this biological process should be precisely regulated and strictly monitored. Moreover, the specific mechanism behind each subtype of the disease that may link the defects of autophagy to PD still remains to be discovered. Considering the complex nature of PD, individualized interventional targets seem to be the most promising method for deciding the right timing and appropriate degree of activation of autophagy.

CONCLUDING REMARKS

Significant progress has been made in understanding the causes of this neurodegenerative disorder. The accumulation of dysfunctional mitochondria and compromised mitophagy have emerged as common features of affected neurons in patients and animal models that may cause the accumulation of misfolded protein aggregates. In addition, aggregation of α-synuclein and deficiency in PD-related genes can impair neuronal mitophagy and mitochondrial homeostasis. It is crucial to find out the key factors and their roles involved in the pathogenesis of different form of PD. Further studies aiming at modulating the process of autophagy accurately and individually may provide novel therapeutic strategies for this widespread disease.

AUTHOR CONTRIBUTIONS

FG, JY, DW, CL, YF, and HW wrote the manuscript. WH and JZ edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31471016 and 81602503), the CAMS Initiative for Innovative Medicine (2016-I2 M-1-008), and the National Key Research and Development Program of China (2016YFA0101001).

REFERENCES

1. de Rijk MC, Breteler MM, Graveland GA, Ott A, Grobbee DE, van der Meché FG, et al. Prevalence of Parkinson's disease in the elderly: the Rotterdam study. *Neurology* (1995) 45:2143–6. doi:10.1212/WNL.45.12.2143
2. Gan-Or Z, Dion PA, Rouleau GA. Genetic perspective on the role of the autophagy-lysosome pathway in Parkinson disease. *Autophagy* (2015) 11:1443–57. doi:10.1080/15548627.2015.1067364
3. Schapira AHV. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* (2008) 7:97–109. doi:10.1016/S1474-4422(07)70327-7
4. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, et al. Molecular definitions of autophagy and related processes. *EMBO J* (2017) 36:1811–36. doi:10.15252/embj.201796697
5. Dauer W, Przedborski S. Parkinson's disease. *Neuron* (2003) 39:889–909. doi:10.1016/S0896-6273(03)00568-3
6. Lang AE, Lozano AM. Parkinson's disease. First of two parts. *N Engl J Med* (1998) 339:1044–53. doi:10.1056/NEJM199810083391506
7. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* (1997) 388:839–40. doi:10.1038/42166
8. Taylor RC, Berendzen KM, Dillin A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. *Nat Rev Mol Cell Biol* (2014) 15:211–7. doi:10.1038/nrm3752
9. Tsigelny IF, Bar-On P, Sharikov Y, Crews L, Hashimoto M, Miller MA, et al. Dynamics of alpha-synuclein aggregation and inhibition of pore-like oligomer development by beta-synuclein. *FEBS J* (2007) 274:1862–77. doi:10.1111/j.1742-4658.2007.05733.x
10. Decressac M, Mattsson B, Weikop P, Lundblad M, Jakobsson J, Björklund A. TFEB-mediated autophagy rescues midbrain dopamine neurons from α-synuclein toxicity. *Proc Natl Acad Sci U S A* (2013) 110:E1817–26. doi:10.1073/pnas.1305623110
11. Tokuda T, Qureshi MM, Ardah MT, Varghese S, Shehab SAS, Kasai T, et al. Detection of elevated levels of α-synuclein oligomers in CSF from patients with Parkinson disease. *Neurology* (2010) 75:1766–72. doi:10.1212/WNL.0b013e3181fd613b
12. Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, et al. In vivo demonstration that alpha-synuclein oligomers are toxic. *Proc Natl Acad Sci U S A* (2011) 108:4194–9. doi:10.1073/pnas.1100976108
13. Giehm L, Svergun DI, Otzen DE, Vestergaard B. Low-resolution structure of a vesicle disrupting alpha-synuclein oligomer that accumulates during fibrillation. *Proc Natl Acad Sci U S A* (2011) 108:3246–51. doi:10.1073/pnas.1013225108
14. Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P. Mitochondrial association of alpha-synuclein causes oxidative stress. *Cell Mol Life Sci* (2008) 65:1272–84. doi:10.1007/s00018-008-7589-1
15. Hayashi T, Rizzuto R, Hajnoczky G, Su T-P. MAM: more than just a housekeeper. *Trends Cell Biol* (2009) 19:81–8. doi:10.1016/j.tcb.2008.12.002
16. Subramaniam SR, Vergnes L, Franich NR, Reue K, Chesselet M-F. Region specific mitochondrial impairment in mice with widespread overexpression of alpha-synuclein. *Neurobiol Dis* (2014) 70:204–13. doi:10.1016/j.nbd.2014.06.017
17. Plotegher N, Gratton E, Bubacco L. Number and brightness analysis of alpha-synuclein oligomerization and the associated mitochondrial morphology alterations in live cells. *Biochim Biophys Acta* (2014) 1840:2014–24. doi:10.1016/j.bbagen.2014.02.013
18. Calì T, Ottolini D, Negro A, Brini M. α-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions. *J Biol Chem* (2012) 287:17914–29. doi:10.1074/jbc.M111.302794
19. Rowland AA, Voeltz GK. Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat Rev Mol Cell Biol* (2012) 13:607–25. doi:10.1038/nrm3440
20. Luth ES, Stavrovskaya IG, Bartels T, Kristal BS, Selkoe DJ. Soluble, prefibrillar α-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction. *J Biol Chem* (2014) 289:21490–507. doi:10.1074/jbc.M113.545749
21. Fountaine TM, Venda LL, Warrick N, Christian HC, Brundin P, Channon KM, et al. The effect of alpha-synuclein knockdown on MPP+ toxicity in models of human neurons. *Eur J Neurosci* (2008) 28:2459–73. doi:10.1111/j.1460-9568.2008.06527.x
22. Ryan BJ, Lourenço-Venda LL, Crabtree MJ, Hale AB, Channon KM, Wade-Martins R. α-Synuclein and mitochondrial bioenergetics regulate tetrahydrobiopterin levels in a human dopaminergic model of Parkinson disease. *Free Radic Biol Med* (2014) 67:58–68. doi:10.1016/j.freeradbiomed.2013.10.008
23. Appel-Cresswell S, Vilarino-Guell C, Encarnacion M, Sherman H, Yu I, Shah B, et al. Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. *Mov Disord* (2013) 28:811–3. doi:10.1002/mds.25421

24. Khalaf O, Fauvet B, Oueslati A, Dikiy I, Mahul-Mellier A-L, Ruggeri FS, et al. The H50Q mutation enhances α-synuclein aggregation, secretion, and toxicity. *J Biol Chem* (2014) 289:21856–76. doi:10.1074/jbc.M114.553297
25. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* (2002) 4:160–4. doi:10.1038/ncb748
26. Perfeito R, Lázaro DF, Outeiro TF, Rego AC. Linking alpha-synuclein phosphorylation to reactive oxygen species formation and mitochondrial dysfunction in SH-SY5Y cells. *Mol Cell Neurosci* (2014) 62:51–9. doi:10.1016/j.mcn.2014.08.002
27. Mougenot A-L, Nicot S, Bencsik A, Morignat E, Verchère J, Lakhdar L, et al. Prion-like acceleration of a synucleinopathy in a transgenic mouse model. *Neurobiol Aging* (2012) 33:2225–8. doi:10.1016/j.neurobiolaging.2011.06.022
28. Luk KC, Kehm VM, Zhang B, O'Brien P, Trojanowski JQ, Lee VMY. Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice. *J Exp Med* (2012) 209:975–86. doi:10.1084/jem.20112457
29. Mattson MP, Gleichmann M, Cheng A. Mitochondria in neuroplasticity and neurological disorders. *Neuron* (2008) 60:748–66. doi:10.1016/j.neuron.2008.10.010
30. De Duve C. The lysosome. *Sci Am* (1963) 208:64–72. doi:10.1038/scientificamerican0563-64
31. Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Res* (2005) 8:3–5. doi:10.1089/rej.2005.8.3
32. Ryan BJ, Hoek S, Fon EA, Wade-Martins R. Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. *Trends Biochem Sci* (2015) 40:200–10. doi:10.1016/j.tibs.2015.02.003
33. International Parkinson Disease Genomics Consortium, Nalls MA, Plagnol V, Hernandez DG, Sharma M, Sheerin U-M, et al. Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. *Lancet* (2011) 377:641–9. doi:10.1016/S0140-6736(10)62345-8
34. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MMK, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* (2004) 304:1158–60. doi:10.1126/science.1096284
35. Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* (2005) 28:57–87. doi:10.1146/annurev.neuro.28.061604.135718
36. Zhang J, Ney PA. NIX induces mitochondrial autophagy in reticulocytes. *Autophagy* (2008) 4:354–6. doi:10.4161/auto.5552
37. Grenier K, McLelland G-L, Fon EA. Parkin- and PINK1-dependent mitophagy in neurons: will the real pathway please stand up? *Front Neurol* (2013) 4:100. doi:10.3389/fneur.2013.00100
38. Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RLA, Kim J, et al. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc Natl Acad Sci U S A* (2010) 107:378–83. doi:10.1073/pnas.0911187107
39. Moore AS, Holzbaur ELF. Spatiotemporal dynamics of autophagy receptors in selective mitophagy. *Autophagy* (2016) 12:1956–7. doi:10.1080/15548627.2016.1212788
40. Ziviani E, Tao RN, Whitworth AJ. Drosophila Parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc Natl Acad Sci U S A* (2010) 107:5018–23. doi:10.1073/pnas.0913485107
41. Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* (2015) 85:257–73. doi:10.1016/j.neuron.2014.12.007
42. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* Parkin mutants. *Proc Natl Acad Sci U S A* (2003) 100:4078–83. doi:10.1073/pnas.0737556100
43. Pesah Y, Pham T, Burgess H, Middlebrooks B, Verstreken P, Zhou Y, et al. *Drosophila* Parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. *Development* (2004) 131:2183–94. doi:10.1242/dev.01095
44. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, et al. *Drosophila* pink1 is required for mitochondrial function and interacts genetically with Parkin. *Nature* (2006) 441:1162–6. doi:10.1038/nature04779
45. Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, et al. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by Parkin. *Nature* (2006) 441:1157–61. doi:10.1038/nature04788
46. Cha G-H, Kim S, Park J, Lee E, Kim M, Lee SB, et al. Parkin negatively regulates JNK pathway in the dopaminergic neurons of *Drosophila*. *Proc Natl Acad Sci U S A* (2005) 102:10345–50. doi:10.1073/pnas.0500346102
47. Whitworth AJ, Theodore DA, Greene JC, Benes H, Wes PD, Pallanck LJ. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a *Drosophila* model of Parkinson's disease. *Proc Natl Acad Sci U S A* (2005) 102:8024–9. doi:10.1073/pnas.0501078102
48. Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, et al. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Mol Cell* (2009) 33:627–38. doi:10.1016/j.molcel.2009.02.013
49. Heeman B, Van den Haute C, Aelvoet S-A, Valsecchi F, Rodenburg RJ, Reumers V, et al. Depletion of PINK1 affects mitochondrial metabolism, calcium homeostasis and energy maintenance. *J Cell Sci* (2011) 124:1115–25. doi:10.1242/jcs.078303
50. Akundi RS, Huang Z, Eason J, Pandya JD, Zhi L, Cass WA, et al. Increased mitochondrial calcium sensitivity and abnormal expression of innate immunity genes precede dopaminergic defects in Pink1-deficient mice. *PLoS One* (2011) 6:e16038. doi:10.1371/journal.pone.0016038
51. Shin J-H, Ko HS, Kang H, Lee Y, Lee Y-I, Pletinkova O, et al. PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. *Cell* (2011) 144:689–702. doi:10.1016/j.cell.2011.02.010
52. Bonifati V, Rizzu P, Squitieri F, Krieger E, Vanacore N, van Swieten JC, et al. DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. *Neurol Sci* (2003) 24:159–60. doi:10.1007/s10072-003-0108-0
53. Hague S, Rogaeva E, Hernandez D, Gulick C, Singleton A, Hanson M, et al. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. *Ann Neurol* (2003) 54:271–4. doi:10.1002/ana.10663
54. Canet-Avilés RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc Natl Acad Sci U S A* (2004) 101:9103–8. doi:10.1073/pnas.0402959101
55. Zondler L, Miller-Fleming L, Repici M, Gonçalves S, Tenreiro S, Rosado-Ramos R, et al. DJ-1 interactions with α-synuclein attenuate aggregation and cellular toxicity in models of Parkinson's disease. *Cell Death Dis* (2014) 5:e1350. doi:10.1038/cddis.2014.307
56. Andres-Mateos E, Perier C, Zhang L, Blanchard-Fillion B, Greco TM, Thomas B, et al. DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. *Proc Natl Acad Sci U S A* (2007) 104:14807–12. doi:10.1073/pnas.0703219104
57. Lopert P, Patel M. Brain mitochondria from DJ-1 knockout mice show increased respiration-dependent hydrogen peroxide consumption. *Redox Biol* (2014) 2:667–72. doi:10.1016/j.redox.2014.04.010
58. Paisán-Ruíz C, Jain S, Evans EW, Gilks WP, Simón J, van der Brug M, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* (2004) 44:595–600. doi:10.1016/j.neuron.2004.10.023
59. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* (2004) 44:601–7. doi:10.1016/j.neuron.2004.11.005
60. Manzoni C, Mamais A, Dihanich S, Abeti R, Soutar MPM, Plun-Favreau H, et al. Inhibition of LRRK2 kinase activity stimulates macroautophagy. *Biochim Biophys Acta* (2013) 1833:2900–10. doi:10.1016/j.bbamcr.2013.07.020
61. Stafa K, Tsika E, Moser R, Musso A, Glauser L, Jones A, et al. Functional interaction of Parkinson's disease-associated LRRK2 with members of the dynamin GTPase superfamily. *Hum Mol Genet* (2014) 23:2055–77. doi:10.1093/hmg/ddt600
62. Wang X, Yan MH, Fujioka H, Liu J, Wilson-Delfosse A, Chen SG, et al. LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. *Hum Mol Genet* (2012) 21:1931–44. doi:10.1093/hmg/dds003
63. Niu J, Yu M, Wang C, Xu Z. Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via dynamin-like protein. *J Neurochem* (2012) 122:650–8. doi:10.1111/j.1471-4159.2012.07809.x

64. Su Y-C, Qi X. Inhibition of excessive mitochondrial fission reduced aberrant autophagy and neuronal damage caused by LRRK2 G2019S mutation. *Hum Mol Genet* (2013) 22:4545–61. doi:10.1093/hmg/ddt301
65. Papkovskaia TD, Chau K-Y, Inesta-Vaquera F, Papkovsky DB, Healy DG, Nishio K, et al. G2019S leucine-rich repeat kinase 2 causes uncoupling protein-mediated mitochondrial depolarization. *Hum Mol Genet* (2012) 21:4201–13. doi:10.1093/hmg/dds244
66. Yakhine-Diop SMS, Bravo-San Pedro JM, Gómez-Sánchez R, Pizarro-Estrella E, Rodríguez-Arribas M, Climent V, et al. G2019S LRRK2 mutant fibroblasts from Parkinson's disease patients show increased sensitivity to neurotoxin 1-methyl-4-phenylpyridinium dependent of autophagy. *Toxicology* (2014) 324:1–9. doi:10.1016/j.tox.2014.07.001
67. Alegre-Abarrategui J, Christian H, Lufino MMP, Mutihac R, Venda LL, Ansorge O, et al. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. *Hum Mol Genet* (2009) 18:4022–34. doi:10.1093/hmg/ddp346
68. Tong Y, Yamaguchi H, Giaime E, Boyle S, Kopan R, Kelleher RJ, et al. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. *Proc Natl Acad Sci U S A* (2010) 107:9879–84. doi:10.1073/pnas.1004676107
69. Orenstein SJ, Kuo S-H, Tasset I, Arias E, Koga H, Fernandez-Carasa I, et al. Interplay of LRRK2 with chaperone-mediated autophagy. *Nat Neurosci* (2013) 16:394–406. doi:10.1038/nn.3350
70. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* (2004) 305:1292–5. doi:10.1126/science.1101738
71. Martinez-Vicente M, Talloczy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, et al. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *J Clin Invest* (2008) 118:777–88. doi:10.1172/JCI32806
72. Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci* (2010) 30:12535–44. doi:10.1523/JNEUROSCI.1920-10.2010
73. Holemans T, Sørensen DM, van Veen S, Martin S, Hermans D, Kemmer GC, et al. A lipid switch unlocks Parkinson's disease-associated ATP13A2. *Proc Natl Acad Sci U S A* (2015) 112:9040–5. doi:10.1073/pnas.1508220112
74. Dehay B, Ramirez A, Martinez-Vicente M, Perier C, Canron M-H, Doudnikoff E, et al. Loss of P-type ATPase ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration. *Proc Natl Acad Sci U S A* (2012) 109:9611–6. doi:10.1073/pnas.1112368109
75. Vidal RL, Matus S, Bargsted L, Hetz C. Targeting autophagy in neurodegenerative diseases. *Trends Pharmacol Sci* (2014) 35:583–91. doi:10.1016/j.tips.2014.09.002
76. Sarkar S, Ravikumar B, Floto RA, Rubinsztein DC. Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies. *Cell Death Differ* (2009) 16:46–56. doi:10.1038/cdd.2008.110
77. Majumder S, Richardson A, Strong R, Oddo S. Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. *PLoS One* (2011) 6:e25416. doi:10.1371/journal.pone.0025416
78. Miyara M, Kotake Y, Tokunaga W, Sanoh S, Ohta S. Mild MPP(+) exposure impairs autophagic degradation through a novel lysosomal acidity-independent mechanism. *J Neurochem* (2016) 139:294–308. doi:10.1111/jnc.13700
79. Liu K, Shi N, Sun Y, Zhang T, Sun X. Therapeutic effects of rapamycin on MPTP-induced parkinsonism in mice. *Neurochem Res* (2013) 38:201–7. doi:10.1007/s11064-012-0909-8
80. Cooper O, Seo H, Andrabi S, Guardia-Laguarta C, Graziotto J, Sundberg M, et al. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. *Sci Transl Med* (2012) 4:141ra90. doi:10.1126/scitranslmed.3003985
81. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* (2003) 278:25009–13. doi:10.1074/jbc.M300227200
82. Heras-Sandoval D, Pérez-Rojas JM, Hernández-Damián J, Pedraza-Chaverri J. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cell Signal* (2014) 26:2694–701. doi:10.1016/j.cellsig.2014.08.019
83. Malagelada C, Jin ZH, Jackson-Lewis V, Przedborski S, Greene LA. Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. *J Neurosci* (2010) 30:1166–75. doi:10.1523/JNEUROSCI.3944-09.2010
84. He C, Levine B. The Beclin 1 interactome. *Curr Opin Cell Biol* (2010) 22:140–9. doi:10.1016/j.ceb.2010.01.001
85. Ciechomska IA, Goemans GC, Skepper JN, Tolkovsky AM. Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function. *Oncogene* (2009) 28:2128–41. doi:10.1038/onc.2009.60
86. Maiuri MC, Le Toumelin G, Criollo A, Rain J-C, Gautier F, Juin P, et al. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J* (2007) 26:2527–39. doi:10.1038/sj.emboj.7601689
87. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem* (2007) 282:5641–52. doi:10.1074/jbc.M609532200
88. He Q, Koprich JB, Wang Y, Yu W-B, Xiao B-G, Brotchie JM, et al. Treatment with trehalose prevents behavioral and neurochemical deficits produced in an AAV α-synuclein rat model of Parkinson's disease. *Mol Neurobiol* (2016) 53:2258–68. doi:10.1007/s12035-015-9173-7
89. Perucho J, Casarejos MJ, Gomez A, Solano RM, de Yébenes JG, Mena MA. Trehalose protects from aggravation of amyloid pathology induced by isoflurane anesthesia in APP(swe) mutant mice. *Curr Alzheimer Res* (2012) 9:334–43. doi:10.2174/156720512800107573
90. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, et al. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat Med* (2004) 10:148–54. doi:10.1038/nm985
91. Høyer-Hansen M, Jäättelä M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ* (2007) 14:1576–82. doi:10.1038/sj.cdd.4402200
92. Fouillet A, Levet C, Virgone A, Robin M, Dourlen P, Rieusset J, et al. ER stress inhibits neuronal death by promoting autophagy. *Autophagy* (2012) 8:915–26. doi:10.4161/auto.19716
93. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* (2013) 12:703–19. doi:10.1038/nrd3976
94. Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, et al. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J Neurosci* (2009) 29:13578–88. doi:10.1523/JNEUROSCI.4390-09.2009
95. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, et al. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J Clin Invest* (2008) 118:2190–9. doi:10.1172/JCI33585
96. Nascimento-Ferreira I, Nóbrega C, Vasconcelos-Ferreira A, Onofre I, Albuquerque D, Aveleira C, et al. Beclin 1 mitigates motor and neuropathological deficits in genetic mouse models of Machado-Joseph disease. *Brain* (2013) 136:2173–88. doi:10.1093/brain/awt144
97. Jaeger PA, Pickford F, Sun C-H, Lucin KM, Masliah E, Wyss-Coray T. Regulation of amyloid precursor protein processing by the Beclin 1 complex. *PLoS One* (2010) 5:e11102. doi:10.1371/journal.pone.0011102
98. Mariño G, Madeo F, Kroemer G. Autophagy for tissue homeostasis and neuroprotection. *Curr Opin Cell Biol* (2011) 23:198–206. doi:10.1016/j.ceb.2010.10.001

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.

frontiers in Neurology

ORIGINAL RESEARCH
published: 06 April 2018
doi: 10.3389/fneur.2018.00222

Glutathione S-Transferase Alpha 4 Prevents Dopamine Neurodegeneration in a Rat Alpha-Synuclein Model of Parkinson's Disease

*Michael Jewett, Elna Dickson, Kajsa Brolin, Matilde Negrini, Itzia Jimenez-Ferrer and Maria Swanberg**

Translational Neurogenetics Unit, Department of Experimental Medical Science, Wallenberg Neuroscience Center, Lund University, Lund, Sweden

Parkinson's disease (PD) is a common, progressive neurodegenerative disease, which typically presents itself with a range of motor symptoms, like resting tremor, bradykinesia, and rigidity, but also non-motor symptoms such as fatigue, constipation, and sleep disturbance. Neuropathologically, PD is characterized by loss of dopaminergic cells in the substantia nigra pars compacta (SNpc) and Lewy bodies, neuronal inclusions containing α-synuclein (α-syn). Mutations and copy number variations of *SNCA*, the gene encoding α-syn, are linked to familial PD and common *SNCA* gene variants are associated to idiopathic PD. Large-scale genome-wide association studies have identified risk variants across another 40 loci associated to idiopathic PD. These risk variants do not, however, explain all the genetic contribution to idiopathic PD. The rat *Vra1* locus has been linked to neuroprotection after nerve- and brain injury in rats. *Vra1* includes the glutathione *S*-transferase alpha 4 (*Gsta4*) gene, which encodes a protein involved in clearing lipid peroxidation by-products. The DA.VRA1 congenic rat strain, carrying PVG alleles in *Vra1* on a DA strain background, was recently reported to express higher levels of *Gsta4* transcripts and to display partial neuroprotection of SNpc dopaminergic neurons in a 6-hydroxydopamine (6-OHDA) induced model for PD. Since α-syn expression increases the risk for PD in a dose-dependent manner, we assessed the neuroprotective effects of *Vra1* in an α-syn-induced PD model. Human wild-type α-syn was overexpressed by unilateral injections of the rAAV6-α-syn vector in the SNpc of DA and DA.VRA1 congenic rats. *Gsta4* gene expression levels were significantly higher in the striatum and midbrain of DA.VRA1 compared to DA rats at 3 weeks post surgery, in both the ipsilateral and contralateral sides. At 8 weeks post surgery, DA.VRA1 rats suffered significantly lower fiber loss in the striatum and lower loss of dopaminergic neurons in the SNpc compared to DA. Immunofluorescent stainings showed co-expression of Gsta4 with Gfap at 8 weeks suggesting that astrocytic expression of Gsta4 underlies *Vra1*-mediated neuroprotection to α-syn induced pathology. This is the second PD model in which *Vra1* is linked to protection of the nigrostriatal pathway, solidifying Gsta4 as a potential therapeutic target in PD.

Keywords: Parkinson's disease, α-synuclein, dopaminergic neurons, neuroprotection, glutathione *S*-transferase alpha 4, Vra1

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Mattia Volta, EURAC Research, Italy
Mohamed Mosaad Salama, Mansoura University, Egypt

***Correspondence:**
Maria Swanberg
maria.swanberg@med.lu.se

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *01 December 2017*
Accepted: *21 March 2018*
Published: *06 April 2018*

Citation:
Jewett M, Dickson E, Brolin K, Negrini M, Jimenez-Ferrer I and Swanberg M (2018) Glutathione S-Transferase Alpha 4 Prevents Dopamine Neurodegeneration in a Rat Alpha-Synuclein Model of Parkinson's Disease. Front. Neurol. 9:222. doi: 10.3389/fneur.2018.00222

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) resulting in a range of motor and non-motor symptoms. One of the pathological hallmarks of PD is the accumulation of α-synuclein (α-syn) protein, which is abundant in neuronal inclusions termed Lewy bodies and Lewy neurites (1). About 10% of PD cases are familial, and so far, mutations in seven genes have been linked to PD with a recessive or dominant inheritance pattern (2). The remaining 90% are classified as idiopathic PD with a complex etiology, meaning that both genetic and environmental factors contribute to the disease (3, 4). So far, 41 PD risk loci have been confirmed as associated to idiopathic PD (5, 6). There is, however, still a substantial missing heritability, i.e., undiscovered genetic risk factors contributing to PD etiology.

The *Vra1* region on rat chromosome 8 was linked to neuroprotection after ventral root avulsion (VRA) was performed in an intercross between the inbred Dark Agouti (DA) and Piebald Virol Glaxo (PVG.1AV1) rat strains (7). The congenic DA.VRA1 strain, carrying PVG.1AV1 alleles in the neuroprotective *Vra1* region on a DA strain background, was used to fine map *Vra1* and several candidate genes were discovered (8). Glutathione *S*-transferase alpha 4 (Gsta4), a protein involved in the elimination of lipid peroxidation by-products, such as 4-hydroxy-2-nonenal (HNE) (9), was subsequently identified as the strongest candidate gene regulating neurodegeneration in response to VRA (10) and traumatic brain injury in DA.VRA1 congenic rats (11).

Glutathione S-transferase alpha 4 belongs to the alpha class of glutathione *S*-transferases (GSTs). GSTs are a family of isoenzymes involved in cellular detoxification mechanisms including clearance of lipid peroxidation by-products through glutathione (GSH) conjugation (9, 12). Not much is known about the expression patterns of Gsta4 in humans or in rodents, although studies suggest that it is expressed ubiquitously (13, 14). Furthermore, while rat Gsta4 is only 60% homologous with human GSTA4, the two enzymes have similar catalytic affinity to HNE (9), making it a valuable experimental target. Genetic associations have been made between GSTA4 mutations and risk for certain types of cancer (15, 16), but not much is known about the role of GSTA4 in PD. However, HNE has been shown to be significantly elevated in PD brains (17–19), suggesting that GSTA4 is somehow affected and could be a key player in the disease. In order to study the effects of Gsta4 in a PD model that induces high levels of oxidative stress, we recently performed unilateral striatal 6-hydroxydopamine (6-OHDA) lesions in DA and DA.VRA1 rats. At 8 weeks post lesion, DA.VRA1 congenic rats suffered less striatal fiber loss and were more resistant to SNpc neuronal cell death compared to DA rats. In addition, *Gsta4* expression was elevated in the striatum and midbrain of DA.VRA1 rats at 2 days post lesion compared to DA, which is when the first signs of the degenerative process occur after 6-OHDA injections (20), but stabilized already after 7 days. This suggests that *Gsta4* plays a major role in protecting DA.VRA1 rats from a dopaminergic-specific toxin and that it exerts its effects early in the degenerative process (21). The 6-OHDA lesion, however, does not model the α-syn pathology seen in PD.

The genetics linking α-syn to PD is abundant. Mutations in *SNCA* encoding α-syn are linked to monogenic PD (22), and copy-number variation of SNCA is linked to PD in a dose-dependent manner with several duplications (23–32) and triplications (32–34) being reported. In addition, common variants of *SNCA* are associated to idiopathic PD (35). Thus α-syn is clearly implicated in PD etiology and is, therefore, widely used in PD animal models: from transgenic rodent models (36) to viral vector-mediated models (37), with the latter being able to deliver a more consistent and progressive PD-like phenotype (38).

It has been shown that the overexpression of α-syn in rodents through the use of viral vectors leads to a progressive pathology with loss of midbrain dopaminergic neurons (39, 40). In fact, reports have shown that recombinant adeno-associated viral (rAAV) vector-mediated overexpression of α-syn in rats reproduces several of the neuropathological aspects seen in patients (41–43), making it a relevant research model for studying PD. There is also evidence that α-syn activates oxidative stress mechanisms; for example, studies have shown that α-syn overexpression, like 6-OHDA, leads to mitochondrial impairment, which in turn leads to the production of reactive oxygen species (ROS) and lipid peroxidation (44–47).

In this study, we investigated if the *Vra1* locus encoding *Gsta4* mediates neuroprotection after overexpression of human wildtype (WT) α-syn in the rat SNpc. Compared to DA, DA.VRA1 congenic rats displayed higher gene expression levels of *Gsta4* in the striatum and SNpc at 3 weeks after α-syn overexpression. Furthermore, at 8 weeks after α-syn overexpression, we observed less degeneration of dopaminergic fibers in the striatum and their respective cell bodies in the SNpc. Similar to what was previously reported from the 6-OHDA model (21), Gsta4 was expressed in astrocytes in the SNpc at 8 weeks post rAAV injections. These results suggest that the *Vra1* locus protects from α-syn-induced PD-like neurodegeneration and that astrocytes mediate this action through expression of Gsta4.

MATERIALS AND METHODS

Research Model

For this study, we used two different inbred strains of rats: Dark Agouti (DA) and DA.VRA1, a congenic strain developed by transferring Vra1 alleles from the PVG^{av1} strain to a DA background strains as previously described (21). 64 male rats were used in this study (33 DA and 31 DA.VRA1 congenics), weighing approximately 220–250 g. Professor Piehl at the Karolinska Institutet, Stockholm, Sweden generously provided the founders for each strain. 51 (28 DA and 23 DA.VRA1) animals were subjected to unilateral injections of an rAAV6 vector construct to overexpress human WT α-syn, while 13 (5 DA and 8 DA.VRA1) were injected with the same vector construct to overexpress GFP in the midbrain at 12 weeks of age with the following titers: α-syn (1.2E + 14 gc/ml) and GFP (3.2E + 14 gc/ml). The expression of both transgenes is led by the synapsin-1 promoter and enhanced with the woodchuck hepatitis virus posttranscriptional

regulatory element (WPRE) (42). For quantification of dopaminergic neurodgeneration, the rAAV6-GFP-injected animals of both strains were pooled together as one group and abbreviated DA (GFP). This was done because no differences were found between the two strains after O.D. measurements in the striatum and stereological measurements in the SNpc (see Quantification of Dopaminergic Fiber Loss in the Striatum and Quantification of Dopaminergic Cell Loss in SNpc). The rats were given *ad libitum* access to food and water during a 12 h light/dark cycle and housed 2–3 per cage. 32 animals were sacrificed at 3 weeks post surgery for gene expression and immunofluorescence analysis, while 32 others were sacrificed at 8 weeks post surgery for histological analysis. All procedures described were approved by the Ethical Committee for the use of laboratory animals in the Lund/Malmö region.

Surgical Procedure

All surgical procedures were performed as described previously (21). 3 µl of rAAV6-α-syn or -GFP were unilaterally injected in the SNpc, which was targeting using the following coordinates, given in millimeters relative to bregma and dural surface (48): AP = −5.3, ML = −1.7, DV = −7.2. After the procedure, 0.15 ml Metacam (Apoteksbolaget, Sweden) was injected s.c. for postoperative analgesia. All animals were then placed in clean cages on a heated pad for recovery.

Tissue Preparation and Histology

Most tissue preparation and immunostainings were performed as described previously (21) For DAB stainings in this study, the following primary antibodies were used: mouse anti-tyrosine hydroxylase (TH) (1:1,000, Immunostar, Hudson, WI, USA), rabbit anti-vesicular monoamine transporter 2 (VMAT2) (1:4,000, Immunostar Hudson, WI USA), mouse anti-human WT α-syn (1:2,000, Santa Cruz, CA, USA), and chicken anti-GFP (1:20,000 Abcam, Cambridge, UK). The SNpc sections were given an initial antigen-retrieval incubation in Tris/EDTA (pH 9.0) at 80°C for 45 min when stained for TH.

Double immunofluorescence stainings were performed as described previously (21). The primary antibodies used were rabbit anti-GSTA4 (1:100 Antibodies-online GmbH, Aachen, Germany), mouse anti-Gfap (1:1,000, Santa Cruz, CA USA), chicken anti-IBA1 (1:500 Synaptic Systems, Göttingen, Germany), and mouse anti-NeuN (1:1,000 Millipore, Billerica, MA USA) and were incubated together at 4°C. To compare immunofluorescent stainings of midbrain and striatum for Gsta4 and Gfap at 3 and 8 weeks, stainings were performed in parallel and images were taken with the same settings. All images were captured at high-resolution with the confocal Leica SP8 microscope (Leica Microsystems, Wetzlar, Germany).

Quantification of Dopaminergic Fiber Loss in the Striatum

Striatum pictures were acquired as described previously (21). Dorsal (D) striatal TH^+ fiber density was evaluated as optical density (O.D.) by image densitometry at six coronal levels (+1.60, +1.15, +0.70, +0.25, −0.20, −0.75 mm from bregma) using the ImageJ software (https://imagej.nih.gov NIH, USA). The Rodbard calibration function within the software was used to normalize the range of gray-scale (0–255) into O.D. values. Each image was transformed into 8-bit (gray-scale). The contralateral (CL) and ipsilateral (IL) striatum was delineated for each section, and the O.D. values representing the strength of the TH^+ staining from each side were obtained. O.D. values from the corpus callosum were used to correct for non-specific background staining. Finally, the dopaminergic fiber loss was expressed as relative to the CL side versus the intact side for each animal. Three DA rats were excluded from the analysis due to complications during surgery or with tissue processing, leaving 7 DA, and 6 DA.VRA1 for quantification. Striatum divisions between D and ventral (V) are shown in **Figure 2B**.

Quantification of Dopaminergic Cell Loss in SNpc

Dopaminergic neurons in the SNpc were quantified by stereology of TH^+ cells according to the optical fractionator principle using the Stereo Investigator software (MBF Bioscience, USA) as described previously (21). With a Leitz DMRBE microscope (Leica, Germany), a 5× objective was used to delineate the areas of interest for each section, and a 100× oil-immersion objective was used for the cell counting. A frame ratio of 11% was assigned to each slide, and the average mounted section thickness (h) was 24.3 µm (±2.1). The average number of dopaminergic neurons counted in each individual was 286 (±73). A maximal Gundersen coefficient of error (C.E.) (49) of 0.08 was accepted. The counting criteria used matches the one previously used (21). Three animals were excluded from the analysis due to complications during surgery or with tissue processing, leaving 7 DA, and 6 DA.VRA1 for quantification.

Gene Expression Analysis

Animals were sedated and sacrificed at 3 weeks postsurgery as described previously (21). Pieces of right and left striatum and ventral midbrain weighing approximately 30 mg were dissected from the brain and placed in lysing matrix beaded tubes (MP Biomedicals, USA) and immediately stored at −80°C. The RNeasy Mini kit (Qiagen, Germany) was used to extract RNA from these samples, following the supplier's protocol with some variations already mentioned in Jewett et al. (21). Reverse Transcription and Quantitative (RT)-PCR followed using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA) and SSoAdvanced Universal SYBR green Supermix (BioRad, USA), respectively. qPCR was performed with this protocol: 5 µl Supermix + 0.5 µl of each primer + 4 µl cDNA for each sample. Sample amplification followed this 3-step protocol (1. 30 s at 95°C; 2. 60 s at 62°C for 39 cycles; 3. 5 min at 68°C) with the following primers (5′-3′): *Gsta4* (fw: GACCGTCCTGAAGTTCTAGTGA, rev: TGCCTCTGGAATGCTCTGT), *gapdh* (fw: CAACTCCC TCAAGATTGTCAGCAA, rev: GGCATGGACTGTGGTCATGA) and β-*actin* (fw: AAGTCCCTCACCCTCCCAAAAG, rev: AAGCAATGCTGTCACCTTCCC). Levels of *Gsta4* gene expression were calculated using $2^{-\Delta\Delta Cq}$ (50) and normalized relating

each value to CL DA of within the respective brain regions (striatum and SNpc).

Statistical Analysis

All statistics were performed with GraphPad Prism (version 7, La Jolla, CA, USA). Values are expressed as mean ± SD. Due to the low number of animals used for each data set, a Shapiro–Wilk normality test was performed to determine whether to proceed with parametric or non-parametric tests. Stereology and densitometry differences between groups were analyzed using a one-way ANOVA followed by Bonferroni's multiple comparisons *post hoc* test; statistical significance was set at p-value < 0.05. Correlation analysis was performed using the Pearson correlation coefficient (r), statistical significance was set at p-value <0.05, and a 95% confidence interval was used. A one-way ANOVA was used to calculate gene expression differences between groups at each time point, followed by Bonferroni's multiple comparisons *post hoc* test.

RESULTS

DA.VRA1 Rats Present Higher Levels of Gsta4 Gene Expression

Glutathione *S*-transferase alpha 4 has been shown to be upregulated in IL and CL sides of both striatum and midbrain of DA and DA.VRA1 rats at 2 days post striatal 6-OHDA injections, which is when the first signs of neuronal degeneration become evident within that model (20, 21). For this study, we wanted to investigate *Gsta4* expression levels within those same regions at a time point relevant to dopaminergic degeneration within the model of nigral rAAV-α-syn overexpression. We, therefore, chose to assess gene expression of *Gsta4* at 3 weeks after rAAV-mediated α-syn injections in the SNpc using the CL striatum and midbrain regions as internal controls (42). *Gsta4* expression is significantly higher in the striatum (**Figure 1A**) ($p < 0.05$) and midbrain (**Figure 1B**) ($p < 0.01$) of DA.VRA1 compared to DA rats. There are no differences in *Gsta4* expression between the CL and IL side within each strain (**Figures 1A,B**).

DA.VRA1 Congenic Rats Display Less Dopaminergic Fiber Loss After α-Syn Overexpression

The rAAV-α-syn model was chosen because it has been shown to produce partial and progressive degeneration of dopaminergic fibers in the striatum and cell bodies in the SNpc, a hallmark of PD (42). In order to evaluate accurate targeting and expression of the transgenes, striatum and midbrain sections were stained for GFP and human WT α-syn. The histological analysis shows high levels of both GFP and α-syn expression with accurate targeting of the nigrostriatal pathway (**Figure 2A**). Furthermore, our stainings of dopaminergic (TH^+) fibers in the striatum indicate that mainly the dorsal striatum was denervated upon α-syn overexpression. Therefore, the striatum was subdivided into dorsal, mainly innervated by the SN, and ventral, mainly innervated by the ventral tegmental area (51) (**Figure 2B**). Optical densitometry measuring the density of TH^+ fibers of the IL compared to the CL striatum points to a higher proportion of remaining TH^+ fibers in the IL dorsal striatum of DA.VRA1 compared to DA rats [mean (SD): 69 (13) vs. 54 (9)%, $p < 0.023$], with DA(GFP) animals being unaffected (**Figure 2C**). PVG.1AV1 alleles in the *Vra1* locus thus protected striatal dopaminergic fibers of DA.VRA1 congenic rats from α-syn-induced degeneration.

DA.VRA1 Congenic Rats Are Partially Protected From Dopaminergic Cell Loss in SNpc

Midbrain dopaminergic neurons were quantified at 8 weeks post α-syn overexpression and GFP as a control (**Figures 3A–D**). Stereological cell counting performed with TH^+-stained sections shows a reduction in dopaminergic cells in the IL SNpc of both DA and DA.VRA1 congenic rats compared to DA(GFP); however, there was no significant difference in the proportion of remaining TH^+ neurons between DA and DA.VRA1 rats [50 (9) vs. 40 (8)%, $p = 0.06$, **Figure 3B**]. Due to the possibility of TH being downregulated, thus giving an underestimation of dopaminergic neurons, VMAT2 was also used as a

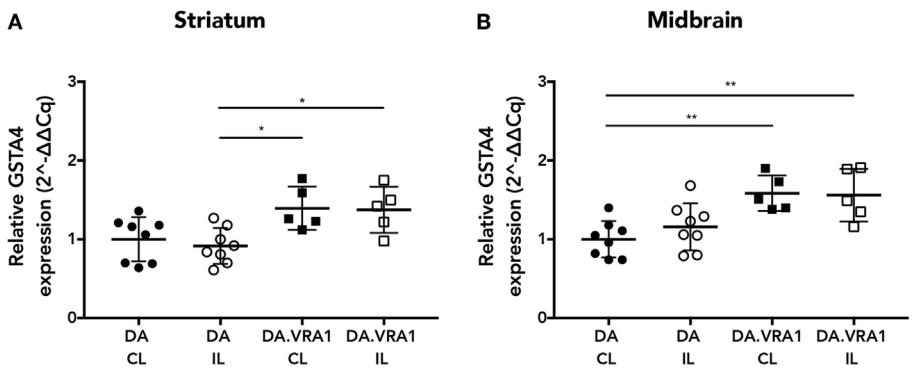


FIGURE 1 | Glutathione *S*-transferase alpha 4 (*Gsta4*) gene expression in the striatum and midbrain after recombinant adeno-associated viral (rAAV)-mediated α-syn overexpression in the substantia nigra pars compacta. *Gsta4* expression was significantly higher in DA.VRA1 compared to DA striatum **(A)** and midbrain **(B)** at 3 weeks post rAAV injection. There was no difference within each strain between ipsilateral (IL) and contralateral (CL) sides. Data were normalized to DA CL mean values for the respective brain region. Mean ± SD, $^*p < 0.05$, $^{**}p < 0.01$.

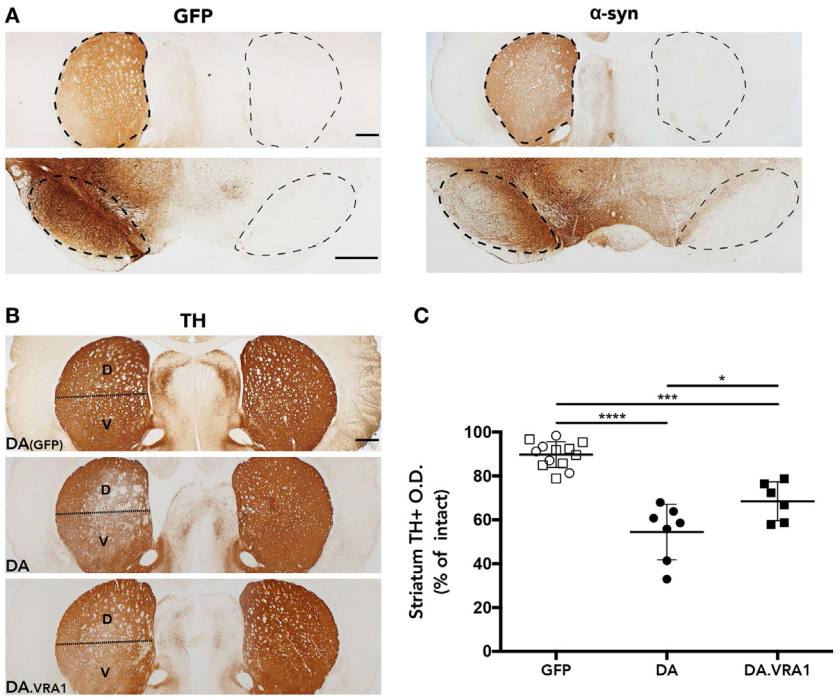


FIGURE 2 | Striatal dopaminergic fibers are protected from α-syn-induced degeneration by alleles in the *Vra1* locus. **(A)** Sample images showing GFP/α-syn transgene expression in the striatum and midbrain 8 weeks post unilateral recombinant adeno-associated viral (rAAV)-GFP/α-syn injections into the substantia nigra pars compacta (SNpc). **(B)** Representative pictures from DA (GFP), DA, and DA.VRA1 rats showing dopaminergic fibers in the striatum stained for tyrosine hydroxylase (TH) at 8 weeks after rAAV-α-syn injection. The lesioned striatum is divided in two parts: dorsal (D), the region receiving most afferent projections from the cells of the SNpc, and ventral (V). **(C)** Optical density quantification of TH^+ fibers in the lesioned relative to intact dorsal striatum at 8 weeks post surgery. DA.VRA1 rats display higher levels of remaining TH^+ fibers in the lesioned striatum compared to DA. Mean ± SD, $p < 0.05$ based on a one-way ANOVA followed by a Bonferroni *post hoc* test. O.D., optical density, scale bars = 500 μm.

dopaminergic marker to stain and count nigral cells. VMAT2 is a molecule essential for recruiting cytosolic dopamine into synaptic vescicles, and is, therefore, considered a reliable marker for dopaminergic cells (52, 53). Indeed, when quantifying $VMAT2^+$ neurons, we can see Vra1-mediated protection of nigral dopaminergic neurons in the IL SNpc of DA.VRA1 congenic vs DA rats [54 (7) vs. 44 (7)%, $p < 0.004$, **Figure 3D**]. In order to verify whether the loss of dopaminergic fibers in the striatum reflects the extent of dopaminergic cell death in both strains, we performed a correlation analysis between the two sets of data. We found a strong positive correlation between striatal TH + fiber density and remaining dopaminergic cells in the SNpc marked with VMAT2 in both strains ($p < 0.002$; $r = 0.8$, **Figure 3E**).

Gsta4 Is Expressed in Midbrain Astrocytes

We have previously observed Gsta4 expression in astrocytes but not in microglia or neurons at 8 weeks after 6-OHDA lesion (21). We made the same evaluation with double fluorescence immunostainings on midbrain sections combining Gsta4 with astrocytic (Gfap), microglial (Iba1), or neuronal (NeuN) markers at 8 weeks after α-syn overexpression (**Figure 4**). The stainings reveal a similar co-localization pattern of Gsta4 with Gfap (**Figures 4A,D,G,G'**) and not Iba1 (**Figures 4B,E,H**) or NeuN (**Figures 4C,F,I**) within this model, thus confirming astrocytic expression of Gsta4. This pattern remains constant in DA(GFP), DA, and DA.VRA1 animals (**Figures 4A–I**). Once again, the co-localization is more clear in the somas of SNpc astrocytes rather than the projections (**Figure 4G'**).

Since the gene expression analysis was performed at 3 weeks, and in order to check for any visible differences between Gsta4 gene and protein expression patterns at this time point, we chose to look at Gsta4 localization at 3 weeks as well. Immunofluorescent stainings for Gsta4 and Gfap were compared between midbrain and striatum sections at 3 and 8 weeks post rAAV-α-syn delivery. The staining intensity for both Gsta4 and Gfap is visibly lower at 3 weeks when compared to 8 weeks (**Figures 5A–D'**). At 3 weeks, Gsta4-stained cell bodies do not stand out compared to the background and less Gfap-positive cells are visible. In addition, no co-localization of Gsta4 is detectable with Gfap (**Figures 5A,B**), NeuN, or Iba1 (data not shown). However, at 8 weeks post rAAV-α-syn delivery, there is clear co-localization of Gsta4 and Gfap in both the midbrain and striatum (**Figures 5C,D**). This suggests a delayed increase in astrocytic Gsta4 expression in response to α-syn overexpression.

DISCUSSION

In this study, we show that PVG alleles in the *Vra1* locus partially protect the nigrostriatal pathway of DA.VRA1 congenic rats

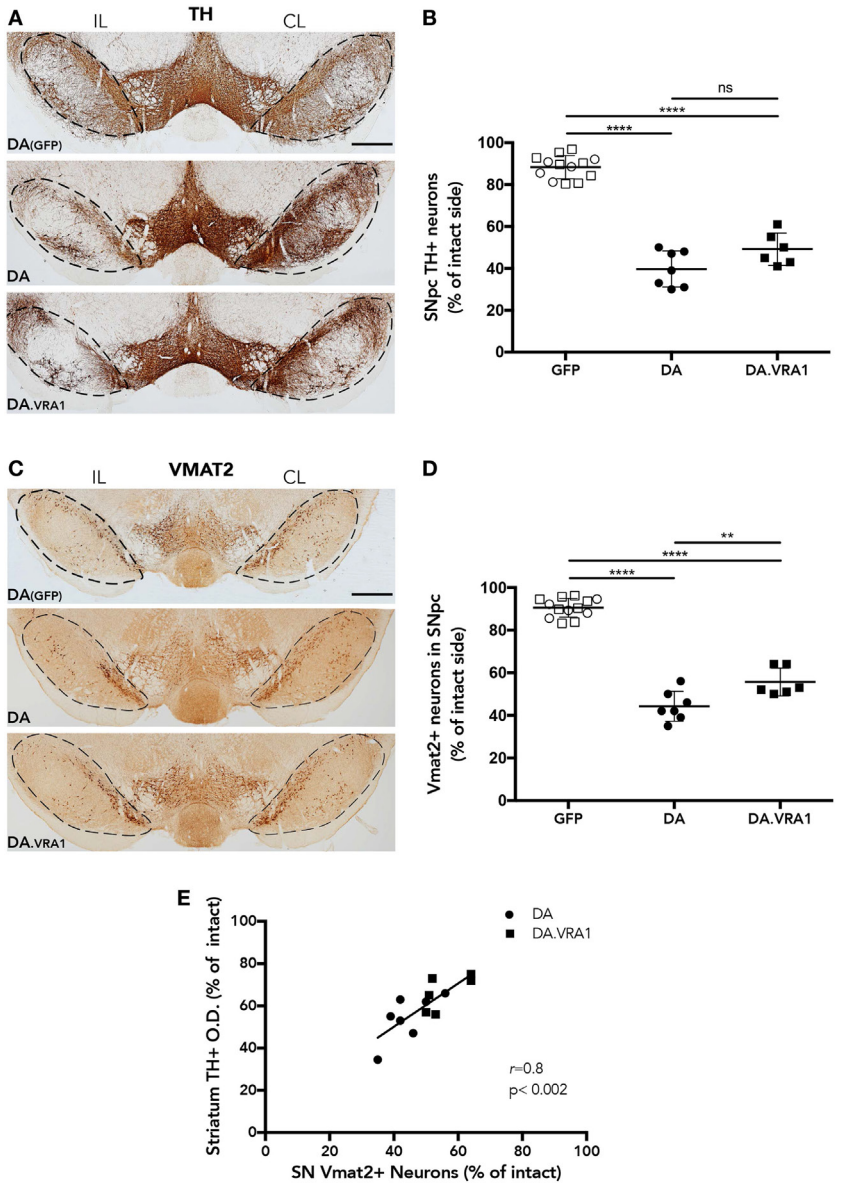


FIGURE 3 | The *Vra1*-locus mediates partial protection of nigral dopaminergic neurons in response to α-syn overexpression. **(A)** Representative images showing midbrain TH+ cells in DA(GFP), DA, and DA.VRA1 rats at 8 weeks post unilateral recombinant adeno-associated viral (rAAV)-α-syn injection. Dashed lines represent the area used for stereological cell counts. **(B)** Stereological quantification of TH+ neurons in the SN shows no significant difference in the percentage of remaining TH+ neurons in the IL side between DA and DA.VRA1. **(C)** Representative images showing midbrain vesicular monoamine transporter 2 (VMAT2)+ cells in DA(GFP), DA, and DA.VRA1 rats at 8 weeks post unilateral rAAV-α-syn injection. **(D)** Stereological quantification of VMAT2+ dopaminergic neurons at 8 weeks post injection shows a similar pattern as for TH+ cells, but with DA.VRA1 congenic rats displaying partial protection to dopaminergic cell loss in the IL substantia nigra pars compacta (SNpc) compared to DA rats. **(E)** The ratio of dopaminergic cells quantified by VMAT2 in the lesioned vs intact SNpc strongly correlates with the relative density of TH+ fibers in the dorsal striatum. Individual data points and mean ± SD are shown. CL, contralateral; IL, ipsilateral; scale bars = 500 μm. $^*p < 0.05$, with one-way ANOVA followed by a Bonferroni *post hoc* test. *r* = Pearson correlation coefficient.

from α-syn-induced neurodegeneration. At 3 weeks after unilateral rAAV-α-syn delivery, *Gsta4* expression levels were higher in both the IL and CL striatum and midbrain of DA.VRA1 rats compared to DA. When quantifying dopaminergic neurodegeneration at 8 weeks, the density of striatal dopaminergic fibers in the lesioned side was significantly higher in the congenic rats compared to DA, and similar evidence of *Vra1*-mediated neuroprotection was observed for midbrain dopaminergic cell bodies by stereological cell counts of TH+ and VMAT2+ neurons. These results are in line with our previous observations where DA.VRA1 rats displayed partial dopaminergic neuroprotection to striatal 6-OHDA lesion (21). However, while the toxin-based model results in dopaminergic loss mediated by the generation of ROS and mitochondrial damage, the current study models

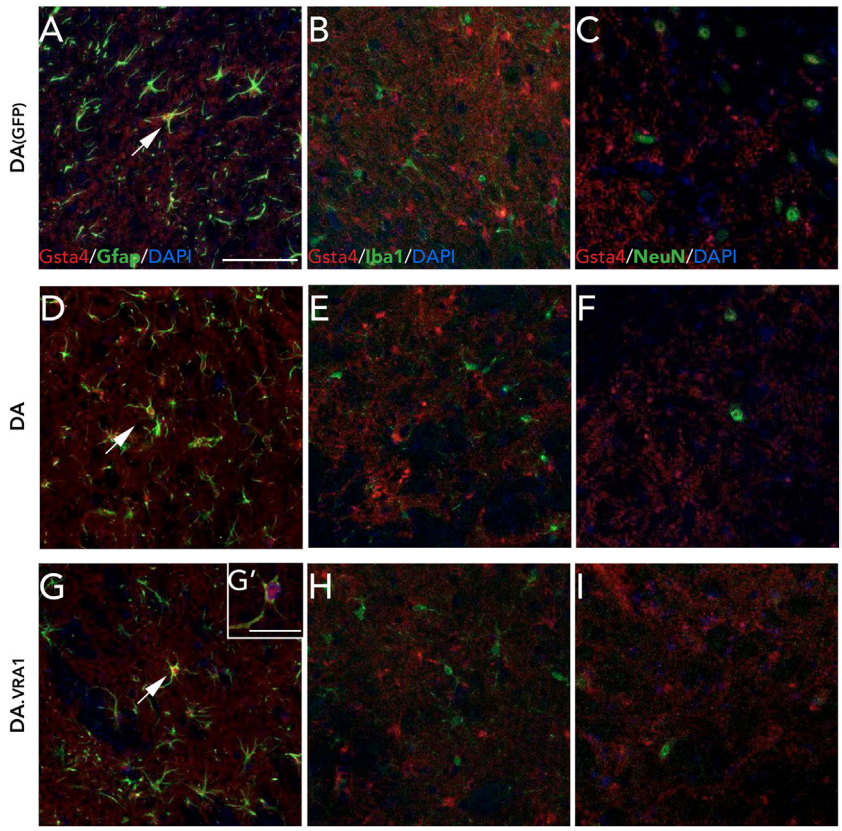


FIGURE 4 | Glutathione *S*-transferase alpha 4 (Gsta4) is expressed in midbrain astrocytes 8 weeks after rAAV-GFP/α-syn injection. Immunofluorescent staining of Gsta4 combined with cell-specific markers for **(A,D,G,G')** astrocytes; Gfap, **(B,E,H)** microglia; Iba1 and **(C,F,I)** neurons; NeuN in DA(GFP), DA, and DA.VRA1 rats. Gsta4 staining co-localized with Gfap **(A,D,G)** but not Iba1 **(B,E,H)** or NeuN **(C,F,I)**, suggesting astrocytic expression. Pictures taken at 20×; scale bar = 20 μm. **(G')** 60× image showing co-localization, with Gsta4 mainly expressed in the soma; scale bar = 100 μm. All markers were combined with the nuclear marker DAPI (blue).

α-syn-induced pathology, similar to that seen in PD patients. In addition, we show that Gsta4 is expressed in the cytoplasm of midbrain and striatal astrocytes at 8 weeks after α-syn overexpression, suggesting that astrocytes play an important role in protecting nearby neurons and neurites from α-syn-induced toxicity.

Our previous work, detailing the neuroprotective effects of Gsta4 after striatal 6-OHDA injections, puts focus on the oxidative stress aspects of PD. The neurodegenerative process of 6-OHDA is thought to be due to accumulation of ROS (20) and high levels of HNE within the affected cells (18, 54). Furthermore, 6-OHDA models have been shown to reproduce progressive and retrograde degeneration of the nigrostriatal pathway, mirroring some aspects of the same degeneration seen in PD patients with mild to moderate stages of the disease (55, 56). However, the striatal 6-OHDA model does not cover other pathogenic mechanisms of PD, such as the production of toxic α-syn species or impaired protein degradation (57). The functional link between α-syn and PD is very strong, with α-syn-containing Lewy bodies being present in both familial and idiopathic PD, and the SNCA gene being both linked to familial PD and associated to the risk of developing idiopathic PD. The rat rAAV-α-syn model employed here is based on the clear link between α-syn and PD-like pathology and complements the 6-OHDA model, which can be considered a model for toxin-induced PD. The rAAV vector used in the current study includes the WPRE element, which amplifies the expression of the transgene and induces unilateral overexpression of α-syn, progressive dopaminergic neurodegeneration, and motor impairment, which peak at 8 weeks postinjections (42). The rAAV-α-syn model also induces more progressive behavioral impairments compared to the striatal 6-OHDA model, probably due to the buildup of toxic α-syn species leading to deficits in synaptic function (57, 58).

With the striatal 6-OHDA model, aiming to examine causality of the neuroprotection observed in DA.VRA1 rats at 8 weeks, we measured *Gsta4* expression at 2 and 7 days post lesion, when the very early signs of neurodegeneration are seen in the striatum (59). In the rAAV-α-syn model, the first signs of dopaminergic dysfunction and cell loss occur at 3 weeks postinjection (42). Therefore, to keep within the same line of thinking for this study, we performed gene expression analysis of *Gsta4* at 3 weeks. *Gsta4* expression was higher in both striatum and midbrain of DA.VRA1 rats compared to DA rats. The strain difference was

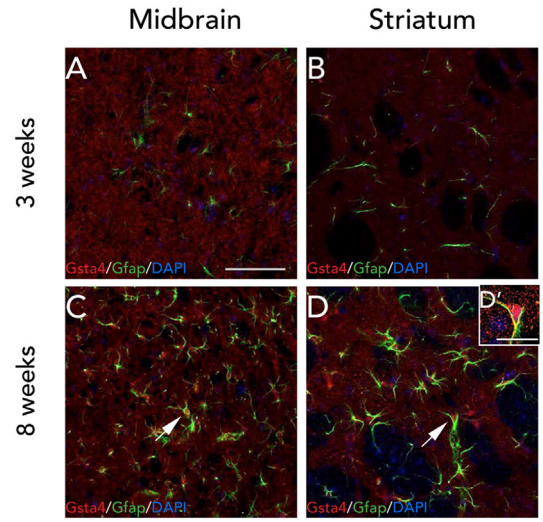


FIGURE 5 | Expression of glutathione S-transferase alpha 4 (Gsta4) and Gfap is increased at 8 weeks after recombinant adeno-associated viral-α-syn injection. Co-immunofluorescent stainings of Gsta4 and Gfap in the midbrain **(A,C)**, and striatum **(B,D)** of a DA.VRA1 congenic rat. Both Gsta4 and Gfap display a lower expression at 3 weeks **(A,B)** compared to 8 weeks **(C,D)**. Pictures taken at 20×, scale bar = 20 µm. **(D')** 60× image showing co-localization of Gsta4 with Gfap; scale bar = 100 µm. Stainings were combined with the nuclear marker DAPI (blue).

seen in both the lesioned and the intact sides, suggesting that α-syn overexpression does not induce *Gsta4* gene expression at this time point. Based on the observation that, along with increased Gfap staining, Gsta4 immunostaining was enhanced at 8 weeks compared to 3 weeks post transgene delivery, there might be a delayed increase in *Gsta4* gene expression after the α-syn transgene overexpression is established. Alternatively, a modest and continuous increase in *Gsta4* gene expression in the DA.VRA1 congenic strain is sufficient to partially protect midbrain dopaminergic cell projections and somas from degeneration.

There is plenty of evidence suggesting that α-syn overexpression increases oxidative stress levels, which is a key feature of PD. Both *in vivo* and *in vitro* models have shown that accumulation of α-syn can lead to mitochondrial dysfunction through the inhibition of Complex 1 (C1), which in turn leads to the production of ROS (60–62). Interestingly, it has also been shown that ROS are a result of depleted glutathione (GSH) in PD brains (63) and low levels of GSH can lead to the decrease of C1 activity (64). Indeed, one important aspect of Gsta4 activity is its ability to catalyze the conjugation of GSH to lipid peroxidation by-products such as HNE (9). Furthermore, a study by Shearn et al. on chronic alcohol consumption in a *Gsta4* null mouse showed that Gsta4 works as a mitochondrial detoxifier (65). This strongly suggests that α-syn toxicity is partly mediated by oxidative stress mechanisms, mainly acting through the mitochondria in dopaminergic cells and involving GSH metabolism. The fact that we see a similar neuroprotective phenotype of DA.VRA1 rats in both the α-syn overexpression model and the striatal 6-OHDA model strongly suggests that the *Vra1* locus encoding Gsta4 regulates key processes in PD-like dopaminergic neurodegeneration. The human ortholog GSTA4 is thus a promising therapeutic target in PD with a complex etiology.

In rat, Gsta proteins have been found to be abundant in astrocytes, the choroid plexus, as well as in endothelial cells and/or astrocytic end feet associated with blood vessels, Purkinje cells, and neurons (66). Therefore, regional differences in the cellular and subcellular distribution of Gsta4 are not unlikely. In our previous work where the *Vra1* locus was found to protect from striatal 6-OHDA lesions, we aimed to uncover the localization of Gsta4 within the affected areas of the rat brain. We found Gsta4 co-expression with the astrocytic marker Gfap, but not with the microglial (Iba1) or the neuronal (NeuN) markers at 8 weeks post injection (21). In the current study, we confirm the astrocytic localization of Gsta4 at 8 weeks in both DA and DA.VRA1 strains. In a nerve injury model, expression of Gsta4 has been shown in spinal motor neurons and not astrocytes (10). Of note, we cannot rule out the possibility that dopaminergic neurons express Gsta4 at levels not detected by immunostainings in our studies.

The relationship between α-syn and astrocytes is well studied. α-syn is found mainly in neurons, but can often accumulate in astrocytes as well, usually after spreading from neurons (67–69), possibly through cell-to-cell transfer (70). A recent study by Lindström et al. points out the important role of astrocytes in α-synucleinopathies. They show that in a co-culture system, astrocytes engulf large amounts of α-syn oligomers but are subsequently not able to degrade them completely, which leads to the formation of inclusions. It suggested that this is most likely brought on by a dysfunctional lysosomal system. Astrocytes also showed signs of mitochondrial damage caused by the accumulation of these α-syn oligomers (71). Furthermore, studies have shown that astrocytes can produce ROS under stressful conditions (72), thus leaving surrounding neurons susceptible to damage (73). This is relevant to the results obtained from DA.VRA1 congenic rats by us (21) and others (10), since ROS production is increased by 6-OHDA (20), α-syn overexpression (61), and in nerve injury models (74)—all environments where DA.VRA1 rats have been shown to express higher levels of *Gsta4*. When adding the fact that astrocytes also have a very high activity and release of GSH, which might be neuroprotective in itself (75), the link between Gsta4 activity and α-syn pathology is strengthened. More work is necessary to uncover the specific mechanisms by which Gsta4 protects from PD-like pathology in rat PD models. For example, a more in-depth analysis of the role of Gsta4 in astrocytic mitochondria might help answer key questions surrounding potential neuroprotective mechanisms.

In conclusion, this is the first report suggesting potential neuroprotective effects of the *Vra1* locus and *Gsta4* in an α-syn-induced PD model. Moreover, this study emphasizes the importance of utilizing animal models with naturally occurring allelic differences in order to gain a better understanding of neurodegenerative diseases with complex traits, such as PD. Gsta4 has now been implicated as a potential neuroprotective agent in both the 6-OHDA and α-syn overexpression PD models, making

the human ortholog a very attractive candidate for future PD therapeutic research.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical Committee for the use of laboratory animals in the Lund/Malmö region.

AUTHOR CONTRIBUTIONS

MJ and MS conceived and designed the experiments; MJ, ED, KB, MN, and IJ-F performed the experiments; MJ, KB, and ED analyzed the data; MJ and MS wrote the paper with contribution from coauthors.

FUNDING

This study was accomplished within MultiPark—a Strategic Research Area at Lund University and supported by the following foundations: Crafoordska Stiftelsen, Kungliga Fysiografiska Sällskapet Nilsson-Ehle fonderna, Greta och Johan Kocks Stiftelser, Stiftelsen Lars Hiertas Minne, Magnus Bergvalls Stiftelse, Segerfalk's stiftelse, Thelma Zoégas stiftelse, Tore Nilsons Stiftelse, Tesdorpf's Stiftelse, and Per-Erik och Ulla Schyberg's stiftelse.

REFERENCES

1. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci U S A* (1998) 95(11): 6469–73. doi:10.1073/pnas.95.11.6469
2. Lill CM. Genetics of Parkinson's disease. *Mol Cell Probes* (2016) 30(6):386–96. doi:10.1016/j.mcp.2016.11.001
3. Ryan SD, Dolatabadi N, Chan SF, Zhang X, Akhtar MW, Parker J, et al. Isogenic human iPSC Parkinson's model shows nitrosative stress-induced dysfunction in MEF2-PGC1alpha transcription. *Cell* (2013) 155(6):1351–64. doi:10.1016/j.cell.2013.11.009
4. Sveinbjornsdottir S. The clinical symptoms of Parkinson's disease. *J Neurochem* (2016) 139(Suppl 1):318–24. doi:10.1111/jnc.13691
5. Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat Genet* (2014) 46(9):989–93. doi:10.1038/ng.3043
6. Chang D, Nalls MA, Hallgrimsdottir IB, Hunkapiller J, van der Brug M, Cai F, et al. A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci. *Nat Genet* (2017) 49(10):1511–6. doi:10.1038/ng.3955
7. Lidman O, Swanberg M, Horvath L, Broman KW, Olsson T, Piehl F. Discrete gene loci regulate neurodegeneration, lymphocyte infiltration, and major histocompatibility complex class II expression in the CNS. *J Neurosci* (2003) 23(30):9817–23.
8. Swanberg M, Harnesk K, Strom M, Diez M, Lidman O, Piehl F. Fine mapping of gene regions regulating neurodegeneration. *PLoS One* (2009) 4(6):e5906. doi:10.1371/journal.pone.0005906
9. Hubatsch I, Ridderstrom M, Mannervik B. Human glutathione transferase A4-4: an alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. *Biochem J* (1998) 330(Pt 1):175–9. doi:10.1042/bj3300175
10. Strom M, Al Nimer F, Lindblom R, Nyengaard JR, Piehl F. Naturally occurring genetic variability in expression of Gsta4 is associated with differential survival of axotomized rat motoneurons. *Neuromolecular Med* (2012) 14(1): 15–29. doi:10.1007/s12017-011-8164-8
11. Al Nimer F, Strom M, Lindblom R, Aeinehband S, Bellander BM, Nyengaard JR, et al. Naturally occurring variation in the glutathione-S-transferase 4 gene determines neurodegeneration after traumatic brain injury. *Antioxid Redox Signal* (2013) 18(7):784–94. doi:10.1089/ars.2011.4440
12. Martinez-Lara E, Siles E, Hernandez R, Canuelo AR, Luisa del Moral M, Jimenez A, et al. Glutathione S-transferase isoenzymatic response to aging in rat cerebral cortex and cerebellum. *Neurobiol Aging* (2003) 24(3):501–9. doi:10.1016/S0197-4580(02)00139-2
13. Singhal SS, Zimniak P, Sharma R, Srivastava SK, Awasthi S, Awasthi YC. A novel glutathione S-transferase isozyme similar to GST 8-8 of rat and mGSTA4-4 (GST 5.7) of mouse is selectively expressed in human tissues. *Biochim Biophys Acta* (1994) 1204(2):279–86. doi:10.1016/0167-4838(94)90019-1
14. Zimniak P, Singhal SS, Srivastava SK, Awasthi S, Sharma R, Hayden JB, et al. Estimation of genomic complexity, heterologous expression, and enzymatic characterization of mouse glutathione S-transferase mGSTA4-4 (GST 5.7). *J Biol Chem* (1994) 269(2):992–1000.
15. Qian J, Jing J, Jin G, Wang H, Wang Y, Liu H, et al. Association between polymorphisms in the GSTA4 gene and risk of lung cancer: a case-control study in a Southeastern Chinese population. *Mol Carcinog* (2009) 48(3): 253–9. doi:10.1002/mc.20478
16. Li CG, Zhao ZM, Hu MG, Liu R. Predictive role of glutathione-S-transferase gene polymorphisms in risk and prognosis of hepatocellular carcinoma. *Asian Pac J Cancer Prev* (2012) 13(7):3247–52. doi:10.7314/APJCP.2012.13.7.3247
17. Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci U S A* (1996) 93(7):2696–701. doi:10.1073/pnas.93.7.2696
18. Selley ML. (E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. *Free Radic Biol Med* (1998) 25(2):169–74. doi:10.1016/S0891-5849(98)00021-5
19. Castellani RJ, Perry G, Siedlak SL, Nunomura A, Shimohama S, Zhang J, et al. Hydroxynonenal adducts indicate a role for lipid peroxidation in neocortical and brainstem Lewy bodies in humans. *Neurosci Lett* (2002) 319(1):25–8. doi:10.1016/S0304-3940(01)02514-9
20. Glinka Y, Gassen M, Youdim MB. Mechanism of 6-hydroxydopamine neurotoxicity. *J Neural Transm Suppl* (1997) 50:55–66. doi:10.1007/978-3-7091-6842-4_7
21. Jewett M, Jimenez-Ferrer I, Swanberg M. Astrocytic expression of GSTA4 is associated to dopaminergic neuroprotection in a rat 6-OHDA model of Parkinson's disease. *Brain Sci* (2017) 7(7):E73. doi:10.3390/brainsci7070073
22. Klein C, Westenberger A. Genetics of Parkinson's disease. *Cold Spring Harb Perspect Med* (2012) 2(1):a008888. doi:10.1101/cshperspect.a008888
23. Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, et al. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* (2004) 364(9440):1167–9. doi:10.1016/s0140-6736(04)17103-1
24. Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P, et al. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* (2004) 364(9440):1169–71. doi:10.1016/s0140-6736(04)17104-3
25. Nishioka K, Hayashi S, Farrer MJ, Singleton AB, Yoshino H, Imai H, et al. Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease. *Ann Neurol* (2006) 59(2):298–309. doi:10.1002/ana.20753
26. Fuchs J, Nilsson C, Kachergus J, Munz M, Larsson EM, Schule B, et al. Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication. *Neurology* (2007) 68(12):916–22. doi:10.1212/01.wnl.0000254458.17630.c5
27. Ahn TB, Kim SY, Kim JY, Park SS, Lee DS, Min HJ, et al. Alpha-synuclein gene duplication is present in sporadic Parkinson disease. *Neurology* (2008) 70(1):43–9. doi:10.1212/01.wnl.0000271080.53272.c7
28. Brueggemann N, Odin P, Gruenewald A, Tadic V, Hagenah J, Seidel G, et al. Re: alpha-synuclein gene duplication is present in sporadic Parkinson disease. *Neurology* (2008) 71(16):1294; author reply 1294. doi:10.1212/01.wnl.0000338439.00992.c7
29. Ikeuchi T, Kakita A, Shiga A, Kasuga K, Kaneko H, Tan CF, et al. Patients homozygous and heterozygous for SNCA duplication in a family with parkinsonism and dementia. *Arch Neurol* (2008) 65(4):514–9. doi:10.1001/archneur.65.4.514

30. Troiano AR, Cazeneuve C, Le Ber I, Bonnet AM, Lesage S, Brice A. Re: alpha-synuclein gene duplication is present in sporadic Parkinson disease. *Neurology* (2008) 71(16):1295; author reply 1295. doi:10.1212/01.wnl.0000338435.78120.0f
31. Uchiyama T, Ikeuchi T, Ouchi Y, Sakamoto M, Kasuga K, Shiga A, et al. Prominent psychiatric symptoms and glucose hypometabolism in a family with a SNCA duplication. *Neurology* (2008) 71(16):1289–91. doi:10.1212/01.wnl.0000327607.28928.e6
32. Ibanez P, Lesage S, Janin S, Lohmann E, Durif F, Destee A, et al. Alpha-synuclein gene rearrangements in dominantly inherited parkinsonism: frequency, phenotype, and mechanisms. *Arch Neurol* (2009) 66(1):102–8. doi:10.1001/archneurol.2008.555
33. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. Alpha-synuclein locus triplication causes Parkinson's disease. *Science* (2003) 302(5646):841. doi:10.1126/science.1090278
34. Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, et al. Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Ann Neurol* (2004) 55(2):174–9. doi:10.1002/ana.10846
35. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet* (2009) 41(12):1308–12. doi:10.1038/ng.487
36. Harvey BK, Wang Y, Hoffer BJ. Transgenic rodent models of Parkinson's disease. *Acta Neurochir Suppl* (2008) 101:89–92. doi:10.1007/978-3-211-78205-7_15
37. Blesa J, Przedborski S. Parkinson's disease: animal models and dopaminergic cell vulnerability. *Front Neuroanat* (2014) 8:155. doi:10.3389/fnana.2014.00155
38. Visanji NP, Brotchie JM, Kalia LV, Koprich JB, Tandon A, Watts JC, et al. Alpha-synuclein-based animal models of Parkinson's disease: challenges and opportunities in a new era. *Trends Neurosci* (2016) 39(11):750–62. doi:10.1016/j.tins.2016.09.003
39. Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, et al. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* (2002) 22(7):2780–91.
40. Kirik D, Annett LE, Burger C, Muzyczka N, Mandel RJ, Bjorklund A. Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease. *Proc Natl Acad Sci U S A* (2003) 100(5):2884–9. doi:10.1073/pnas.0536383100
41. Azeredo da Silveira S, Schneider BL, Cifuentes-Diaz C, Sage D, Abbas-Terki T, Iwatsubo T, et al. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. *Hum Mol Genet* (2009) 18(5):872–87. doi:10.1093/hmg/ddn417
42. Decressac M, Mattsson B, Lundblad M, Weikop P, Bjorklund A. Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of alpha-synuclein in midbrain dopamine neurons. *Neurobiol Dis* (2012) 45(3):939–53. doi:10.1016/j.nbd.2011.12.013
43. Febbraro F, Sahin G, Farran A, Soares S, Jensen PH, Kirik D, et al. Ser129D mutant alpha-synuclein induces earlier motor dysfunction while S129A results in distinctive pathology in a rat model of Parkinson's disease. *Neurobiol Dis* (2013) 56:47–58. doi:10.1016/j.nbd.2013.03.014
44. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, et al. α-Synuclein promotes mitochondrial deficit and oxidative stress. *Am J Pathol* (2000) 157(2):401–10. doi:10.1016/S0002-9440(10)64553-1
45. Esteves AR, Arduino DM, Swerdlow RH, Oliveira CR, Cardoso SM. Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids. *Antioxid Redox Signal* (2009) 11(3):439–48. doi:10.1089/ars.2008.2247
46. Rocha EM, De Miranda B, Sanders LH. Alpha-synuclein: pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. *Neurobiol Dis* (2017) 109(Pt B):249–57. doi:10.1016/j.nbd.2017.04.004
47. Tapias V, Hu X, Luk KC, Sanders LH, Lee VM, Greenamyre JT. Synthetic alpha-synuclein fibrils cause mitochondrial impairment and selective dopamine neurodegeneration in part via iNOS-mediated nitric oxide production. *Cell Mol Life Sci* (2017) 74(15):2851–74. doi:10.1007/s00018-017-2541-x
48. George Paxinos CW. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press (2007).
49. Gundersen HJ, Jensen EB, Kieu K, Nielsen J. The efficiency of systematic sampling in stereology – reconsidered. *J Microsc* (1999) 193(Pt 3):199–211. doi:10.1046/j.1365-2818.1999.00457.x
50. Livak and Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001) 25(4), 402–408. doi:10.1006/meth.2001.1262
51. Voorn P, Vanderschuren LJ, Groenewegen HJ, Robbins TW, Pennartz CM. Putting a spin on the dorsal-ventral divide of the striatum. *Trends Neurosci* (2004) 27(8):468–74. doi:10.1016/j.tins.2004.06.006
52. Takahashi N, Miner LL, Sora I, Ujike H, Revay RS, Kostic V, et al. VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci U S A* (1997) 94(18):9938–43. doi:10.1073/pnas.94.18.9938
53. Miller GW, Erickson JD, Perez JT, Penland SN, Mash DC, Rye DB, et al. Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. *Exp Neurol* (1999) 156(1):138–48. doi:10.1006/exnr.1998.7008
54. Smith MP, Cass WA. Oxidative stress and dopamine depletion in an intrastriatal 6-hydroxydopamine model of Parkinson's disease. *Neuroscience* (2007) 144(3):1057–66. doi:10.1016/j.neuroscience.2006.10.004
55. Kirik D, Rosenblad C, Bjorklund A. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp Neurol* (1998) 152(2):259–77. doi:10.1006/exnr.1998.6848
56. Deumens R, Blokland A, Prickaerts J. Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp Neurol* (2002) 175(2):303–17. doi:10.1006/exnr.2002.7891
57. Venda LL, Cragg SJ, Buchman VL, Wade-Martins R. Alpha-synuclein and dopamine at the crossroads of Parkinson's disease. *Trends Neurosci* (2010) 33(12):559–68. doi:10.1016/j.tins.2010.09.004
58. Decressac M, Mattsson B, Bjorklund A. Comparison of the behavioural and histological characteristics of the 6-OHDA and alpha-synuclein rat models of Parkinson's disease. *Exp Neurol* (2012) 235(1):306–15. doi:10.1016/j.expneurol.2012.02.012
59. Rosenblad C, Kirik D, Bjorklund A. Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. *Exp Neurol* (2000) 161(2):503–16. doi:10.1006/exnr.1999.7296
60. Junn E, Mouradian MM. Human alpha-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. *Neurosci Lett* (2002) 320(3):146–50. doi:10.1016/S0304-3940(02)00016-2
61. Vali S, Chinta SJ, Peng J, Sultana Z, Singh N, Sharma P, et al. Insights into the effects of alpha-synuclein expression and proteasome inhibition on glutathione metabolism through a dynamic in silico model of Parkinson's disease: validation by cell culture data. *Free Radic Biol Med* (2008) 45(9):1290–301. doi:10.1016/j.freeradbiomed.2008.08.002
62. Moon HE, Paek SH. Mitochondrial dysfunction in Parkinson's disease. *Exp Neurobiol* (2015) 24(2):103–16. doi:10.5607/en.2015.24.2.103
63. Jenner P, Olanow CW. The pathogenesis of cell death in Parkinson's disease. *Neurology* (2006) 66(10 Suppl 4):S24–36. doi:10.1212/WNL.66.10_suppl_4.S24
64. Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. *J Parkinsons Dis* (2013) 3(4):461–91. doi:10.3233/jpd-130230
65. Shearn CT, Fritz KS, Shearn AH, Saba LM, Mercer KE, Engi B, et al. Deletion of GSTA4-4 results in increased mitochondrial post-translational modification of proteins by reactive aldehydes following chronic ethanol consumption in mice. *Redox Biol* (2016) 7:68–77. doi:10.1016/j.redox.2015.11.013
66. Johnson JA, el Barbary A, Kornguth SE, Brugge JF, Siegel FL. Glutathione S-transferase isoenzymes in rat brain neurons and glia. *J Neurosci* (1993) 13(5):2013–23.
67. Croisier E, Graeber MB. Glial degeneration and reactive gliosis in alpha-synucleinopathies: the emerging concept of primary gliodegeneration. *Acta Neuropathol* (2006) 112(5):517–30. doi:10.1007/s00401-006-0119-z
68. Braak H, Sastre M, Del Tredici K. Development of alpha-synuclein immunoreactive astrocytes in the forebrain parallels stages of intraneuronal pathology in sporadic Parkinson's disease. *Acta Neuropathol* (2007) 114(3):231–41. doi:10.1007/s00401-007-0244-3
69. Fellner L, Stefanova N. The role of glia in alpha-synucleinopathies. *Mol Neurobiol* (2013) 47(2):575–86. doi:10.1007/s12035-012-8340-3
70. Angot E, Steiner JA, Lema Tome CM, Ekstrom P, Mattsson B, Bjorklund A, et al. Alpha-synuclein cell-to-cell transfer and seeding in grafted dopaminergic neurons in vivo. *PLoS One* (2012) 7(6):e39465. doi:10.1371/journal.pone.0039465
71. Lindstrom et al. Extensive uptake of alpha-synuclein oligomers in astrocytes results in sustained intracellular deposits and mitochondrial damage. *Mol Cell Neurosci*, (2017) 82:143–156. doi:10.1016/j.mcn.2017.04.009

72. Sheng WS, Hu S, Feng A, Rock RB. Reactive oxygen species from human astrocytes induced functional impairment and oxidative damage. *Neurochem Res* (2013) 38(10):2148–59. doi:10.1007/s11064-013-1123-z
73. Sugiyama K, Brunori A, Mayer ML. Glial uptake of excitatory amino acids influences neuronal survival in cultures of mouse hippocampus. *Neuroscience* (1989) 32(3):779–91. doi:10.1016/0306-4522(89)90298-4
74. Lieven CJ, Hoegger MJ, Schlieve CR, Levin LA. Retinal ganglion cell axotomy induces an increase in intracellular superoxide anion. *Invest Ophthalmol Vis Sci* (2006) 47(4):1477–85. doi:10.1167/iovs.05-0921
75. Heales SJ, Lam AA, Duncan AJ, Land JM. Neurodegeneration or neuroprotection: the pivotal role of astrocytes. *Neurochem Res* (2004) 29(3):513–9. doi:10.1023/B:NERE.0000014822.69384.0f

Conflict of Interest Statement: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

frontiers in Neurology

ORIGINAL RESEARCH
published: 09 April 2018
doi: 10.3389/fneur.2018.00208

Inhibition of mTORC1 Signaling Reverts Cognitive and Affective Deficits in a Mouse Model of Parkinson's Disease

Débora Masini, Alessandra Bonito-Oliva†, Maëlle Bertho and Gilberto Fisone**

Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

Non-motor symptoms, including cognitive deficits and affective disorders, are frequently diagnosed in Parkinson's disease (PD) patients and are only partially alleviated by dopamine replacement therapy. Here, we used a 6-hydroxydopamine (6-OHDA) mouse model of PD to examine the effects exerted on non-motor symptoms by inhibition of the mammalian target of rapamycin complex 1 (mTORC1), which is involved in the control of protein synthesis, cell growth, and metabolism. We show that rapamycin, which acts as an allosteric inhibitor of mTORC1, counteracts the impairment of novel object recognition. A similar effect is produced by PF-4708671, an inhibitor of the downstream target of mTORC1, ribosomal protein S6 kinase (S6K). Rapamycin is also able to reduce depression-like behavior in PD mice, as indicated by decreased immobility in the forced swim test. Moreover, rapamycin exerts anxiolytic effects, thereby reducing thigmotaxis in the open field and increasing exploration of the open arm in the elevated plus maze. In contrast to rapamycin, administration of PF-4708671 to PD mice does not counteract depression- and anxiety-like behaviors. Altogether, these results identify mTORC1 as a target for the development of drugs that, in combination with standard antiparkinsonian agents, may widen the efficacy of current therapies for the cognitive and affective symptoms of PD.

Keywords: Parkinson's disease, mammalian target of rapamycin, rapamycin, ribosomal protein S6 kinase, PF-4708671, depression, anxiety, cognition

OPEN ACCESS

Edited by:
Xiaobo Mao,
Johns Hopkins University,
United States

Reviewed by:
Peter Fuhr,
University Hospital of Basel,
Switzerland
Paolo Calabresi,
University of Perugia, Italy

****Correspondence:***
Débora Masini
debora.masini@ki.se;
Gilberto Fisone
gilberto.fisone@ki.se

†Present address:
Alessandra Bonito-Oliva,
Laboratory of Chemical Biology and Signal Transduction, The Rockefeller University, New York, NY, United States

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *30 November 2017*
Accepted: *16 March 2018*
Published: *09 April 2018*

Citation:
Masini D, Bonito-Oliva A, Bertho M and Fisone G (2018) Inhibition of mTORC1 Signaling Reverts Cognitive and Affective Deficits in a Mouse Model of Parkinson's Disease. Front. Neurol. 9:208. doi: 10.3389/fneur.2018.00208

INTRODUCTION

Cognitive impairment and affective disorders are frequently diagnosed in patients with Parkinson's disease (PD) and represent a major clinical challenge, in addition to the classic motor symptoms (1–5). Dementia develops in about 40% of PD patients and is often preceded by mild cognitive impairments, which compromise attentional, executive, and visuospatial functions. These latter ailments often develop before the onset of cardinal motor symptoms and are present in about 20% of PD patients at the time of diagnosis (4, 5). A significant proportion of PD patients are also affected by anxiety and depression, which appear in the early stages of the disease and are often refractory to dopamine replacement therapies (1, 3). Non-motor symptoms represent a serious challenge to the quality of life for both patients and their families, prompting the search for more effective therapies.

The mammalian target of rapamycin (mTOR) signaling pathway is involved in multiple aspects of cognitive processes. mTOR is the key catalytic component of two large multimeric complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2) (6, 7). mTORC1 regulates a variety of cellular functions, including cell growth and proliferation, autophagy and protein synthesis, whereas mTORC2 participates in the control of cytoskeletal dynamics and cell size.

Two of the main downstream targets of mTORC1, the ribosomal protein S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding protein (4E-BP), promote mRNA translation *via* activation of downstream initiation and elongation factors (8–10). Activation of these signaling components modulates synaptic plasticity and affects cognition through spatial and temporal coordination of protein synthesis. Thus, mTORC1 signaling is required for long-term potentiation in the hippocampus, and for memory formation and consolidation (11, 12).

Excessive activation of mTORC1 is linked to intellectual disabilities, including tuberous sclerosis (13, 14), fragile X syndrome [(15) but see also (16)] and Down syndrome (17). Notably, the cognitive impairment observed in animal models of tuberous sclerosis and Down syndrome is counteracted by rapamycin, a selective inhibitor of mTORC1 (13, 14, 18).

Dysregulated mTOR transmission is also implicated in affective disorders. The current prevailing hypothesis is that decreased mTORC1 activity in different cortical regions is associated with depression whereas augmented mTORC1 activity, such as that produced by the NMDA receptor agonist ketamine, reverts these conditions (19–22). However, studies in animal models have shown that subchronic administration of rapamycin reduces depressive-like behaviors (23), prompting further analysis of the actions of this drug on emotional deficits.

In this study, we used a mouse model to examine the effects of rapamycin and PF-4708671, a selective S6K inhibitor (24), to counteract memory impairment, depressive- and anxiety-like behaviors associated with PD. Our results indicate that inhibition of mTORC1 with rapamycin may represent a potential approach to the combined treatment of these disorders.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (3 months old; 25–30 g; Jackson Laboratory, ME, USA) were housed under a 12 light-dark cycle with food and water *ad libitum*. All experiments were carried out in accordance with the guidelines of Research Ethics Committee of Karolinska Institutet and Swedish Animal Welfare Agency. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

6-hydroxydopamine hydrochloride (6-OHDA; Sigma-Aldrich, Stockholm, Sweden) was dissolved in 0.02% ascorbic acid in saline at a concentration of 4 μg/μL and injected directly into the dorsal striatum. Rapamycin (LC Laboratories, Woburn, MA, USA) was dissolved in 5% dimethyl sulfoxide (DMSO), 5% Tween20, 15% polyethylene glycol (PEG), and distilled water to a final concentration of 5 mg/kg, and administered intraperitoneally (i.p.) in a volume of 2 mL/kg for three consecutive days, and then 30 min preceding the open field (OF), the elevated plus maze (EPM), and the forced swim test (FST). Rapamycin was also injected 30 min prior to all phases of the novel object recognition (NOR) test. PF-4708671 (MedChem Express, Monmouth Junction, USA) was dissolved in 17% DMSO, 10% Tween80, in saline and injected (50 mg/kg in 5 mL/kg volume, i.p.) 1 h prior to each experiment (OF, EPM, FST, familiarization and test phases of NOR).

6-OHDA Lesion

Mice were anesthetized with 4% isofluorane and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) equipped with a heating pad to maintain normothermia. All animals were injected subcutaneously with 0.1 mg/kg of Temgesic before surgery. Partial dopamine depletion was induced by injecting each striatum with 1 μL of 6-OHDA according to the following coordinates (mm): anteroposterior + 0.6; mediolateral ± 2.2; and dorsoventral −3.2 (25). Control mice received a sham lesion, consisting of bilateral injections (1 μL of vehicle). After surgery, the animals were allowed to recover for 3 weeks.

Behavioral Tests

Each mouse was subjected to sequential tests performed according to their increasing averseness (i.e., OF, NOR, EPM, and FST). Each test was separated by 4–7 days, during which animals were left undisturbed.

Open Field (OF) and Thigmotaxis

In this test, the preferential exploration of the peripheral zone of the OF, referred to as thigmotaxis, is considered an index of anxiety (26–28). Thigmotaxis was evaluated in a box (38 cm × 38 cm × 28 cm) divided into peripheral and central zones (defined as body center beyond 10 cm from wall). Each mouse was allowed to explore the apparatus for 15 min and its behavior was recorded by a video camera connected to an automated tracking system (Ethovision XT-10, Noldus, The Netherlands). The percentage of time spent by the animals exploring the center zone of the apparatus was measured and represented as a time course scatter plot (30 s time sampling intervals, one-zero sampling method). Curve fitting with nonlinear regression was used to generate trend lines. The cumulative time in the center zone was calculated as percentage of the total experimental time.

Novel Object Recognition (NOR)

The NOR test is based on the natural preference of rodents for novel objects and is commonly employed to assess memory function (27, 28). Mice were first habituated for 3 days (20 min/day) to the experimental cage (38 cm × 38 cm plastic chamber). On the familiarization phase (day 4), two identical objects (white plastic cylinders 3 cm high and 1 cm radius) were placed in the back left and right corners of the cage, 10 cm from the walls. Mice were placed near the wall opposite to the objects and allowed to explore for 15 min. During the test (day 5), one of the two (familiar) objects was replaced with a novel object (plastic orange object of comparable size). Mice were placed in the apparatus and left free to explore for 5 min. The experiment was video-recorded and object exploration (time during which the mouse nose was in contact with the object or directed toward it at a distance ≤ 2 cm) was measured by an observer blind to groups and treatments. Two measures were considered: (1) the total exploration time (s) spent by the animal interacting with the two objects during the test and (2) the exploration time (%) spent by the animal interacting with the novel object over the total exploration time

(e.g., [novel/(familiar + novel)] × 100) during the test. Example tracings shown in the NOR test were generated by plotting the *x*,*y* positions of the nose-point, detected every 0.2 s and color coded according to the behavior being assessed.

Elevated Plus Maze (EPM)

The EPM test is commonly used to evaluate anxiety-like behavior in mice. The test is based on the natural preference of rodents for closed spaces, and the propensity to avoid the open arms is considered an index of anxiety (29). The apparatus is composed of four gray plastic arms, arranged as a cross and located 40 cm above the plane of a laboratory bench. Two arms, opposite to each other, are enclosed by lateral walls (70 cm × 6 cm × 40 cm) and the other arms are without walls (70 cm × 6 cm × 0.75 cm); the closed and open arms delimit a small square area (6 cm × 6 cm) named center. Each mouse was placed in the center of the maze facing one of the open arms away from the experimenter, and its behavior was video-recorded for 5 min. The time spent by the mice in each of the three compartments (open, close, center zones) and the distance covered were measured with Ethovision XT-10. Head dips, defined as events in which the mouse nose-point was beyond the borders of the maze, were scored. Position heat maps were generated by averaging the proportion of track found in each location per animal. Range of colors was calculated by comparing the location frequencies of all subjects, with images representing the group averages.

Forced Swim Test (FST)

The FST is a standard paradigm to evaluate depression-like behavior in rodents (30). In this test, each mouse was placed in a glass cylinder (25 cm in height and 17 cm in diameter), filled up to 16 cm with water at a temperature of 23°C and let swim for 10 min. At the end of the test, the mouse was removed from the cylinder, gently dried, and placed in a new cage on a warm pad for at least 20 min. The test was video-recorded and analyzed both manually, by an observer blind to groups, and automatically (Ethovision XT-10). The immobility time (defined as the time spent by the mouse floating, with only minimal movements to keep the head above the water surface) was measured and considered as an index of depression. Climbing was defined by forceful thrash movements of the forelimbs against the walls of the cylinder and concomitant displacement of body center <1.6 cm below water surface (calibrated to animal body size).

Statistical Analyses

Behavioral data were analyzed with one-way ANOVA (multiple groups) or two-way repeated measures ANOVA (multiple groups with two measures per subject), and *post hoc* comparisons between groups were made with Fisher's LSD test or Holm–Sidak's test, respectively. Data that did not comply with normality assumptions (Brown–Forsythe test for SD) were analyzed with Kruskal–Wallis test, followed by Dunn's multiple comparison test. $p \leq 0.05$ were considered significant and all data are presented as mean ± SEM.

RESULTS

Previous work showed that mice with a partial 6-OHDA lesion of the dopaminergic system display memory deficit and affective disturbances reminiscent of early stage PD (31–33). In the first series of experiments, this model was employed to examine the effects of rapamycin and PF-4708671 on the disruption of long-term memory. Four groups of animals were used: sham-lesion (Sham) mice treated with vehicle, 6-OHDA lesion (Lesion) mice treated with vehicle, Lesion mice treated with rapamycin (Lesion Rapa), and Lesion mice treated with PF-4708671 (Lesion PF).

As shown in **Figure 1**, the 6-OHDA lesion abolished the ability of the mice to distinguish between a familiar and a novel object.

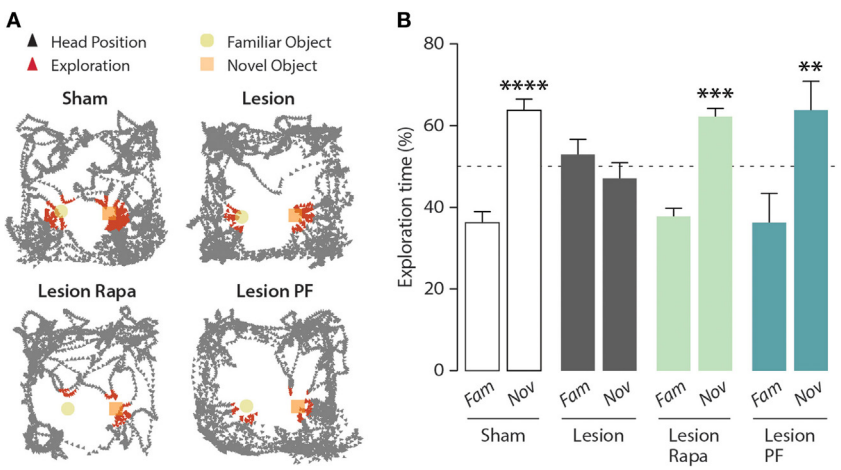


FIGURE 1 | Inhibition of mammalian target of rapamycin complex 1, or its downstream target S6 kinase, rescues novel object recognition (NOR) memory in a mouse model of Parkinson's disease. NOR test performance in control (Sham) mice treated with vehicle ($n = 16$), and Lesion mice treated with vehicle ($n = 12$), rapamycin (Lesion Rapa, $n = 16$), or PF-4708671 (Lesion PF, $n = 8$). **(A)** Representative traces of mice performing the NOR test. Triangles indicate the head position during the test (in black) and during exploratory behavior (in red). **(B)** Time spent exploring the familiar (Fam) or novel (Nov) object over a 5 min test. Data are expressed as percent of total exploration time and represented as mean ± SEM. **** $p = 0.0001$, *** $p = 0.0003$, and ** $p = 0.003$ vs. Fam within groups (two-way ANOVA followed by Fisher's *post hoc* comparison).

Subchronic administration of rapamycin, starting 4 days preceding the test, reverted the impairment of NOR produced by partial dopamine depletion.

mTORC1 regulates two major downstream effector targets involved in the modulation of protein synthesis: 4E-BP and S6K (6, 7). In order to determine the contribution of these two signaling components, we compared the effect of rapamycin, which prevents mTORC1-mediated regulation of both 4E-BP and S6K, with that of the selective S6K inhibitor PF-4708671 (24). Similar to rapamycin, PF-4708671 reverted the impairment of NOR observed in the Lesion group. Two-way ANOVA indicated significant group × object interaction ($F_{3,48} = 5.10$, $p = 0.004$), Fisher's *post hoc* comparison (**Figure 1B**).

It should be noted that, under these experimental conditions, Lesion mice treated with PF-4708671 showed a 60% reduction in the overall object exploration, as compared with the other groups. However, this reduction was not accompanied by reduced motor activity and, importantly, did not affect their ability to perform the task (data not shown).

We next examined the effect of rapamycin and PF-4708671 on the depression-like behavior produced by partial dopamine depletion. As previously reported (31), Lesion mice displayed increased immobility in the FST (**Figures 2A,B**). Time course analysis (2 min bins) indicated that the highest immobility time of Lesion mice occurred in the second half of the test (significant group × time interaction two-way repeated measures ANOVA, $F_{3,44} = 13.02$, $p < 0.0001$, followed by Holm–Sidak's *post hoc* comparison: bin 8 $p = 0.007$, bin 10 $p = 0.02$) (**Figure 2A**). Cumulative analysis showed that the increase in immobility time produced by the 6-OHDA lesion was reverted by rapamycin, but not by PF-4708671 (one-way ANOVA, $F_{3,44} = 13.01$, $p < 0.0001$, followed by Fisher's *post hoc* comparison) (**Figure 2B**).

The depression-like response in the FST was further analyzed by measuring climbing (**Figure 2C**), swimming time (**Figure 2D**),

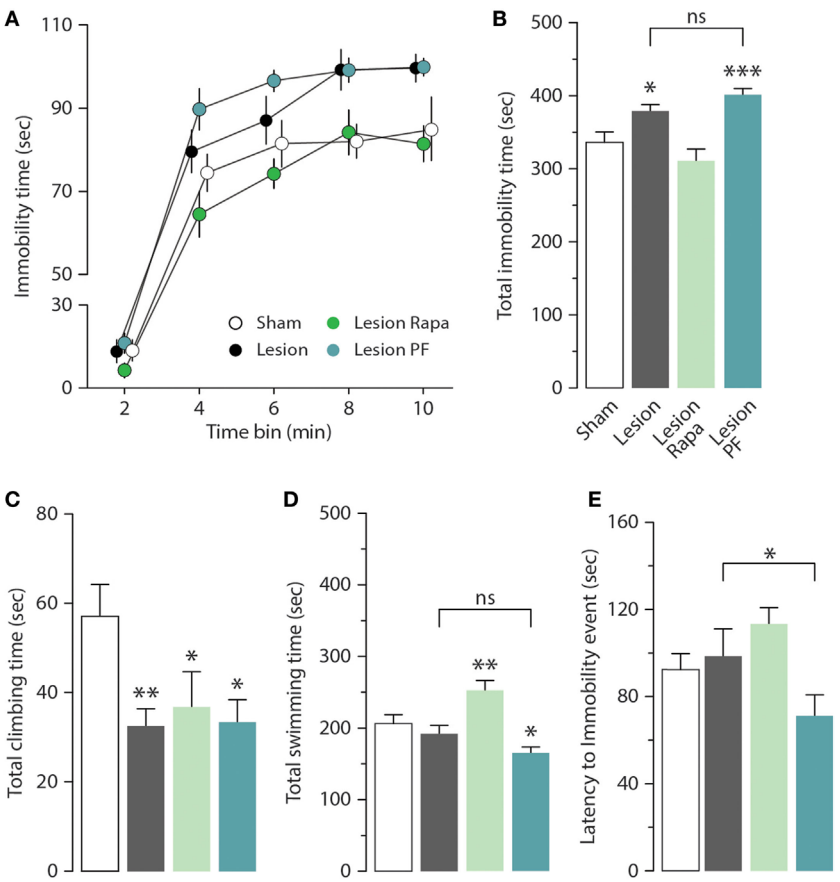


FIGURE 2 | Rapamycin reverts depression-like behavior in a mouse model of Parkinson's disease. Immobility time in the forced swim test (FST) was measured in Sham ($n = 12$), and Lesion mice treated with vehicle ($n = 12$), rapamycin (Lesion Rapa, $n = 12$), or PF-4708671 (Lesion PF, $n = 12$). **(A)** Time course (2 min bins) over the 10 min FST test. Repeated measures two-way ANOVA followed by Holm–Sidak's *post hoc* indicated a significant difference $p = 0.02$ for Lesion vs. Sham and $p = 0.0006$ for Lesion PF vs. Sham. **(B)** Total immobility time (s) during the 10-min FST test. * $p = 0.01$ and *** $p = 0.0002$ vs. Sham (one-way ANOVA followed by Fisher's *post hoc* comparison). **(C)** Total time (s) spent climbing. * $p = 0.03$ and 0.01, ** $p = 0.007$ vs. Sham (one-way ANOVA followed by Fisher's *post hoc* test). **(D)** Total time (s) spent swimming. * $p = 0.01$, ** $p = 0.006$ vs. Sham (one-way ANOVA followed by Fisher's *post hoc* comparison). **(E)** Latency (s) to first immobility event. * $p = 0.04$ Lesion vs. Lesion PF (one-way ANOVA followed by Fisher's *post hoc* comparison). Data are presented as mean ± SEM. Groups and treatments are as indicated in (B).

and latency to the first immobility event (**Figure 2E**). We found that climbing activity was reduced in 6-OHDA lesion mice (one-way ANOVA, $F_{3,44} = 3.29$, $p = 0.03$, followed by Fisher's *post hoc* comparison), with no effect on swimming time (one-way ANOVA, $F_{3,44} = 10.52$, $p < 0.0001$, followed by Fisher's *post hoc* comparison), or latency to immobility (one-way ANOVA, $F_{3,44} = 3.43$, $p = 0.02$ followed by Fisher's *post hoc* comparison). Treatment with rapamycin increased swimming time (**Figure 2D**) without affecting climbing activity (**Figure 2C**) or latency to immobility (**Figure 2E**). PF-4708671 did not modify the performance of 6-OHDA lesion mice with regard to climbing and swimming (**Figures 2C,D**), and reduced latency to immobility (**Figure 2E**).

The mouse model of PD utilized in this study displays anxiety-like behavior in multiple paradigms (31, 33). In this study, we used the OF test to evaluate thigmotaxis as an index of anxiety in Sham and Lesion mice treated with vehicle, rapamycin or PF-4708671. The number of visits to the center zone of the OF (measured as visits/30 s, during a period of 15 min) is increased in all groups over time (two-way repeated measures ANOVA indicates a significant effect of time $F_{28,1232} = 16.14$, $p < 0.0001$) (**Figure 3A**, left panel). We also observed a significant effect of treatment during the course of the experiment (two-way repeated measures ANOVA, group × time interaction, $F_{84,1232} = 1.41$, $p = 0.0098$). Best-fitting curves showed that Sham mice began exploring the center zone of the OF earlier than Lesion mice (**Figure 3A**, left panel). In line with this measurement, thigmotaxis was increased in Lesion mice, as indicated by reduced time spent in the center zone (one-way ANOVA, $F_{3,44} = 3.59$, $p = 0.02$ followed by Fisher's *post hoc* comparison) (**Figure 3A**, right panel). The increase in thigmotaxis observed in Lesion mice was reverted by rapamycin, but not by PF-4708671. Neither lesion nor drug treatments affected the distance (m) covered by the animals or their average speed (cm/s) (**Figure 3B**).

Mice were further tested for anxiety-like behavior in the EPM apparatus. Heat maps with group average were generated to allow visualization of exploration patterns in response to the different treatments (**Figure 4A**). In line with previous work, Lesion mice spent significantly less time in the open arms of the EPM compared with Sham mice (31, 33). We observed that this effect was reversed when Lesion mice were treated with rapamycin (Kruskal–Wallis, $p = 0.006$, followed by Dunn's *post hoc* comparison). A partial reduction of the effect of the 6-OHDA lesion was also observed in response to PF-4708671 (Dunn's *post hoc* comparison, Lesion vs. Lesion PF, $p = 0.03$) (**Figure 4B**, left panel).

The similarity of the heat maps generated from Lesion and Lesion PF mice prompted a further analysis of the activity of each experimental group in the open arm of the EPM, to better understand the effects of rapamycin and PF-4708671. Analysis of the distance moved (cm) in the open arms showed no difference in motor activity, which should be regarded as a marker of exploratory behavior, between Lesion, and Lesion PF mice (Kruskal–Wallis, $p < 0.0001$, followed by Dunn's *post hoc* comparison) (**Figure 4B**, right panel). Notably, the reduced exploration of the open arm observed in these mice cannot be explained by a decrease in overall mobility since this parameter was comparable in all groups, during the 5-min test (**Figure 4C**). In line with these observations, Lesion PF mice spent more time in the center of the apparatus in comparison to the other groups (Kruskal–Wallis, $p = 0.01$, followed by Dunn's *post hoc*) (**Figure 4A**, cf. red color).

In the EPM test, head dip events are regarded as an additional indication of reduced anxiety (34). Therefore, we measured this behavior with automated tracking of nose-point position (**Figure 4D**). The total duration of head dip events (s) and their number were reduced in Lesion mice and these effects were reverted by rapamycin, but not by PF-4708671 (for head dip duration: one-way ANOVA, $F_{3,44} = 3.63$, $p = 0.02$ followed by Fisher's *post hoc* comparison; for head dip number: one-way ANOVA, $F_{3,44} = 5.51$, $p = 0.002$, followed by Fisher's *post hoc* comparison)

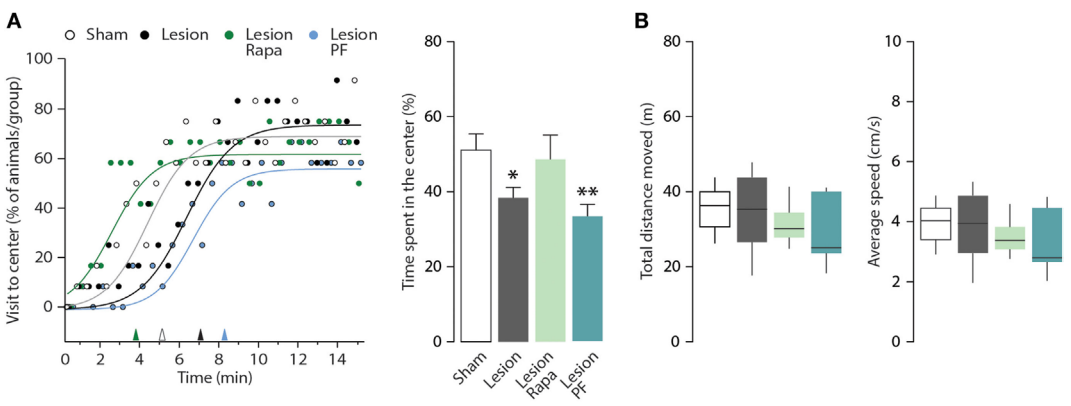


FIGURE 3 | Rapamycin counteracts thigmotaxis in a mouse model of Parkinson's disease. Open field (OF) performance of Sham ($n = 12$), and Lesion mice treated with vehicle ($n = 12$), rapamycin (Lesion Rapa, $n = 12$), or PF-4708671 (Lesion PF, $n = 12$) during the 15-min test. **(A)** Left panel shows the percentage of animals that visited the center of the OF, expressed as time course. The performance is graphed as 30-s time points (circles) and best-fitting curves. Triangles on the *x*-axis indicate the latency (min) for 50% of the animals in each group to explore the center zone of the OF. Right panel shows the time spent in the center zone, expressed as percent over the total duration of the test. Data are presented as mean ± SEM. * $p = 0.05$, ** $p = 0.007$ vs. Sham (one-way ANOVA followed by Fisher's *post hoc* comparison). **(B)** Tukey whiskers plots with the median, 10th, 25th, 70th, and 90th percentiles showing total distance moved in the OF (m) and average speed (cm/s). One-way ANOVA showed no differences between groups. Groups and treatments are as indicated in (A).

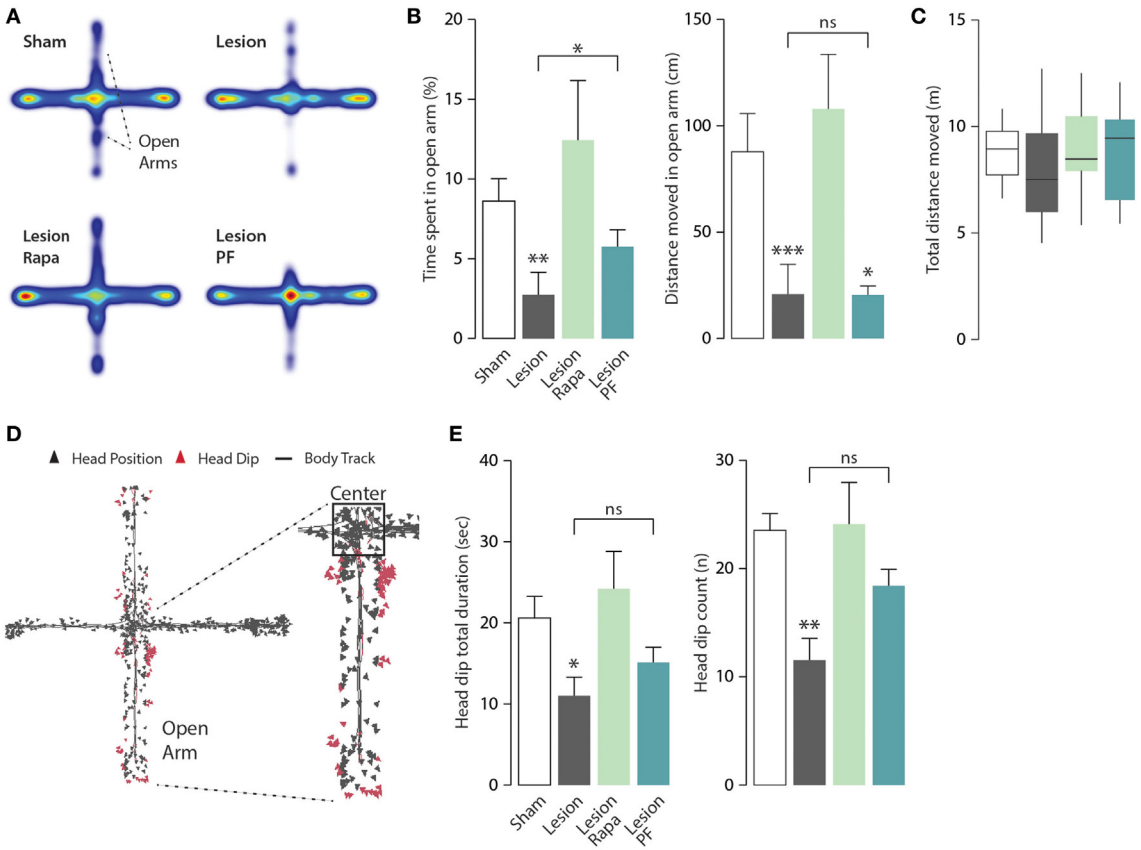


FIGURE 4 | Rapamycin abolishes anxiety-like behavior in a mouse model of Parkinson's disease. Elevated plus maze (EPM) test performance in Sham mice treated with vehicle (n = 12), and Lesion mice treated with vehicle (n = 12), rapamycin (Lesion Rapa, n = 12), or PF-4708671 (Lesion PF, n = 12). **(A)** Group average heat map locations where range of colors was calculated comparing location frequencies over all subjects. **(B)** Time spent in the EPM open arm expressed as percent of total time (left panel) and total distance moved (cm) (right panel) during the 5-min test. ** p = 0.005, * p = 0.03 vs. Sham for time, and *** p = 0.0005, * p = 0.048 vs. Sham for distance (Kruskal–Wallis test followed by Dunn's *post hoc* comparison). **(C)** Tukey whiskers plots with the median, 10th, 25th, 70th, and 90th percentiles showing total distance (m) covered in the EPM. One-way ANOVA showed no differences between groups. Groups and treatments are as indicated in (B). **(D)** Head dip analysis carried out by tracking nose-point position, as an additional marker of anxiety. Triangles indicate the head position during the test with head dip events in red. **(E)** Total duration (s) of head dips (left panel) and number of events (right panel). Data are presented as mean ± SEM. * p = 0.03 and ** p = 0.002 vs. Sham group (one-way ANOVA followed by Fisher's *post hoc* comparison).

(**Figure 4E**). No effect was found in the average duration of individual head dip events. Altogether, the analyses of anxiety-like behaviors indicate that rapamycin, but not PF-4708671, abolishes anxiety-like behaviors in experimental parkinsonism.

DISCUSSION

This study shows that inhibition of mTORC1, or its downstream target S6K, counteracts the memory deficit observed in a mouse model of early stage PD. It also shows that depression- and anxiety-like behaviors are eliminated by mTORC1 inhibition, but not by selective blockade of the mTORC1 downstream target, S6K.

The mouse model used in this study is based on a partial bilateral lesion with 6-OHDA, leading to 65–75% loss of dopaminergic nigrostriatal innervation and striatal dopamine levels (31, 32). These reductions reproduce an early stage of PD, characterized by mild changes in gate dynamics (31), which are unlikely to interfere with the assessment of cognitive and affective parameters.

We found that subchronic administration of rapamycin, which effectively reduces mTORC1 activity in the brain (35), abolishes the impairment of long-term NOR produced by a partial lesion of the dopamine system. Rapamycin acts by preventing the phosphorylation of S6K and 4E-BP, which in turn regulate two parallel signaling branches implicated in the control of protein synthesis, and in multiple aspects of synaptic plasticity and memory (11, 12). Our results indicate that selective inhibition of S6K with PF-4708671 is sufficient to rescue memory performance. Interestingly, PF-4708671 has also been shown to rescue hippocampal long-term potentiation and counteract behavioral abnormalities in mouse models of Angelman and fragile X syndromes (36, 37).

Rapamycin and PF-4708671 have been previously reported to re-establish cognitive performance in pathological models

characterized by abnormal mTORC1 signaling and protein translation (38, 39). Such dysregulation has not been demonstrated in the model of PD utilized in this study; thus, mTORC1, or S6K inhibition, is likely to correct memory deficits independently of a preexisting condition of mTORC1 hyperactivation. In line with this possibility, a clinical study showed that administration of the rapamycin analog everolimus, following cardiac transplant, a condition which is not associated with abnormal mTORC1 regulation, results in a significant improvement of memory and affective performance (40). Interestingly, this effect was proposed to occur, at least in part, through reduction of brain inflammation, which is commonly associated with neurodegenerative disorders including PD (41).

In addition to cognitive impairment, the mouse model utilized in this study reproduces affective symptoms typically observed in PD patients, such as depression and anxiety. We found that rapamycin counteracts the depression-like behavior manifested by PD mice as increased immobility in the FST. Notably, we observed that the anti-depressant effect of rapamycin is exerted by promoting swimming, but not climbing, which is regarded as a behavioral component related to motor stimulation rather than anti-depressant properties (42).

The finding that rapamycin reduces depression-like behavior contrasts with previous studies indicating that reduced mTOR signaling is associated with depression (19–22). In this regard, our results are more in line with the observation that subchronic administration of rapamycin, albeit at higher doses than those used in the present study, exerts anti-depressant effects in the FST and tail suspension tests (23).

In contrast to the results obtained with rapamycin, we did not observe any decrease in immobility time in the FST when PD mice were treated with PF-4708671. This suggests that the anti-depressant action of rapamycin depends on concomitant inhibition of the 4E-BP and S6K signaling cascades or that additional alternative mechanisms downstream of mTORC1 are required. For instance, rapamycin may reduce depression by promoting autophagy, which is negatively regulated by mTORC1 through inhibition of the mammalian autophagy-initiating kinase Ulk1 (43). In support of this possibility, several agents exerting anti-depressant actions, including lithium, citalopram, and trehalose, have been shown to induce autophagy (44–46).

Rapamycin counteracts the anxiety-like behavior observed in the mouse model of PD. In particular, this drug normalizes the time spent by PD mice in the center zone of the OF, thereby reducing thigmotaxis. A similar normalization was also observed in the EPM test, in which rapamycin increased the propensity of PD mice to explore the open arm of the apparatus. Selective inhibition of S6K with PF-4708671 did not produce a reduction of anxiety-like behaviors comparable to that observed with rapamycin. Thus, PF-4708671 did not reduce thigmotaxis in the OF and only partially reverted anxiety-like behavior in the EPM test. Although PF-4708671 increased the time spent by PD mice in the exposed area of the apparatus, it failed to induce a full exploration of the open arm. Moreover, and in contrast with rapamycin, administration of PF-4708671 did not counteract the reduction in head dip behavior, which is regarded as another indicator of anxiety. Altogether, these results indicate that rapamycin is capable of fully rescuing affective behavior in a mouse model of PD, and that this effect likely requires blockade of multiple downstream targets of mTORC1.

In conclusion, we show that inhibition of mTORC1 with rapamycin effectively counteracts memory deficit and mood disorders in a model of PD. We also show that inhibition of S6K, a well-characterized target of mTORC1, partially reproduces these effects by rescuing memory performance. Further studies will be necessary to fully characterize the action of rapamycin and identify additional components of the mTORC1 signaling machinery that may represent additional targets for the treatment of psychiatric symptoms associated with PD.

ETHICS STATEMENT

All experiments were carried out in accordance with the guidelines of Research Ethics Committee of Karolinska Institutet and Swedish Animal Welfare Agency.

AUTHOR CONTRIBUTIONS

GF conceived the project. DM and ABO designed experiments with contributions from all authors. DM and MB performed experiments and statistical analysis. GF and DM wrote the manuscript with contributions from all authors. GF supervised all aspects of the work.

FUNDING

This study was supported by the Swedish Research Council (grant number 2015-02886) and Karolinska Institutet Doctoral (KID) program (to GF). DM was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), Ministry of Education in Brazil.

REFERENCES

1. Barone P. Neurotransmission in Parkinson's disease: beyond dopamine. *Eur J Neurol* (2010) 17:364–76. doi:10.1111/j.1468-1331.2009.02900.x
2. Chaudhuri KR, Odin P. The challenge of non-motor symptoms in Parkinson's disease. *Prog Brain Res* (2010) 184:325–41. doi:10.1016/S0079-6123(10)84017-8
3. Barone P. Treatment of depressive symptoms in Parkinson's disease. *Eur J Neurol* (2011) 18(Suppl 1):11–5. doi:10.1111/j.1468-1331.2010.03325.x
4. Svenningsson P, Westman E, Ballard C, Aarsland D. Cognitive impairment in patients with Parkinson's disease: diagnosis, biomarkers, and treatment. *Lancet Neurol* (2012) 11:697–707. doi:10.1016/S1474-4422(12)70152-7
5. Yang Y, Tang BS, Guo JF. Parkinson's disease and cognitive impairment. *Parkinsons Dis* (2016) 2016:6734678. doi:10.1155/2016/6734678
6. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* (2004) 18:1926–45. doi:10.1101/gad.1212704
7. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149:274–93. doi:10.1016/j.cell.2012.03.017
8. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev* (2001) 15:2852–64. doi:10.1101/gad.912401
9. Raught B, Peiretti F, Gingras AC, Livingstone M, Shahbazian D, Mayeur GL, et al. Phosphorylation of eucaryotic translation initiation factor 4B Ser422

is modulated by S6 kinases. *EMBO J* (2004) 23:1761–9. doi:10.1038/sj.emboj.7600193
10. Holz MK, Ballif BA, Gygi SP, Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* (2005) 123:569–80. doi:10.1016/j.cell.2005.10.024
11. Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N. Translational control of long-lasting synaptic plasticity and memory. *Neuron* (2009) 61:10–26. doi:10.1016/j.neuron.2008.10.055
12. Santini E, Huynh TN, Klann E. Mechanisms of translation control underlying long-lasting synaptic plasticity and the consolidation of long-term memory. *Prog Mol Biol Transl Sci* (2014) 122:131–67. doi:10.1016/B978-0-12-420170-5.00005-2
13. Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, Kwiatkowski DJ, et al. Reversal of learning deficits in a Tsc2± mouse model of tuberous sclerosis. *Nat Med* (2008) 14:843–8. doi:10.1038/nm1788
14. Ehninger D. From genes to cognition in tuberous sclerosis: implications for mTOR inhibitor-based treatment approaches. *Neuropharmacology* (2013) 68:97–105. doi:10.1016/j.neuropharm.2012.05.015
15. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, Mcbride SM, Klann E, et al. Dysregulation of mTOR signaling in fragile X syndrome. *J Neurosci* (2010) 30:694–702. doi:10.1523/JNEUROSCI.3696-09.2010
16. Ronesi JA, Huber KM. Homer interactions are necessary for metabotropic glutamate receptor-induced long-term depression and translational activation. *J Neurosci* (2008) 28:543–7. doi:10.1523/JNEUROSCI.5019-07.2008
17. Troca-Marin JA, Alves-Sampaio A, Montesinos ML. An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse model of Down's syndrome. *J Neurosci* (2011) 31:9445–55. doi:10.1523/JNEUROSCI.0011-11.2011
18. Andrade-Talavera Y, Benito I, Casanas JJ, Rodriguez-Moreno A, Montesinos ML. Rapamycin restores BDNF-LTP and the persistence of long-term memory in a model of down's syndrome. *Neurobiol Dis* (2015) 82:516–25. doi:10.1016/j.nbd.2015.09.005
19. Howell KR, Kutiyanawalla A, Pillai A. Long-term continuous corticosterone treatment decreases VEGF receptor-2 expression in frontal cortex. *PLoS One* (2011) 6:e20198. doi:10.1371/journal.pone.0020198
20. Jernigan CS, Goswami DB, Austin MC, Iyo AH, Chandran A, Stockmeier CA, et al. The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry* (2011) 35:1774–9. doi:10.1016/j.pnpbp.2011.05.010
21. Duman RS, Li N, Liu RJ, Duric V, Aghajanian G. Signaling pathways underlying the rapid antidepressant actions of ketamine. *Neuropharmacology* (2012) 62:35–41. doi:10.1016/j.neuropharm.2011.08.044
22. Yu JJ, Zhang Y, Wang Y, Wen ZY, Liu XH, Qin J, et al. Inhibition of calcineurin in the prefrontal cortex induced depressive-like behavior through mTOR signaling pathway. *Psychopharmacology (Berl)* (2013) 225:361–72. doi:10.1007/s00213-012-2823-9
23. Cleary C, Linde JA, Hiscock KM, Hadas I, Belmaker RH, Agam G, et al. Antidepressive-like effects of rapamycin in animal models: implications for mTOR inhibition as a new target for treatment of affective disorders. *Brain Res Bull* (2008) 76:469–73. doi:10.1016/j.brainresbull.2008.03.005
24. Pearce LR, Alton GR, Richter DT, Kath JC, Lingardo L, Chapman J, et al. Characterization of PF-4708671, a novel and highly specific inhibitor of p70 ribosomal S6 kinase (S6K1). *Biochem J* (2010) 431:245–55. doi:10.1042/BJ20101024
25. Franklin KBJ, Paxinos G. *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press (1997).
26. Simon P, Dupuis R, Costentin J. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behav Brain Res* (1994) 61:59–64. doi:10.1016/0166-4328(94)90008-6
27. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process* (2012) 13:93–110. doi:10.1007/s10339-011-0430-z
28. Grayson B, Leger M, Piercy C, Adamson L, Harte M, Neill JC. Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. *Behav Brain Res* (2015) 285:176–93. doi:10.1016/j.bbr.2014.10.025
29. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* (1987) 92:180–5. doi:10.1007/BF00177912
30. Castagne V, Moser PC, Porsolt RD. Preclinical behavioral models for predicting antipsychotic activity. *Adv Pharmacol* (2009) 57:381–418. doi:10.1016/S1054-3589(08)57010-4
31. Bonito-Oliva A, Masini D, Fisone G. A mouse model of non-motor symptoms in Parkinson's disease: focus on pharmacological interventions targeting affective dysfunctions. *Front Behav Neurosci* (2014) 8:290. doi:10.3389/fnbeh.2014.00290
32. Bonito-Oliva A, Pignatelli M, Spigolon G, Yoshitake T, Seiler S, Longo F, et al. Cognitive impairment and dentate gyrus synaptic dysfunction in experimental parkinsonism. *Biol Psychiatry* (2014) 75:701–10. doi:10.1016/j.biopsych.2013.02.015
33. Masini D, Lopes-Aguiar C, Bonito-Oliva A, Papadia D, Andersson R, Fisahn A, et al. The histamine H3 receptor antagonist thioperamide rescues circadian rhythm and memory function in experimental parkinsonism. *Transl Psychiatry* (2017) 7:e1088. doi:10.1038/tp.2017.58
34. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* (2007) 2:322–8. doi:10.1038/nprot.2007.44
35. Santini E, Heiman M, Greengard P, Valjent E, Fisone G. Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA-induced dyskinesia. *Sci Signal* (2009) 2:ra36. doi:10.1126/scisignal.2000308
36. Bhattacharya A, Mamcarz M, Mullins C, Choudhury A, Boyle RG, Smith DG, et al. Targeting translation control with p70 S6 kinase 1 inhibitors to reverse phenotypes in fragile X syndrome mice. *Neuropsychopharmacology* (2016) 41:1991–2000. doi:10.1038/npp.2015.369
37. Sun J, Liu Y, Tran J, O'neal P, Baudry M, Bi X. mTORC1-S6K1 inhibition or mTORC2 activation improves hippocampal synaptic plasticity and learning in Angelman syndrome mice. *Cell Mol Life Sci* (2016) 73:4303–14. doi:10.1007/s00018-016-2269-z
38. Troca-Marin JA, Alves-Sampaio A, Montesinos ML. Deregulated mTOR-mediated translation in intellectual disability. *Prog Neurobiol* (2012) 96:268–82. doi:10.1016/j.pneurobio.2012.01.005
39. Costa-Mattioli M, Monteggia LM. mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nat Neurosci* (2013) 16:1537–43. doi:10.1038/nn.3546
40. Lang UE, Heger J, Willbring M, Domula M, Matschke K, Tugtekin SM. Immunosuppression using the mammalian target of rapamycin (mTOR) inhibitor everolimus: pilot study shows significant cognitive and affective improvement. *Transplant Proc* (2009) 41:4285–8. doi:10.1016/j.transproceed.2009.08.050
41. Phani S, Loike JD, Przedborski S. Neurodegeneration and inflammation in Parkinson's disease. *Parkinsonism Relat Disord* (2012) 18(Suppl 1):S207–9. doi:10.1016/S1353-8020(11)70064-5
42. Costa AP, Vieira C, Bohner LO, Silva CF, Santos EC, De Lima TC, et al. A proposal for refining the forced swim test in Swiss mice. *Prog Neuropsychopharmacol Biol Psychiatry* (2013) 45:150–5. doi:10.1016/j.pnpbp.2013.05.002
43. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13:132–41. doi:10.1038/ncb2152
44. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol* (2005) 170:1101–11. doi:10.1083/jcb.200504035
45. Zschocke J, Zimmermann N, Berning B, Ganal V, Holsboer F, Rein T. Antidepressant drugs diversely affect autophagy pathways in astrocytes and neurons – dissociation from cholesterol homeostasis. *Neuropsychopharmacology* (2011) 36:1754–68. doi:10.1038/npp.2011.57
46. Kara NZ, Toker L, Agam G, Anderson GW, Belmaker RH, Einat H. Trehalose induced antidepressant-like effects and autophagy enhancement in mice. *Psychopharmacology (Berl)* (2013) 229:367–75. doi:10.1007/s00213-013-3119-4

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.

frontiers in Neurology

REVIEW
published: 09 April 2018
doi: 10.3389/fneur.2018.00228

Modeling Parkinson's Disease in *Drosophila*: What Have We Learned for Dominant Traits?

Yulan Xiong and Jianzhong Yu**

Department of Anatomy and Physiology, Kansas State University College of Veterinary Medicine, Manhattan, KS, United States

Parkinson's disease (PD) is recognized as the second most common neurodegenerative disorder after Alzheimer's disease. Unfortunately, there is no cure or proven disease modifying therapy for PD. The recent discovery of a number of genes involved in both sporadic and familial forms of PD has enabled disease modeling in easily manipulable model systems. Various model systems have been developed to study the pathobiology of PD and provided tremendous insights into the molecular mechanisms underlying dopaminergic neurodegeneration. Among all the model systems, the power of *Drosophila* has revealed many genetic factors involved in the various pathways, and provided potential therapeutic targets. This review focuses on *Drosophila* models of PD, with emphasis on how *Drosophila* models have provided new insights into the mutations of dominant genes causing PD and what are the convergent mechanisms.

Keywords: Parkinson's disease, *Drosophila*, modeling, leucine-rich repeat kinase 2, α-synuclein, glucocerebrosidase, vacuolar protein sorting 35

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Matteo Bologna, Sapienza Università di Roma, Italy
Paolo Calabresi, University of Perugia, Italy

****Correspondence:***
Yulan Xiong
yulanxiong@ksu.edu;
Jianzhong Yu
jianzhongyu@ksu.edu

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *09 January 2018*
Accepted: *23 March 2018*
Published: *09 April 2018*

Citation:
Xiong Y and Yu J (2018) Modeling Parkinson's Disease in Drosophila: What Have We Learned for Dominant Traits? Front. Neurol. 9:228. doi: 10.3389/fneur.2018.00228

INTRODUCTION

Parkinson's disease (PD) is recognized as the most common movement disorder and the second most common neurodegenerative disorder after Alzheimer's disease (1). The classical motor features including akinesia, resting tremor, muscle rigidity, and postural imbalance are clinical symptoms in PD patients, and the none-motor features including cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain, and fatigue also frequently occur (1). The progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta is the cause for the cardinal symptoms (2). Although the majority of PD cases appear to be sporadic, the identification of causative genes that cause familial forms of PD has led to important insights into the pathogenesis of this progressive neurodegenerative disease (3). To date, genes encoding α-synuclein (α-Syn), leucine-rich repeat kinase 2 (LRRK2), Parkin, phosphatase and tensin homolog deleted on chromosome 10-induced putative kinase 1 (PINK1), DJ-1, vacuolar protein sorting 35 (VPS35), and glucocerebrosidase (GBA), among others are associated with genetic forms of PD that closely resemble idiopathic PD (3–8). Among these genes, *LRRK2*, *α-synuclein*, *GBA*, and *VPS35* are the dominant traits, and *parkin*, *DJ-1*, and *PINK1* are the recessive genes. Various model systems have been developed to study the function of PD-causing genes

Abbreviations: PD, Parkinson's disease; LRRK2, leucine-rich repeat kinase 2; PINK1, phosphatase and tensin homolog deleted on chromosome 10-induced putative kinase 1; VPS35, vacuolar protein sorting 35; GBA, glucocerebrosidase; DA, dopaminergic; SNpc, substantia nigra pars compacta; EMS, ethyl methanesulfonate; SJ1, synaptojanin 1; EndoA, endophilin A; GSK3β, glycogen synthase kinase 3β; GWASs, genome-wide association studies; LBs, Lewy bodies; α-Syn, α-synuclein; UPS, ubiquitin-protease system; HDAC6, histone deacetylase 6; TRAP1, tumor necrosis factor receptor associated protein-1; ER, endoplasmic reticulum; GlcSph, glucosylceramide (GlcCer) and glucosylsphingosine; GD, Gaucher's disease; UPR, unfolded protein response.

and provided tremendous insights into the molecular mechanisms underlying DA neurodegeneration. While few of genetic models in rodents recapitulate the cardinal features of PD, the power of *Drosophila* has revealed many genetic factors involved in the various pathways, and provided potential therapeutic targets. Here, we focus on the dominant genes causing PD. We discuss how *Drosophila* models have provided new insights into the mutations of dominant genes causing PD and what are the convergent mechanisms.

Drosophila AS A MODEL IN THE STUDY OF PD

Drosophila melanogaster, as a non-mammalian animal, provides a simple, yet powerful, *in vivo* system to model PD pathobiology. *Drosophila* has well-defined nerve system. Particularly, in adult brain, *Drosophila* has distinct DA neuronal clusters including about 200 DA neurons and displays complicated behaviors mimicking some human behaviors which are DA dependent. Both transgenic and knockout approaches have been utilized to develop *Drosophila* models of PD. The *Drosophila* Gal4/UAS system is a powerful tool for targeted transgene expression and has been used to direct selective expression of mutant PD genes. As a simple organism, *Drosophila* provides great advantages in conducting genome-wide modifier screenings and high-throughput drug screenings. Modifier screenings allow analyses of genome-wide genetic interactions based on the modification of given phenotypes and further identify components of diverse signaling pathways involved in PD pathogenesis.

Several steps can be taken to establish and utilize *Drosophila* models to study PD:

(1) Develop *Drosophila* lines carrying mutant PD genes,
(2) Characterize the phenotypes of the *Drosophila* models and determine whether they recapitulate the pathogenesis of the disease,
(3) Explore the molecular mechanisms underlying the phenotypes,
(4) Identify genetic modifiers that suppress or enhance the disease phenotypes through genetic screenings, and dissect the signaling pathways and pathogenic mechanisms involved in pathogenesis,
(5) Screen for drug candidates,
(6) Study the impact of environmental (e.g., toxin) or aging influence in combination with genetic factors on the pathogenesis of PD.

MODELING LRRK2-ASSOCIATED PD IN *Drosophila*

Mutations in the *LRRK2* gene (PARK8, dardarin) is the most common known genetic cause of PD and cause late-onset, autosomal dominant PD with age-related penetrance and clinical features identical to late-onset sporadic PD (9, 10). LRRK2 is a large multifunctional protein about 280 kD. It includes two important enzymatic domains, which are a GTPase domain and a kinase domain, and several protein interaction domains including a LRRK2-specific repeat domain, a leucine-rich repeat, and a WD40 repeat (11–13). Disease causing mutations are found in both enzymatic domains, indicating their importance in disease pathogenesis (11, 12). The most prevalent LRRK2 mutation, G2019S, within the kinase domain, accounts for ~1% of sporadic late-onset PD and 5–6% of familial PD worldwide (14). In North African Arabs and Ashkenazi Jews, the frequency of LRRK2–G2019S mutation can be as high as 30–40% in PD patients (15, 16). Patients with the G2019S mutation exhibit Lewy bodies (LBs) in most cases and incomplete penetrance even at advanced ages (1). However, mutations in the GTPase domain such as R1441 C/G often vary on LB pathology and exhibit nearly complete penetrance (9, 10). This suggests that these pathogenic mutations may cause disease *via* distinct pathogenic pathways/mechanisms. Tremendous work suggests LRRK2 GTPase and kinase enzyme activities may reciprocally regulate each other to direct LRRK2 functions in diverse cellular signaling pathways (17, 18). LRRK2 is demonstrated to be involved in protein translation, vesicle trafficking, mitochondrial function, lysosomal–autophagy, and cytoskeletal dynamics (13, 18–22). However, how LRRK2 mutations cause neurodegeneration in PD still need to be defined. To this end, various animal models of LRRK2-associated PD have been generated (23–25). Among these models, LRRK2 *Drosophila* models have provided unique and critical insights on LRRK2 functions (**Table 1**).

LRRK2 *Drosophila* Models

LRRK2 Knockout *Drosophila* Models

Drosophila has a single human LRRK2 homolog, dLRRK. Residues changed by PD-causing mutations in human LRRK2 are highly conserved in *dLRRK* (**Table 1**). To study the physiological function of endogenous LRRK2, *dLRRK* loss-of-function mutant fly lines have been generated. One major line used in the studies is LRRK^{e03680} from the Exelixis Collection at the Harvard Medical School. It was generated with piggyBac element insertion in the intron between exon 5 and exon 6 of *dLRRK* gene. In characterization of dLRRK knockout *Drosophila* model on PD related pathogenesis, one study reported knockout of dLRRK exhibited a decrease in tyrosine hydroxylase (TH) immunostaining, shrunken DA neurons, and locomotor activity deficits (26), while three studies reported that the homozygous mutant flies developed unchanged number and pattern of DA neurons as well as a normal life span (27–29). Furthermore, the sensitivity of those dLRRK2 knockout flies response to oxidative stress have been examined. Wang et al. showed that *dLRRK* mutant flies are selectively sensitive to H_2O_2 (27). By contrast, a report by Imai et al. demonstrated that *dLRRK* knockout flies are relatively resistant to paraquat and H_2O_2 treatment (28). Thus, the exact role of dLRRK in PD-related pathogenesis remains elusive. As the majority of studies supported that *dLRRK* is not required for DA neurons survival and this is consistent with the results from LRRK2 knockout rodents (mice or rats), the general agreement is that LRRK2-induced neuronal toxicity is from a gain-of-function but not a loss-of-function mechanism.

LRRK2 Transgenic *Drosophila* Models

Patients carrying heterozygou s or homozygous LRRK2 pathogenic mutations have similar disease risk and progression, supporting

TABLE 1 | *Drosophila* models for leucine-rich repeat kinase 2 (LRRK2)-associated Parkinson's disease.

Genetic manipulations	References	LRRK2 variants	Neurodegeneration	Motor activities/life span	Other functions
Knockout	(26)	dLRRK	Tyrosine hydroxylase (TH) neurons: no changes TH neurons shrunken TH staining ↓	Locomotor activity ↓	ND
	(27)	dLRRK	No changes	Life span ↓	Sensitive to hydrogen peroxide, not to paraquat, rotenone, and β-mercaptoethanol
	(28)	dLRRK	TH neurons: no changes; DA content ↑	Life span ↓ Fertility ↓ Malformed abdomen	Hydrogen peroxide ↓ Paraquat ↓
	(29)	dLRRK	ND	Locomotor activity ↓	ND
Transgenic	(26)	dLRRK	No changes	No changes	ND
	(30)	hLRRK2	TH neurons ↓ No response to L-DOPA Retinal degeneration	Locomotor activity ↓ Life span ↓ Response to L-DOPA	ND
		hG2019S	TH neurons ↓↓ No response to L-DOPA Retinal degeneration	Locomotor activity ↓ Life span ↓ Response to L-DOPA	ND
	(28)	dLRRK	No changes	ND	No changes
		dY1383C dI1915T	TH neurons: no changes TH staining ↓ DA content ↓	ND	Hydrogen peroxide ↑ Paraquat ↑
	(31)	hLRRK2 (at 29°C)	TH neurons ↓ Retinal degeneration	Locomotor activity: 10 days ↓ 20 days ↑ Life span ↑ Fertility ↑	Rotenone ↑
		hI1122V (at 29°C) hY1699C (at 29°C) hI2020T (at 29°C)	TH neurons ↓ the most with I2020T Retinal degeneration	Locomotor activity: 10 days ↓ 20 days ↑ Life span ↑ in hY1699C, hI2020T Fertility ↑ in hI1122V, hI2020T	Rotenone ↑
	(32)	hLRRK2	No changes	No changes	No changes
		hG2019S hY1699C hG2385R	TH neurons ↓	Locomotor activity ↓ Life span ↓	hG2019S, hG2385R ↑ hY1699C no change
	(33)	hLRRK2	ND	Locomotor activity: no changes Life span ↓	Dendritic ends ↓
		hG2019S	TH neurons ↓	Locomotor activity ↓↓ Life span ↓↓	Dendritic ends ↓↓ Axon degeneration ↑
		hR1441C	ND	Locomotor activity ↓	Dendritic ends ↓
		hG2385R		Life span ↓	
	(34)	hLRRK2	ND	ND	Visual function: no changes
		hG2019S	ND	ND	Visual function ↓
		hI1122V hR1441C hY1383C hI1915T hI2020T hG2385R hG2019/K 1906M	ND	ND	Visual function: no changes

(*Continued*)

TABLE 1 | Continued

Genetic manipulations	References	LRRK2 variants	Neurodegeneration	Motor activities/life span	Other functions
	(35)	hLRRK2 hG2019S	ND	Locomotor activity: no changes	Axon transport: no changes
		hR1441C hY1699C	ND	Locomotor activity ↓	Axon transport ↓
		dR1069C dY1383C	ND	Locomotor activity ↓	Axon transport ↓
	(36)	hG2019S hI2020T	ND	Bradykinesia, akinesia, hypokinesia, and increased tremor	Proboscis extension response ↓
		hR1441C hG2019/K 1906M	ND	No changes	No changes

ND, not determined; O/E, overexpression; ↑, increased; ↓, decreased.

LRRK2 dominant nature (1) (**Table 1**). Indeed, in contrast to *dLRRK* loss-of-function mutant, overexpression of either human LRRK2 (hLRRK2) or dLRRK pathogenic mutations in flies leads to an age-dependent DA neuronal loss and DA-responsive locomotor deficits (28, 30–33, 36, 37). Notably, different LRRK2 mutations cause different phenotypes of the degeneration. One study demonstrated that dopaminergic expression of LRRK2 G2019S led to non-autonomous neurodegeneration in visual system (34). This degeneration is specific to G2019S mutation and dependent on kinase activity. Another report showed that GTPase-COR domain mutations R1441C or Y1699C, but not G2019S, preferentially inhibits axonal transport in *Drosophila* and causes locomotor deficits (35). This suggests that the defects depend on LRRK2 GTPase activity (35). Recently, Cording et al. reported that expressing either the G2019S or I2020T but not R1441C, or kinase dead LRRK2 in DA neurons reduces proboscis extension response, with bradykinesia, akinesia, and tremor (36). These studies support the possibility that different LRRK2 pathogenic mutations act at distinct pathways and cause disease *via* distinct pathogenic mechanisms. The LRRK2 transgenic fly models support the gain-of-function of LRRK2 in PD pathogenesis.

LRRK2 *Drosophila* Models Reveal LRRK2 Functions in PD

LRRK2 Functions in Vesicular Trafficking

Vesicular trafficking has been implicated to play crucial roles in neurodegeneration (38). LRRK2 *Drosophila* models have provided extensive evidence of potential roles for LRRK2 in various vesicle trafficking processes including endocytosis, ER-Golgi and retromer trafficking, and autophagy–lysosomal pathways (39). dLRRK was reported to localize to the membranes of late endosomes and lysosomes, physically and functionally interacts with Rab7, a key mediator of late endosomal transport and lysosome biogenesis (40). Nonsense alleles in dLRRK induced by ethyl methanesulfonate causes striking defects in the autophagy–lysosomal pathways (41). Furthermore, LRRK2 has been shown to interact with clathrin-light chains to limit Rac1 activation on endosomes (42). Importantly, studies in *Drosophila* show that LRRK2 phosphorylates endophilin A (EndoA), a central component of synaptic endocytosis, and synaptojanin 1 (SJ1), a synaptic vesicle protein which was recently linked to recessive PD (37, 43–45). LRRK2 regulates EndoA and SJ1 by phosphorylation at synapses, which facilitates synaptic endocytosis through clathrin uncoating at the synaptic terminals. In addition, LRRK2's role in retromer and ER–Golgi trafficking was highlighted by genetic interactions between LRRK2 and VPS35, Rab7L1, ArfGAP1 in *Drosophila* (46–49). Moreover, dLRRK has been demonstrated to regulate axonal transport and Golgi outpost dynamics in dendrites through the golgin Lava lamp (35, 50). Taken together, these studies strongly support the roles of LRRK2 in vesicular trafficking processes, which may provide potential mechanisms for α-Syn accumulation in LRRK2-associated PD.

LRRK2 Functions in Protein Translation Machinery

The first evidence of LRRK2 function in protein translation was demonstrated in *Drosophila* (28). In this study, both dLRRK and human LRRK2 can phosphorylate eukaryotic initiation factor 4E-binding protein (4E-BP), a negative regulator of eukaryotic initiation factor 4E-mediated protein translation and a key mediator downstream of mTOR signaling to various stress responses (28). The notion that LRRK2 functions in protein translation was further strengthened by the study from the same group that LRRK2 interacts with the microRNA pathway to regulate protein synthesis (51). However, these studies were done in *Drosophila* system and need to be extended to mammalian systems. Subsequently, Martin and colleagues, using LRRK2 *Drosophila* model and human DA neurons, demonstrated that LRRK2 phosphorylates ribosome protein s15 to regulate protein translation and mediate LRRK2-induced neurodegeneration (52). Recently, Penney et al. showed that LRRK2 targets Furin1 to promote cap-dependent translation, which is required for LRRK2 synaptic function (53). Taken together, these findings support convergent evidence that LRRK2 regulates protein translation machinery directly or indirectly, which could be a potential therapeutic avenue for LRRK2-associated PD.

LRRK2 Functions in Dendritic Degeneration and Synaptic Dysfunction

Mutant LRRK2 functions in dendritic degeneration were first revealed by the evidence that LRRK2 G2019S induces

mislocalization of the axonal protein tau in dendrites and in turn causes dendrite degeneration (33). This may act through tau phosphorylation by the glycogen synthase kinase 3β, which is promoted by LRRK2 G2019S (33). In addition, LRRK2 regulates synaptic morphology through phosphorylation of Futsch at the presynaptic compartments and interaction with 4E-BP at the postsynaptic site of the *Drosophila* neuromuscular junctions (NMJs) (54). Furthermore, a recent finding indicates that loss of dLRRK disrupts the retrograde synaptic compensation while overexpression of either dLRRK or hLRRK2 can induce a retrograde enhancement of presynaptic release (53). This regulation of synaptic homeostasis might act through a mechanism that LRRK2 promotes cap-dependent translation (53). These studies suggest that LRRK2 might regulate synaptic function in neural circuits.

LRRK2 *Drosophila* Models as Platforms to Identify Potential Therapeutic Compounds

The genetic LRRK2 *Drosophila* models provide promising *in vivo* platforms for inhibitor identification and validation, and drug development. It has shown that sorafenib, curcumin, or GW5074 significantly suppressed LRRK2 PD-like phenotypes in *Drosophila* (55, 56). Melatonin attenuates hLRRK2-induced synaptic dysfunction and sleep disorders (57). Although candidate compounds have been used in these studies, they open the possibility of performing compound screens. Recently, Lin et al. identified compounds from the FDA-approved licensed drug library that could rescue LRRK2-induced neurite degeneration, motor disability, and DA neuron loss (58). Of 640 compounds, lovastatin had the highest lipophilicity, which facilitates crossing the blood–brain barrier (58). These studies provide significant steps toward the development of new drugs for treatment of LRRK2-associated PD.

MODELING α-Syn-ASSOCIATED PD IN *Drosophila*

The discovery of the first missense mutation A53T in the *SNCA* gene in 1997 (59) and the insoluble aggregated α-Syn forms as the major component of LBs, a pathological hallmark of PD (60), heralded a new era in PD research. Since then, more *SNCA* pathogenic mutations as well as multiplications of *SNCA* have been identified as genetic causes of PD [review in Ref (61).]. In addition, multiple genome-wide association studies have identified *SNCA* as a risk factor for sporadic PD (62, 63). These findings revealed a central role of *SNCA* in PD. *SNCA* encodes α-Syn protein, a small protein with 140 amino acid residues. It is highly soluble and enriched at presynaptic terminals, where it binds lipids and regulates synaptic vesicle release and it has a propensity of self-aggregate to form oligomeric species and LB-like fibrils (64, 65). Multiple evidence suggest that oligomeric species of α-Syn, which are precursors for higher-order fibrillar aggregates in LBs, are pathogenically toxic and the culprits for neuronal degeneration (66). Recently, prion-like behavior of α-Syn has attracted a lot of attention and been debated in playing an important role in the pathogenesis of PD (67, 68). Although *Drosophila* have no homolog of *SNCA*, pathogenic mutations and multiplication of *SNCA* causing PD with dominant inheritance pattern implicates a toxic gain-of-function mechanism, which led to suitable transgenic modeling in fly by overexpressing wild-type or mutant α-Syn (69) (**Table 2**).

α-Syn Transgenic *Drosophila* Models

Feany and Bender first developed α-Syn transgenic *Drosophila* models by overexpressing either wild-type or familial mutants A53T and A30P of human α-Syn using the conventional Gal4/USA expression system (70). These models recapitulate the essential features of PD: adult-onset loss of DA neurons, filamentous intraneuronal inclusions containing α-Syn and locomotor dysfunction (70). In an independent study, Auluck et al. confirmed the phenotypes reported by Feany and Bender (71). In addition to these phenotypes, Chen et al. demonstrated olfactory deficits and elevated anxiety in a α-Syn transgenic *Drosophila* model expressing A30P (79, 80). There are deficits in two out of three olfactory modalities, odor discrimination and tested-olfactory acuity. A30P expression in dopamine neurons is necessary for both acuity and discrimination deficits. Gajula Balija et al. showed the non-motor symptoms such as an abnormal sleep-like behavior, locomotor deficits, and abnormal circadian periodicity when targeted expression of pre-fibrillar α-Syn mutants in a subset of serotonergic and DA neurons (78). In 2017, the Feany group expended the scope of their previous α-Syn transgenic *Drosophila* models using a binary expression system, the Q system, which relies on the transcriptional activation by the *Neurospora* protein QF2 to activate transgene expression (81). This new α-Syn *Drosophila* model shows robust neurodegeneration, early-onset locomotor deficits, and abundant α-Syn aggregation (81). Although there is some discrepancy over the strength of the phenotypes (73, 82), the α-Syn transgenic *Drosophila* models are widely used to delineate underlying pathogenic mechanisms and identify novel proteins mediating α-Syn toxicity.

α-Syn *Drosophila* Models Reveal α-Syn Functions in PD

α-Syn Aggregation and Misfolding in α-Syn-Induced Neurotoxicity

Accumulating evidence revealed α-Syn aggregation and misfolding plays a central role in the pathogenesis of PD and synucleinopathies. Wild type or mutant α-Syn has been demonstrated to be aggregated as inclusions when overexpressing in flies (70, 71, 81). Structurally engineered α-Syn mutants with an increased oligomerization propensity increase neurotoxicity in *Drosophila* (76). Truncation of α-Syn contributes to aggregation and LB formation. Expression of α-Syn with a deletion of NAC domain (α-Syn Δ71–82) did not show evidence of α-Syn aggregation and any DA neurodegeneration, suggesting the essential role of NAC domain of α-Syn in aggregation and toxicity (75). By contrast, a C-terminal truncated α-Syn has ability to promote aggregation and enhance neurotoxicity (75). Interestingly, calpain-cleaved α-Syn fragments with similar molecular weight to truncated α-Syn have been identified in the PD/DLB patient

TABLE 2 | Transgenic *Drosophila* models of α-Syn-associated Parkinson's disease.

Transgenic systems	References	α-Syn variants	Neurodegeneration	Motor/non-motor activities and life span	Cellular functions
	(70)	α-Syn-WT A30P A53T	Tyrosine hydroxylase (TH) neurons ↓ Retinal degeneration ↑	Locomotor activity ↓	Filamentous intraneuronal inclusions containing α-Syn
	(71)	α-Syn-WT A30P A53T	TH neurons ↓	ND	Lewy body- and LN-like inclusions, Hsp70 protected against α-Syn–induced dopaminergic neuronal degeneration
	(72)	α-Syn-WT	ND	ND	Phosphorylation of α-Syn at S129 ↑
		A30P			Phosphorylation of α-Syn at S129 ↑↑
		A53T			Phosphorylation of α-Syn at S129 ↑↑↑
	(73)	α-Syn-WT A30P A53T	TH neurons: no changes No retinal degeneration	Locomotor activity: no changes	ND
	(74)	α-Syn-W	TH neurons ↓ Retinal degeneration ↑	ND	ND
		S129A	TH neuron: no changes No retinal degeneration		
		S129D	TH neurons ↓↓ Retinal degeneration ↑↑		
Gal4/UAS system	(75)	α-Syn-WT	TH neurons ↓	ND	α-Syn aggregation ↑
		α-SynΔ71–82aa	TH neurons: no changes		No α-Syn aggregation
		Syn 1–120aa	TH neurons ↓↓		α-Syn aggregation ↑↑
	(76)	α-Syn-WT	TH neurons: no changes	Locomotor activity: no changes	Soluble oligomers of α-Syn *in vitro*
		A30P A53T A56P	TH neurons ↓	Locomotor activity ↓	Soluble oligomers of α-Syn *in vitro* ↑
		A30P/A56P/A76P (TP)	TH neurons ↓↓	Locomotor activity ↓↓	Soluble oligomers of α-Syn *in vitro* ↑↑
	(77)	α-Syn-WT	TH neurons ↓ Retinal degeneration ↑	ND	Soluble oligomers of α-Syn ↑
		Y125F/Y133F/ Y136 F (YF)	TH neurons ↓↓ Retinal degeneration ↑↑		Soluble oligomers of α-Syn ↑↑
		S129D	TH neurons ↓↓ Retinal degeneration ↑↑		Soluble oligomers of α-Syn ↑↑
	(78)	α-Syn-WT	ND	Sleep behavior normal Circadian locomotor activity defects ↑	ND
		A53T		Sleep behavior abnormal ↑ Circadian locomotor activity defects ↑	
		A30P/A56P/ A76P (TP)	ND	Sleep behavior abnormal ↑↑ Circadian locomotor activity defects ↑↑	ND
	(79)	A30P	ND	Olfactory deficits	ND
	(80)	A30P	TH neurons ↓	Locomotor activity ↓ Anxiety ↑	ND
Q system	(81)	α-Syn-WT	TH neurons ↓↓	Locomotor activity ↓↓	α-Syn aggregation ↑↑

ND, not determined; ↑, increased; ↓, decreased.

brains and α-Syn-expressing flies (83). This suggests the physiological and pathological importance of the truncated α-Syn.

Protein quality control systems including molecular chaperones and protein degradation function as a defense mechanism against protein misfolding and aggregation. Identification of suppressors in these systems further supports a critical role of toxic oligomers and aggregation in α-Syn-induced neurotoxicity. Histone deacetylase 6 suppresses α-Syn-induced DA neurodegeneration and promotes the formation of α-Syn inclusions by reducing α-Syn oligomers (84). Auluck et al.

demonstrated that increasing the level of chaperone Hsp70 ameliorated the toxicity of α-Syn to DA neurons while a reduction in chaperone activity enhanced α-Syn-induced DA neuronal loss in *Drosophila* system (71). In addition, decreased level of the mitochondrial chaperone protein tumor necrosis factor receptor associated protein-1 enhanced A53T-α-Syn-induced age-dependent DA neuron loss in fly (85). Molecular chaperones assist proper protein folding and thus protect against α-Syn misfolding and aggregation. If proteins have been misfolded and aggregated, they are cleared by degradation. The ubiquitin-protease system and the autophagy-lysosome systems are two major protein degradation systems. Using *Drosophila* and cell culture systems, Lee et al. demonstrated that co-expression of ubiquitin can rescue α-Syn-induced neurotoxicity. This neuroprotection is dependent on the formation of lysine 48 polyubiquitin linkage, which is known to target protein degradation (86). This observation is further strengthened by evidence that overexpression of the ubiquitin ligase Nedd4 can rescue α-Syn-induced degenerative phenotype in fly (87). Furthermore, the deubiquitinase Usp8 interacted and partly colocalized with α-Syn, and deubiquitinated K63-linked chains on α-Syn. Knockdown of Usp8 in fly reduced α-Syn levels and α-Syn-induced toxicity (88). In addition, Cullen et al. showed that the defect of cathepsin D, a major lysosomal aspartyl protease, enhanced α-Syn-induced neurodegeneration *in vivo* in *Drosophila* (89). Taken together, these results confirmed that protein quality control systems function as a protection mechanism against α-Syn aggregation and misfolding.

α-Syn Phosphorylation Controls Neurotoxicity Inclusion Formation

Phosphorylation of α-Syn plays a key role in the PD pathogenesis. Phosphorylation at Ser129 is the one extensively phosphorylated in brain tissues from PD patients and related disorders, suggesting a role for Ser129 phosphorylation in disease pathogenesis (90, 91). In transgenic flies, it has been demonstrated that human α-Syn is phosphorylated at Ser129, and phosphorylation increases with age as DA neurons degenerate, mimicking the pathogenic phenomena in PD patient (72). Later on, Chen and Feany generated transgenic flies carrying the mutations at S129 of α-Syn (S129A to block phosphorylation and S129D to mimic phosphorylation) (74). Using these transgenic lines, they demonstrated phosphorylation of S129 is critical for α-Syn-induced DA neuron degeneration, and blocking S129 phosphorylation increases inclusion formation (74). As increased number of inclusion bodies correlates with reduced toxicity, this study suggested inclusion bodies might have protective function. Recently, the Feany group reported that tyrosine and serine phosphorylation of α-Syn have opposing effects: levels of soluble oligomeric species of α-Syn were increased by serine 129 phosphorylation and decreased by tyrosine 125 phosphorylation, suggesting detrimental effects of S129 phosphorylation and a neuroprotective action of T125 phosphorylation (77). These studies reveal that phosphorylation of α-Syn plays an important role in α-Syn-induced inclusion body formation and DA neurodegeneration.

α-Syn Functions in Vesicular Trafficking

The first evidence of α-Syn functions in trafficking in animal models has been reported by Cooper et al. using a combination of a genetic screening in yeast and validation in α-Syn transgenic *Drosophila* models (92). In this study, Rab1 rescues the neuron loss in the flies (92). Recently, using *Drosophila* models of α-Syn toxicity, several reports have implicated α-Syn functions in vesicular trafficking particularly through the small GTPase Rab proteins. The endosomal recycling factor Rab11 was demonstrated to modulate synaptic vesicle size, decrease α-Syn aggregation and ameliorate several α-Syn-dependent phenotypes (93). Rab7, regulating trafficking of late endosomes and autophagosomes, and Rab8, modulating post-Golgi vesicle trafficking, rescue the locomotor deficit in α-Syn flies (94, 95). Notably, other PD genes such as LRRK2 and PINK1 have also recently been linked to Rab proteins (48, 96–98). Thus, determination of the precise mechanisms of Rabs-mediated functions in PD pathogenesis is warranted.

α-Syn Functions in Mitochondrial Dysfunction and Oxidative Stress

Oxidative stress and mitochondrial dysfunction have been proposed as important causative factors for the progression of PD. Botella et al. found that DA neurons are specifically sensitive to an environmental oxidative insult (hyperoxia) induced oxidative stress. The mutant forms of α-Syn enhanced the toxicity under this stress in the *Drosophila* model (99). In addition, the co-expression of Cu/Zn superoxide dismutase protects against mutant α-Syn-induced DA neuronal loss (99). The same group also demonstrated that dopamine, which produces reactive oxygen species, might be involved in the α-Syn-induced neurotoxicity through oxidative stress (100). Furthermore, α-Syn-induced neuronal death in *Drosophila* is enhanced by the mutants that promote glutathione synthesis and conjugation (101). Natural antioxidants attenuate locomotor deficits of α-Syn transgenic flies (102). GPI, an enzyme in glucose metabolism, acts as neuroprotection from α-Syn proteotoxicity in flies (103). Recently, Feany group provided evidence that the interaction of α-Syn with spectrin initiates pathological alteration of the actin cytoskeleton and downstream neurotoxicity, and consequent mitochondrial dysfunction through altered Drp1 localization (81). These results suggest that oxidative stress and mitochondrial dysfunction are features of α-Syn toxicity.

α-Syn *Drosophila* Models as Platforms to Identify Potential Therapeutic Compounds

Based on the functions and mechanisms revealed by α-Syn *Drosophila* models, several pharmacological interventions have been developed in *Drosophila* to ameliorate α-Syn toxicity. Geldamamycin, an Hsp90 inhibitor and chaperone inductor, was able to protect α-Syn-expressing neurons in *Drosophila* (104). Nicotinamide, the principal form of niacin (vitamin B3), has been demonstrated to improve the motor dysfunction in α-Syn transgenic flies through improvement of oxidative mitochondrial dysfunction (105). The dopamine agonists pergolide, bromocriptine, and

2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine (SK&F 38393), D-519, and D-520 were substantially effective on improvement of locomotor function of α-Syn flies (106, 107). Atropine, the prototypical muscarinic cholinergic receptor antagonist, was effective (106). A potent dopamine D_2/D_3 receptor agonist D-607 exhibited significant neuroprotection in a *Drosophila* model of synucleinopathy (108). In addition, HDAC inhibitors such as sodium butyrate or SAHA, and SIRT2 inhibitors have been identified to protect against α-Syn-induced neurotoxicity in flies (109, 110). Taken together, these studies suggest that protein quality control systems, oxidative stress, mitochondrial function, and DA biosynthesis pathways are potential targets for developing therapeutic agents for α-Syn toxicity.

MODELING GBA-ASSOCIATED PD IN *Drosophila*

Heterozygous mutations in glucocerebrosidase (GCase, encoded by *GBA1* gene) are recently emerging to be the most common known genetic risk factor for PD (111). GCase is a lysosomal protein and homozygous mutations cause Gaucher's disease, a lysosomal storage disorder (112). As a lysosomal enzyme, GCase is synthesized in the endoplasmic reticulum (ER). At ER, it undergoes N-linked glycosylation on four asparagines. After correctly folded, it processes to the Golgi for further modifications on its N-linked glycans, and finally it traffics to the lysosomes (113). GCase cleaves the β-glucosyl linkage of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph). Mutations in *GBA* cause accumulation of lipid substrates of GCase such as GlcCer and GlcSph (114). Recent reports demonstrated that mutations in GBA not only contribute to the occurrence of PD but also lead to more significant and rapid cognitive decline in PD (115). Most disease causing mutations of GBA are thought to be dominant-negative mutations that lead to the GBA deficiency, compromised GlcCer metabolism and the subsequent failure of lysosomal mediated degradation of GBA substrates and α-Syn. A severe heterozygous mutation L444P and a mild heterozygous mutation N370S are the most common mutations of GBA in PD. Mutations L444P and N370S cause ER stress, decreased lysosomal GCase, and accumulation of α-Syn aggregates (116, 117). *Drosophila* as one of the major model systems so far for studying GBA-associated PD has revealed significant insights (**Table 3**).

TABLE 3 | *Drosophila* models of glucocerebrosidase (GBA)-associated Parkinson's disease.

Genetic manipulations	References	GBA variants	Neurodegeneration	Motor activities/ life span	Cellular functions
Knockout/ knockdown	(118)	dGBA1a$^{-/-}$	ND	Locomotor activity: no changes Life span ↑	ND
		dGBA1b$^{-/-}$ dGBA1a,b$^{-/-}$	Tyrosine hydroxylase (TH) neurons ↓	Locomotor activity ↓↓ Life span ↓↓	Changes in lipid metabolism, accumulation of substrate GlcCer, deficits in lysosomal–autophagy pathway, and abnormality of mitochondria
	(119)	dGBA1a,b$^{-/-}$(ΔTT)	TH neurons: no changes Other neurodegeneration	Locomotor activity ↓ Life span ↓ Memory deficits	Ubiquitinated proteins ↑ α-Syn aggregates ↑ α-Syn expression does not enhance the phenotypes
	(120, 121)	dGBA1a,b$^{-/-}$	TH neurons ↓	Life span ↓	GCase activity ↓
	(122)	dGBA1a-RNAi	TH neurons ↓ Retinal degeneration	Locomotor activity ↓	Proteinase K-resistant α-Syn accumulation when crossed with α-Syn flies
Transgenic	(120, 121)	hGBA-WT	No changes	No changes	No changes
		hN370S hL444P	TH level ↓	Locomotor activity ↓ Life span ↓	GCase activity ↓ ER stress ↑
	(123)	hGBA-WT	ND	ND	Neurodevelopment in fly eyes: no changes ER stress: no changes
		hR120W			Neurodevelopment in fly eyes: no changes ER stress ↓
		HRecNcil (L444P + A456V + V460V)			Neurodevelopmental defects in fly eyes ↑ ER stress ↓
	(124)	hGBA-WT	TH neurons: no changes	Locomotor activity: no changes	ER stress ↑
		hN370S hL444P	TH neurons ↓	Locomotor activity ↓	GCase activity ↓ compared with WT ER stress ↑

ND, not determined; ↑, increased; ↓, decreased.

GBA *Drosophila* Models

GBA Knockout/Knockdown *Drosophila* Models

Drosophila has two homologs of the human *GBA1* gene, CG31148 and CG31414, which are referred to *dGBA1a* and *dGBA1b*, respectively, and shares 32% amino acid identity. These two genes are found on the same chromosome with the *CG31413* gene in between and show differential tissue expression. *dGBA1b* is expressed in the adult brain at low levels as well as in the adult fat body, whereas *dGBA1a* is predominantly expressed in the adult fly gut but not in the adult brain (FlyAtlas) (125). As GBA mutations in PD show a dominant-negative function, the loss-of-function of GBA was investigated in *Drosophila* by either knockdown or knockout dGBA1.

Kinghorn et al. generated dGBA1a, dGBA1b single knockouts (dGBA1a$^{-/-}$, dGBA1b$^{-/-}$) or dGBA1a/b double knockouts (dGBA1a,b$^{-/-}$) using ends-out homologous recombination (118). dGBA1b$^{-/-}$ and dGBA1a,b$^{-/-}$ showed a significantly decreased survival and age-dependent locomotor deficits compared with control flies while dGBA1a$^{-/-}$ showed opposite phenotype with increased survival and without significant effect on climbing ability over time. As *dGBA1b* is expressed in the adult brain, the study was focusing on dGBA1b$^{-/-}$ and dGBA1a,b$^{-/-}$, both of which showed similar phenotypes (118). Knockout of dGBA resulted in changes in lipid metabolism, accumulation of substrate GlcCer, deficits in lysosomal–autophagy pathway, neurodegeneration, and abnormality of mitochondria. Importantly, mTOR inhibitor rapamycin partially ameliorated the lifespan, locomotor, and starvation phenotypes in dGBA deficient flies (118). Another group used publicly available transposon insertions in dGBA1a and dGBA1b to create deletion of dGBA1 (GBA1Δ^{TT}) (119). Using this approach, they removed the majority (first 433aa) of dGBA1b, small potion (33 codons) of c-terminal of dGBA1a, and the whole *CG31413* gene in between. Consistent with the study by Kinghorn et al. GBA1Δ^{TT} homozygotes exhibit shortened lifespan, behavioral phenotypes, memory deficits and neurodegeneration but no DA neuronal loss (119). GBA1Δ^{TT} homozygotes increased accumulation of ubiquitinated proteins and α-Syn aggregates. However, α-Syn expression does not enhance GBA1Δ^{TT} fly phenotypes (119). In addition to these two knockout dGBA1 fly lines, Maor et al. took advantage of two other fly lines, each of which has a minos insertion in dGBA1a and dGBA1b, respectively, to cause premature termination of dGBA1a and dGBA1b. By crossing these two lines, double heterozygous flies have been generated. This fly model exhibited about 30% decrease in GCase activity and has decreased TH immunoreactivity, shortened lifespan, and an age-dependent DA neurodegeneration (120, 121). Besides the knockouts of dGBA1, Suzuki et al. used transgenic RNAi flies to knock down dGBA1a and dGBA1b (122). dGBA1a-RNAi flies exhibited a bout 80–90% decrease in GCase activity while dGBA1b-RNAi flies only showed about 20% decrease. Thus, the study focused on dGBA1a-RNAi flies, which exacerbated the locomotor dysfunction, loss of DA neurons, retinal degeneration, and accumulation of proteinase K-resistant α-Syn in α-Syn-expressing flies (122).

Both knockdown and knockout of dGBA1 in fly have been consistently shown shortened lifespan, behavioral phenotypes and accumulation of α-Syn aggregates, despite of different phenotypes in DA neurodegeneration. These GBA fly models provide useful platforms for further study of GBA function in PD.

GBA Transgenic *Drosophila* Models

Heterozygous mutations L444P and N370S are the most common and thought to be dominant-negative mutations of GBA in PD. To investigate GBA functions in PD, transgenic *Drosophila* expressing human WT, N370S and L444P were generated (120, 121, 124). N370S and L444P transgenic flies exhibited significant decreased GCase activity by 82 and 75%, respectively, compared with GBA WT transgenic flies despite equivalent expression levels of GBA protein (124). N370S and L444P transgenic flies consistently showed shortened life span, a progressive climbing defect, increased level of ER stress and DA neurodegeneration (121, 123, 124). This suggests that those transgenic flies can recapitulate some PD signs.

GBA *Drosophila* Models Reveal GBA Functions in PD

Two major functions of GBA have been implicated in GBA *Drosophila* models. One is the function in ER stress and unfolded protein response (UPR) in the ER. Mutant GCase are recognized as misfolded proteins and undergo various degrees of ER associated degradation. The accumulation of midfolded molecules in the ER activate signaling events known as UPR (120). Immunostaining in GBA transgenic flies revealed that a significant amount of GCase colocalized with ER and N370S and L444P caused abnormal aggregates and swelling within the ER (124). To measure UPR activation, an ER stress reporter transgene Xbp1 was used. N370S and L444P GBA mutations induced significant higher Xbp1 level compared to WT GBA flies, suggesting of an increased level of ER stress (120, 124). Another function revealed by GBA fly models is in lysosomal-autophagic pathway. Using LysoTracker and LC3 as markers to monitor lysosomal and autophagic pathology, Kinghorn et al. demonstrated that enlarged and abnormal lysosomes and accumulated Atg8, the fly LC3 homolog, were present in dGBA knockout fly brains (118). The probable downstream effects of lysosomal-autophagic dysfunction could be the accumulation of p62, a marker for lysosomal-autophagic degradation, and polyubiquitinated proteins (118). While these studies provide significant phenotyping investigation on GBA functions, the detailed molecular mechanisms are still largely unknown. Using these GBA *Drosophila* models, further dissection of the molecules involved in these pathways is warranted.

GBA *Drosophila* Models as Platforms to Identify Potential Therapeutic Compounds

The clear evidence showed that mutant GCase causes increased ER stress and activated UPR in fly, therefore removal of mutant misfolded GCase by pharmacological chaperones from the ER should at least partially rescue the phenotype. Two chaperones, ambroxol and isofagomine, were previously used to increase amount and lysosomal activity of mutant GCase (126–129). Indeed, ambroxol and isofagomine reduced ER stress, and

reversed locomotor deficits in GBA mutant flies *in vivo* (121, 124). This suggests that removal of mutant misfolded GCase from the ER may alleviate PD symptoms. Small chaperones can cross the blood–brain barrier, bind to GCase and stabilize proper folding and ensure delivery to lysosomes. Thus, small chaperones may be applicable for GBA-associated PD.

MODELING VPS35-ASSOCIATED PD IN *Drosophila*

Mutations in the *VPS35* gene encoding a core subunit of a heteropentameric complex referred to the retromer have recently emerged as a new cause of late-onset, autosomal dominant familial PD (7, 8). The mutation, D620N, has so far been unambiguously identified to cause PD. The VPS35 protein functions as a core component of the retromer, a protein complex that associates with the endosome to facilitate recycling of transmembrane protein cargoes from both endosome-to-Golgi and endosome-to-plasma membrane transport (130). The retromer is a highly conserved multi-protein complex, the core of which consists of the subunits VPS35, VPS29, and VPS26. The only identifiable VPS35 homolog in *Drosophila* is encoded by CG5625.

VPS35 *Drosophila* Models

VPS35 Knockout/Knockdown *Drosophila* Models

Two lines of null mutation in *VPS35* were generated by either imprecise excision of a P-element inserted at the 5′ end of CG5625 (P[EPgy2]CG5625EY^{14200}), or by a deletion of nearly 2 kb, which removes the first three exons including the translation start site (*VPS35*^{MH20}) (**Table 4**). Both mutants die at late larval or pupal stages, indicating the essential function of VPS35. VPS35-null mutants and RNAi lines (the Vienna *Drosophila* RNAi Center) were consistently demonstrated to reduce Wingless secretion but not Hedgehog signaling by reducing the recycle of Wntless from endosomes to the trans-Golgi network (131–133). Loss of VPS35 inhibits scavenger receptor ligand endocytosis, causes signaling defects at the NMJ, and leads to over proliferation of blood cells in larvae, which suggests VPS35 has tumor suppressor properties (134). Mechanistically, the endocytic and signaling defects of VPS35 mutants maybe due to VPS35 negatively regulation of actin polymerization (134). As these studies were at early stages before mutations of VPS35 has been identified to associate with PD, the PD pathological phenotypes in VPS35 knockout or knockdown mutants were not investigated. In recent studies, knockdown of VPS35 in *Drosophila* induced the accumulation of the detergent-insoluble α-Syn in the brain and exacerbated locomotor deficits, compound eye disorganization, and interommatidial bristle loss in α-Syn transgenic flies (135). These findings indicate that the retromer may play a crucial role in α-Syn degradation. The loss of *Drosophila* VPS35 (dVPS35) affects synaptic vesicle recycling, DA synaptic release and sleep behavior (46). The manipulation of *Drosophila* LRRK2 dLRRK together with Rab5 and Rab11 improves the VPS35 synaptic phenotypes (46). Taken together, VPS35 knockout/knockdown *Drosophila* models mimic some pathogenesis of PD, indicating that these fly models could be useful platforms to study VPS35-associated PD.

VPS35 Transgenic *Drosophila* Models

Vacuolar protein sorting 35-linked PD is inherited as a dominant trait, which may imply that the mutation of VPS35 has a

TABLE 4 | *Drosophila* models of vacuolar protein sorting 35 (VPS35)-associated Parkinson's disease.

Genetic manipulations	References	VPS35 variants	Neurodegeneration	Motor activities/ life span	Cellular functions
Knockdown	(135)	dVPS35 siRNA in α-Syn transgenic fly	ND	Locomotor activity ↓	Accumulation of the detergent-insoluble α-Syn, cathepsin D activity ↓ Mild eye disorganization
Knockout	(46)	dVPS35$^{-/-}$	Tyrosine hydroxylase (TH) neurons: no changes	ND	Defects on synaptic vesicle recycling, dopaminergic synaptic release and sleep behavior associated with dopaminergic activity; genetic interaction with leucine-rich repeat kinase 2 and Rab5, Rab
	(136)	dVPS35$^{-/-}$	ND	Locomotor activity ↓	Synaptic overgrowth ↓
Transgenic	(137)	hVPS35-WT hL774M	TH neurons: no changes	No changes	Sensitive to rotenone: no changes
		hD620N hP316S	TH neurons ↓	Locomotor activity ↓ Life span ↓	Sensitive to rotenone ↑
	(136)	dVPS35-WT dD650N dR550W dL800M hVPS35-WT hD620N hR524W hL774M	No retinal degeneration	Locomotor activity: no changes Life span: no changes	D620N mutation confers a partial loss of function; VPS35 genetically interacts with Parkin

ND, not determined; ↑, increased; ↓, decreased.

gain-of-function toxicity (**Table 4**). One study demonstrated that VPS35 D620N transgenic flies led to late-onset loss of DA neurons, locomotor deficits, shortened lifespans, and increased sensitivity to a PD-linked environmental toxin, rotenone (137). However, Malik and colleagues did not find evidence of dominant toxicity from any variants including the pathogenic D620N mutation, even with aging. By a definitive test to determine whether transgene expression can rescue endogenous VPS35 mutant phenotypes, they concluded that the D620N mutation confers a partial loss of function (136). This notion is further supported by other studies in fly or mouse systems that VPS35 DN mutation acts as a dominant-negative function (47, 48, 138, 139).

VPS35 *Drosophila* Models Reveal VPS35 Functions in PD

To date, VPS35 *Drosophila* models have revealed three major functions of VPS35 in trafficking pathways in neuronal system. First, VPS35 regulates synaptic vesicle endocytosis through the endosomal pathway. Loss of VPS35 increases the number of synaptic boutons of the NMJ in larval motor neurons (46, 134). It has been demonstrated that VPS35 cooperates with LRRK2 to regulate synaptogenesis, synaptic dynamics and endocytosis, and synaptic vesicles regeneration through the Rab-mediated endocytic pathway (46). Importantly, it in turn regulates DA activity and survival, a key element of PD etiology (46). Second, VPS35 mediates endolysosomal and Golgi apparatus sorting. Wild-type VPS35, but not a familial PD-associated mutant form, can rescue LRRK2 led to endolysosomal and Golgi apparatus sorting defects (48). In addition, it has been reported by several groups that VPS35 functions in endosome-to-Golgi retrieval are required for Wingless secretion (131–133). However, whether this function is related to DA neurodegeneration is unknown. Third, VPS35 functions in lysosomal degradation pathway. VPS35 dysfunction impairs the maturation of a lysosome protease cathepsin D in regulating the proteolytic pathway that is important for α-Syn metabolism, and in turn exacerbates neurotoxicity and causes eye degeneration and motor disability (135). These findings indicate that VPS35 may play a crucial role in α-Syn degradation and might thereby contribute to the pathogenesis of the disease. While it remains unclear if these functions are causally for DA neurodegeneration caused by VPS35 PD mutant, these studies have provided important insights into cellular pathways that are perturbed by VPS35 mutations in neurons.

CONVERGENT MECHANISMS

Dissecting genetic interaction among PD genes will be crucial to establish convergent functional pathways of these genes or risk factors. *Drosophila* as a classic genetic model provides powerful tools to study genetic interactions between different genes. Genetic dissection revealed that LRRK2 interacts with other PD genes or risk factors such as Parkin, DJ-1, PINK1, VPS35, and RAB7L (31, 46–48) and implicated several potential functions. Genetic interaction between LRRK2 and VPS35 or Rab7L indicates LRRK2 function in retromer and lysosomal pathways (46–48). Genetic interaction between LRRK2 and Parkin or PINK1 indicates LRRK2 function in mitochondria dysfunction and also suggests that dominant PD genes may act *via* common pathways with the recessive PD genes (31). Furthermore, VPS35 genetically interacts with *Parkin* but interestingly not with *PINK1*(136). Notably, α-Syn, LRRK2, and PINK1 have recently been linked to Rab proteins (48, 92–98), and the manipulation of *Drosophila* LRRK2 dLRRK together with Rab5 and Rab11 improves the VPS35 synaptic phenotypes (46). All the studies are convergent to implicate an important emerging role for defects in trafficking pathways. The accumulation of altered proteins including α-Syn and damaged mitochondria ultimately might overwhelm the disposal mechanisms, in turn cause DA neurodegeneration.

CONCLUDING REMARKS

While the rodent models generally attack more attention and efforts on studying human disorders because of their high conservation of basal ganglia circuit with human, modeling PD in rodents using genetics has been viewed as difficult (23, 140). The rodent models of PD could not fulfill all the key features of PD (140). The reason that the rodents are "imperfect" for modeling PD might be compensatory mechanisms in the rodents, and/or incomplete penetrance of some PD gene mutations such as LRRK2 disease causing mutations in human, and/or the combination effects of non-cell-autonomous and cell-autonomous processes (23, 140).

To this caveat, *Drosophila* models have provided significant contributions to our understanding of the mechanisms of PD pathogenesis in a comparatively short time frame and cost effective mode. Overexpression of PD dominant traits (LRRK2 and α-Syn) or knockout of dominant-negative genes (GBA and VPS35) in fly has been consistently demonstrated to mimic the essential PD signs such as DA neurodegeneration and behavioral deficits. Based on these fly models, genetic modifiers and small molecular compounds have been rapidly identified. Moreover, the combination roles of the genetic and environmental factors such as oxidative stress have been explored in PD. The important functions of LRRK2 in trafficking and protein translation, the critical contribution of α-Syn aggregation and phosphorylation, were initially discovered in fly. The *Drosophila* models so far are one of the major model systems to study GBA function in PD. Thus, the use of *Drosophila* models opened tremendous opportunities to explore the basic function of disease causing genes and to model the disease pathogenesis.

However, *Drosophila* is a relatively simple model organism, far less complex brain circuit than humans. For example, *Drosophila* does not have α-Syn homolog and a true human LRRK2 homolog. α-Syn neuropathology in the form of LBs is the hallmark of PD pathogenesis. Whether α-Syn is required for developing PD models has been raised. In addition, *Drosophila* has limited cell death effectors. Some aspects of human diseases may not be evident in fly. Thus, validation of findings from *Drosophila* to mammalian systems, including rodent models, human postmortem tissue,

and human DA neuronal cultures, is warranted. Therefore, the strategy would be to identify the basic aspects of the underlying disease mechanisms using simple organism model systems such as *Drosophila* and further characterize and validate the findings in mammalian conditions.

AUTHOR CONTRIBUTIONS

YX and JY analyzed the literatures and wrote the manuscript.

FUNDING

This work was supported by grants from NIH/NIA K01-AG046366, the William N. & Bernice E. Bumpus Foundation Innovation Awards, the Kansas INBRE: P20 GM103418, American Parkinson Disease Association (APDA) Research Grant, Johnson Cancer Research Center Innovative Research Awards and Equipment Awards at Kansas State University, start-up fund and SUCCESS-FYI Intramural Grant from Kansas State University College of Veterinary Medicine.

REFERENCES

1. Lees AJ, Hardy J, Revesz T. Parkinson's disease. *Lancet* (2009) 373:2055–66. doi:10.1016/S0140-6736(09)60492-X
2. Savitt JM, Dawson VL, Dawson TM. Diagnosis and treatment of Parkinson disease: molecules to medicine. *J Clin Invest* (2006) 116:1744–54. doi:10.1172/JCI29178
3. Martin I, Dawson VL, Dawson TM. Recent advances in the genetics of Parkinson's disease. *Annu Rev Genomics Hum Genet* (2011) 12:301–25. doi:10.1146/annurev-genom-082410-101440
4. Chartier-Harlin MC, Dachsel JC, Vilarino-Guell C, Lincoln SJ, Lepretre F, Hulihan MM, et al. Translation initiator EIF4G1 mutations in familial Parkinson disease. *Am J Hum Genet* (2011) 89:398–406. doi:10.1016/j.ajhg.2011.08.009
5. Scholz SW, Jeon BS. GBA mutations and Parkinson disease: when genotype meets phenotype. *Neurology* (2015) 84:866–7. doi:10.1212/WNL.0000000000001321
6. Vilarino-Guell C, Rajput A, Milnerwood AJ, Shah B, Szu-Tu C, Trinh J, et al. DNAJC13 mutations in Parkinson disease. *Hum Mol Genet* (2014) 23:1794–801. doi:10.1093/hmg/ddt570
7. Vilarino-Guell C, Wider C, Ross OA, Dachsel JC, Kachergus JM, Lincoln SJ, et al. VPS35 mutations in Parkinson disease. *Am J Hum Genet* (2011) 89:162–7. doi:10.1016/j.ajhg.2011.06.001
8. Zimprich A, Benet-Pages A, Struhal W, Graf E, Eck SH, Offman MN, et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. *Am J Hum Genet* (2011) 89:168–75. doi:10.1016/j.ajhg.2011.06.008
9. Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* (2004) 44:595–600. doi:10.1016/j.neuron.2004.10.023
10. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* (2004) 44:601–7. doi:10.1016/j.neuron.2004.11.005
11. Cookson MR. The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. *Nat Rev Neurosci* (2010) 11:791–7. doi:10.1038/nrn2935
12. Mata IF, Wedemeyer WJ, Farrer MJ, Taylor JP, Gallo KA. LRRK2 in Parkinson's disease: protein domains and functional insights. *Trends Neurosci* (2006) 29:286–93. doi:10.1016/j.tins.2006.03.006
13. Xiong Y, Dawson VL, Dawson TM. LRRK2 GTPase dysfunction in the pathogenesis of Parkinson's disease. *Biochem Soc Trans* (2012) 40:1074–9. doi:10.1042/BST20120093
14. Healy DG, Falchi M, O'Sullivan SS, Bonifati V, Durr A, Bressman S, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *Lancet Neurol* (2008) 7:583–90. doi:10.1016/S1474-4422(08)70117-0
15. Benamer HT, de Silva R. LRRK2 G2019S in the north African population: a review. *Eur Neurol* (2010) 63:321–5. doi:10.1159/000279653
16. Lesage S, Patin E, Condroyer C, Leutenegger AL, Lohmann E, Giladi N, et al. Parkinson's disease-related LRRK2 G2019S mutation results from independent mutational events in humans. *Hum Mol Genet* (2010) 19:1998–2004. doi:10.1093/hmg/ddq081
17. Berwick DC, Harvey K. LRRK2 signaling pathways: the key to unlocking neurodegeneration? *Trends Cell Biol* (2011) 21(5):257–65. doi:10.1016/j.tcb.2011.01.001
18. Islam MS, Moore DJ. Mechanisms of LRRK2-dependent neurodegeneration: role of enzymatic activity and protein aggregation. *Biochem Soc Trans* (2017) 45:163–72. doi:10.1042/BST20160264
19. Cookson MR. LRRK2 pathways leading to neurodegeneration. *Curr Neurol Neurosci Rep* (2015) 15:42. doi:10.1007/s11910-015-0564-y
20. Martin I, Kim JW, Dawson VL, Dawson TM. LRRK2 pathobiology in Parkinson's disease. *J Neurochem* (2014) 131:554–65. doi:10.1111/jnc.12949
21. Nikonova EV, Xiong Y, Tanis KQ, Dawson VL, Vogel RL, Finney EM, et al. Transcriptional responses to loss or gain of function of the leucine-rich repeat kinase 2 (LRRK2) gene uncover biological processes modulated by LRRK2 activity. *Hum Mol Genet* (2012) 21:163–74. doi:10.1093/hmg/ddr451
22. Xiong Y, Coombes CE, Kilaru A, Li X, Gitler AD, Bowers WJ, et al. GTPase activity plays a key role in the pathobiology of LRRK2. *PLoS Genet* (2010) 6:e1000902. doi:10.1371/journal.pgen.1000902
23. Xiong Y, Dawson TM, Dawson VL. Models of LRRK2-associated Parkinson's disease. *Adv Neurobiol* (2017) 14:163–91. doi:10.1007/978-3-319-49969-7_9
24. Xiong Y, Neifert S, Karuppagounder SS, Liu Q, Stankowski JN, Lee BD, et al. Robust kinase- and age-dependent dopaminergic and norepinephrine neurodegeneration in LRRK2 G2019S transgenic mice. *Proc Natl Acad Sci U S A* (2018) 115(7):1635–40. doi:10.1073/pnas.1712648115
25. Xiong Y, Neifert S, Karuppagounder SS, Stankowski JN, Lee BD, Grima JC, et al. Overexpression of Parkinson's disease-associated mutation LRRK2 G2019S in mouse forebrain induces behavioral deficits and alpha-synuclein pathology. *eNeuro* (2017) 4(2). doi:10.1523/ENEURO.0004-17.2017
26. Lee SB, Kim W, Lee S, Chung J. Loss of LRRK2/PARK8 induces degeneration of dopaminergic neurons in *Drosophila*. *Biochem Biophys Res Commun* (2007) 358:534–9. doi:10.1016/j.bbrc.2007.04.156
27. Wang D, Tang B, Zhao G, Pan Q, Xia K, Bodmer R, et al. Dispensable role of *Drosophila* ortholog of LRRK2 kinase activity in survival of dopaminergic neurons. *Mol Neurodegener* (2008) 3:3. doi:10.1186/1750-1326-3-3
28. Imai Y, Gehrke S, Wang HQ, Takahashi R, Hasegawa K, Oota E, et al. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in *Drosophila*. *EMBO J* (2008) 27:2432–43. doi:10.1038/emboj.2008.163
29. Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, Whitworth AJ. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat Neurosci* (2009) 12:1129–35. doi:10.1038/nn.2372
30. Liu Z, Wang X, Yu Y, Li X, Wang T, Jiang H, et al. A *Drosophila* model for LRRK2-linked parkinsonism. *Proc Natl Acad Sci U S A* (2008) 105:2693–8. doi:10.1073/pnas.0708452105
31. Venderova K, Kabbach G, Abdel-Messih E, Zhang Y, Parks RJ, Imai Y, et al. Leucine-rich repeat kinase 2 interacts with Parkin, DJ-1 and PINK-1 in a *Drosophila melanogaster* model of Parkinson's disease. *Hum Mol Genet* (2009) 18:4390–404. doi:10.1093/hmg/ddp394
32. Ng CH, Mok SZ, Koh C, Ouyang X, Fivaz ML, Tan EK, et al. Parkin protects against LRRK2 G2019S mutant-induced dopaminergic neurodegeneration in *Drosophila*. *J Neurosci* (2009) 29:11257–62. doi:10.1523/JNEUROSCI.2375-09.2009
33. Lin CH, Tsai PI, Wu RM, Chien CT. LRRK2 G2019S mutation induces dendrite degeneration through mislocalization and phosphorylation of tau by recruiting autoactivated GSK3ss. *J Neurosci* (2010) 30:13138–49. doi:10.1523/JNEUROSCI.1737-10.2010
34. Hindle S, Afsari F, Stark M, Middleton CA, Evans GJ, Sweeney ST, et al. Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to

non-autonomous visual neurodegeneration, accelerated by increased neural demands for energy. *Hum Mol Genet* (2013) 22:2129–40. doi:10.1093/hmg/ddt061
35. Godena VK, Brookes-Hocking N, Moller A, Shaw G, Oswald M, Sancho RM, et al. Increasing microtubule acetylation rescues axonal transport and locomotor deficits caused by LRRK2 Roc-COR domain mutations. *Nat Commun* (2014) 5:5245. doi:10.1038/ncomms6245
36. Cording AC, Shiaelis N, Petridi S, Middleton CA, Wilson LG, Elliott CJH. Targeted kinase inhibition relieves slowness and tremor in a *Drosophila* model of LRRK2 Parkinson's disease. *NPJ Parkinsons Dis* (2017) 3:34. doi:10.1038/s41531-017-0036-y
37. Islam MS, Nolte H, Jacob W, Ziegler AB, Putz S, Grosjean Y, et al. Human R1441C LRRK2 regulates the synaptic vesicle proteome and phosphoproteome in a *Drosophila* model of Parkinson's disease. *Hum Mol Genet* (2016) 25:5365–82. doi:10.1093/hmg/ddw352
38. Abeliovich A, Gitler AD. Defects in trafficking bridge Parkinson's disease pathology and genetics. *Nature* (2016) 539:207–16. doi:10.1038/nature20414
39. Hewitt VL, Whitworth AJ. Mechanisms of Parkinson's disease: lessons from *Drosophila*. *Curr Top Dev Biol* (2017) 121:173–200. doi:10.1016/bs.ctdb.2016.07.005
40. Dodson MW, Zhang T, Jiang C, Chen S, Guo M. Roles of the *Drosophila* LRRK2 homolog in Rab7-dependent lysosomal positioning. *Hum Mol Genet* (2012) 21:1350–63. doi:10.1093/hmg/ddr573
41. Dodson MW, Leung LK, Lone M, Lizzio MA, Guo M. Novel ethyl methanesulfonate (EMS)-induced null alleles of the *Drosophila* homolog of LRRK2 reveal a crucial role in endolysosomal functions and autophagy in vivo. *Dis Model Mech* (2014) 7:1351–63. doi:10.1242/dmm.017020
42. Schreij AM, Chaineau M, Ruan W, Lin S, Barker PA, Fon EA, et al. LRRK2 localizes to endosomes and interacts with clathrin-light chains to limit Rac1 activation. *EMBO Rep* (2015) 16:79–86. doi:10.15252/embr.201438714
43. Matta S, Van Kolen K, da Cunha R, van den Bogaart G, Mandemakers W, Miskiewicz K, et al. LRRK2 controls an EndoA phosphorylation cycle in synaptic endocytosis. *Neuron* (2012) 75:1008–21. doi:10.1016/j.neuron.2012.08.022
44. Soukup SF, Kuenen S, Vanhauwaert R, Manetsberger J, Hernandez-Diaz S, Swerts J, et al. A LRRK2-dependent endophilinA phosphoswitch is critical for macroautophagy at presynaptic terminals. *Neuron* (2016) 92:829–44. doi:10.1016/j.neuron.2016.09.037
45. Soukup SF, Verstreken P. EndoA/Endophilin-A creates docking stations for autophagic proteins at synapses. *Autophagy* (2017) 13:971–2. doi:10.1080/15548627.2017.1286440
46. Inoshita T, Arano T, Hosaka Y, Meng H, Umezaki Y, Kosugi S, et al. Vps35 in cooperation with LRRK2 regulates synaptic vesicle endocytosis through the endosomal pathway in *Drosophila*. *Hum Mol Genet* (2017) 26:2933–48. doi:10.1093/hmg/ddx179
47. Linhart R, Wong SA, Cao J, Tran M, Huynh A, Ardrey C, et al. Vacuolar protein sorting 35 (Vps35) rescues locomotor deficits and shortened lifespan in *Drosophila* expressing a Parkinson's disease mutant of leucine-rich repeat kinase 2 (LRRK2). *Mol Neurodegener* (2014) 9:23. doi:10.1186/1750-1326-9-23
48. MacLeod DA, Rhinn H, Kuwahara T, Zolin A, Di Paolo G, McCabe BD, et al. RAB7L1 interacts with LRRK2 to modify intraneuronal protein sorting and Parkinson's disease risk. *Neuron* (2013) 77:425–39. doi:10.1016/j.neuron.2012.11.033
49. Xiong Y, Yuan C, Chen R, Dawson TM, Dawson VL. ArfGAP1 is a GTPase activating protein for LRRK2: reciprocal regulation of ArfGAP1 by LRRK2. *J Neurosci* (2012) 32:3877–86. doi:10.1523/JNEUROSCI.4566-11.2012
50. Lin CH, Li H, Lee YN, Cheng YJ, Wu RM, Chien CT. Lrrk regulates the dynamic profile of dendritic Golgi outposts through the golgin Lava lamp. *J Cell Biol* (2015) 210(3):471–83. doi:10.1083/jcb.201411033
51. Gehrke S, Imai Y, Sokol N, Lu B. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature* (2010) 466:637–41. doi:10.1038/nature09191
52. Martin I, Kim JW, Lee BD, Kang HC, Xu JC, Jia H, et al. Ribosomal protein s15 phosphorylation mediates LRRK2 neurodegeneration in Parkinson's disease. *Cell* (2014) 157:472–85. doi:10.1016/j.cell.2014.01.064
53. Penney J, Tsurudome K, Liao EH, Kauwe G, Gray L, Yanagiya A, et al. LRRK2 regulates retrograde synaptic compensation at the *Drosophila* neuromuscular junction. *Nat Commun* (2016) 7:12188. doi:10.1038/ncomms12188
54. Lee S, Liu HP, Lin WY, Guo H, Lu B. LRRK2 kinase regulates synaptic morphology through distinct substrates at the presynaptic and postsynaptic compartments of the *Drosophila* neuromuscular junction. *J Neurosci* (2010) 30:16959–69. doi:10.1523/JNEUROSCI.1807-10.2010
55. Liu Z, Hamamichi S, Lee BD, Yang D, Ray A, Caldwell GA, et al. Inhibitors of LRRK2 kinase attenuate neurodegeneration and Parkinson-like phenotypes in *Caenorhabditis elegans* and *Drosophila* Parkinson's disease models. *Hum Mol Genet* (2011) 20:3933–42. doi:10.1093/hmg/ddr312
56. Yang D, Li T, Liu Z, Arbez N, Yan J, Moran TH, et al. LRRK2 kinase activity mediates toxic interactions between genetic mutation and oxidative stress in a *Drosophila* model: suppression by curcumin. *Neurobiol Dis* (2012) 47:385–92. doi:10.1016/j.nbd.2012.05.020
57. Sun X, Ran D, Zhao X, Huang Y, Long S, Liang F, et al. Melatonin attenuates hLRRK2-induced sleep disturbances and synaptic dysfunction in a *Drosophila* model of Parkinson's disease. *Mol Med Rep* (2016) 13:3936–44. doi:10.3892/mmr.2016.4991
58. Lin CH, Lin HI, Chen ML, Lai TT, Cao LP, Farrer MJ, et al. Lovastatin protects neurite degeneration in LRRK2-G2019S parkinsonism through activating the Akt/Nrf pathway and inhibiting GSK3beta activity. *Hum Mol Genet* (2016) 25(10):1965–78. doi:10.1093/hmg/ddw068
59. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* (1997) 276:2045–7. doi:10.1126/science.276.5321.2045
60. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* (1997) 388:839–40. doi:10.1038/42166
61. Koprich JB, Kalia LV, Brotchie JM. Animal models of alpha-synucleinopathy for Parkinson disease drug development. *Nat Rev Neurosci* (2017) 18:515–29. doi:10.1038/nrn.2017.75
62. Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat Genet* (2014) 46:989–93. doi:10.1038/ng.3043
63. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet* (2009) 41:1308–12. doi:10.1038/ng.487
64. Bendor JT, Logan TP, Edwards RH. The function of alpha-synuclein. *Neuron* (2013) 79:1044–66. doi:10.1016/j.neuron.2013.09.004
65. Wong YC, Krainc D. alpha-Synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. *Nat Med* (2017) 23:1–13. doi:10.1038/nm.4269
66. Bengoa-Vergniory N, Roberts RF, Wade-Martins R, Alegre-Abarrategui J. Alpha-synuclein oligomers: a new hope. *Acta Neuropathol* (2017) 134:819–38. doi:10.1007/s00401-017-1755-1
67. Brundin P, Melki R. Prying into the prion hypothesis for Parkinson's disease. *J Neurosci* (2017) 37:9808–18. doi:10.1523/JNEUROSCI.1788-16.2017
68. Surmeier DJ, Obeso JA, Halliday GM. Parkinson's disease is not simply a prion disorder. *J Neurosci* (2017) 37:9799–807. doi:10.1523/JNEUROSCI.1787-16.2017
69. Mizuno H, Fujikake N, Wada K, Nagai Y. alpha-Synuclein transgenic *Drosophila* as a model of Parkinson's disease and related synucleinopathies. *Parkinsons Dis* (2010) 2011:212706. doi:10.4061/2011/212706
70. Feany MB, Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* (2000) 404:394–8. doi:10.1038/35006074
71. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* (2002) 295:865–8. doi:10.1126/science.1067389
72. Takahashi M, Kanuka H, Fujiwara H, Koyama A, Hasegawa M, Miura M, et al. Phosphorylation of alpha-synuclein characteristic of synucleinopathy lesions is recapitulated in alpha-synuclein transgenic *Drosophila*. *Neurosci Lett* (2003) 336:155–8. doi:10.1016/S0304-3940(02)01258-2
73. Pesah Y, Burgess H, Middlebrooks B, Ronningen K, Prosser J, Tirunagaru V, et al. Whole-mount analysis reveals normal numbers of dopaminergic neurons following misexpression of alpha-synuclein in *Drosophila*. *Genesis* (2005) 41:154–9. doi:10.1002/gene.20106
74. Chen L, Feany MB. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a *Drosophila* model of Parkinson disease. *Nat Neurosci* (2005) 8:657–63. doi:10.1038/nn1443

75. Periquet M, Fulga T, Myllykangas L, Schlossmacher MG, Feany MB. Aggregated alpha-synuclein mediates dopaminergic neurotoxicity in vivo. *J Neurosci* (2007) 27:3338–46. doi:10.1523/JNEUROSCI.0285-07.2007
76. Karpinar DP, Balija MB, Kugler S, Opazo F, Rezaei-Ghaleh N, Wender N, et al. Pre-fibrillar alpha-synuclein variants with impaired beta-structure increase neurotoxicity in Parkinson's disease models. *EMBO J* (2009) 28: 3256–68. doi:10.1038/emboj.2009.257
77. Chen L, Periquet M, Wang X, Negro A, McLean PJ, Hyman BT, et al. Tyrosine and serine phosphorylation of alpha-synuclein have opposing effects on neurotoxicity and soluble oligomer formation. *J Clin Invest* (2009) 119:3257–65. doi:10.1172/JCI39088
78. Gajula Balija MB, Griesinger C, Herzig A, Zweckstetter M, Jackle H. Pre-fibrillar alpha-synuclein mutants cause Parkinson's disease-like non-motor symptoms in *Drosophila*. *PLoS One* (2011) 6:e24701. doi:10.1371/journal.pone.0024701
79. Chen AY, Wilburn P, Hao X, Tully T. Walking deficits and centrophobism in an alpha-synuclein fly model of Parkinson's disease. *Genes Brain Behav* (2014) 13:812–20. doi:10.1111/gbb.12172
80. Chen AY, Xia S, Wilburn P, Tully T. Olfactory deficits in an alpha-synuclein fly model of Parkinson's disease. *PLoS One* (2014) 9:e97758. doi:10.1371/journal.pone.0097758
81. Ordonez DG, Lee MK, Feany MB. alpha-Synuclein induces mitochondrial dysfunction through spectrin and the actin cytoskeleton. *Neuron* (2018) 97(1):108–124.e6. doi:10.1016/j.neuron.2017.11.036
82. Whitworth AJ, Wes PD, Pallanck LJ. *Drosophila* models pioneer a new approach to drug discovery for Parkinson's disease. *Drug Discov Today* (2006) 11:119–26. doi:10.1016/S1359-6446(05)03693-7
83. Dufty BM, Warner LR, Hou ST, Jiang SX, Gomez-Isla T, Leenhouts KM, et al. Calpain-cleavage of alpha-synuclein: connecting proteolytic processing to disease-linked aggregation. *Am J Pathol* (2007) 170:1725–38. doi:10.2353/ajpath.2007.061232
84. Du G, Liu X, Chen X, Song M, Yan Y, Jiao R, et al. *Drosophila* histone deacetylase 6 protects dopaminergic neurons against {alpha}-synuclein toxicity by promoting inclusion formation. *Mol Biol Cell* (2010) 21:2128–37. doi:10.1091/mbc.E10-03-0200
85. Butler EK, Voigt A, Lutz AK, Toegel JP, Gerhardt E, Karsten P, et al. The mitochondrial chaperone protein TRAP1 mitigates alpha-synuclein toxicity. *PLoS Genet* (2012) 8:e1002488. doi:10.1371/journal.pgen.1002488
86. Lee FK, Wong AK, Lee YW, Wan OW, Chan HY, Chung KK. The role of ubiquitin linkages on alpha-synuclein induced-toxicity in a *Drosophila* model of Parkinson's disease. *J Neurochem* (2009) 110:208–19. doi:10.1111/j.1471-4159.2009.06124.x
87. Davies SE, Hallett PJ, Moens T, Smith G, Mangano E, Kim HT, et al. Enhanced ubiquitin-dependent degradation by Nedd4 protects against alpha-synuclein accumulation and toxicity in animal models of Parkinson's disease. *Neurobiol Dis* (2014) 64:79–87. doi:10.1016/j.nbd.2013.12.011
88. Alexopoulou Z, Lang J, Perrett RM, Elschami M, Hurry ME, Kim HT, et al. Deubiquitinase Usp8 regulates alpha-synuclein clearance and modifies its toxicity in Lewy body disease. *Proc Natl Acad Sci U S A* (2016) 113:E4688–97. doi:10.1073/pnas.1523597113
89. Cullen V, Lindfors M, Ng J, Paetau A, Swinton E, Kolodziej P, et al. Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo. *Mol Brain* (2009) 2:5. doi:10.1186/1756-6606-2-5
90. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* (2002) 4:160–4. doi:10.1038/ncb748
91. Saito Y, Kawashima A, Ruberu NN, Fujiwara H, Koyama S, Sawabe M, et al. Accumulation of phosphorylated alpha-synuclein in aging human brain. *J Neuropathol Exp Neurol* (2003) 62:644–54. doi:10.1093/jnen/62.6.644
92. Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, et al. Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. *Science* (2006) 313:324–8. doi:10.1126/science.1129462
93. Breda C, Nugent ML, Estranero JG, Kyriacou CP, Outeiro TF, Steinert JR, et al. Rab11 modulates alpha-synuclein-mediated defects in synaptic transmission and behaviour. *Hum Mol Genet* (2015) 24:1077–91. doi:10.1093/hmg/ddu521
94. Dinter E, Saridaki T, Nippold M, Plum S, Diederichs L, Komnig D, et al. Rab7 induces clearance of alpha-synuclein aggregates. *J Neurochem* (2016) 138:758–74. doi:10.1111/jnc.13712
95. Yin G, Lopes da Fonseca T, Eisbach SE, Anduaga AM, Breda C, Orcellet ML, et al. alpha-Synuclein interacts with the switch region of Rab8a in a Ser129 phosphorylation-dependent manner. *Neurobiol Dis* (2014) 70:149–61. doi:10.1016/j.nbd.2014.06.018
96. Lai YC, Kondapalli C, Lehneck R, Procter JB, Dill BD, Woodroof HI, et al. Phosphoproteomic screening identifies Rab GTPases as novel downstream targets of PINK1. *EMBO J* (2015) 34:2840–61. doi:10.15252/embj.201591593
97. Purlyte E, Dhekne HS, Sarhan AR, Gomez R, Lis P, Wightman M, et al. Rab29 activation of the Parkinson's disease-associated LRRK2 kinase. *EMBO J* (2018) 37(1):1–18. doi:10.15252/embj.201798099
98. Steger M, Tonelli F, Ito G, Davies P, Trost M, Vetter M, et al. Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases. *Elife* (2016) 5:e12813. doi:10.7554/eLife.12813
99. Botella JA, Bayersdorfer F, Schneuwly S. Superoxide dismutase overexpression protects dopaminergic neurons in a *Drosophila* model of Parkinson's disease. *Neurobiol Dis* (2008) 30:65–73. doi:10.1016/j.nbd.2007.11.013
100. Bayersdorfer F, Voigt A, Schneuwly S, Botella JA. Dopamine-dependent neurodegeneration in *Drosophila* models of familial and sporadic Parkinson's disease. *Neurobiol Dis* (2010) 40:113–9. doi:10.1016/j.nbd.2010.02.012
101. Trinh K, Moore K, Wes PD, Muchowski PJ, Dey J, Andrews L, et al. Induction of the phase II detoxification pathway suppresses neuron loss in *Drosophila* models of Parkinson's disease. *J Neurosci* (2008) 28:465–72. doi:10.1523/JNEUROSCI.4778-07.2008
102. Jahromi SR, Haddadi M, Shivanandappa T, Ramesh SR. Attenuation of neuromotor deficits by natural antioxidants of *Decalepis hamiltonii* in transgenic *Drosophila* model of Parkinson's disease. *Neuroscience* (2015) 293:136–50. doi:10.1016/j.neuroscience.2015.02.048
103. Knight AL, Yan X, Hamamichi S, Ajjuri RR, Mazzulli JR, Zhang MW, et al. The glycolytic enzyme, GPI, is a functionally conserved modifier of dopaminergic neurodegeneration in Parkinson's models. *Cell Metab* (2014) 20:145–57. doi:10.1016/j.cmet.2014.04.017
104. Auluck PK, Meulener MC, Bonini NM. Mechanisms of suppression of {alpha}-synuclein neurotoxicity by geldanamycin in *Drosophila*. *J Biol Chem* (2005) 280:2873–8. doi:10.1074/jbc.M412106200
105. Jia H, Li X, Gao H, Feng Z, Li X, Zhao L, et al. High doses of nicotinamide prevent oxidative mitochondrial dysfunction in a cellular model and improve motor deficit in a *Drosophila* model of Parkinson's disease. *J Neurosci Res* (2008) 86:2083–90. doi:10.1002/jnr.21650
106. Pendleton RG, Parvez F, Sayed M, Hillman R. Effects of pharmacological agents upon a transgenic model of Parkinson's disease in *Drosophila melanogaster*. *J Pharmacol Exp Ther* (2002) 300:91–6. doi:10.1124/jpet.300.1.91
107. Yedlapudi D, Joshi GS, Luo D, Todi SV, Dutta AK. Inhibition of alpha-synuclein aggregation by multifunctional dopamine agonists assessed by a novel in vitro assay and an in vivo *Drosophila* synucleinopathy model. *Sci Rep* (2016) 6:38510. doi:10.1038/srep38510
108. Das B, Rajagopalan S, Joshi GS, Xu L, Luo D, Andersen JK, et al. A novel iron (II) preferring dopamine agonist chelator D-607 significantly suppresses alpha-syn- and MPTP-induced toxicities in vivo. *Neuropharmacology* (2017) 123:88–99. doi:10.1016/j.neuropharm.2017.05.019
109. Kontopoulos E, Parvin JD, Feany MB. Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Hum Mol Genet* (2006) 15:3012–23. doi:10.1093/hmg/ddl243
110. Outeiro TF, Kontopoulos E, Altmann SM, Kufareva I, Strathearn KE, Amore AM, et al. Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* (2007) 317:516–9. doi:10.1126/science.1143780
111. Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med* (2009) 361:1651–61. doi:10.1056/NEJMoa0901281
112. Aflaki E, Westbroek W, Sidransky E. The complicated relationship between Gaucher disease and parkinsonism: insights from a rare disease. *Neuron* (2017) 93:737–46. doi:10.1016/j.neuron.2017.01.018
113. Erickson AH, Ginns EI, Barranger JA. Biosynthesis of the lysosomal enzyme glucocerebrosidase. *J Biol Chem* (1985) 260:14319–24.
114. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. *Lancet* (2008) 372:1263–71. doi:10.1016/S0140-6736(08)61522-6

115. Barkhuizen M, Anderson DG, Grobler AF. Advances in GBA-associated Parkinson's disease – pathology, presentation and therapies. *Neurochem Int* (2016) 93:6–25. doi:10.1016/j.neuint.2015.12.004
116. Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, et al. Gaucher disease glucocerebrosidase and alpha-synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell* (2011) 146:37–52. doi:10.1016/j.cell.2011.06.001
117. Siebert M, Sidransky E, Westbroek W. Glucocerebrosidase is shaking up the synucleinopathies. *Brain* (2014) 137:1304–22. doi:10.1093/brain/awu002
118. Kinghorn KJ, Gronke S, Castillo-Quan JI, Woodling NS, Li L, Sirka E, et al. A *Drosophila* model of neuronopathic Gaucher disease demonstrates lysosomal-autophagic defects and altered mTOR signalling and is functionally rescued by rapamycin. *J Neurosci* (2016) 36:11654–70. doi:10.1523/JNEUROSCI.4527-15.2016
119. Davis MY, Trinh K, Thomas RE, Yu S, Germanos AA, Whitley BN, et al. Glucocerebrosidase deficiency in *Drosophila* results in alpha-synuclein-independent protein aggregation and neurodegeneration. *PLoS Genet* (2016) 12:e1005944. doi:10.1371/journal.pgen.1005944
120. Maor G, Rencus-Lazar S, Filocamo M, Steller H, Segal D, Horowitz M. Unfolded protein response in Gaucher disease: from human to *Drosophila*. *Orphanet J Rare Dis* (2013) 8:140. doi:10.1186/1750-1172-8-140
121. Maor G, Cabasso O, Krivoruk O, Rodriguez J, Steller H, Segal D, et al. The contribution of mutant GBA to the development of Parkinson disease in *Drosophila*. *Hum Mol Genet* (2016) 25:2712–27. doi:10.1093/hmg/ddw129
122. Suzuki M, Fujikake N, Takeuchi T, Kohyama-Koganeya A, Nakajima K, Hirabayashi Y, et al. Glucocerebrosidase deficiency accelerates the accumulation of proteinase K-resistant alpha-synuclein and aggravates neurodegeneration in a *Drosophila* model of Parkinson's disease. *Hum Mol Genet* (2015) 24:6675–86. doi:10.1093/hmg/ddv372
123. Suzuki T, Shimoda M, Ito K, Hanai S, Aizawa H, Kato T, et al. Expression of human Gaucher disease gene GBA generates neurodevelopmental defects and ER stress in *Drosophila* eye. *PLoS One* (2013) 8:e69147. doi:10.1371/journal.pone.0069147
124. Sanchez-Martinez A, Beavan M, Gegg ME, Chau KY, Whitworth AJ, Schapira AH. Parkinson disease-linked GBA mutation effects reversed by molecular chaperones in human cell and fly models. *Sci Rep* (2016) 6:31380. doi:10.1038/srep31380
125. Robinson SW, Herzyk P, Dow JA, Leader DP. FlyAtlas: database of gene expression in the tissues of *Drosophila melanogaster*. *Nucleic Acids Res* (2013) 41:D744–50. doi:10.1093/nar/gks1141
126. Ambrosi G, Ghezzi C, Zangaglia R, Levandis G, Pacchetti C, Blandini F. Ambroxol-induced rescue of defective glucocerebrosidase is associated with increased LIMP-2 and saposin C levels in GBA1 mutant Parkinson's disease cells. *Neurobiol Dis* (2015) 82:235–42. doi:10.1016/j.nbd.2015.06.008
127. Bendikov-Bar I, Maor G, Filocamo M, Horowitz M. Ambroxol as a pharmacological chaperone for mutant glucocerebrosidase. *Blood Cells Mol Dis* (2013) 50:141–5. doi:10.1016/j.bcmd.2012.10.007
128. Migdalska-Richards A, Daly L, Bezard E, Schapira AH. Ambroxol effects in glucocerebrosidase and alpha-synuclein transgenic mice. *Ann Neurol* (2016) 80:766–75. doi:10.1002/ana.24790
129. Narita A, Shirai K, Itamura S, Matsuda A, Ishihara A, Matsushita K, et al. Ambroxol chaperone therapy for neuronopathic Gaucher disease: a pilot study. *Ann Clin Transl Neurol* (2016) 3:200–15. doi:10.1002/acn3.292
130. Williams ET, Chen X, Moore DJ. VPS35, the retromer complex and Parkinson's disease. *J Parkinsons Dis* (2017) 7:219–33. doi:10.3233/JPD-161020
131. Belenkaya TY, Wu Y, Tang X, Zhou B, Cheng L, Sharma YV, et al. The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. *Dev Cell* (2008) 14:120–31. doi:10.1016/j.devcel.2007.12.003
132. Franch-Marro X, Wendler F, Guidato S, Griffith J, Baena-Lopez A, Itasaki N, et al. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nat Cell Biol* (2008) 10:170–7. doi:10.1038/ncb1678
133. Port F, Kuster M, Herr P, Furger E, Banziger C, Hausmann G, et al. Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nat Cell Biol* (2008) 10:178–85. doi:10.1038/ncb1687
134. Korolchuk VI, Schutz MM, Gomez-Llorente C, Rocha J, Lansu NR, Collins SM, et al. *Drosophila* Vps35 function is necessary for normal endocytic trafficking and actin cytoskeleton organisation. *J Cell Sci* (2007) 120:4367–76. doi:10.1242/jcs.012336
135. Miura E, Hasegawa T, Konno M, Suzuki M, Sugeno N, Fujikake N, et al. VPS35 dysfunction impairs lysosomal degradation of alpha-synuclein and exacerbates neurotoxicity in a *Drosophila* model of Parkinson's disease. *Neurobiol Dis* (2014) 71:1–13. doi:10.1016/j.nbd.2014.07.014
136. Malik BR, Godena VK, Whitworth AJ. VPS35 pathogenic mutations confer no dominant toxicity but partial loss of function in *Drosophila* and genetically interact with parkin. *Hum Mol Genet* (2015) 24:6106–17. doi:10.1093/hmg/ddv322
137. Wang HS, Toh J, Ho P, Tio M, Zhao Y, Tan EK. In vivo evidence of pathogenicity of VPS35 mutations in the *Drosophila*. *Mol Brain* (2014) 7:73. doi:10.1186/s13041-014-0073-y
138. Ishizu N, Yui D, Hebisawa A, Aizawa H, Cui W, Fujita Y, et al. Impaired striatal dopamine release in homozygous Vps35 D620N knock-in mice. *Hum Mol Genet* (2016) 25:4507–17. doi:10.1093/hmg/ddw279
139. Munsie LN, Milnerwood AJ, Seibler P, Beccano-Kelly DA, Tatarnikov I, Khinda J, et al. Retromer-dependent neurotransmitter receptor trafficking to synapses is altered by the Parkinson's disease VPS35 mutation p.D620N. *Hum Mol Genet* (2015) 24:1691–703. doi:10.1093/hmg/ddu582
140. Dawson TM, Ko HS, Dawson VL. Genetic animal models of Parkinson's disease. *Neuron* (2010) 66:646–61. doi:10.1016/j.neuron.2010.04.034

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.

frontiers in Neurology

REVIEW
published: 04 May 2018
doi: 10.3389/fneur.2018.00295

Alpha-Synuclein: From Early Synaptic Dysfunction to Neurodegeneration

Veronica Ghiglieri[1,2], *Valeria Calabrese*[2] *and Paolo Calabresi*[2,3]*

[1] *Dipartimento di Filosofia, Scienze Sociali, Umane e della Formazione, Università degli Studi di Perugia, Perugia, Italy,* [2] *Laboratorio di Neurofisiologia, Fondazione Santa Lucia, IRCCS, Rome, Italy,* [3] *Clinica Neurologica, Dipartimento di Medicina, Università degli Studi di Perugia, Ospedale Santa Maria della Misericordia di Perugia, Perugia, Italy*

Over the last two decades, many experimental and clinical studies have provided solid evidence that alpha-synuclein (α-syn), a small, natively unfolded protein, is closely related to Parkinson's disease (PD) pathology. To provide an overview on the different roles of this protein, here we propose a synopsis of seminal and recent studies that explored the many aspects of α-syn. Ranging from the physiological functions to its neurodegenerative potential, the relationship with the possible pathogenesis of PD will be discussed. Close attention will be paid on early cellular and molecular alterations associated with the presence of α-syn aggregates.

Keywords: synucleinopathy, experimental parkinsonism, neurodegeneration, synaptic plasticity, protein aggregation

OPEN ACCESS

Edited by:
Graziella Madeo,
National Institutes of Health (NIH), United States

Reviewed by:
Marina Pizzi,
University of Brescia, Italy
Mohamed Mosaad Salama,
Mansoura University, Egypt

***Correspondence:**
Paolo Calabresi
paolo.calabresi@unipg.it

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *11 January 2018*
Accepted: *17 April 2018*
Published: *04 May 2018*

Citation:
Ghiglieri V, Calabrese V and Calabresi P (2018) Alpha-Synuclein: From Early Synaptic Dysfunction to Neurodegeneration. Front. Neurol. 9:295. doi: 10.3389/fneur.2018.00295

THE MANY ROLES OF ALPHA-SYNUCLEIN (α-syn)

Alpha-synuclein is a 140 aminoacid protein, encoded by the *SNCA* gene on human chromosome 4. This protein is mainly expressed in presynaptic sites at several neurotransmitter systems in the central nervous system (CNS) (1). Despite its ubiquitous distribution through many areas involved in complex behaviors, α-syn pathology does not impact on all brain sites of expression, but rather shows a prevalent effect in selective vulnerable sites (1, 2). Moreover, α-syn is highly present in red blood cells (3) and in other extra CNS tissues (4, 5), indicating a wide range of actions of this protein throughout the body.

Although α-syn is gaining increasing consideration as a critical factor in Parkinson's disease (PD) pathophysiology and 20 years of research have been spent in the attempt to unravel the physiological roles of this protein, its mechanisms of action are still unclear and so are the complex dynamics that characterize its flexibility to adapt and the tendency to become toxic.

α-syn exists in a dynamic balance between monomeric and oligomeric states, which are not easily prone to form fibrils in physiological conditions. Interestingly, its structure predicts the multifunctional properties that have been attributed to this protein (6). As a result, this structural flexibility allows α-syn to adopt a wide range of conformations depending on the environment and binding partners (7, 8). In fact, α-syn can either relate to intracellular and membrane proteins with its enzymatic activity or interact with lipid surfaces and organize membrane activities through steric mechanisms.

Given its prevalent localization at presynaptic sites, the first function described for α-syn was its chaperone function and in particular its ability in controlling exocytosis through management of synaptic vesicle pool and trafficking. Accordingly, mutations of the SNCA gene coding for α-syn leads to functional alterations of SNAP REceptor (SNARE) proteins, a family of receptors that binds the soluble *N*-ethylmaleimide sensitive fusion attachment proteins (SNAP) receptor (SNARE) proteins and regulates their assembly (9). Another presynaptic target for α-syn is the DA active trasporter (DAT) (10, 11).

Upon interaction with lipidic surfaces α-syn binding causes the formation of an amphipathic alpha-helix that in physiologic conditions does not cross the bilayer. Under specific stimulations, oligomers of α-syn may form membrane pores that may dissipate the transmembrane potential, dysregulating ion gradients (12, 13).

Several strategies exist to ensure the prevention of α-syn oligomerization (14–17), including complex hydrophobic interactions between C- and N-tails of the protein (16, 18, 19). Interestingly, α-syn possesses a polar C terminal tail able to interact with the hydrophobic region of a separate denatured protein, sharing structural and functional homology with other molecular chaperones. Thus, the extreme flexibility of this protein also relies on the ability of α-syn to auto assemble and act as an intramolecular chaperone (20). In agreement, α-syn truncated at the C-terminus lacks this auto-chaperone property (21) and aggregates at an increased rate compared with the full-length counterpart (14, 15, 21, 22). Despite its crucial contribution to ensure a good orchestration of processes at the active zones, α-syn translocates late to the terminals during development (23) and its absence seems to be not detrimental for synaptogenesis, indicating that its function is rather essential for stressful and sustained activity over time during the long life of a neuron (24, 25). All these characteristics strongly argue for a critical role in neurotransmitter release and synaptic plasticity. A feature that makes multifunctional α-syn as much enigmatic as difficult to counteract in pathological settings is that, like many other disease-associated misfolding proteins, its absence is less detrimental than its accumulation (26, 27). In physiological conditions, during proteins translation, polypeptides fold under control of chaperones. Errors in assembly are frequent and become more common with aging but they are usually limited by several quality control mechanisms that target denatured and misfolded proteins to degradation (28–30). Given the complex management of this protein expression and the high versatility of its functions, failures in these homeostatic steps do not simply bring to an abnormal gain of function but rather to a potent trigger for a series of neurodegenerative cascades in the intracellular environment. The possibility that residual physiological functions and compensative mechanisms are in act during degeneration, complicates therapeutic approaches and adds unpredictability to possible manipulation of α-syn functions.

α-syn OLIGOMERS AND FIBRILLARY AGGREGATES: PATHOLOGICAL IMPLICATIONS

An increasing body of evidence from studies carried out in animal models and in patients support the hypothesis that the processes underlying α-syn proteostasis have central roles in the pathogenesis of PD. This concept dates back to 20 years ago when two discoveries provided support for a role of possible link between α-syn mutations and PD. The first report was the identification of a missense mutation of this gene (31) causing a form of early-onset familial PD by Polymeropoulos and his research team. In the same year, Spillantini's group provided experimental evidence that α-syn is the primary structural component of Lewy bodies (LB), intracytoplasmatic inclusions of α-syn aggregates, which are considered the main pathological hallmark of PD (32). Shortly after, also sporadic idiopathic forms of PD were found associated with the presence of LB in the brain parenchyma (33).

In the last years, physiological and pathological functions of α-syn and other misfolding proteins have been investigated in relation with other known aspects of the disease, to explore possible causal relationships. For PD, many risk factors have been identified that include both environmental and genetic causes. Oxidative stress, mitochondrial dysfunctions, neuroinflammation, point mutations, multiplications, and specific polymorphisms are genetic determinants that may cooperate to create ideal conditions for developing PD.

Interestingly, these factors are also determinants that impact on the predisposition of α-syn to exert toxicity.

Despite the existence of redundant quality control systems to ensure a correct assembly of α-syn and the ability of other synucleins to inhibit and control oligomerization of α-syn, this protein may express its neurotoxic potential when soluble monomers initially form oligomers, then progressively combine to form small protofibrils and further aggregate in large, insoluble α-syn fibrils forming LB (34, 35). Although its natural propensity to balance between a soluble and membrane-bound state and its plasticity of conformation, acute triggers of accumulation and aggregation of α-syn can be manifold like overproduction of the protein, failure in the molecular system that cleave misfolded forms, exposure to pH changes, oxidative stress, and mitochondrial overwork.

More chance for aggregation is offered by a variety of post-translational covalent modifications (8) potentially promoting conformational changes that make α-syn more prone to aggregation. For example, tyrosine nitration (Tyr125) and truncation of α-syn at the C-terminus are frequently found in α-syn pathological aggregates and have been shown to promote fibrillation *in vitro* (36, 37).

Finally, a progressive, age-related decline of efficiency in the in proteolytic mechanisms might play a synergistic role in the accumulation of α-syn (38, 39). These observations are consistent with data showing increased levels of α-syn in nigral dopaminergic neurons during normal aging (40).

In the healthy brain, intracellular homeostasis of α-syn is ensured by the combined actions of the ubiquitin–proteasome (UP) system and the lysosomal autophagy system (LAS) with the latter more involved in the clearance of oligomeric assemblies (38). Any failure in these systems is a potential trigger to overproduction and accumulation of α-syn forms, although compensatory mechanisms and additional proteases can take control over the protein maturation (38, 41). An aspect that complicates the scenario is that accumulation of α-syn may itself inhibit these homeostatic systems (42, 43) and reduce chaperoning of misfolded forms, enrolling the whole compartment into a vicious cycle that rapidly and uncontrollably triggers multiple neurodegeneration pathways. Accordingly, several mutations associated with genetic forms of PD are associated with reduced LAS function.

Analysis of LB has been indicative of the post-translational modifications mostly associated with pathogenic forms of α-syn (44). Among them, phosphorylation is probably the most studied

modification since Ser129 phosphorylated α-syn is thought to be the dominant form of α-syn in LB (45). In support of this prevalence, a recent proteomics study quantified cortical expression levels of various α-syn forms from PD cases and controls (46).

It remains unclear, however, whether phosphorylation of α-syn impacts the fibrillation process (47). The role of nitration and oxidation in favoring toxic species is more clearly demonstrated in decreasing the tendency of α-syn to form fibrils and stabilizing oligomers, leading to enhanced toxicity (48, 49). Nitration of α-syn at specific residues has been characterized in brains from patients with synucleinopathies (50). Oxidized α-syn may result by way of oxidized derivatives of DA leading to a decrease in fibril formation and a subsequent increase in protofibril accumulation (51).

Truncated α-syn species have been found in LB associated with an increased tendency to form fibrils *in vitro* and with increased toxicity in overexpressing laboratory animals (52, 53) even if evidence of correlation with human disease are scarce (46).

The pathological relevance of α-syn species is extensively debated (44) and stabilization of the amyloid pathway is a main focus of research. It has been proposed that toxic species could be either amyloid-like insoluble fibrils, as the ones found in LB, although more evidence would support a key role for soluble oligomers or protofibrils (35). Several groups have investigated the different states of α-syn aggregation and thoroughly examined the functional consequences of aggregate-associated toxicity producing conflicting results (35, 44). However, the general concept is that α-syn exists under various conformational shapes and oligomeric states in a dynamic balance, modulated by factors either accelerating or inhibiting fibril formation. Genetic mutations related to PD have a role in determining the pattern of expression of the various aggregates (54–56), although the identification and characterization of the toxic α-syn species remain incomplete.

Strategies to counteract α-syn toxicity range from increasing protein clearance, which might be enhanced by stimulating autophagy, to act on α-syn post-translational modifications (44). Also, approaches targeting α-syn aggregation with inhibitors (57–59) or by inducing either passive or active immunization against α-syn species have shown promises in several transgenic mouse models of PD (60–62).

However, limits of this approach have been recently discussed (44). Given the incomplete knowledge of possible cellular roles of oligomers and the many functions covered by this protein, the precise α-syn species to target remains unclear. It is possible to hypothesize that modest presence of specific α-syn aggregates can be useful for the cell as part of compensative mechanism that is still unclear, and that their elimination could be harmful and accelerate instead of counteracting the disease process.

DA NEURONS VULNERABILITY TO α-syn

Relevant to the impact of protein misfolding in brain functions, an aspect that still puzzles researchers in the field of neurodegenerative diseases is the selective vulnerability of certain population of neurons to a wide range of insults.

In PD, dopaminergic neurons of the substantia nigra pars compacta (SNc) show selective neurodegeneration and cell death with reduction of dopamine (DA) levels in the striatum and impairment of several basal ganglia functions. The mechanism by which α-syn injures dopaminergic neurons remains to be fully established.

Alpha-synuclein is related to DA neurons for its ability to modulate DA homeostasis in synapses and to bind and influence the activity of DAT (63–65), although the implicated mechanisms are still debated (66–68). This protein is also an important modulator of DA metabolism as it controls DA synthesis by reducing the phosphorylation state of tyrosine hydroxylase and stabilizing it in its inactive state (69). Accordingly, absence of α-syn exerts considerable impact on the dopaminergic system because it causes decreased striatal DA levels and reduced DAT function (70). Lack of α-syn is also associated with decreased DA striatal uptake (71), reduced number of TH-positive terminals as well as of nigral DA cells (72).

However, the sensitivity of DA neurons to α-syn toxicity does not only depend on the possible lack of support to DA metabolism, but on the intrinsic and selective vulnerability of these neurons to excitotoxic challenge.

A recent review discusses the common traits of neurons of SNc neurons and other nuclei most vulnerable to PD pathology, offering an interesting point of view (73). The authors posit that SNc neurons share particular vulnerability to oxidative stress with cells of other brain nuclei involved in arousal responses and in the control of sensorimotor networks, needed for surviving behaviors such as vigilance, escape, and attack. SNc DA neurons possess at least two characteristics that make them particularly vulnerable to excitotoxic insult.

First, these neurons display an extensive length of branched axons that offer a high number of transmitter release sites. This diffuse axonal arbor might be functional to the coordination of the activity in spatially distributed networks, such as the basal ganglia. However, mitochondrial stress is elevated in the axons of SNc DA neurons and this is one reason why these neurons show increased vulnerability.

Second, DA neurons also have spontaneous activity and act as autonomous pacemakers. Their activity is characterized by large oscillations in intracellular calcium (Ca^{2+}) concentration that are driven by the opening of voltage-dependent Cav1 Ca^{2+} channels (also known as L-type Ca^{2+} channels) to ensure a rhythmic (2–10 Hz) spiking (74–76). This ability is associated to low intrinsic Ca^{2+} buffering and requires a strict control of Ca-mediated processes from intracellular stores, promoting Ca^{2+} entry into the mitochondria (77, 78) as well as oxidative phosphorylation and production of ATP (79). All these events are needed to fulfill bioenergetic needs (79, 80) and to avoid undesired compensative activation of ATP-sensitive potassium channels, which would silence ongoing neuronal activity.

Substantia nigra pars compacta cells and other neurons of brain nuclei involved in sensorimotor integration are endowed with this complex set of feedforward control mechanisms that ensure to rapidly implement a correct strategy in response to environmental challenge. A price for this adaptive ability is the vulnerability of the system to age, genetic mutations, or environmental toxins that may increase production of reactive oxygen species that can impair proteostasis, cause accumulation DNA damages, particularly in mitochondria. When mitochondrial

dysfunction reaches a level in which mitophagy is impaired, also cellular autophagic processes are affected and UP and LAS systems are compromised. Accordingly, in rodents, SNc, locus coeruleus, and dorsal motor nucleus of the vagus neurons (which are the only ones that have been studied at this level) manifest a basal mitochondrial oxidant stress in the somatodendritic region that is attributable to the feedforward control of oxidative phosphorylation of ATP (78, 81–83). On this line, a recent paper by Burbulla and colleagues (84) analyzed the synergistic detrimental effect of increased levels of α-syn, dopaminergic receptor stimulation, and mitochondrial dysfunction in mice showing functional inactivation of DJ-1, modeling an early-onset genetic form of PD. Interestingly, mice with both DA neuron-specific overexpression of human α-syn A53T (85) and constitutive DJ-1 deficiency show increased levels of oxidized DA in nigral neurons and decreased lysosomal activity compared with mice bearing the single DJ-1 mutation.

All these data support the concept that α-syn induces exacerbation of a Ca^{2+} dyshomeostasis in DA neurons. The paper by Luo and coworkers provided experimental evidence for this link by studying the potential effects of increased α-syn levels on processes downstream of the Ca^{2+}-signaling pathway, demonstrating the contribution of a new calcium-dependent pathway in the dopaminergic neuronal loss (86). A possible explanation resides in the combination of the α-syn oligomers property to trigger Ca^{2+} influx and the intrinsic physiological characteristics of DA neurons. This neuronal population is in fact characterized by pacemaker activity that, as described above, depends on a complex homeostatic regulation, which involves the activity of L-type calcium channels (87), bringing the DA neuron on the edge of triggering neurodegenerative pathways. Another study that has been instrumental in deciphering the link between DA neurons and α-syn is the paper by Feng et al. (88) demonstrating that in particular conditions, like overexpression of wild-type (WT) α-syn, oligomers causes the formation of pore-like structures throughout the membrane acting as non-selective channels. This was associated with increase in membrane conductance and with cell death (88).

BEYOND THE BRAAK HYPOTHESIS

The prevalent belief on the progression of PD neurodegeneration is based on the observation of an ascending pattern of its clinical manifestations that identifies the disease phase. The idea is that toxic species of α-syn progressively reach more brain regions over the course of the disease, as suggested by Braak and coworkers (89), starting from peripheral body dysfunctions and olfactory impairment through central brainstem functions to end with alterations of higher functions over years or decades following the first exposure to stressors.

In this theoretical framework, the speculation that prodromal symptoms of PD (hyposmia, constipation, and autonomic dysfunctions) might be due to peripheral seeding of α-syn aggregates gained a broad consideration in the field. Indeed, in prodromal phases, inflammation in the gastrointestinal tract or in the olfactory system may trigger the formation of α-syn aggregates (90). This concept is supported by recent evidence obtained in *Snca*-overexpressing mice suggesting a role of gut microbiota in hosting immune and inflammation response linked to α-syn pathology, associated with motor deficits (91).

Thus, α-syn would be released into the synaptic cleft, endocytosed by neighboring neurons, and seed aggregation of endogenous α-syn once inside their new cellular host (92–94). However, this is a much-debated issue and, although many studies support the notion of a spreading of α-syn pathology through a prion-like activity, recent analyses have challenged this theory demonstrating that the distribution of pathology in the brains of PD patients is not consistent with this model. The prion-like nature of α-syn was postulated around a decade ago after the observation of the development of LB-like intracellular inclusions in grafted DA neurons of PD patients who received a transplantation of embryonic mesencephalic grafts 11, 14, and 16 years earlier (95–97). The hypothesis of a "host-to-the-graft" transmission of LB led to the concept that α-syn oligomers may spread from cell to cell through axonal transport and exocytosis, aggregate into LB, and then transferred to other neurons.

A recent study by Peelaerts and colleagues investigated whether different forms of α-syn aggregates are genuine protein strains with a given role and a specific impact on animal physiology (98), based on the hypothesis that different strains could account for the different clinicopathological traits within synucleinopathies (99, 100). The authors propose that α-syn exists and exerts its detrimental effects, in different strains leading to different aggregates that cause as many distinct synucleinopathies (PD, dementia with LB, multiple system atrophy) (98, 101). The most relevant insights from this study are that (1) the dynamic nature of α-syn species is reflected into distinct competencies in the various species that could account for different phenotypes; (2) α-syn strains amplify *in vivo*; and (3) α-syn assemblies cross the blood–brain barrier after intravenous injection.

Although findings in support of the prion-like hypothesis are numerous, an increasing number of studies have recently challenged this vision. Two interesting reviews thoroughly discuss limits of the data collected in support of the ascending theory of α-syn pathology rather supporting a threshold theory to explain controversial data. One of the most important point of the "threshold theory" (102) stems from the simple consideration of PD as a global systemic disease supported by many genetic, cellular, and functional data. The fact that invalidates the ascending theory regards the evidence that brainstem and peripheral neurons are more resistant to insults and less prone to neurodegeneration compared to DA neurons (103–105) and capable of regeneration (106, 107). One explanation of the early dysfunction of brainstem and enteric neurons is due to their low threshold of functional reserve in contrast with the resilience of central neurons as part of widespread interconnected networks that ensure a good degree of compensation and redundancy to conserve higher functions (108, 109). Indeed, central networks have a great ability to compensate for an impaired function of a given central brain area, resulting in a late appearance of motor and cognitive symptoms. The authors propose that parallel pathological events in PD occur at similar rates resulting in the first symptoms pertaining to a peripheral nervous system alteration, due to an earlier functional threshold in the autonomic nervous system compared with midbrain dopaminergic circuitry. This threshold function explains the progression of early symptoms in PD.

INFLAMMATION AND IMMUNE RESPONSE IN PD

In many disorders of the CNS, a key aspect of neurodegeneration is neuroinflammation. In PD, abnormal functions of astrocytes and increase in soluble inflammatory cytokines from microglia and immune cells have been proposed as a critical player that together with glutamate-mediated excitotoxicity becomes major determinants for pathophysiology. In nigrostriatal degeneration, inflammatory response is invariably associated with α-syn-mediated events. Glial cells, which cover a wide range of functions in support of neuron development, maintenance, and survival, seem to be critically involved at many levels in the neurodegenerative spreading of the disease pathology. These studies have provided evidence supporting CNS immune resident cells role in PD (110). Activation of some glial components, such as astrocytes, however, is not limited to final phases of the inflammation process but it has been recently supposed to play a relevant part in initiating the pathology (111). Conversely, although it is well known that microglia plays a role in abnormal plasticity by its ability to produce inflammation mediators (112), it is less clear if microgliosis is instrumental to disease pathogenesis or a secondary event following the ongoing neurodegeneration and a primary role remains to be defined (113).

Functionally, all neuronal activities require an intact glial function provided by both astrocytes and microglia, which become essential for neurons enrolled into intense synaptic activity, such as DA SNc neurons. Astrocytes, besides their role as structural, metabolic, and trophic support, are directly involved in synaptic transmission, ensuring a proper communication, and avoiding abnormal stimulation of extrasynaptic receptors. While astrocytes can be considered an essential component of an operational synaptic surveillance, microglia is in charge of the immune surveillance in the brain. Abnormal microglia activation was found in autopsy brain tissues from PD patients and in experimental parkinsonism (110, 114–117) and many recent papers have focused on the roles of microglia in PD pathology [reviewed in Ref. (113)] bringing support to the notion that neurodegenerative processes and inflammation coexist and cooperate at the same time to respond to brain insults, and that these events do not just occur in series as the disease progresses. For example, when an immune response is initiated by microglia, astrocytes surround the area, creating a barrier to prevent the spread of toxic signals into the surrounding tissue (118). Neuroinflammation and immune response, including autoimmune activity, share molecular pathways initiated by cellular elements during degeneration.

A recent review paper by Booth and colleagues (111) has provided an extensive overview of the studies that link astrocytes alterations and PD, with particular attention to the monogenic forms of disease in which genetic mutations affect the functions of both principal neurons and astrocytes. A recent transcriptome study demonstrated that of 17 genes that have been implicated in PD, 8 are also expressed in astrocytes and are essential for their homeostasis (119). Although SNCA gene shows a low astrocytic expression, it has been suggested that even a modest presence of α-syn might be challenging for their function. In fact, α-syn initiates and regulates astrocyte activation in response to inflammatory stimuli. Also, astrocytes have been reported to take up aggregated alpha syn. The most fascinating aspect of astrocyte involvement in neuronal degeneration, relevant to PD, is that astrocytes change in shape and function to provide support to DA neurons under intense stressful conditions (120–122). Astrocytes are also able to take up circulating DA precursor L-DOPA to release DA (123), as they express enzymes and the complete machinery for its metabolism, suggesting a close relationship to DA systems.

It has been recently shown that in 6-hydroxydopamine-lesioned rats, modeling late stage PD, marked astrocytosis and microglial activation accompany neurodegeneration over time as the damage progresses, being strikingly visible in striatal samples 2 months after the lesion. Interestingly, following a repetitive transcranial magnetic stimulation (TMS) treatment, reduction of intense astrogliosis and microgliosis was associated with, and may underlie, recovery of corticostriatal plasticity. Such TMS-mediated recovery of glial morphology and function was associated with selective increase of DA in dorsolateral striatum of treated parkinsonian animals (124).

These data are in agreement with studies showing that microglia is implicated in the production of neurotrophins, interleukins, proinflammatory, and antiinflammatory cytokines (114–117) and with studies linking TMS beneficial effects with stabilization of microglia, reduction of neuroinflammation biomarkers (110, 125). Midbrain DA neurons, α-syn, and immune response are also linked together by their involvement in altered Ca^{2+} homeostasis. The paper by Luo et al. (86) demonstrated that in DA neurons of A53T α-syn transgenic mice dysregulation of intracellular Ca^{2+} activates the calcineurin pathway that, in turn, increases the translocation rate of the nuclear factor of activated T cells (NFAT) from cytosolic to nuclear compartments. This is associated with the expression of cytokine genes, in human T cells and enhanced cell death in SNc. Inhibition of calcineurin renormalizes the mitochondrial Ca^{2+} fluxes rescuing the α-syn-induced loss of primary mesencephalic DA neuron cultures.

In support of the relationships between immune system and α-syn pathology, an interesting study reported significantly higher service levels of antibodies against monomeric α-syn in patients at early phases of disease. This paper postulates that autoimmunity responses take part in a compensative attempt of neuroprotection (126). A recent paper by Shalash and collaborators has shown that α-syn autoantibodies (AIAs) can be a promising avenue in the field of peripheral biomarkers compared patients with PD, to patients with AD along with controls (127). These molecules are also produced in gender-dependent fashion, across the lifespan during the development and can be detected in healthy young subjects, with titers similar to healthy adults (128). This might suggest that autoantibodies production might be optimized over time in response to environmental stimuli.

A series of studies by Sulzer and coworkers further supports the existence of a link between α-syn and immune response. During PD neuroinflammation, the blood–brain barrier becomes permeable to immune cells recruited into the CNS by massive proinflammatory cytokine production from microglia (129, 130). A recent paper by this group shows that this process triggers an immune response against identified α-syn epitopes in PD patients who presents specific major histocompatibility complex alleles.

In particular, two antigenic regions have been identified: the first near the N terminus (called Y39 region) and the second near the C terminus, the well-known S129 region, containing the amino acid residue S129, whose phosphorylation has been associated with α-syn pathology (131).

EARLY SYNAPTIC ALTERATIONS PRECEDING NEURODEGENERATION

Although many advances have been made in deciphering the mechanisms by which α-syn triggers neurodegenerative pathways, the ability of mammalian brain to compensate for loss of functions still constitutes a main obstacle in a readily identification of the disease's traits. As a consequence, a still unmet medical need is to find increasingly reliable functional biomarkers of disease that may bring to an earlier diagnosis and, possibly, predict disease trajectory. To this aim, current research is focusing on cerebrospinal fluid biomarkers and early synaptic alterations. While advances in the research of peripheral biomarkers have been extensively reviewed elsewhere (132), we will focus on the other front with a main question to address: which measurable synaptic changes can be predictive of PD neurodegeneration?

An ideal condition to explore subtle alteration of synaptic activity before neuronal degeneration is offered by animal models of disease in which expression of α-syn is genetically altered. In these models, it is possible to follow the synapse development at different time points along the disease progression and simultaneously study associated motor deficits to find increasingly more sensitive behavioral tests. Both presynaptic and postsynaptic modifications, recently reviewed by Burre, have been associated with α-syn pathology (20). However, given their specific localization at nerve terminals, presynaptic alterations were soon predicted and investigated, leading to seminal papers demonstrating that altered α-syn interferes with SNARE protein assembly, with an associated reduction in exocytosis and DA release (9) thus affecting the activity of the release machinery, although the extent by which α-syn affects neurotransmitter release is debated (20). Relevant to its presynaptic effects, it is noteworthy that α-syn also interacts with other synaptic proteins, such as Synapsin III, a protein that, similar to other members of the synapsin family, plays essential roles in neurotransmitter release, but has an extrasynaptic localization. Interestingly, it has been reported that synapsin III interacts with α-syn in both physiological and pathological conditions, further increasing the complex pattern of presynaptic actions of α-syn pathology (20, 133–136). It seems to be clear, in fact, that due to its ability to mobilize among different sectors of the active zone upon stimulation (137, 138), α-syn dynamic interactions with SNARE, lipidic raft and DAT are highly dependent on the neural activity. Any alteration in this well-tuned machinery is therefore associated with neurotransmission alterations, which may have less impact in basal neurotransmission, but become critical during intense neuronal activity and over their long lifetime. α-syn can also permeabilize lipid membranes through the formation of cations permeable transmembrane pores, thus altering membrane conductances, and increase the risk for altered calcium homeostasis (12, 139).

This effect of α-syn was also studied in cell systems overexpressing the protein (140, 141). Using whole-cell patch-clamp recordings, Feng and coworkers (88) measured ion leakage upon the application of an electrical potential in a dopaminergic cell line. These effects were associated with a modest but significant time-dependent increase in cell death, demonstrating a link between α-syn pathology and conductance changes.

However, while DA release machinery alterations were a primary expected effect of α-syn toxicity, more recent papers have focused on the postsynaptic counterpart of this pathological scenario. Altered activity and distribution of postsynaptic density components have only recently been explored but may be promising tools to detect subtle but measurable changes at the core of this synaptopathy.

Since cognitive alterations have been observed as prodromal PD symptoms early plastic alterations have been first explored in the hippocampus. In 2012, Costa and colleagues studied CA1 hippocampal plasticity in a transgenic mouse model for α-syn aggregation obtained by the expression of human α-syn 120 under the control of the tyrosine hydroxylase promoter (α-syn 120 mice) and leading to the formation of pathological inclusions in the SNc and olfactory bulb and to a reduction in striatal DA levels (142, 143). In a presymptomatic motor stage characterized by spatial memory alterations, CA1 hippocampal pyramidal neurons of α-syn 120 mice show a reduced ability to respond to a high-frequency stimulation with a form of long-lasting plasticity expressed in this area and dependent on DA D1 and NMDA receptors stimulation, called long-term potentiation (LTP). Postsynaptic density modifications were associated with plastic changes as NMDA receptor subunit composition was found changed with a significant decrease of GluN2A/GluN2B subunit ratio. This effect was due to decreased DA release as L-DOPA was able to rescue synaptic functions. Overall, these results first demonstrated that, similar to human condition, cognitive deficit precede motor symptoms with postsynaptic mechanisms (143). In support of this notion, other studies have shown that α-syn plays a role in NMDA receptor trafficking in other brain areas (144–147), suggesting that postsynaptic actions of α-syn impact on intracellular events relevant for synaptic plasticity.

Given the possibility that in pathological conditions α-syn species (monomer, oligomers, and fibrils) may also act extracellularly thus possibly inducing postsynaptic effects, *in vitro* models have been developed to clarify the role of extracellular α-syn in hippocampal plasticity alterations. On this line, the group of Outeiro conducted a series of studies to demonstrate the effects of extracellular α-syn oligomers. The study carried out by Diogenes and colleagues shows that different species of α-syn have distinct effects on synaptic activity. In particular, among oligomers, monomers, and fibrils, only prolonged incubation with oligomers in healthy rodent brain slices were able to increase basal synaptic transmission through a mechanism dependent on NMDA receptor activation, accompanied by an increase in the expression of GluR2-lacking AMPA receptors. In these slices, stimulation with a theta-burst pattern was not able to induce LTP without a previous application of a low-frequency stimulation indicating a saturation effect underlying impairment of LTP (148). Interestingly, these detrimental effects were counteracted

by adenosine A2a receptor antagonists, known for their neuroprotective role in PD therapy, and were not observed in animals lacking A2aR. Moreover, blockade of these receptors was able to reduce α-syn aggregates (149).

Another evidence of the link between α-syn overexpression and NMDA receptor dysfunction comes from a recent study of the same group as a further support to the concept that many elements in the postsynaptic compartment play important roles in predisposing the synapse to NMDA-dependent excitotoxicity mediated by its interaction with α-syn aggregates. Using an *in vitro* approach, the authors demonstrated that LTP alterations are caused by the abnormal activity of cellular prion protein, known to act as a cell surface-binding partner for soluble oligomeric protein and to interact with NMDA receptors at postsynaptic density. This protein, when engaged in pathological interactions with α-syn, mediates Ca^{2+} dyshomeostasis and synaptic dysfunction through a mechanism involving Fyn kinase phosphorylation, which is tightly regulated by mGluR5 *via* adenosine A2A receptors. In turn, activated Fyn phosphorylates Y1472 residue of GluN2B-expressing NMDA receptors with consequent excitotoxic effects (150).

Although these studies greatly contributed to the understanding of α-syn synaptic effects, the effects of α-syn oligomers on the functional activity of the striatum have been explored only recently. In fact, the striatum represents the most interesting target for PD therapy since it is the main recipient of dopaminergic nigral neurons, whose activity is impaired by α-syn-mediated toxicity (151). For this reason, transgenic animals overexpressing altered forms of α-syn, such as the truncated human α-syn 1–120 or the WT human α-syn, may be valuable models to assess specific aspects of the pathogenesis of synucleinopathies and to analyze the cell type-specific alterations of striatal synaptic plasticity in the initial phase of the disease.

The first report to show alterations in corticostriatal plasticity associated with α-syn overexpression was provided by an *ex vivo* study performed in slices from mice overexpressing a truncated form of α-syn at a late symptomatic stage of disease (152). But, it was few years later that, taking advantage of the

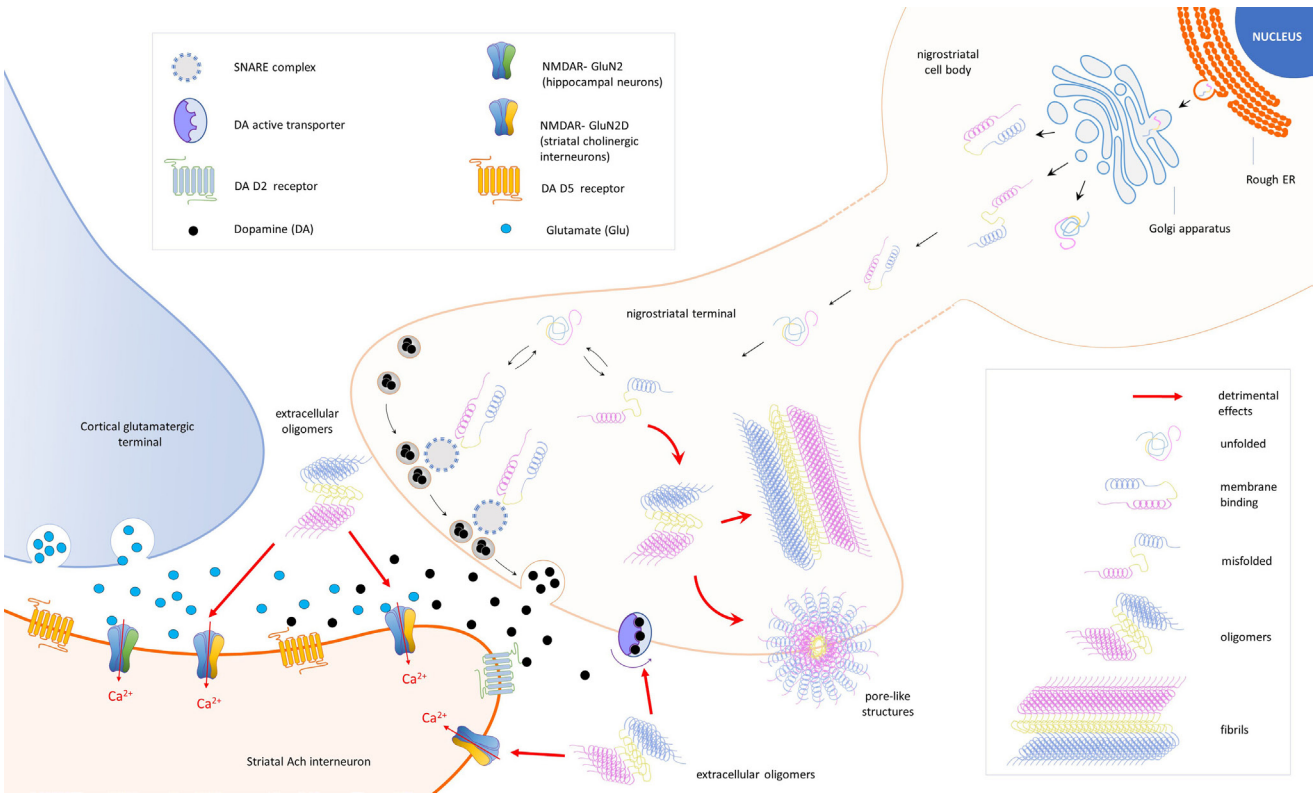


FIGURE 1 | Schematic representation of the cellular and synaptic detrimental actions mediated by different forms of the protein alpha-synuclein (α-syn). In nigral neurons, endoplasmic reticulum (rough ER), *SNCA* transcripts are translated into native α-syn proteins, which are assembled in the Golgi apparatus and released in different conformations. Due to its auto-chaperone activity, α-syn exists in a dynamic balance between monomeric unfolded and amphipathic alpha-helix (membrane binding) state, adopting a range of conformations depending on the environment and binding partners. During the assembly process, misfolding proteins might be also produced (misfolded) and escape detection and clearance by intracellular quality control systems. After synapse maturation, α-syn migrates to nerve terminals and interacts with intracellular proteins [SNAP REceptor (SNARE) complex] and the dopamine (DA) active trasporter to ensure a correct control of neurotransmission. Misfolded α-syn may combine into oligomers that, under specific stimulations, form transmembrane pore-like structures able to alter membrane conductances. Overexpression of α-syn exacerbates pathological events and culminates with the formation of fibrillar aggregates (fibrils), a major component of Lewy bodies. Extracellular α-syn oligomers interfere with the expression of long-term potentiation, a form of synaptic plasticity mediated by *N*-methyl-D-aspartate receptors (NMDAR) in striatal cholinergic interneurons. A direct interaction between α-syn and the GluN2D subunit has been demonstrated in three different models of experimental parkinsonism.

TABLE 1 | Summary of the findings on the role of α-syn in the distinct aspects contributing to pathogenesis of Parkinson's disease (PD).

	Reference	Findings	Experimental conditions
Neurodegeneration	(22)	*C-terminally truncated alpha-synuclein* (α-syn), particularly α-syn (1–120), assembles into filaments morphologically very similar to those seen in neurodegenerative conditions	Ala[30]Pro α-syn, Ala[53]Thr α-syn, α-syn (1–110), α-syn (1–120), and α-syn (1–130) subcloned into the bacterial expression vector pRK172 and expressed in *Escherichia coli* BL21(DE3)
	(52)	α-syn lacking residues 71–82 (*α-synΔ71–82*) are unable to aggregate and show no dopaminergic neurotoxicity, whereas truncated C-terminal α-syn (*α-synΔ120*) has a moderate role in influencing both aggregation and toxicity	Transgenic *Drosophila* modified to obtain α-synΔ71–82 or α-synΔ120
	(40)	*Nigrostriatal α-syn* levels increase with age causing inclusion bodies to form in nigral neurons and drive dopamine (DA) levels over a symptomatic threshold	Human and non-human primate models
	(41)	*A53T* α-syn, but not ΔDQ/A53T, causes toxicity in primary cortical neurons through chaperone activity dysfunction and aberrant macroautophagy activation	Stable rat PC12 and human SH-SY5Y cells inducibly expressing human wild-type (WT) α-syn, ΔDQ/WT α-syn, A53T α-syn, and ΔDQ/A53T α-syn
	(53)	Co-expression of human full-length α-syn (*αsynFL*) and C-terminally truncated human α-syn (*αsynΔ110*) can augment the accumulation of pathological αsynFL protein and lead to dopaminergic cell death	Adult Sprague-Dawley rats injected in the SN with viral vector rAAV5-asynFL + rAAV5-asynD110
	(42)	WT α-syn overexpression causes a decrease in LC3-II levels impairing autophagy which increases accumulation of aggregate-prone proteins and sensitizes the cell to proapoptotic assaults	Human neuroblastoma cells (SKNSH), human cervical carcinoma cells (HeLa), and human embryonic kidney cells (HEK293)
	(43)	The introduction of small amounts of pre-formed *α-syn fibrils* into α-syn-expressing cells results in aggregation of endogenously expressed α-syn and the formation of insoluble aggregates which persist even when soluble α-syn levels are substantially reduced, indicating their refractoriness to clearance	Mammalian and primary neuronal cell cultures—HEK293 cells stably expressing WT or A53T α-syn
	(88)	*α-syn overexpression* results in increased oligomer production and formation of membrane-bound α-syn-containing pores that induce increase in membrane conductance, determining cell death	MN9DwtsynIR-Esgfp (MN9Dsyn) cells were derived and engineered from mouse embryonic mesencephalon
	(13)	Penetration of α-syn into membranes gives rise to the formation of *annular pore-like oligomer structures* with the ability to increase cell permeability and calcium influx	Cell culture of neuronal cells expressing WT or mutant A53T α-syn
	(86)	α-syn activates *calcineurin* (*CN*), mediating the translocation of NFATc3, which contributes to the loss of neurons. Administration of CN inhibitor cyclosporine A rescues the α-syn-induced loss of primary mDA neuron cultures	HEK293 cells transfected with WT and A53T α-syn cDNAs
	(98)	Exogenous α-syn strains seed the assembly of endogenous α-syn. Differently from α-syn *oligomers*, α-syn *fibrils* and *ribbons* remain in place after crossing the blood–brain barrier. Distinct α-syn assemblies can affect neurotransmission after acute exposure, but only fibrillar α-syn exhibits perpetual behavioral and aggravated neurotoxic phenotypes *in vivo*	Intracerebral injections of exogenous α-syn in Wistar rats
Oxidative stress	(48)	*Nitration of Tyr residues* appears to prevent fibrillogenesis from soluble α-syn proteins. Oxidative stress causes soluble α-syn to form covalently linked dimers and higher oligomers and allows for fibril formation and stabilization	WT and Tyr to Phe mutant recombinant human α-syn proteins expressed in *E. coli* BL21(DE3) RIL cells
	(49)	*Nitration* effectively inhibits fibrillation of α-syn. The addition of low concentration of nitrated α-syn inhibits the fibrillation of non-modified α-syn	Human WT α-syn was expressed using *E. coli* BL21
Immune response	(126)	Autoimmune response to α-syn can serve as a valid *biomarker*, reflecting the progressive brain neurodegeneration and impaired α-syn homeostasis occurring in PD	Analysis of human PD patients' serum
	(91)	Changes in human microbiota are correlated to the motor and *gastrointestinal parkinsonian dysfunctions* by impacting neuroinflammation and α-syn aggregation. *Gut bacteria* from PD patients promote enhanced motor impairment when transplanted into α-syn overexpressing mice	Germ free male α-syn overexpressing and WT colonized with fecal microbes from PD patients and healthy controls

(*Continued*)

TABLE 1 | Continued

	Reference	Findings	Experimental conditions
	(131)	Peptides derived from two regions of α-syn (Y39 and S129) produce immune responses in patients with PD which are enacted mostly by IL-5-secreting $CD4^+$ T cells, as well as IFNγ -secreting $CD8^+$ cytotoxic T cells. The Y39 antigenic region is strikingly close to the α-syn mutations that cause PD	Genome sequencing of PD patients
Synaptic alterations	(23)	The *double α/β synuclein deletion*, but not single ones, decreases DA levels, and impairs synaptic parameters (structure of synapse, neurotransmitters release, mobilization of synaptic vesicles, or forms of short- and long-term synaptic plasticity)	$\alpha^{+/+}\ \beta^{+/+}$, $\alpha^{-/-}\ \beta^{+/+}$, $\alpha^{+/+}\ \beta^{-/-}$, $\alpha^{-/-}\ \beta^{-/-}$-syn mice
	(25)	In aged *α-syn null mice* a dramatic effect on synapse structure, a decrease conduction velocity and a lower neuronal excitability are observed. Modest overexpression of human α-syn in young mice causes a decrement in neurotransmission similar to aged α-syn null mice	αβγ-syn triple KO mice
	(63)	In dopaminergic neurons, intracellular α-syn induces a DA active trasporter *(DAT)-mediated inward current* extracellular Na^+ independent but transmembrane Cl^- gradient sensitive, which is eliminated by DAT antagonist GBR12935 and is absent with intracellular heat-inactivated α-syn. These changes are paralleled by an α-syn-dependent decrease in rate of DAT-mediated substrate uptake. The membrane potential of cells overexpressing α-syn rest at more depolarized state, disrupting cell homeostasis	Primary neuronal culture of acutely dissociated TH:RFP mouse midbrain DA neurons
	(64)	DA uptake and DAT distribution in striatal membranes are dysregulated in *young mice* overexpressing A53T α-syn. Uptake of DA through DAT is normalized in *older animals* where bioavailability of *A53T α-syn* is reduced and expression of β-syn and γ-syn increased. The normalization of DA uptake with aging may relate to a shift in modulation of DAT from α-syn to other synucleins	Transgenic mice expressing mutant A53T α-syn
	(153)	Overexpression of either truncated or full-length human *α-syn affects plasticity of* cholinergic interneurons (*ChIs*), but not of spiny projection neurons, resulting in mild cognitive and motor deficits, mimicking early PD. Acute application of exogenous human α-syn oligomers to striatal slices of control animals impairs long-term potentiation of ChIs by targeting *GluN2D*-expressing *N*-methyl-D-aspartate receptors (NMDARs). Subchronic L-DOPA restores synaptic plasticity in ChIs, suggesting that a long-term dopaminergic activation is required to compensate for the complex molecular effects induced by DA denervation on NMDAR subunits	Transgenic mice expressing truncated human α-syn (1–120) and Sprague-Dawley rats injected with the adeno-associated viral vector (AAV) carrying WT human α-syn (AAV-α-syn)
	(154)	Early versus optimal motor learning changes *striatal DAT levels*. DAT-regulated activation of the D1 pathway in the dorsolateral striatum, during early-stage of incremental motor learning, cooperates with D1 pathway activation to prevent premature shifting to habit learning. Overexpression of α-syn impairs motor learning by altering DAT expression, before leading to DA neuronal loss and bradykinesia	CD1 mice model of PD by performing bilateral injections of recombinant adeno-associated viral vector (rAAV)-hu-α-syn and rAAVGFP in the SNpc/VTA (ventral tegmental area)

gradual progression of the disease offered by genetic models, Tozzi and coworkers investigated α-syn-mediated alterations in plasticity by studying both spiny projection neurons (SPNs) and cholinergic interneurons (ChIs) in two different models of early PD: the mice transgenic for truncated human α-syn (1–120) and the rat injected with the adeno-associated viral vector carrying WT human α-syn in the SNc (153). In a presymptomatic stage, before any neuronal degeneration, procedural learning deficit was associated in both rodent models with selective impairment of LTP in ChIs but not in SPNs. Similar to what observed in the hippocampus, also here a direct interaction between α-syn and NMDA receptors is suggested, as the loss of LTP in striatal ChIs was dependent on a direct interaction of α-syn with GluN2D-expressing NMDA receptors, which are selectively expressed in this class of interneurons. This link has also been studied in an *in vitro* model. Bath incubation of corticostriatal slices of healthy animals with α-syn oligomers caused the same synaptic alterations that were not rescued by exogenous DA or a D1-like receptor agonist, suggesting that the blockade on synaptic plasticity is not mediated by an α-syn-mediated interference with DA release. These alterations correlate with the behavioral pattern observed, mimicking the early phase of PD, and are in line with

what observed in PD patients, in which mild cognitive alterations associated with cholinergic dysfunction, frequently precede overt motor symptoms.

It is noteworthy that micromolar concentration of α-syn oligomers were found effective in determining hippocampal pathology, while nanomolar concentration were sufficient to induce striatal alterations. Taken together, these data suggest that increasing concentrations of α-syn may progressively affect NMDA receptor-mediated functions on distinct neuronal populations, indicating that the vulnerability to this protein may be cell type specific and region specific. On this view, early dysfunction of the striatal cholinergic system, occurring at very low concentrations, represents a possible functional marker of the disease. On the same line of research, a recent study by Giordano and coworkers provided an important link between presynaptic and postsynaptic actions of α-syn and contribute to the reconstruction of a comprehensive view of the many faces of α-syn pathology (154).

Using an animal model of PD, in which animals overexpress human WT α-syn in the midbrain neurons, the authors demonstrate that very early stages are associated with reduced striatal DAT and impaired acquisition of performance plateau in the rotarod task. Interestingly, behavioral impairment has a unique electrophysiological correlate that depends on DAT alteration. In fact, while a form of plasticity, the long-term depression (LTD) is equally expressed at corticostriatal synapses of both WT and α-syn mice before and after exposure to exercise through accelerated rotarod test, healthy animals show an interesting switch from LTD to LTP during the acquisition phase of motor learning. Mice overexpressing human α-syn do not show this shift in plasticity that is instrumental to acquire motor habits and perform correctly. This early training-induced shift from LTD to LTP, and the achievement of a good performance, is impaired in control animals pretreated with DAT inhibitor GBR-12909. These findings, in line with previous studies (155–159), further suggest that early signs of synucleinopathy do not necessarily correlate with DA neuronal loss and support the notion that a reorganization of cellular plasticity within the dorsal striatum is necessary for the acquisition of a motor skill, and it depends on an intact dopaminergic transmission, controlled by DAT, which is impaired early by nigral overexpression of human α-syn.

CONCLUSION

Taken together, all these findings return a complex but increasingly clear scenario designed by the many roles of α-syn (**Figure 1**; **Table 1**).

Future studies will be needed to further investigate how endogenous molecules interact with different α-syn conformations. In particular, based on the majority of data reviewed here, we expect that more effort will be aimed at explaining the mechanisms underlying distinct cell-type and region-specific α-syn-mediated NMDA receptor alterations. Moreover, in light of the latter study and of many reports supporting the neuroprotective effect of intense exercise in newly designed rehabilitation programs (160, 161), a promising link to explore will be the interaction between α-syn aggregation and experience.

AUTHOR CONTRIBUTIONS

VG wrote and revised the manuscript, prepared the figure and reviewed the final version; VC revised the manuscript, prepared the review table and proofread the final version; PC conceived and designed the manuscript, edited, and proofread the final version of the paper.

FUNDING

This work was supported by grants from Progetto di Ricerca di Interesse Nazionale (PRIN) 2015 (prot. 2015FNWP34) (to PC), Ricerca Finalizzata RF-2013-02356215 (to PC) and by grants from the Fresco Parkinson Institute to New York University School of Medicine and The Marlene and Paolo Fresco Institute for Parkinson's and Movement Disorders, which were made possible with support from Marlene and Paolo Fresco (to PC and to VG).

REFERENCES

1. Jakes R, Spillantini MG, Goedert M. Identification of two distinct synucleins from human brain. *FEBS Lett* (1994) 345:27–32. doi:10.1016/0014-5793(94)00395-5
2. Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Yoshimoto M, et al. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc Natl Acad Sci U S A* (1993) 90:11282–6. doi:10.1073/pnas.90.23.11282
3. Nakai M, Fujita M, Waragai M, Sugama S, Wei J, Akatsu H, et al. Expression of alpha-synuclein, a presynaptic protein implicated in Parkinson's disease, in erythropoietic lineage. *Biochem Biophys Res Commun* (2007) 358:104–10. doi:10.1016/j.bbrc.2007.04.108
4. Askanas V, Engel WK, Alvarez RB, Mcferrin J, Broccolini A. Novel immunolocalization of alpha-synuclein in human muscle of inclusion-body myositis, regenerating and necrotic muscle fibers, and at neuromuscular junctions. *J Neuropathol Exp Neurol* (2000) 59:592–8. doi:10.1093/jnen/59.7.592
5. Ltic S, Perovic M, Mladenovic A, Raicevic N, Ruzdijic S, Rakic L, et al. Alpha-synuclein is expressed in different tissues during human fetal development. *J Mol Neurosci* (2004) 22:199–204. doi:10.1385/JMN:22:3:199
6. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of alpha-synuclein: from structure and toxicity to therapeutic target. *Nat Rev Neurosci* (2013) 14:38–48. doi:10.1038/nrn3406
7. Jain N, Bhasne K, Hemaswasthi M, Mukhopadhyay S. Structural and dynamical insights into the membrane-bound alpha-synuclein. *PLoS One* (2013) 8:e83752. doi:10.1371/journal.pone.0083752
8. Gallegos S, Pacheco C, Peters C, Opazo CM, Aguayo LG. Features of alpha-synuclein that could explain the progression and irreversibility of Parkinson's disease. *Front Neurosci* (2015) 9:59. doi:10.3389/fnins.2015.00059
9. Garcia-Reitbock P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, et al. SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. *Brain* (2010) 133:2032–44. doi:10.1093/brain/awq132
10. Sidhu A, Wersinger C, Vernier P. Alpha-synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. *FEBS Lett* (2004) 565:1–5. doi:10.1016/j.febslet.2004.03.063
11. Bellucci A, Collo G, Sarnico I, Battistin L, Missale C, Spano P. Alpha-synuclein aggregation and cell death triggered by energy deprivation and dopamine overload are counteracted by D2/D3 receptor activation. *J Neurochem* (2008) 106:560–77. doi:10.1111/j.1471-4159.2008.05406.x

12. Tosatto L, Andrighetti AO, Plotegher N, Antonini V, Tessari I, Ricci L, et al. Alpha-synuclein pore forming activity upon membrane association. *Biochim Biophys Acta* (2012) 1818:2876–83. doi:10.1016/j.bbamem.2012.07.007
13. Tsigelny IF, Sharikov Y, Wrasidlo W, Gonzalez T, Desplats PA, Crews L, et al. Role of alpha-synuclein penetration into the membrane in the mechanisms of oligomer pore formation. *FEBS J* (2012) 279:1000–13. doi:10.1111/j.1742-4658.2012.08489.x
14. Murray IV, Giasson BI, Quinn SM, Koppaka V, Axelsen PH, Ischiropoulos H, et al. Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. *Biochemistry* (2003) 42:8530–40. doi:10.1021/bi027363r
15. Hoyer W, Cherny D, Subramaniam V, Jovin TM. Impact of the acidic C-terminal region comprising amino acids 109–140 on alpha-synuclein aggregation in vitro. *Biochemistry* (2004) 43:16233–42. doi:10.1021/bi048453u
16. Bertoncini CW, Jung YS, Fernandez CO, Hoyer W, Griesinger C, Jovin TM, et al. Release of long-range tertiary interactions potentiates aggregation of natively unstructured alpha-synuclein. *Proc Natl Acad Sci U S A* (2005) 102:1430–5. doi:10.1073/pnas.0407146102
17. Levitan K, Chereau D, Cohen SI, Knowles TP, Dobson CM, Fink AL, et al. Conserved C-terminal charge exerts a profound influence on the aggregation rate of alpha-synuclein. *J Mol Biol* (2011) 411:329–33. doi:10.1016/j.jmb.2011.05.046
18. Dedmon MM, Lindorff-Larsen K, Christodoulou J, Vendruscolo M, Dobson CM. Mapping long-range interactions in alpha-synuclein using spin-label NMR and ensemble molecular dynamics simulations. *J Am Chem Soc* (2005) 127:476–7. doi:10.1021/ja044834j
19. Zhou W, Long C, Reaney SH, Di Monte DA, Fink AL, Uversky VN. Methionine oxidation stabilizes non-toxic oligomers of alpha-synuclein through strengthening the auto-inhibitory intra-molecular long-range interactions. *Biochim Biophys Acta* (2010) 1802:322–30. doi:10.1016/j.bbadis.2009.12.004
20. Burre J. The synaptic function of alpha-synuclein. *J Parkinsons Dis* (2015) 5:699–713. doi:10.3233/JPD-150642
21. Souza JM, Giasson BI, Lee VM, Ischiropoulos H. Chaperone-like activity of synucleins. *FEBS Lett* (2000) 474:116–9. doi:10.1016/S0014-5793(00)01563-5
22. Crowther RA, Jakes R, Spillantini MG, Goedert M. Synthetic filaments assembled from C-terminally truncated alpha-synuclein. *FEBS Lett* (1998) 436:309–12. doi:10.1016/S0014-5793(98)01146-6
23. Chandra S, Fornai F, Kwon HB, Yazdani U, Atasoy D, Liu X, et al. Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions. *Proc Natl Acad Sci U S A* (2004) 101:14966–71. doi:10.1073/pnas.0406283101
24. Bisaglia M, Mammi S, Bubacco L. Structural insights on physiological functions and pathological effects of alpha-synuclein. *FASEB J* (2009) 23:329–40. doi:10.1096/fj.08-119784
25. Greten-Harrison B, Polydoro M, Morimoto-Tomita M, Diao L, Williams AM, Nie EH, et al. Alphabetagamma-synuclein triple knockout mice reveal age-dependent neuronal dysfunction. *Proc Natl Acad Sci U S A* (2010) 107:19573–8. doi:10.1073/pnas.1005005107
26. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. Alpha-synuclein locus triplication causes Parkinson's disease. *Science* (2003) 302:841. doi:10.1126/science.1090278
27. Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P, et al. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* (2004) 364:1169–71. doi:10.1016/S0140-6736(04)17104-3
28. Hightower LE. Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell* (1991) 66:191–7. doi:10.1016/0092-8674(91)90611-2
29. Sherman MY, Goldberg AL. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* (2001) 29:15–32. doi:10.1016/S0896-6273(01)00177-5
30. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* (2002) 295:1852–8. doi:10.1126/science.1068408
31. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* (1997) 276:2045–7. doi:10.1126/science.276.5321.2045
32. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* (1997) 388:839–40. doi:10.1038/42166
33. Mezey E, Dehejia AM, Harta G, Tresser N, Suchy SF, Nussbaum RL, et al. Alpha synuclein is present in Lewy bodies in sporadic Parkinson's disease. *Mol Psychiatry* (1998) 3:493–9. doi:10.1038/sj.mp.4000446
34. Kim C, Lee SJ. Controlling the mass action of alpha-synuclein in Parkinson's disease. *J Neurochem* (2008) 107:303–16. doi:10.1111/j.1471-4159.2008.05612.x
35. Melki R. Role of different alpha-synuclein strains in synucleinopathies, similarities with other neurodegenerative diseases. *J Parkinsons Dis* (2015) 5:217–27. doi:10.3233/JPD-150543
36. Oueslati A, Fournier M, Lashuel HA. Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. *Prog Brain Res* (2010) 183:115–45. doi:10.1016/S0079-6123(10)83007-9
37. Chavarria C, Souza JM. Oxidation and nitration of alpha-synuclein and their implications in neurodegenerative diseases. *Arch Biochem Biophys* (2013) 533:25–32. doi:10.1016/j.abb.2013.02.009
38. Xilouri M, Brekk OR, Stefanis L. Alpha-synuclein and protein degradation systems: a reciprocal relationship. *Mol Neurobiol* (2013) 47:537–51. doi:10.1007/s12035-012-8341-2
39. Kaushik S, Cuervo AM. Proteostasis and aging. *Nat Med* (2015) 21:1406–15. doi:10.1038/nm.4001
40. Chu Y, Kordower JH. Age-associated increases of alpha-synuclein in monkeys and humans are associated with nigrostriatal dopamine depletion: is this the target for Parkinson's disease? *Neurobiol Dis* (2007) 25:134–49. doi:10.1016/j.nbd.2006.08.021
41. Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L. Aberrant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. *PLoS One* (2009) 4:e5515. doi:10.1371/journal.pone.0005515
42. Winslow AR, Chen CW, Corrochano S, Acevedo-Arozena A, Gordon DE, Peden AA, et al. Alpha-synuclein impairs macroautophagy: implications for Parkinson's disease. *J Cell Biol* (2010) 190:1023–37. doi:10.1083/jcb.201003122
43. Tanik SA, Schultheiss CE, Volpicelli-Daley LA, Brunden KR, Lee VM. Lewy body-like alpha-synuclein aggregates resist degradation and impair macroautophagy. *J Biol Chem* (2013) 288:15194–210. doi:10.1074/jbc.M113.457408
44. Dehay B, Bourdenx M, Gorry P, Przedborski S, Vila M, Hunot S, et al. Targeting alpha-synuclein for treatment of Parkinson's disease: mechanistic and therapeutic considerations. *Lancet Neurol* (2015) 14:855–66. doi:10.1016/S1474-4422(15)00006-X
45. Anderson JP, Walker DE, Goldstein JM, De Laat R, Banducci K, Caccavello RJ, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem* (2006) 281:29739–52. doi:10.1074/jbc.M600933200
46. Kellie JF, Higgs RE, Ryder JW, Major A, Beach TG, Adler CH, et al. Quantitative measurement of intact alpha-synuclein proteoforms from post-mortem control and Parkinson's disease brain tissue by intact protein mass spectrometry. *Sci Rep* (2014) 4:5797. doi:10.1038/srep05797
47. Paleologou KE, Schmid AW, Rospigliosi CC, Kim HY, Lamberto GR, Fredenburg RA, et al. Phosphorylation at Ser-129 but not the phosphomimics S129E/D inhibits the fibrillation of alpha-synuclein. *J Biol Chem* (2008) 283:16895–905. doi:10.1074/jbc.M800747200
48. Norris EH, Giasson BI, Ischiropoulos H, Lee VM. Effects of oxidative and nitrative challenges on alpha-synuclein fibrillogenesis involve distinct mechanisms of protein modifications. *J Biol Chem* (2003) 278:27230–40. doi:10.1074/jbc.M212436200
49. Yamin G, Uversky VN, Fink AL. Nitration inhibits fibrillation of human alpha-synuclein in vitro by formation of soluble oligomers. *FEBS Lett* (2003) 542:147–52. doi:10.1016/S0014-5793(03)00367-3
50. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, et al. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* (2000) 290:985–9. doi:10.1126/science.290.5493.985
51. Conway KA, Rochet JC, Bieganski RM, Lansbury PT Jr. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* (2001) 294:1346–9. doi:10.1126/science.1063522
52. Periquet M, Fulga T, Myllykangas L, Schlossmacher MG, Feany MB. Aggregated alpha-synuclein mediates dopaminergic neurotoxicity in vivo. *J Neurosci* (2007) 27:3338–46. doi:10.1523/JNEUROSCI.0285-07.2007

53. Ulusoy A, Febbraro F, Jensen PH, Kirik D, Romero-Ramos M. Co-expression of C-terminal truncated alpha-synuclein enhances full-length alpha-synuclein-induced pathology. *Eur J Neurosci* (2010) 32:409–22. doi:10.1111/j.1460-9568.2010.07284.x
54. Conway KA, Harper JD, Lansbury PT. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat Med* (1998) 4:1318–20. doi:10.1038/3311
55. Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury PT Jr. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc Natl Acad Sci U S A* (2000) 97:571–6. doi:10.1073/pnas.97.2.571
56. Karpinar DP, Balija MB, Kugler S, Opazo F, Rezaei-Ghaleh N, Wender N, et al. Pre-fibrillar alpha-synuclein variants with impaired beta-structure increase neurotoxicity in Parkinson's disease models. *EMBO J* (2009) 28:3256–68. doi:10.1038/emboj.2009.257
57. Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, et al. EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proc Natl Acad Sci U S A* (2010) 107:7710–5. doi:10.1073/pnas.0910723107
58. Wagner J, Ryazanov S, Leonov A, Levin J, Shi S, Schmidt F, et al. Anle138b: a novel oligomer modulator for disease-modifying therapy of neurodegenerative diseases such as prion and Parkinson's disease. *Acta Neuropathol* (2013) 125:795–813. doi:10.1007/s00401-013-1114-9
59. Levin J, Schmidt F, Boehm C, Prix C, Botzel K, Ryazanov S, et al. The oligomer modulator anle138b inhibits disease progression in a Parkinson mouse model even with treatment started after disease onset. *Acta Neuropathol* (2014) 127:779–80. doi:10.1007/s00401-014-1265-3
60. Masliah E, Rockenstein E, Mante M, Crews L, Spencer B, Adame A, et al. Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease. *PLoS One* (2011) 6:e19338. doi:10.1371/journal.pone.0019338
61. Bae EJ, Lee HJ, Rockenstein E, Ho DH, Park EB, Yang NY, et al. Antibody-aided clearance of extracellular alpha-synuclein prevents cell-to-cell aggregate transmission. *J Neurosci* (2012) 32:13454–69. doi:10.1523/JNEUROSCI.1292-12.2012
62. Mandler M, Valera E, Rockenstein E, Weninger H, Patrick C, Adame A, et al. Next-generation active immunization approach for synucleinopathies: implications for Parkinson's disease clinical trials. *Acta Neuropathol* (2014) 127:861–79. doi:10.1007/s00401-014-1256-4
63. Swant J, Goodwin JS, North A, Ali AA, Gamble-George J, Chirwa S, et al. Alpha-synuclein stimulates a dopamine transporter-dependent chloride current and modulates the activity of the transporter. *J Biol Chem* (2011) 286:43933–43. doi:10.1074/jbc.M111.241232
64. Oaks AW, Frankfurt M, Finkelstein DI, Sidhu A. Age-dependent effects of A53T alpha-synuclein on behavior and dopaminergic function. *PLoS One* (2013) 8:e60378. doi:10.1371/journal.pone.0060378
65. Butler B, Goodwin S, Saha K, Becker J, Sambo D, Davari P, et al. Dopamine transporter activity is modulated by alpha-synuclein. *J Biol Chem* (2015) 290(49):29542–54. doi:10.1074/jbc.A115.639880
66. Lee FJ, Liu F, Pristupa ZB, Niznik HB. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *FASEB J* (2001) 15:916–26. doi:10.1096/fj.00-0334com
67. Wersinger C, Prou D, Vernier P, Sidhu A. Modulation of dopamine transporter function by alpha-synuclein is altered by impairment of cell adhesion and by induction of oxidative stress. *FASEB J* (2003) 17:2151–3. doi:10.1096/fj.03-0152fje
68. Wersinger C, Sidhu A. Attenuation of dopamine transporter activity by alpha-synuclein. *Neurosci Lett* (2003) 340:189–92. doi:10.1016/S0304-3940(03)00097-1
69. Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* (2002) 22:3090–9. doi:10.1523/JNEUROSCI.22-08-03090.2002
70. Al-Wandi A, Ninkina N, Millership S, Williamson SJ, Jones PA, Buchman VL. Absence of alpha-synuclein affects dopamine metabolism and synaptic markers in the striatum of aging mice. *Neurobiol Aging* (2010) 31:796–804. doi:10.1016/j.neurobiolaging.2008.11.001
71. Chadchankar H, Ihalainen J, Tanila H, Yavich L. Decreased reuptake of dopamine in the dorsal striatum in the absence of alpha-synuclein. *Brain Res* (2011) 1382:37–44. doi:10.1016/j.brainres.2011.01.064
72. Garcia-Reitboeck P, Anichtchik O, Dalley JW, Ninkina N, Tofaris GK, Buchman VL, et al. Endogenous alpha-synuclein influences the number of dopaminergic neurons in mouse substantia nigra. *Exp Neurol* (2013) 248:541–5. doi:10.1016/j.expneurol.2013.07.015
73. Surmeier DJ, Obeso JA, Halliday GM. Selective neuronal vulnerability in Parkinson disease. *Nat Rev Neurosci* (2017) 18:101–13. doi:10.1038/nrn.2016.178
74. Mercuri NB, Bonci A, Calabresi P, Stratta F, Stefani A, Bernardi G. Effects of dihydropyridine calcium antagonists on rat midbrain dopaminergic neurones. *Br J Pharmacol* (1994) 113:831–8. doi:10.1111/j.1476-5381.1994.tb17068.x
75. Puopolo M, Raviola E, Bean BP. Roles of subthreshold calcium current and sodium current in spontaneous firing of mouse midbrain dopamine neurons. *J Neurosci* (2007) 27:645–56. doi:10.1523/JNEUROSCI.4341-06.2007
76. Guzman JN, Sanchez-Padilla J, Chan CS, Surmeier DJ. Robust pacemaking in substantia nigra dopaminergic neurons. *J Neurosci* (2009) 29:11011–9. doi:10.1523/JNEUROSCI.2519-09.2009
77. Hayashi T, Rizzuto R, Hajnoczky G, Su TP. MAM: more than just a housekeeper. *Trends Cell Biol* (2009) 19:81–8. doi:10.1016/j.tcb.2008.12.002
78. Guzman JN, Sanchez-Padilla J, Wokosin D, Kondapalli J, Ilijic E, Schumacker PT, et al. Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1. *Nature* (2010) 468:696–700. doi:10.1038/nature09536
79. Balaban RS. The role of $Ca^{(2+)}$ signaling in the coordination of mitochondrial ATP production with cardiac work. *Biochim Biophys Acta* (2009) 1787:1334–41. doi:10.1016/j.bbabio.2009.05.011
80. Budd SL, Nicholls DG. Mitochondria in the life and death of neurons. *Essays Biochem* (1998) 33:43–52. doi:10.1042/bse0330043
81. Putzier I, Kullmann PH, Horn JP, Levitan ES. Cav1.3 channel voltage dependence, not Ca^{2+} selectivity, drives pacemaker activity and amplifies bursts in nigral dopamine neurons. *J Neurosci* (2009) 29:15414–9. doi:10.1523/JNEUROSCI.4742-09.2009
82. Goldberg JA, Guzman JN, Estep CM, Ilijic E, Kondapalli J, Sanchez-Padilla J, et al. Calcium entry induces mitochondrial oxidant stress in vagal neurons at risk in Parkinson's disease. *Nat Neurosci* (2012) 15:1414–21. doi:10.1038/nn.3209
83. Sanchez-Padilla J, Guzman JN, Ilijic E, Kondapalli J, Galtieri DJ, Yang B, et al. Mitochondrial oxidant stress in locus coeruleus is regulated by activity and nitric oxide synthase. *Nat Neurosci* (2014) 17:832–40. doi:10.1038/nn.3717
84. Burbulla LF, Song P, Mazzulli JR, Zampese E, Wong YC, Jeon S, et al. Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in Parkinson's disease. *Science* (2017) 357:1255–61. doi:10.1126/science.aam9080
85. Chen L, Xie Z, Turkson S, Zhuang X. A53T human alpha-synuclein overexpression in transgenic mice induces pervasive mitochondria macroautophagy defects preceding dopamine neuron degeneration. *J Neurosci* (2015) 35:890–905. doi:10.1523/JNEUROSCI.0089-14.2015
86. Luo J, Sun L, Lin X, Liu G, Yu J, Parisiadou L, et al. A calcineurin- and NFAT-dependent pathway is involved in alpha-synuclein-induced degeneration of midbrain dopaminergic neurons. *Hum Mol Genet* (2014) 23:6567–74. doi:10.1093/hmg/ddu377
87. Surmeier DJ, Guzman JN, Sanchez-Padilla J. Calcium, cellular aging, and selective neuronal vulnerability in Parkinson's disease. *Cell Calcium* (2010) 47:175–82. doi:10.1016/j.ceca.2009.12.003
88. Feng LR, Federoff HJ, Vicini S, Maguire-Zeiss KA. Alpha-synuclein mediates alterations in membrane conductance: a potential role for alpha-synuclein oligomers in cell vulnerability. *Eur J Neurosci* (2010) 32:10–7. doi:10.1111/j.1460-9568.2010.07266.x
89. Braak H, Del Tredici K, Rub U, De Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* (2003) 24:197–211. doi:10.1016/S0197-4580(02)00065-9
90. Lema Tome CM, Tyson T, Rey NL, Grathwohl S, Britschgi M, Brundin P. Inflammation and alpha-synuclein's prion-like behavior in Parkinson's disease – is there a link? *Mol Neurobiol* (2013) 47:561–74. doi:10.1007/s12035-012-8267-8
91. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell* (2016) 167:1469–80.e1412. doi:10.1016/j.cell.2016.11.018
92. Angot E, Steiner JA, Hansen C, Li JY, Brundin P. Are synucleinopathies prion-like disorders? *Lancet Neurol* (2010) 9:1128–38. doi:10.1016/S1474-4422(10)70213-1

93. Brundin P, Melki R, Kopito R. Prion-like transmission of protein aggregates in neurodegenerative diseases. *Nat Rev Mol Cell Biol* (2010) 11:301–7. doi:10.1038/nrm2873
94. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, et al. Parkinson disease. *Nat Rev Dis Primers* (2017) 3:17013. doi:10.1038/nrdp.2017.13
95. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med* (2008) 14:504–6. doi:10.1038/nm1747
96. Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat Med* (2008) 14:501–3. doi:10.1038/nm1746
97. Mendez I, Vinuela A, Astradsson A, Mukhida K, Hallett P, Robertson H, et al. Dopamine neurons implanted into people with Parkinson's disease survive without pathology for 14 years. *Nat Med* (2008) 14:507–9. doi:10.1038/nm1752
98. Peelaerts W, Bousset L, Van Der Perren A, Moskalyuk A, Pulizzi R, Giugliano M, et al. Alpha-synuclein strains cause distinct synucleinopathies after local and systemic administration. *Nature* (2015) 522:340–4. doi:10.1038/nature14547
99. Bousset L, Pieri L, Ruiz-Arlandis G, Gath J, Jensen PH, Habenstein B, et al. Structural and functional characterization of two alpha-synuclein strains. *Nat Commun* (2013) 4:2575. doi:10.1038/ncomms3575
100. Guo JL, Covell DJ, Daniels JP, Iba M, Stieber A, Zhang B, et al. Distinct alpha-synuclein strains differentially promote tau inclusions in neurons. *Cell* (2013) 154:103–17. doi:10.1016/j.cell.2013.05.057
101. Lee SJ, Masliah E. Neurodegeneration: aggregates feel the strain. *Nature* (2015) 522:296–7. doi:10.1038/nature14526
102. Engelender S, Isacson O. The threshold theory for Parkinson's disease. *Trends Neurosci* (2017) 40:4–14. doi:10.1016/j.tins.2016.10.008
103. Laiwand R, Werman R, Yarom Y. Time course and distribution of motoneuronal loss in the dorsal motor vagal nucleus of guinea pig after cervical vagotomy. *J Comp Neurol* (1987) 256:527–37. doi:10.1002/cne.902560405
104. Lams BE, Isacson O, Sofroniew MV. Loss of transmitter-associated enzyme staining following axotomy does not indicate death of brainstem cholinergic neurons. *Brain Res* (1988) 475:401–6. doi:10.1016/0006-8993(88)90635-X
105. Sofroniew MV, Isacson O. Distribution of degeneration of cholinergic neurons in the septum following axotomy in different portions of the fimbria-fornix: a correlation between degree of cell loss and proximity of neuronal somata to the lesion. *J Chem Neuroanat* (1988) 1:327–37.
106. Engel AK, Kreutzberg GW. Neuronal surface changes in the dorsal vagal motor nucleus of the guinea pig in response to axotomy. *J Comp Neurol* (1988) 275:181–200. doi:10.1002/cne.902750203
107. Fernandez E, Pallini R, Lauretti L, Marchese E, Gangitano C, Del Fa A, et al. Levocarnitine acetyl prevents cell death following long-term section of the vagus nerve in rats. *Int J Clin Pharmacol Res* (1992) 12:289–97.
108. Alexander GE, Delong MR, Strick PL. Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu Rev Neurosci* (1986) 9:357–81. doi:10.1146/annurev.ne.09.030186.002041
109. DeLong MR, Wichmann T. Basal ganglia circuits as targets for neuromodulation in Parkinson disease. *JAMA Neurol* (2015) 72:1354–60. doi:10.1001/jamaneurol.2015.2397
110. Cullen CL, Young KM. How does transcranial magnetic stimulation influence glial cells in the central nervous system? *Front Neural Circuits* (2016) 10:26. doi:10.3389/fncir.2016.00026
111. Booth HDE, Hirst WD, Wade-Martins R. The role of astrocyte dysfunction in Parkinson's disease pathogenesis. *Trends Neurosci* (2017) 40:358–70. doi:10.1016/j.tins.2017.04.001
112. Centonze D, Muzio L, Rossi S, Furlan R, Bernardi G, Martino G. The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. *Cell Death Differ* (2010) 17:1083–91. doi:10.1038/cdd.2009.179
113. Rocha NP, De Miranda AS, Teixeira AL. Insights into neuroinflammation in Parkinson's disease: from biomarkers to anti-inflammatory based therapies. *Biomed Res Int* (2015) 2015:628192. doi:10.1155/2015/628192
114. McGeer PL, McGeer EG. Glial cell reactions in neurodegenerative diseases: pathophysiology and therapeutic interventions. *Alzheimer Dis Assoc Disord* (1998) 12(Suppl 2):S1–6. doi:10.1097/00002093-199803001-00001
115. Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E, Isacson O. Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. *Eur J Neurosci* (2002) 15:991–8. doi:10.1046/j.1460-9568.2002.01938.x
116. McGeer PL, Schwab C, Parent A, Doudet D. Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration. *Ann Neurol* (2003) 54:599–604. doi:10.1002/ana.10728
117. Orr CF, Rowe DB, Mizuno Y, Mori H, Halliday GM. A possible role for humoral immunity in the pathogenesis of Parkinson's disease. *Brain* (2005) 128:2665–74. doi:10.1093/brain/awh625
118. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol* (2010) 119:7–35. doi:10.1007/s00401-009-0619-8
119. Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron* (2016) 89:37–53. doi:10.1016/j.neuron.2015.11.013
120. Tsai MJ, Lee EH. Characterization of L-DOPA transport in cultured rat and mouse astrocytes. *J Neurosci Res* (1996) 43:490–5. doi:10.1002/(SICI)1097-4547(19960215)43:4<490::AID-JNR10>3.0.CO;2-6
121. Inyushin MY, Huertas A, Kucheryavykh YV, Kucheryavykh LY, Tsydzik V, Sanabria P, et al. L-DOPA uptake in astrocytic endfeet enwrapping blood vessels in rat brain. *Parkinsons Dis* (2012) 2012:321406. doi:10.1155/2012/321406
122. Oliva I, Fernandez M, Martin ED. Dopamine release regulation by astrocytes during cerebral ischemia. *Neurobiol Dis* (2013) 58:231–41. doi:10.1016/j.nbd.2013.06.007
123. Asanuma M, Miyazaki I, Murakami S, Diaz-Corrales FJ, Ogawa N. Striatal astrocytes act as a reservoir for L-DOPA. *PLoS One* (2014) 9:e106362. doi:10.1371/journal.pone.0106362
124. Cacace F, Mineo D, Viscomi MT, Latagliata EC, Mancini M, Sasso V, et al. Intermittent theta-burst stimulation rescues dopamine-dependent corticostriatal synaptic plasticity and motor behavior in experimental parkinsonism: possible role of glial activity. *Mov Disord* (2017) 32:1035–46. doi:10.1002/mds.26982
125. Sasso V, Bisicchia E, Latini L, Ghiglieri V, Cacace F, Carola V, et al. Repetitive transcranial magnetic stimulation reduces remote apoptotic cell death and inflammation after focal brain injury. *J Neuroinflammation* (2016) 13:150. doi:10.1186/s12974-016-0616-5
126. Yanamandra K, Gruden MA, Casaite V, Meskys R, Forsgren L, Morozova-Roche LA. Alpha-synuclein reactive antibodies as diagnostic biomarkers in blood sera of Parkinson's disease patients. *PLoS One* (2011) 6:e18513. doi:10.1371/journal.pone.0018513
127. Shalash A, Salama M, Makar M, Roushdy T, Elrassas HH, Mohamed W, et al. Elevated serum alpha-synuclein autoantibodies in patients with Parkinson's disease relative to Alzheimer's disease and controls. *Front Neurol* (2017) 8:720. doi:10.3389/fneur.2017.00720
128. Kuhn I, Rogosch T, Schindler TI, Tackenberg B, Zemlin M, Maier RF, et al. Serum titers of autoantibodies against alpha-synuclein and tau in child- and adulthood. *J Neuroimmunol* (2018) 315:33–9. doi:10.1016/j.jneuroim.2017.12.003
129. Brochard V, Combadiere B, Prigent A, Laouar Y, Perrin A, Beray-Berthat V, et al. Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. *J Clin Invest* (2009) 119:182–92. doi:10.1172/JCI36470
130. Cebrian C, Loike JD, Sulzer D. Neuroinflammation in Parkinson's disease animal models: a cell stress response or a step in neurodegeneration? *Curr Top Behav Neurosci* (2015) 22:237–70. doi:10.1007/7854_2014_356
131. Sulzer D, Alcalay RN, Garretti F, Cote L, Kanter E, Agin-Liebes J, et al. T cells from patients with Parkinson's disease recognize alpha-synuclein peptides. *Nature* (2017) 546:656–61. doi:10.1038/nature22815
132. Eusebi P, Giannandrea D, Biscetti L, Abraha I, Chiasserini D, Orso M, et al. Diagnostic utility of cerebrospinal fluid alpha-synuclein in Parkinson's disease: a systematic review and meta-analysis. *Mov Disord* (2017) 32:1389–400. doi:10.1002/mds.27110
133. Kile BM, Guillot TS, Venton BJ, Wetsel WC, Augustine GJ, Wightman RM. Synapsins differentially control dopamine and serotonin release. *J Neurosci* (2010) 30:9762–70. doi:10.1523/JNEUROSCI.2071-09.2010

134. Porton B, Wetsel WC, Kao HT. Synapsin III: role in neuronal plasticity and disease. *Semin Cell Dev Biol* (2011) 22:416–24. doi:10.1016/j.semcdb.2011.07.007
135. Zaltieri M, Grigoletto J, Longhena F, Navarria L, Favero G, Castrezzati S, et al. Alpha-synuclein and synapsin III cooperatively regulate synaptic function in dopamine neurons. *J Cell Sci* (2015) 128:2231–43. doi:10.1242/jcs.157867
136. Longhena F, Faustini G, Varanita T, Zaltieri M, Porrini V, Tessari I, et al. Synapsin III is a key component of alpha-synuclein fibrils in Lewy bodies of PD brains. *Brain Pathol* (2018). doi:10.1111/bpa.12587
137. Fortin DL, Nemani VM, Voglmaier SM, Anthony MD, Ryan TA, Edwards RH. Neural activity controls the synaptic accumulation of alpha-synuclein. *J Neurosci* (2005) 25:10913–21. doi:10.1523/JNEUROSCI.2922-05.2005
138. Fortin DL, Nemani VM, Nakamura K, Edwards RH. The behavior of alpha-synuclein in neurons. *Mov Disord* (2010) 25(Suppl 1):S21–6. doi:10.1002/mds.22722
139. Kim HY, Cho MK, Kumar A, Maier E, Siebenhaar C, Becker S, et al. Structural properties of pore-forming oligomers of alpha-synuclein. *J Am Chem Soc* (2009) 131:17482–9. doi:10.1021/ja9077599
140. Zakharov SD, Hulleman JD, Dutseva EA, Antonenko YN, Rochet JC, Cramer WA. Helical alpha-synuclein forms highly conductive ion channels. *Biochemistry* (2007) 46:14369–79. doi:10.1021/bi701275p
141. Di Pasquale E, Fantini J, Chahinian H, Maresca M, Taieb N, Yahi N. Altered ion channel formation by the Parkinson's-disease-linked E46K mutant of alpha-synuclein is corrected by GM3 but not by GM1 gangliosides. *J Mol Biol* (2010) 397:202–18. doi:10.1016/j.jmb.2010.01.046
142. Tofaris GK, Garcia-Reitbock P, Humby T, Lambourne SL, O'Connell M, Ghetti B, et al. Pathological changes in dopaminergic nerve cells of the substantia nigra and olfactory bulb in mice transgenic for truncated human alpha-synuclein(1-120): implications for Lewy body disorders. *J Neurosci* (2006) 26:3942–50. doi:10.1523/JNEUROSCI.4965-05.2006
143. Costa C, Sgobio C, Siliquini S, Tozzi A, Tantucci M, Ghiglieri V, et al. Mechanisms underlying the impairment of hippocampal long-term potentiation and memory in experimental Parkinson's disease. *Brain* (2012) 135:1884–99. doi:10.1093/brain/aws101
144. Cheng F, Li X, Li Y, Wang C, Wang T, Liu G, et al. Alpha-synuclein promotes clathrin-mediated NMDA receptor endocytosis and attenuates NMDA-induced dopaminergic cell death. *J Neurochem* (2011) 119:815–25. doi:10.1111/j.1471-4159.2011.07460.x
145. Chen Y, Yang W, Li X, Li X, Yang H, Xu Z, et al. Alpha-synuclein-induced internalization of NMDA receptors in hippocampal neurons is associated with reduced inward current and $Ca^{(2+)}$ influx upon NMDA stimulation. *Neuroscience* (2015) 300:297–306. doi:10.1016/j.neuroscience.2015.05.035
146. Navarria L, Zaltieri M, Longhena F, Spillantini MG, Missale C, Spano P, et al. Alpha-synuclein modulates NR2B-containing NMDA receptors and decreases their levels after rotenone exposure. *Neurochem Int* (2015) 85–86:14–23. doi:10.1016/j.neuint.2015.03.008
147. Yang J, Hertz E, Zhang X, Leinartaite L, Lundius EG, Li J, et al. Overexpression of alpha-synuclein simultaneously increases glutamate NMDA receptor phosphorylation and reduces glucocerebrosidase activity. *Neurosci Lett* (2016) 611:51–8. doi:10.1016/j.neulet.2015.11.023
148. Diogenes MJ, Dias RB, Rombo DM, Vicente Miranda H, Maiolino F, Guerreiro P, et al. Extracellular alpha-synuclein oligomers modulate synaptic transmission and impair LTP via NMDA-receptor activation. *J Neurosci* (2012) 32:11750–62. doi:10.1523/JNEUROSCI.0234-12.2012
149. Ferreira DG, Batalha VL, Vicente Miranda H, Coelho JE, Gomes R, Goncalves FQ, et al. Adenosine A2A receptors modulate alpha-synuclein aggregation and toxicity. *Cereb Cortex* (2015) 27:718–30. doi:10.1093/cercor/bhv268
150. Ferreira DG, Temido-Ferreira M, Miranda HV, Batalha VL, Coelho JE, Szego EM, et al. Alpha-synuclein interacts with PrP(C) to induce cognitive impairment through mGluR5 and NMDAR2B. *Nat Neurosci* (2017) 20:1569–79. doi:10.1038/nn.4648
151. Janezic S, Threlfell S, Dodson PD, Dowie MJ, Taylor TN, Potgieter D, et al. Deficits in dopaminergic transmission precede neuron loss and dysfunction in a new Parkinson model. *Proc Natl Acad Sci U S A* (2013) 110:E4016–25. doi:10.1073/pnas.1309143110
152. Tozzi A, Costa C, Siliquini S, Tantucci M, Picconi B, Kurz A, et al. Mechanisms underlying altered striatal synaptic plasticity in old A53T-alpha synuclein overexpressing mice. *Neurobiol Aging* (2012) 33:1792–9. doi:10.1016/j.neurobiolaging.2011.05.002
153. Tozzi A, De Iure A, Bagetta V, Tantucci M, Durante V, Quiroga-Varela A, et al. Alpha-synuclein produces early behavioral alterations via striatal cholinergic synaptic dysfunction by interacting with GluN2D N-methyl-D-aspartate receptor subunit. *Biol Psychiatry* (2016) 79:402–14. doi:10.1016/j.biopsych.2015.08.013
154. Giordano N, Iemolo A, Mancini M, Cacace F, De Risi M, Latagliata EC, et al. Motor learning and metaplasticity in striatal neurons: relevance for Parkinson's disease. *Brain* (2017) 141(2):505–20. doi:10.1093/brain/awx351
155. Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, et al. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* (2002) 22:2780–91. doi:10.1523/JNEUROSCI.22-07-02780.2002
156. Decressac M, Mattsson B, Bjorklund A. Comparison of the behavioural and histological characteristics of the 6-OHDA and alpha-synuclein rat models of Parkinson's disease. *Exp Neurol* (2012) 235:306–15. doi:10.1016/j.expneurol.2012.02.012
157. Decressac M, Mattsson B, Lundblad M, Weikop P, Bjorklund A. Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of alpha-synuclein in midbrain dopamine neurons. *Neurobiol Dis* (2012) 45:939–53. doi:10.1016/j.nbd.2011.12.013
158. Oliveras-Salva M, Van Der Perren A, Casadei N, Stroobants S, Nuber S, D'hooge R, et al. rAAV2/7 vector-mediated overexpression of alpha-synuclein in mouse substantia nigra induces protein aggregation and progressive dose-dependent neurodegeneration. *Mol Neurodegener* (2013) 8:44. doi:10.1186/1750-1326-8-44
159. Song LK, Ma KL, Yuan YH, Mu Z, Song XY, Niu F, et al. Targeted overexpression of alpha-synuclein by rAAV2/1 vectors induces progressive nigrostriatal degeneration and increases vulnerability to MPTP in mouse. *PLoS One* (2015) 10:e0131281. doi:10.1371/journal.pone.0131281
160. Frazzitta G, Maestri R, Ghilardi MF, Riboldazzi G, Perini M, Bertotti G, et al. Intensive rehabilitation increases BDNF serum levels in parkinsonian patients: a randomized study. *Neurorehabil Neural Repair* (2014) 28:163–8. doi:10.1177/1545968313508474
161. Hou L, Chen W, Liu X, Qiao D, Zhou FM. Exercise-induced neuroprotection of the nigrostriatal dopamine system in Parkinson's disease. *Front Aging Neurosci* (2017) 9:358. doi:10.3389/fnagi.2017.00358

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.

frontiers in Neurology

SYSTEMATIC REVIEW
published: 01 June 2018
doi: 10.3389/fneur.2018.00347

Zebrafish as an Animal Model for Drug Discovery in Parkinson's Disease and Other Movement Disorders: A Systematic Review

Rita L. Vaz[1,2], Tiago F. Outeiro[3,4,5,6] and Joaquim J. Ferreira[7,8,9]**

[1] TechnoPhage, SA, Lisboa, Portugal, [2] Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal, [3] Department of Experimental Neurodegeneration, Center for Nanoscale Microscopy and Molecular Physiology of the Brain, University Medical Center Göttingen, Göttingen, Germany, [4] Department of Experimental Neurodegeneration, Center for Biostructural Imaging of Neurodegeneration, University Medical Center Göttingen, Göttingen, Germany, [5] CEDOC, Chronic Diseases Research Centre, Faculdade de Ciências Médicas, NOVA Medical School, Universidade NOVA de Lisboa, Lisboa, Portugal, [6] The Medical School, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, United Kingdom, [7] Faculdade de Medicina, Instituto de Medicina Molecular, Universidade de Lisboa, Lisboa, Portugal, [8] Laboratory of Clinical Pharmacology and Therapeutics, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal, [9] CNS-Campus Neurológico Sénior, Torres Vedras, Portugal

OPEN ACCESS

Edited by:
Antonio Pisani, Università degli Studi di Roma Tor Vergata, Italy

Reviewed by:
Graziella Madeo, National Institutes of Health (NIH), United States
Alessandro Tozzi, University of Perugia, Italy
Fibrosis Gardoni, Università degli Studi di Milano, Italy

***Correspondence:**
Tiago F. Outeiro
touteir@gwdg.de;
Joaquim J. Ferreira
jferreira@medicina.ulisboa.pt

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *01 February 2018*
Accepted: *30 April 2018*
Published: *01 June 2018*

Citation:
Vaz RL, Outeiro TF and Ferreira JJ (2018) Zebrafish as an Animal Model for Drug Discovery in Parkinson's Disease and Other Movement Disorders: A Systematic Review. Front. Neurol. 9:347. doi: 10.3389/fneur.2018.00347

Movement disorders can be primarily divided into hypokinetic and hyperkinetic. Most of the hypokinetic syndromes are associated with the neurodegenerative disorder Parkinson's disease (PD). By contrast, hyperkinetic syndromes encompass a broader array of diseases, including dystonia, essential tremor, or Huntington's disease. The discovery of effective therapies for these disorders has been challenging and has also involved the development and characterization of accurate animal models for the screening of new drugs. Zebrafish constitutes an alternative vertebrate model for the study of movement disorders. The neuronal circuitries involved in movement in zebrafish are well characterized, and most of the associated molecular mechanisms are highly conserved. Particularly, zebrafish models of PD have contributed to a better understanding of the role of several genes implicated in the disease. Furthermore, zebrafish is a vertebrate model particularly suited for large-scale drug screenings. The relatively small size of zebrafish, optical transparency, and lifecycle, are key characteristics that facilitate the study of multiple compounds at the same time. Several transgenic, knockdown, and mutant zebrafish lines have been generated and characterized. Therefore, it is central to critically analyze these zebrafish lines and understand their suitability as models of movement disorders. Here, we revise the pathogenic mechanisms, phenotypes, and responsiveness to pharmacotherapies of zebrafish lines of the most common movement disorders. A systematic review of the literature was conducted by including all studies reporting the characterization of zebrafish models of the movement disorders selected from five bibliographic databases. A total of 63 studies were analyzed, and the most relevant data within the scope of this review were gathered. The majority (62%) of the studies were focused in the characterization of zebrafish models of PD. Overall, the zebrafish models included display conserved biochemical and neurobehavioral features of the phenomenology in humans. Nevertheless, in light

of what is known for all animal models available, the use of zebrafish as a model for drug discovery requires further optimization. Future technological developments alongside with a deeper understanding of the molecular bases of these disorders should enable the development of novel zebrafish lines that can prove useful for drug discovery for movement disorders.

Keywords: drug discovery, hyperkinesia, hypokinesia, movement disorders, zebrafish models

INTRODUCTION

Movement disorders are a heterogeneous group of neurological conditions characterized by the inability to produce or control movement. The typical clinical features include either paucity of voluntary movements, referred to as hypokinesia, bradykinesia and akinesia, or excess of movement, commonly denoted as hyperkinesia, dyskinesia, and abnormal involuntary movements (1). These two major groups have been dynamic, including different categories over time. Most movement disorders lack effective pharmacological therapies, because their complex etiology and pathological mechanisms remain largely unknown. This complicates the development of adequate animal models and, ultimately, of therapeutic compounds. In this context, zebrafish (*Danio rerio*) (**Figure 1**) has become an attractive tool for drug discovery. Zebrafish presents a compromise between the scalability of invertebrate models and overall homology to vertebrates. In the last 20 years, several zebrafish models of brain disorders have been generated (2). Many discoveries were reported, but an overall analysis of zebrafish as an alternative model of movement disorders is lacking. Therefore, the scope of this review was to systematically analyze the latest developments in the generation and characterization of zebrafish models of the most common movement disorders. This highlights the translational value of zebrafish to model these diseases and, ultimately, for drug discovery. The pathogenic mechanisms, disease hallmarks, phenotypic effects of mutations or neurotoxins, and responsiveness to pharmacological interventions are covered for zebrafish models of two hypokinetic and five hyperkinetic disorders (**Table 1**).

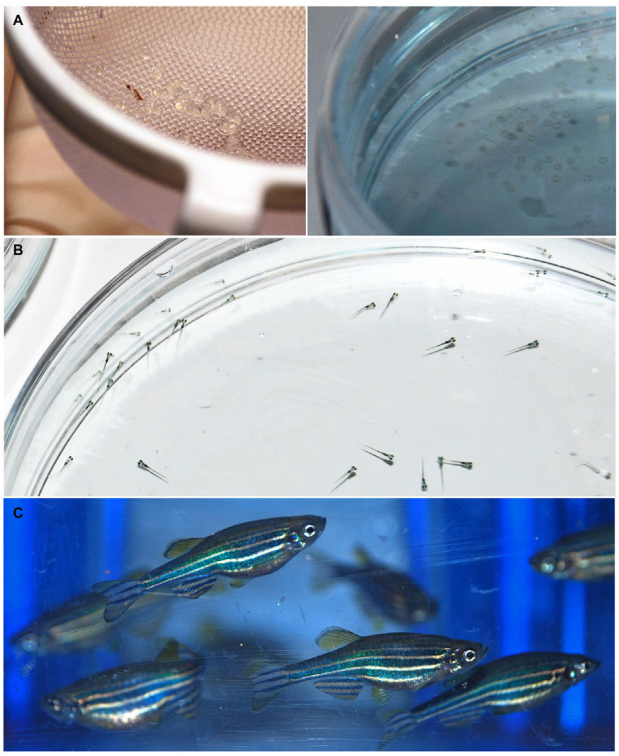


FIGURE 1 | Representative images of zebrafish at embryonic **(A)**, larval **(B)**, and adult **(C)** stage.

Zebrafish as a Model for Translational Research

The utilization of zebrafish for drug discovery increased in the beginning of the twenty-first century (**Figure 2**) (3, 4). Due to its small size and fast reproduction, zebrafish is suitable for large-scale *in vivo* assays. Drug administration is facilitated through the aqueous environment, and the efficacy, bioavailability and toxicity can be readily determined. Importantly, zebrafish is a vertebrate, in contrast to other commonly used organisms, such as *Drosophila melanogaster* or *Caenorhabditis elegans*, in which the anatomical similarity with humans is much lower (5). The optical transparency is another advantage of this teleost, as it enables the direct observation of cellular and physiological processes *in vivo* and in real time. These and other practical features rendered zebrafish the mainstream model for investigation in developmental biology. In addition, it is now also widely used as a disease model and, more recently, it became an important tool for the screening of drugs (**Figure 3**) (6).

Despite the evident differences between fish and mammals, zebrafish hold genomic and physiological homology to humans (7). Moreover, the genome of zebrafish is sequenced and available for annotation in databases. The genome of zebrafish includes orthologs of 71% of human genes, and a high degree of conservation in the functional properties of many of the encoded proteins (8). Physiological and anatomical homology is also evidenced in most of the organs, including the nervous system (7). The basic anatomical structure, the cellular populations, and the chemistry of the zebrafish and human nervous system are evolutionarily conserved. The nervous system of zebrafish is anatomically divided into the fore-, mid-, and hindbrain, including the diencephalon, telencephalon, cerebellum, and spinal cord (2, 6). The blood–brain barrier (BBB) is structurally and functionally similar to that of higher vertebrates and developed by 3 days post fertilization (dpf) (9, 10).

TABLE 1 | Etiology, phenomenology, and pharmacotherapies of the movement disorders covered in this review.

	Disease	Etiology	Pathological hallmarks	Motor symptoms	Pharmacotherapy	Disease-modifying therapy	Reference
Hypokinesias	Parkinson's disease	Environmental factors Genes: SNCA, leucine-rich repeat kinase 2 (LRRK2), DJ-1, VPS35, P1NK1, Parkin Genetic polymorphisms	Dopaminergic cell loss Lewy bodies	Bradykinesia Resting tremor Postural instability Muscular rigidity	**Levodopa** Dopamine agonists Monoamine oxidase inhibitors Catechol-*o*-methyl transferases inhibitors	**None** Isradipine[a] Caffeine[a] Nicotine[a] Inosine[a] Ab and vaccines[a] Nilotinib[a]	(27)
	Progressive supranuclear palsy	MAPT polymorphisms (tau protein) Gene: MAPT Genetic risk factors: STX6, EIF2AK3, and MOBP	Diffuse neuronal loss (cortex, globus, pallidus, subthalamic nucleus, and substantia nigra) Neurofibrillary tangles or neuropil threads (with tau protein) in basal ganglia and brainstem	Unexplained falls Unsteady gait Bradykinesia Ocular motor deficits	**None** Levodopa (few) Taxane (microtubule-stabilizing drug) Tau Ab and vaccines Salsalate (tau acetylation inhibitor)	None	(114)
Hyperkinesias	Dystonia	Environmental factors Genes: TOR1A, THAP1, DYT13, DYT21, (GCH1), (SGCE), (ATP1A3, PRKRA, SLC6A3) Polymorphisms DYT7	Malfunction of basal ganglia Over-excitability of sensorimotor cortex	Excessive, uncontrolled muscle contractions Muscle spasms Abnormal posture	Levodopa (dopa-responsive dystonia) Dopamine agonists (dopa-responsive dystonia) **Anticholinergics** **Antidopaminergics** Clonazepam Acetazolamide Morphine sulfate Sodium oxybate Levetiracetam Zonisamide	None	(118–120)
	Chorea in Huntington's disease	Gene: huntingtin (HTT)	GABAergic MSN loss Intranuclear inclusions of HTT	Chorea Dystonia Bradykinesia Incoordination	**Tetrabenazine** (VMTA2 inhibitor) Haloperidol Fluphenazine Olanzapine	**None** Coenzyme Q10[a] Creatine[a] Dimebon[a] Ethyl eicosapentaenoate (Miraxion)[a] Minocycline[a] Ab and vaccines[a]	(120, 134)
	Stereotypies in Rett syndrome	Gene: MECP2	Reduction of brain volume Abnormally small, densely packed neurons with reduced dendritic complexity and synapse density	Loss of hand skills Gait abnormality Postural instability Hand stereotypies	**None** Glutamate modulators (dextromethorphan and ketamine)[a] Monoamine modulators (desipramine and sarizotan)[a] Neurotrophic factors (fingolimod, mecasermin, and trofinetide)[a] Metabolic factors (lovastatin)[a] Modulators of mitochondrial function (EPI-743 and triheptanoin)[a]	None	(155)

(*Continued*)

TABLE 1 | Continued

Disease	Etiology	Pathological hallmarks	Motor symptoms	Pharmacotherapy	Disease-modifying therapy	Reference
Essential tremor	Unknown genetic and environmental factors	Potentially abnormal cerebellar-thalamic outflow pathways	Progressive active tremor	**Propranolol** (β-adrenergic blocker) **Primidone** (anticonvulsant)	None	(120, 169, 170)
Tics in Tourette's syndrome	Environmental factors Risk genetic polymorphisms	Potentially impaired cortico–striato–thalamocortical circuits	Chronic motortics	**Dopamine receptor blocking drugs** (fluphenazine, haloperidol, and risperidone) **Monoamine-depleting drugs** (tetrabenazine)	None	(172)

[a]*Clinical trails.*
Bold: gold standard pharmacotherapy.

Dissection of the Monoaminergic System in Zebrafish

Specifically, the monoaminergic system is involved in the adjustment of movement and is predominantly conserved in vertebrates (**Figure 4**) (11). The tyrosine hydroxylase (TH) is an important marker of catecholaminergic neurons. Two genes, *th1* and *th2*, encode the TH enzyme in zebrafish, and both proteins are highly similar to the mammalian TH (12). The neuronal populations that express TH1 can be found in the olfactory bulb, telencephalon, diencephalon, locus coeruleus, and caudal lobe (13). The neurons that express TH2 are found in the ventral preoptic region, hypothalamus and colocalize with TH1-positive neurons in the diencephalic dopaminergic cluster. The dopamine transporter (DAT) is also detected in this neuronal population (14). The major difference of the zebrafish catecholaminergic system is the absence of dopaminergic neuronal populations in the midbrain. The diencephalic dopaminergic cluster located in the posterior tuberculum of zebrafish has been suggested to be the functional homolog of substantia nigra in mammals (15). Like in mammals, the noradrenergic population is predominantly located in the locus coeruleus of zebrafish. In turn, the catecholamines, dopamine and noradrenaline, are detectable in zebrafish larvae with 5 dpf (16). Zebrafish encode one monoamine oxidase (MAO) with homology to the human MAO-A and MAO-B, and two putative catechol-*o*-methyl transferases (17, 18). The catecholaminergic receptors and transporters are also conserved among vertebrates. Eight subtypes of the dopamine receptor (17), five alpha-2-adrenergic receptors, and one noradrenaline transporter (19) have been identified in zebrafish so far.

The serotonergic and histaminergic systems of zebrafish also present homologies with the corresponding circuits in mammals. The zebrafish serotonergic neuronal groups can be found in the raphe nuclei, pretectum, posterior paraventricular organ, vagal lobe, and reticular formation (20). Particularly, the serotonergic pretectal and paraventricular neuronal populations are not found in higher vertebrates. Three orthologs of the mammalian serotonin receptors have been identified in the genome of zebrafish, along with two genes, slc6a4a and slc6a4b, that encode serotonin transporters (21, 22). The zebrafish histaminergic system consists of a posterior hypothalamic neuronal cluster, from which all histaminergic projections derive (11). Histamine can be detected at 100 hpf (23), and three histamine receptors have been identified in zebrafish (24).

Overall, the expression of monoaminergic proteins and the spatial distribution of monoaminergic neuronal populations are well characterized. Although zebrafish suffered genome duplication, it seems that the distribution of the duplicated proteins is complementary to that observed in mammals. This helps to explain why, despite the consistent differences, the drugs that target transporters, receptors or enzymes involved in the modulation of neurotransmitters have rather conserved effects (25, 26).

METHODS

The aim of this review was to analyze the studies reported to date on the use of zebrafish models for movement disorders,

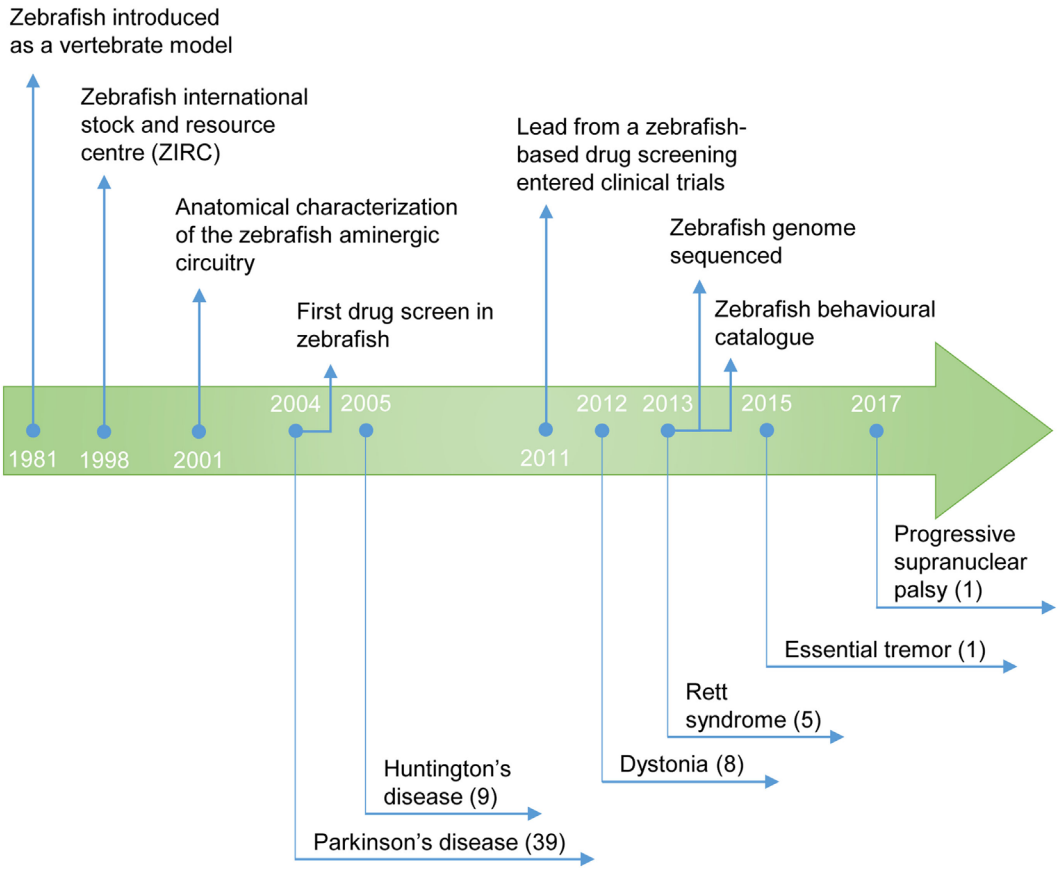


FIGURE 2 | Timeline of the use of zebrafish as a model for the study of movement disorders and drug discovery. The publication year of the first study describing a zebrafish model of the movement disorder is highlighted. (#) Number of studies published to date.

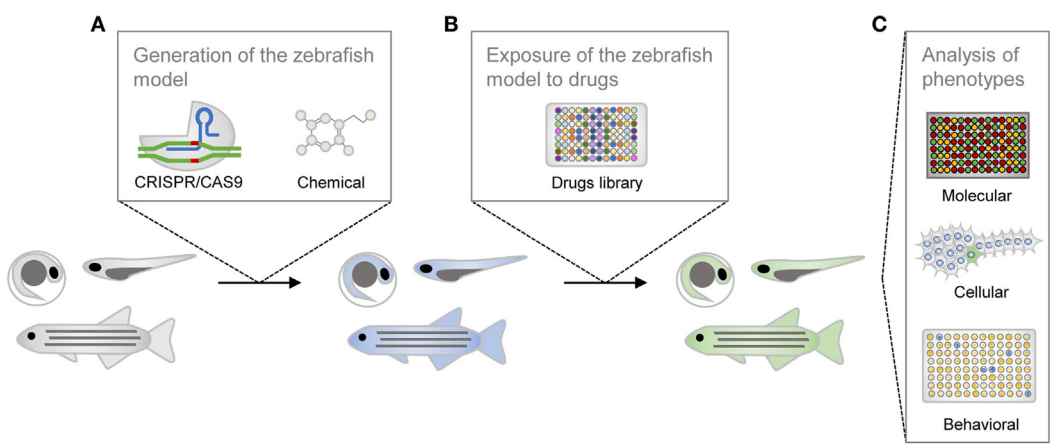


FIGURE 3 | Schematic representation of the drug screening process in zebrafish. The zebrafish line is generated with genetic or chemical tools **(A)**, is incubated with compounds from the library of small molecules **(B)**, and is then phenotypically characterized **(C)**.

and assess their potential for modeling the human pathologies. To achieve this, we conducted a systematic search of the literature using BiomedCentral, EBSCO host, PubMed/Medline, ScienceDirect, and Web of knowledge, in August and September 2017. The following search strategy was used for each of the five bibliographic databases: Title, abstract, keywords, or topic: ("Parkinson's disease" OR "Parkinsons disease" OR "parkinsonism") AND ("zebrafish"); ("progressive supranuclear palsy"

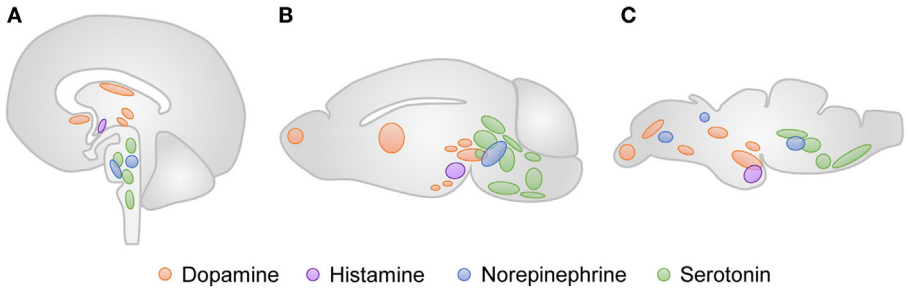


FIGURE 4 | Neuronal clusters that modulate movement in vertebrates. The approximate anatomical location of the dopaminergic (orange), histaminergic (purple), noradrenergic (blue), and serotonergic (green) regions are represented for human **(A)**, rodent **(B)**, and zebrafish **(C)** brains.

OR "supranuclear palsy") AND ("zebrafish"); ("dystonia" OR "dystonic") AND ("zebrafish"); ("Tremor" OR "Tremors") AND ("zebrafish"); ("Tourette's syndrome" OR "Tourettes syndrome") AND ("zebrafish"); ("Huntington's disease" OR "Huntingtons disease") AND ("zebrafish"); ("Rett syndrome" OR "Rett" OR "RTT") AND ("zebrafish"). All dates were included in the search criteria. Only published, peer-reviewed studies written in English were considered. The studies with the description of the phenomenology of zebrafish models of movement disorders were included in the review, through scrutiny of the title and abstract of the papers identified during the systematic search. Studies with no description of the pathological mechanisms AND/OR phenotypes of the diseases were excluded from analysis.

For Parkinson's disease (PD), a total of 39 studies (**Figure 2**) met the inclusion criteria, from 110 returned by BiomedCentral, 111 by EBSCO host, 221 by PubMed/Medline, 4,015 by ScienceDirect, and 181 by Web of knowledge. For progressive supranuclear palsy (PSP), 1 study fulfilled the inclusion criteria, from 8 returned by BiomedCentral, 2 by EBSCO host, 1 by PubMed/Medline, 207 by ScienceDirect, and 2 by Web of knowledge. For dystonia, 8 studies were in agreement with the inclusion criteria, from 15 returned by BiomedCentral, 7 by EBSCO host, 11 by PubMed/Medline, 363 by ScienceDirect, and 15 by Web of knowledge. For tremor, 1 study met the inclusion criteria, from 23 returned by BiomedCentral, 6 by EBSCO host, 8 by PubMed/Medline, 618 by ScienceDirect, and 9 by Web of knowledge. For Tourette's syndrome, no study fulfilled the inclusion criteria, from 7 returned by BiomedCentral, 1 by PubMed/Medline, 245 by ScienceDirect, and 2 by Web of knowledge. For Huntington's disease (HD), 9 studies were in line with the inclusion criteria, from 54 returned by BiomedCentral, 22 by EBSCO host, 31 by PubMed/Medline, 964 by ScienceDirect, and 52 by Web of knowledge. Finally, for Rett syndrome (RTT), 5 studies met the inclusion criteria, from 20 returned by BiomedCentral, 5 by PubMed/Medline, 638 by ScienceDirect, and 13 by Web of knowledge.

ZEBRAFISH AS A MODEL OF HYPOKINETIC MOVEMENT DISORDERS

Parkinson's disease and parkinsonism represent the most frequent hypokinetic syndromes. These include akinesia (inability to initiate voluntary movements), bradykinesia (slowness of voluntary movements), gait and balance disturbances (falls), freezing phenomenon (absence or marked reduction of forward stepping during walking), and rigidity (resistance to externally imposed joint movements).

Parkinson's Disease

Parkinson's disease is the most prevalent movement disorder, affecting 100–200 per 100,000 people (27). The etiology of PD is a combination of genetic and environmental factors that, at some point during disease progression, lead to dopaminergic cell loss in the substantia nigra pars compacta and to the accumulation of protein inclusions known as Lewy bodies. These inclusions are primarily composed of the protein α-synuclein (**Table 1**). Mutations in the alpha-synuclein (*snca*), leucine-rich repeat kinase 2 (*lrrk2*), *vps35*, PTEN induced putative kinase 1 (*pink1*), parkinsonism associated deglycase (*dj-1*), and parkin RBR E3 ubiquitin protein ligase (*parkin*) are associated with familial cases. However, the vast majority of the cases (~90–95%) are affected by sporadic PD. The treatment of motor symptoms aims at replacing dopamine and includes levodopa, among other dopaminergic modulators (**Table 1**). There are still no disease-modifying agents for PD, but several drugs are under clinical trials.

Chemical Zebrafish Models of PD

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)-Induced Models

Parkinson's disease is the most studied movement disorder in zebrafish (**Table 2**). The effects of exposure to MPTP, known to cause loss of dopaminergic neurons and parkinsonism in humans (28), have been studied in zebrafish at all developmental stages (embryonic, larval, and adulthood). MPTP causes specific loss of dopaminergic neurons, a decrease of the dopamine, norepinephrine, and serotonin levels, and motility impairments in zebrafish larvae (29–32). By labeling monoaminergic neurons with GFP, Wen et al. (33) showed that the toxic effects of MPTP are more severe in the dopaminergic neurons located in the posterior tuberculum of the ventral diencephalon, corresponding to the mammalian midbrain dopaminergic neurons (33). Other neuronal clusters, including the serotonergic, also seem to be affected, but in less extent (32). By contrast, MPP+, the metabolite of

TABLE 2 | General characteristics of the zebrafish models of hypokinetic movement disorders reviewed.

Disease	Zebrafish model	Pathological hallmarks	Motor phenotype	Responsiveness to pharmacotherapies and disease-modifying therapies	Observations	Reference
Parkinson's disease	Chemical					
	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine	Decrease of dopamine levels Loss of dopaminergic neurons	Deficits in evoked swimming response (bradykinesia) Decrease in total distance moved and swimming velocity (bradykinesia) Increase in number and duration of freezing episodes (dyskinesia)	Selegiline rescues neuronal and motor impairments		(29–33, 35–37)
	6-Hydroxydopamine	Decrease of dopamine levels Loss of dopaminergic neurons	Decrease in total distance moved and swimming velocity (bradykinesia)	Levodopa + carbidopa rescue motor impairments		(36, 40, 43)
	Paraquat	Decrease of dopamine levels	Decrease in total distance moved (bradykinesia)	ND	Only at doses higher than LC_{50} Variable toxic effects	(48, 49, 55, 56)
	Rotenone	Decrease of dopamine levels	Decrease in time swimming at high velocity (bradykinesia)	ND		(59)
	Cytotoxic metabolite of metronidazole	Decrease of dopamine levels Loss of dopaminergic neurons	Decrease in total distance moved and swimming velocity (bradykinesia) Increase in resting time and decrease in active time	ND	Metabolization driven by nitroreductase, expressed under the control of *dat* promoter	(64)
	Titanium dioxide nanoparticles	Loss of dopaminergic neurons	Decrease in total distance moved (bradykinesia)	ND		(65)
	Ziram	Loss of dopaminergic neurons	Decrease in total distance moved (bradykinesia)	Apomorphine rescues motor impairments		(74)
	Genetic					
	β- or γ1-Synucleins knockdown	Decrease of dopamine levels Delayed development of dopaminergic neurons	Decrease in swimming velocity (bradykinesia)	ND		(69)
	γ1-Synuclein overexpression	Synuclein aggregates	ND	ND	Developmental defects	(74)
	Human α-synuclein overexpression	Synuclein aggregates	ND	ND	100% Lethal at 10 days post fertilization	(75)
	Pink1 knockdown	Loss of dopaminergic neurons	Deficits in evoked swimming response (bradykinesia) Decrease in total distance moved (bradykinesia)	ND	Developmental defects	(76, 77, 82)
	Parkin knockdown	Loss of dopaminergic neurons	No changes in total distance moved	ND		(87)
	DJ-1 knockdown	No loss of dopaminergic neurons	ND	ND		(97)

(*Continued*)

TABLE 2 | Continued

Disease	Zebrafish model	Pathological hallmarks	Motor phenotype	Responsiveness to pharmacotherapies and disease-modifying therapies	Observations	Reference
	ΔWD40-LRRK2	Loss of dopaminergic neurons	Decrease in total distance moved (bradykinesia)	Levodopa rescues motor impairments		(101)
	LRRK2 knockdown	Synuclein aggregates Loss of dopaminergic neurons	ND	ND	Developmental defects	(102)
	FBXO7 knockdown	Loss of dopaminergic neurons	Decrease in swimming velocity (bradykinesia)	Apomorphine rescues motor impairments	Developmental defects	(108)
	ATP13A2 knockdown	ND	Decrease in swimming velocity (bradykinesia)	ND	Developmental defects	(109)
Progressive supranuclear palsy (PSP)	A152T-tau overexpression	Increased accumulation of tau and neurofibrillary tangle formation Neuronal death	Deficits in evoked swimming response (bradykinesia)	ND		(115)

ND, not determined.

MPTP, does not affect the serotonergic cluster, indicating a more specific action (32). MPP+ inhibits the multi-subunit enzyme complex I of the mitochondrial electron chain and impairs mitochondrial respiration (34). The subacute exposure to a low dose of MPP+ causes dramatic retrograde mitochondrial transport, before the appearance of neuronal and locomotor changes in zebrafish larvae (35). This suggests that MPP+ damages the mitochondria that are transported back to the cell body for degradation. This mechanism of bioenergetic homeostasis seems to be impaired at higher concentrations of MPP+. The MPTP-induced parkinsonian-like phenotype can be reverted by L-deprenyl (selegiline), an MAO-B inhibitor, and nomifensine, a DAT inhibitor (29, 30, 32). This indicates that the transport of MPTP and its conversion into the active metabolite, MPP+, is mediated by the same mechanisms in zebrafish and in mammals (34). Although selegiline is used as an anti-parkinsonian agent, its restorative effects in MPTP-lesioned zebrafish do not totally mimic the therapeutic effects in PD patients.

In adult zebrafish, MPTP induces a reduction of the levels of dopamine and norepinephrine, which results in marked decrease of the motor performance (31, 36). MPTP-lesioned adult zebrafish exhibit a significant decrease of the swimming velocity (bradykinesia), an erratic swimming pattern and increase of the freezing episodes (dyskinesia) (37). Strikingly, there is no reduction in the number of dopaminergic neurons, neither the activation of pathways that lead to cell death (31, 36). In mice, the activity of the TH decreases after exposure to MPTP (38). This could in part explain the loss of dopamine and norepinephrine in zebrafish, despite the lack of cellular death. In turn, MPTP could cause a transient loss of function of the dopaminergic neurons, instead of its death. A proteomic analysis in MPTP-lesioned zebrafish revealed altered transcriptional regulation of several genes, including *lrrk2*, *dj-1*, *park2*, and *pink1*. In addition, the expression of 73 proteins, some of which associated with neurological pathways, was also changed (37). For instance, the neurofilament light polypeptide-like (NEFL) protein, involved in the glutamatergic and GABAergic signaling in presynaptic nerve terminals, was found downregulated in the brain of MPTP-lesioned zebrafish. As demonstrated in zebrafish larvae, selegiline induces recovery of the PD-like symptoms in MPTP-lesioned adult zebrafish (37). On the other hand, adult zebrafish submitted to an MPP+ injection do not exhibit abnormal phenotype, in contrast to zebrafish larvae (31). MPP+ cannot cross the mammalian BBB (39), suggesting that in adult zebrafish the mature BBB prevents the entry of MMP+ into the central nervous system (CNS). The differences observed in the several developmental stages could result from a lower sensitivity of adult zebrafish to the neurotoxin. The access of MPTP to the brain is very different in each of the developmental stages, which in part could result from the distinct routes of administration adopted. Larvae were exposed to the neurotoxin through the water, because of its extreme permeability, whereas, adults received a single intramuscular or intraperitoneal injection.

6-Hydroxydopamine (6-OHDA)-Induced Models

6-Hydroxydopamine is a hydroxylated compound of dopamine that has been extensively used to induce dopaminergic lesions

in rodents (34). Intramuscular injection of 6-OHDA causes a decrease of the dopamine and norepinephrine levels in adult zebrafish (36). Motor impairments are also observed, despite the lack of loss of dopaminergic neurons. By contrast, when administered into the ventral diencephalon of adult zebrafish, 6-OHDA induces significant ablation (>85%) of dopaminergic neurons in the posterior tuberculum and two other dopaminergic clusters, causing bradykinesia (40). The neuronal population in the olfactory bulb is one of the clusters affected. This mimics the phenotype in rats that exhibit olfactory impairments when lesioned with 6-OHDA into the substantia nigra (41). The inability to cross the BBB, explains the different phenotypes observed when 6-OHDA is administered intramuscularly or intracranially in zebrafish. A recovery of the dopaminergic neurons was observed 30 days post-lesion, which can be attributed to the neuro-regenerative capacity of the adult zebrafish brain (42). Therefore, the highly regenerative nature of zebrafish compromises the study of the progressive degenerative process in PD. On the other hand, the intracerebral administration of 6-OHDA revealed to be a laborious and meticulous protocol.

In the zebrafish larval stage, exposure to 6-OHDA in the water induces a decrease in the expression of TH, reduction in the locomotor activity and anxiogenic behavior (43). The locomotor impairments can be rescued by vitamin E, minocycline, and levodopa + carbidopa, the most effective drug used in patients with PD. Vitamin E is also able to normalize the expression of TH, while minocycline attenuates the increase of the expression of TNF-α and *cd11b* mRNA in 6-OHDA-lesioned zebrafish larvae. Vitamin E has antioxidant properties (44) and minocycline has shown anti-neuroinflammatory activity in rodents (45). This suggests that the oxidative stress and inflammatory process induced by 6-OHDA in zebrafish larvae share functional features with mammals.

Paraquat-Induced Models

Chronic exposure to pesticides, used in agriculture, has been recognized as a risk factor for development of parkinsonian syndromes. Paraquat induces oxidative stress and cytotoxicity in neurons (46). This herbicide is structurally similar to MPP+ and is associated with increased risk of developing PD (47). In adult zebrafish, the systemic administration of paraquat causes locomotor changes, but no anxiety-like behavior (48). In a different study, anxiolytic and aggressive behavior were observed, but no motor impairments in paraquat-lesioned adult zebrafish (49). While the former mimics the locomotor impairments, the later resembles the anxiogenic behavior in paraquat-lesioned rodents (50–52). The authors suggested that the results may be influenced by the genotype or gender of fish. Adult zebrafish lesioned with paraquat also reveal impairments in spatial memory, a decrease in the ratio of DOPAC/dopamine levels, a decrease in the expression of DAT, lowered mitochondrial viability, and an increase in the expression of antioxidant enzymes (48, 49). TH expression and reactive oxygen species (ROS) levels were unaltered. Despite the lack of cellular hallmarks of PD, the neurobehavioral syndrome in zebrafish is very similar to the one observed in paraquat-lesioned rodents (50, 51, 53). The neurotoxic effects of paraquat in this model are also variable (50, 51, 54).

Alternatively, when added to the water, paraquat seems to induce no parkinsonian-like phenotypes in larvae and adult zebrafish. These exhibit a normal number of dopaminergic neurons, normal behavior, and no developmental defects (31). Instead, when the LC_{50} of paraquat is used, zebrafish larvae exhibit a reduction of the dopamine and serotonin levels, activation of antioxidant and oxidative stress related genes, and distinct macrophage activation and migration (55, 56). In addition, significant increase of apoptotic cells in the head, trunk, and tail, and motor deficits are observed. However, the authors did not rule out general toxicity, leaving doubts about the specificity of the phenotypes observed. In addition, the toxic effects of paraquat seem to have high variability. Zebrafish larvae with 48 hpf exposed to 600 mg/L of paraquat do not show morphological defects, while 0.04 and 100 mg/L of paraquat were determined as LC50 in zebrafish larvae with 18 and 72 hpf, respectively. The successful induction of dopaminergic neuronal death by pesticides in rodents has also been striking. While paraquat is associated with high variability (57), rotenone induces weakly reproducible phenotypes and high mortality rates (58).

Rotenone-Induced Models

Exposure to rotenone has been linked to a higher risk of PD (47). In zebrafish, the phenotypes induced by rotenone are incongruent. One study found no cellular or behavioral parkinsonian-like phenotypes in larvae and adult zebrafish exposed to rotenone (31). By contrast, using the same concentration of the pesticide, time of exposure, and route of administration, a different study reported that adult zebrafish have decreased levels of dopamine and TH, deficits in motor function, anxiety and depression-like behavior, and olfactory dysfunction (59). Differences in TH expression and motor performance could be justified by the different protocols used to determine each of the parameters. In one study, the authors determined mRNA expression of TH by *in situ* hybridization and locomotor performance through the mean velocity of swimming. In the other, the authors analyzed TH expression by western blot and motor capacity through monitorization of freezing, swimming at low speed, and swimming at high speed. The difference between rotenone and vehicle treated zebrafish was detectable only at high speed swimming. The phenotypes observed are characteristic of PD patients (60, 61), and some mirror the phenotypes found in rodents (62, 63).

Other Neurotoxic Agents

Other less conventional methods have been used to induce dopaminergic neurotoxicity in zebrafish. This is the case of the transgenic line that expresses the reporter cyan fluorescent protein and the nitroreductase enzyme under the control of the *dat* promoter [Tg(dat:CFP-NTR)]. When transgenic larvae are exposed to metronidazole, the nitroreductase metabolizes it into a cytotoxic product that activates the apoptotic pathway and induces dopaminergic cell loss. The process can be monitored in real time, by detection of the reporter protein. Tg(dat:CFP-NTR) zebrafish larvae exposed to metronidazole show a reduction of the number of neurons in several dopaminergic clusters. This

coincides with a decrease of dopamine levels and the appearance of locomotor impairments (64). The zebrafish line seems to maintain a persistent decrease of dopaminergic neurons that lasts longer than the other toxin-induced models.

Alternatively, a recent study showed that titanium dioxide nanoparticles (TiO_2 NPs) cause parkinsonian-like phenotypes in zebrafish larvae (65). Exposure to TiO_2 NPs induces premature hatching and abnormal development, but no lethality. TiO_2 NPs accumulate in the brain of zebrafish larvae, resulting in the generation of ROS, loss of dopaminergic neurons, cell death in the hypothalamus and locomotor impairments. An increase in the expression of *pink1*, *parkin*, and *uchl1* genes was also observed. Surprisingly, the authors also described an increase of the expression of the *α-synuclein* gene. Since zebrafish lack the ortholog of the human α-synuclein, the authors must have wanted to refer to the other two synucleins expressed in zebrafish (66). In rats, TiO_2 NPs accumulate in the brain, stimulate oxidative stress and inflammatory responses, and cause impairments in the CNS (67). Despite the similarities of the phenotypes observed in zebrafish with the molecular and cellular mechanisms in PD, there is still no association of TiO_2 NPs with increased risk of PD.

Genetic Zebrafish Models of PD

Synucleins

Among the several zebrafish genes with homology to human PD genes, an ortholog of the human α-synuclein appears not to be present in the zebrafish genome (68). Instead, zebrafish express three synuclein isoforms, β-, γ1-, and γ2-synucleins, that seem to compensate the absence of α-synuclein. Functionally, the zebrafish γ1-synuclein appears to be the closest to the human α-synuclein. Knockdown of the β- or γ1-synucleins induces motor impairments in zebrafish, which are even more severe when the expression of both synucleins is abrogated (69). Zebrafish lacking both synucleins have an abnormal development of the dopaminergic system, including delayed differentiation of dopaminergic neurons and reduced levels of dopamine. The phenotype can be reverted by the expression of human α-synuclein. Strikingly, the knockdown of the zebrafish β- and γ1-synucleins results in phenotypes that recapitulate the aspects observed in rodents lacking all synucleins (70–73). In zebrafish, overexpression of γ1-synuclein leads to the formation of neuronal aggregates and neurotoxicity, similarly to the human α-synuclein (74). On the other hand, downregulation of γ1-synuclein protects zebrafish from the toxicity of ziram. Exposure to ziram dramatically increases the risk to develop PD. This pesticide causes loss of dopaminergic neurons and impaired swimming behavior in zebrafish (74). Treatment with apomorphin recues the motor impairments. Moreover, CLR01, an inhibitor of amyloidogenic proteins self-assembly, protects zebrafish against ziram-induced neurotoxicity. These data suggest that ziram might induce toxicity on dopaminergic neurons through the formation of γ1-synuclein toxic oligomers. Still, none of the above zebrafish lines exhibit as a severe phenotype as zebrafish overexpressing human α-synuclein. During embryonic development, this line presents neuronal apoptosis, which results in severe deformities and death within 48–72 h (75). The neurotoxic effect of α-synuclein is mediated by the inhibition of the ubiquitin proteasome system and accumulation of α-synuclein. Treatment with CLR01 reduces the aggregation of α-synuclein and neuronal apoptosis, increasing viability. The devastating effects of the overexpression of human α-synuclein may hinder the successful generation of transgenic zebrafish lines. Perhaps, by restricting the expression of the protein to the dopaminergic neurons or to a transient manner could decrease the lethality, while maintaining the pathological mechanisms.

PTEN Induced Putative Kinase 1 (PINK1)

The gene *PINK1* is implicated in genetic and sporadic cases of PD. Morpholino knockdown of the zebrafish PINK1 ortholog has added evidence to the importance of this gene in the control of oxidative stress and mitochondrial function. Downregulation or total abrogation of the expression of PINK1 results in mitochondrial dysfunction that leads to augmented levels of ROS and activation of the apoptotic signaling pathway in zebrafish (76). The PINK1 null mutant zebrafish line also presents mitochondrial impairments (77). The zebrafish Pink1 influences the expression of other proteins that are critical contributors to the pathogenic process. For instance, the activity of the mitochondrial protein GSK3β is increased and its inhibition, with LiCl and SB216763, partially rescues the phenotypes in PINK1 morphant zebrafish (76). TigarB, the zebrafish ortholog of the human glycolysis and apoptosis regulator Tigar, is also markedly increased in Pink1 null mutants (77). Tigar has been identified as a negative regulator of mitophagy, considered to be crucial in the pathogenesis of early-onset PD (78). The expression of other 177 genes, from the hypoxia-inducible factor (HIF) signaling, TGFβ-signaling, and several key toxicological responses (mitochondrial dysfunction, RAR activation, and biogenesis of mitochondria), is also altered (79). Particularly, the HIF pathway is the most affected pathway in PINK1 knockdown zebrafish. This is known to participate in the regulation of oxidative stress and neuronal differentiation *in vitro* (80, 81).

Whereas, the molecular mechanisms seem to be consistent, the phenotypes induced by the alteration of Pink1 expression in zebrafish vary. PINK1 morphant zebrafish exhibit general developmental delay, severe mispatterning of the axonal scaffold, and moderate decrease of the number of neurons, mainly in the dopaminergic system (76). The phenotype could be rescued by wild-type human *pink1* mRNA. Further supporting these results, PINK1 knockdown zebrafish show changes in neuronal patterning and axonal projections (82). This line also presents mild loss of dopaminergic neurons in the diencephalon, which leads to spontaneous or evoked locomotor impairments. Motor performance could be rescued by the dopamine agonist SFK-38393. Moreover, the expression of exogenous PINK1 rescued all phenotypes. By contrast, a subsequent study reported no morphological or behavioral deficits in PINK1 morphant zebrafish (83). Instead, increased vulnerability to MPTP-induced toxicity was observed. The authors described a reduction in the expression of both, *th1* and *th2* mRNA forms, but normal levels of *dat* mRNA. Although there

was a mild decrease in the number of TH-positive neurons in the dopaminergic diencephalic cluster, the normal levels of *dat* suggest that the downregulation of PINK1 may cause a decline in important mRNAs and proteins, instead of neuronal death. Still, the increased susceptibility to MPTP strengthens the importance of PINK1 in oxidative stress. In accordance, the exposure to H_2O_2 dramatically increases the expression of *pink1* mRNA in zebrafish, which can be reverted by the antioxidant L-glutathione reduced (84). In *PINK1* mutant zebrafish larvae, the loss of dopaminergic neurons is more evident and accompanied by marked microglial activation (77). In fact, PINK1 deficiency causes different phenotypes in mammals, as well. In humans, mutations *in PINK1* can result in early-onset PD and are associated with mitochondrial dysfunction (85). In turn, *PINK1* knockout mice have a mild phenotype, with no neuronal death, no changes in the levels of striatal dopamine, nor in the number and morphology of mitochondria (86). Interestingly, it seems that PINK1 knockdown zebrafish recapitulate better than mice the human phenotypes.

Parkin RBR E3 Ubiquitin Protein Ligase (Parkin)

Mutations in PINK1 and Parkin are implicated in mitochondrial dysfunction and seem to share several pathogenic mechanisms in PD. Consistently, Parkin knockdown zebrafish exhibit a phenotype that resembles PINK1 knockdown zebrafish. Abrogation of the expression of Parkin leads to impaired mitochondrial function, specific loss of dopaminergic neurons in the posterior tuberculum, and increased sensitivity to the toxic effects of MPP+ (87). Neither the serotonergic nor the motor neurons are affected, and the extent of dopaminergic loss is not enough to cause behavioral defects. Parkin knockdown zebrafish present two important phenotypes, mitochondrial dysfunction and dopaminergic cells loss, described in PD patients with mutations in *Parkin* (88, 89). Once again, it seems that the zebrafish model mimics better than mice models the pathological mechanisms in humans. *Parkin* knockout mice do not show any robust morphological changes, neither increased susceptibility to MPP+ (90, 91). Notwithstanding, morpholino-mediated knockdown of the zebrafish *Parkin* generates very dissimilar phenotypes. In a different study, no loss of dopaminergic neurons, neither morphological nor behavioral alterations, was observed upon *Parkin* knockdown in zebrafish (92). This result may be the consequence of a partial ablation (around 50%) of Parkin expression. Still, this line maintained the increased vulnerability to stress-induced cell death. Importantly, the authors also described that the overexpression of Parkin in a transgenic zebrafish line protects from proteotoxic stress-induced cell death. Similarly to PINK1, Parkin is suggested to have a protective role in PD (93). In zebrafish, it seems that the presenilin-associated rhomboid-like (PARL) protein is also part of the PINK1 and Parkin pathway. PARL is a component of the mitochondrial membrane involved in mitochondrial morphology and apoptosis. Morpholino knockdown of both zebrafish paralogs, *parla* and *parlb*, results in high mortality, whereas loss of PARLb leads to the mildest phenotype (94). Loss of one of the PARL's results in mild neurodegeneration and disarranged dopaminergic neurons. Although changes in survival were not reported, generalized cell death was observed. Interestingly, the phenotype can be rescued by human *parl* mRNA and by zebrafish and human *pink1* mRNA. The *PARL* gene has been linked to familial cases of PD. PARL is suggested to be important in the normal trafficking and processing of PINK1 and Parkin in mitochondria (95).

DJ-1

Mutations in *DJ-1* are associated with early-onset PD. The inactivation of DJ-1 in zebrafish leads to an increase in the expression of p53 and Bax, but no cellular or morphological changes (96, 97). Moreover, the concomitant knockdown of *DJ-1* and *mdm2*, a negative regulator of p53, results in dopaminergic neuronal death (96). This suggests that p53 may mediate cell loss in the absence of DJ-1. DJ-1 knockdown zebrafish exhibit loss of dopaminergic neurons after exposure to H_2O_2 and to the proteasome inhibitor MG132. The phenotype can be prevented with pharmacological inhibition of p53, by pifithrin-alpha (96, 97). This demonstrates that DJ-1 knockdown zebrafish are susceptible to programmed cell death and that DJ-1 may mediate the stress response machinery. In accordance, DJ-1-deficient mice only exhibit dopaminergic cell death after toxin exposure (98). The p53-glycerylaldehyde-3-phosphate dehydrogenase (GAPDH)–Bax pathway has been suggested to be involved in PD (99, 100).

Leucine-Rich Repeat Kinase 2 (LRRK2)

The phenotypes of LRRK2 morphant zebrafish have been characterized. The first study describing the consequences of the inhibition of the expression of LRRK2, showed embryonic lethality and severe developmental defects, such as brain developmental retardation, in zebrafish (101). A more recent study, described neuronal loss, affecting the dopaminergic system, upregulation of the expression of β-*synuclein*, *Park13*, and *SOD1*, and β-synuclein aggregation in the CNS (102). The authors also described a wide range of organ abnormalities but did not report such overt toxicity as the former study. On the other hand, the deletion of the WD40 domain of LRRK2 (ΔWD40-LRRK2) causes little impact on embryonic development, in zebrafish (101). Instead, this mutant zebrafish line presents a reduction and disorganization of the axonal tracts, predominantly in the midbrain. In addition, significant loss of the diencephalic dopaminergic neurons and locomotor defects were observed. The phenotype can be rescued by zebrafish and human *lrrk2* mRNA overexpression. The administration of levodopa rescues the motor impairments, but not neurodegeneration, in line with the therapeutic effects in humans. Surprisingly, a subsequent study was not able to replicate the phenotypes described in the ΔWD40-LRRK2 zebrafish line (103). Nevertheless, it has been reported that mutations in the LRRK2-WD40 domain increase neuronal apoptosis under cellular stress (104). In mice, *LRRK2* knockout and G2019S *LRRK2* transgenesis do not induce neuropathological abnormalities, but LRRK2 seems to interfere with normal neurite outgrowth (105, 106).

Other PD Genes

Genes implicated in atypical PD, have also been characterized in zebrafish. Park15 is caused by loss of function of the protein encoded by the gene *fbxo7* (107). Morpholino knockdown of the zebrafish Fbxo7 ortholog results in abnormal patterning and loss of dopaminergic neurons, which lead to severe motor impairments (108). Treatment with apomorphine, a dopamine agonist, can revert the locomotor defects. Surprisingly, the human *fbxo7* mRNA failed to rescue the morphological phenotypes. This observation was justified with an atypical timing and localization of expression, as compared with the expression of the Fbxo7 endogenous zebrafish gene. Fbxo7 morphant zebrafish also exhibit developmental defects, as heart deformations, suggesting that Fbxo7 must have an important role in zebrafish development. The zebrafish ortholog of another gene linked to atypical PD, *atp13a2*, has also shown a crucial role during embryonic development. Complete abrogation of the expression of ATP13A2 leads to embryonic lethality, whereas partial knockdown results in abnormal splicing of *atp13a2* mRNA and obvious behavioral impairments (109). The results are in line with experiments in mice, which have also revealed the importance of ATP13A2 during the early stages of embryonic development and neurogenesis (110).

Effect of Dopaminergic Modulators in Zebrafish

When assessing the validity of an animal model, beyond the pathological hallmarks of the disease, it is important to explore the pharmacologically evoked changes. Studies that report such experiments in zebrafish models of PD are scarce, but there exist some reports exploring the effects of drugs known to modulate movement on healthy zebrafish. For instance, haloperidol and chlorpromazine, two dopamine receptor antagonists, have been tested on zebrafish larvae. The suppression of the dopaminergic signaling by both compounds induces akinetic-like behavior (111). Another study supporting these data showed that the selective dopamine agonists, SFK-38393 and quinpirole, increase motor activity (112). By contrast, the dopamine antagonists, SCH-23390 and haloperidol, decrease motor activity in zebrafish larvae. Interestingly, the non-selective dopamine agonist, apomorphine, and dopamine antagonist, butaclamol, induce biphasic dose-response patterns. This may be attributed to the action of the drugs on multiple dopaminergic receptors. On the other hand, the dopamine antagonists, SCH-23390 and haloperidol, induce different dose-response profiles dependent on the lighting conditions. The authors suggested that the blockade of dopamine receptors in the retinal ganglion cells may have perturbed the adaptation to light/dark conditions. Alternatively, the effects of haloperidol were studied in catalepsy (muscular rigidity). Similarly to rats, it was observed that haloperidol causes the increase of catalepsy in zebrafish (113). This can be reverted by bromocriptine and pramipexole, two dopamine agonists commonly used to improve rigidity. Importantly, this study introduced a new core motor symptom of PD on zebrafish, in alternative to bradykinesia. All the above-mentioned studies demonstrated that the drugs that target the dopaminergic system in mammals elicit similar outcomes in zebrafish, suggesting that the underlying mechanisms that regulate movement are shared by both models. Nevertheless, the demonstration that the phenotypes observed in zebrafish actually result from modulation of the dopaminergic pathway is needed.

Overall, chemical and genetic zebrafish models of PD reproduce several of the biochemical, neurochemical, morphological, and neurobehavioral features of the disease in humans. Importantly, the pharmacological response to drugs used in the clinic is also conserved. The limitations inherent to each model do not seem to surpass the limitations also described in rodents and, in some cases, zebrafish resemble better than rodents, the human features. Finally, the zebrafish genes orthologs to the human genes associated with PD seem to be particularly conserved in terms of sequence and function, as well as, the role of the respective protein in the cellular pathways.

Other Parkinsonian Syndromes

Progressive supranuclear palsy is a PD-plus syndrome associated with tau neuropathology, which affects about 5–7 per 100,000 people (114). The neuropathological hallmarks include the presence of neurofibrillary tangles (insoluble 4-repeat tau protein) or neuropil threads in the basal ganglia and brainstem. Neuronal loss is diffuse, affecting different neuronal structures (**Table 1**). Initially, the clinical presentation is heterogeneous but tends to develop to unsteady gait, bradykinesia, unexplained falls, and ocular motor deficits (vertical supranuclear gaze palsy is used to confirm the diagnose). Most of the cases of PSP are sporadic and associated with polymorphisms in the gene that encodes the tau protein, *MAPT*. Mutations in the *MAPT* gene have been identified in several familial cases, as well, but are rarer.

Recently, zebrafish was used to assess the functional and biochemical consequences of a tau variant, p.A152T, identified to increase the risk of PSP in a cohort study (115). The A152T-tau transgenic zebrafish exhibit increased accumulation and phosphorylation of tau, with formation of neurofibrillary tangles (**Table 2**). The phenotype possibly results from impairments in the proteasome system. The overexpression of A152T-tau also causes neurodegeneration, associated with behavioral deficits in zebrafish. The phenomenology is compatible with the phenotypes observed in A152T transgenic rodent models (116, 117). The use of zebrafish to model PSP is at its beginning and needs further developments. Nevertheless, the data described so far indicate that zebrafish models of PSP may exhibit several neuropathological hallmarks of the disease. Effective pharmacotherapeutic options for PSP are null at the moment, and zebrafish may help to boost the discovery of new drugs.

ZEBRAFISH AS A MODEL OF HYPERKINETIC MOVEMENT DISORDERS

Hyperkinetic movement disorders have a more diverse phenomenology and include tremors, dystonia (sustained, repetitive, and patterned muscle contractions), tics (sudden, rapid, repetitive, and non-rhythmic movements), chorea (brief, irregular, abrupt,

and non-repetitive movements), and stereotypies (repetitive or ritualistic movements), among others.

Dystonia

Dystonia is a common and clinically heterogeneous disorder, which can be manifested as an isolated clinical condition (primary dystonias) or associated with other neurological disorders (secondary dystonias) (118). The cause is diverse and includes genetic and environmental factors (**Table 1**). This phenomenon is believed to result from malfunction of the basal ganglia and, consequently, abnormal plasticity of the sensorimotor cortex (119). The pharmacotherapy for dystonia is solely symptomatic, mostly empirical, and adapted to each case (**Table 1**) (120). In the last 5 years, zebrafish have been used to understand the mechanisms of dystonia.

Genetic Zebrafish Models of Dystonia

The most common cause of early-onset primary dystonia is a mutation in the *TOR1A* gene (121). In zebrafish, the ortholog gene, *tor1*, is not essential for early development of the motor system. The morpholino-mediated knockdown zebrafish line presents normal viability, morphology, development, and behavior (**Table 3**) (122). Zebrafish TOR1 may have an important role in later events, but the transient effect of morpholino-mediated knockdown did not allow to confirm this fact. Accordingly, in *TOR1A* knockout mice, the first phenotypic abnormalities are only observed in a later developmental stage (123). Other genes implicated in dystonia are involved in neuronal development and brain maturation in zebrafish. For instance, dystonia in early childhood can be caused by an autosomal recessive mutation in the pantothenate kinase 2 (*PANK2*) gene (124). Morpholino knockdown of the zebrafish ortholog perturbs the neuronal development and brain morphology and induces hydrocephalus (125). The phenotype can be rescued by pantethine and coenzyme A. To further test the implication of mutations identified in the *col6a3* gene of subjects with primary dystonia, a study found that morpholino knockdown of the zebrafish ortholog gene causes deficits in axonal outgrowth (126). The authors suggested that Col6a3 may participate in the structural organization of neurons. Therefore, its disruption can hamper the establishment of correct neuronal circuitries and synaptic remodeling processes, during brain development and maturation. Finally, knockdown of the ortholog of the human *Atp1a3* leads to brain ventricle dilation and depolarization of Rohon–Beard neurons in zebrafish (127). Although response to tactile stimuli and motility are altered, the dopaminergic neurons seemed to be unaffected. Mutations in the *Atp1a3* are implicated in rapid-onset dystonia parkinsonism (RDP) (128). Ventricle dilation is not observed in patients with RDP, but numerous symptoms reported in dystonic patients suggest the involvement of the somatosensory system. In turn, the depolarization of Rohon–Beard neurons in zebrafish is indicative of altered neuronal excitability, also described in rats (129).

In metabolic disorders, the risk to develop dystonia increases when the manganese homeostasis is compromised. In zebrafish, the mutation of the orthologs of the human manganese transporters, *slc30a10* and *slc39a14*, results in manganese accumulation in the brain (130, 131). This leads to impaired dopaminergic and GABAergic signaling. Changes in the swimming pattern are also visible upon exposure to manganese. The phenotype can be reverted by chelation therapy and iron supplementation, currently used in the clinical practice. Maple syrup urine disease is another metabolic disorder, caused by mutations in the dihydrolipoamide branched-chain transacylase E2 (*DBT)* gene, which can result in severe dystonia. In zebrafish, disruption of the ortholog gene results in elevated levels of branched-chain amino acids (BCAA) (132). This phenomenon is also evidenced in mammalian models and patients. The increase of BCAA leads to the dysregulation of the neurotransmitter glutamate in the brain and spinal cord of zebrafish, which probably contributes to the progressive aberrant motility behavior evidenced. This phenotype was suggested to represent severe dystonia in zebrafish larvae.

Chemical Zebrafish Models of Dystonia

Zebrafish are sensitive to neurotoxic drugs that may cause dystonia. Matrine and sophocarpine, two drugs responsible for poisoning juvenile and infant patients, induce growth retardation in zebrafish (133). Exposure to these drugs also led to changes in spontaneous movements and locomotor performance. The authors suggested that this phenotype results from the neurotoxic effects of the drugs but did not show evidence of neurotoxicity.

In summary, it appears that the heterogeneous nature of dystonia can be reproduced in zebrafish. Zebrafish offers an excellent opportunity to understand the pathogenic mechanisms behind the vast number of genetic and environmental factors linked to dystonia. Nevertheless, this heterogeneity seems to result in a large number of studies, with minor characterization and no consolidation of the observed phenotypes. This may undermine a correct judgment about the validity of zebrafish as a vertebrate model of dystonia. Most certainly, further studies are essential.

Chorea in Huntington's Disease

Chorea is the most common symptom of HD, which affects around 4–10 per 100,000 people in the western world (134). HD is an autosomal-dominant neurodegenerative disorder, which results from abnormal expansion of the CAG repeat in the huntingtin (*HTT*) gene (polyglutamine disease) (**Table 1**). The pathogenesis of HD results from the toxic effects of the mutant HTT RNA and protein, HTT aggregation (intranuclear inclusions of abnormal HTT are pathological hallmark) and impairments in protein homeostasis and clearance. These events lead to the death of GABAergic medium spiny neurons in the striatum. Chorea can be improved by tetrabenazine, and the therapeutic effects of other drugs are only empirical (120). There is currently no disease-modifying treatment for HD.

Genetic Zebrafish Models of HD

The zebrafish ortholog of the human HTT only encodes four glutamines, compared with up to 35 in humans (135). The zebrafish HTT protein is essential for iron, lipid, and cholesterol

TABLE 3 | General characteristics of the zebrafish models of hyperkinetic movement disorders reviewed.

Disease	Zebrafish model	Pathological hallmarks	Motor phenotype	Responsiveness to pharmacotherapies and disease-modifying therapies	Observations	Reference
Dystonia	TOR1A knockdown	No loss of dopaminergic neurons	No changes in swimming velocity or active time	ND		(122)
	PANK2 knockdown	ND	ND	ND	Developmental defects	(125)
	COL6A3 knockdown	ND	ND	ND	Deficits in axonal outgrowth	(126)
	ATP1A3 knockdown	No loss of dopaminergic neurons Altered neuronal excitability	Deficits in evoked swimming response	ND		(127)
	SLC30A10 and SLC39A14 mutation	Impaired dopaminergic and GABAergic signaling	Decrease in total distance moved and locomotor activity	Chelation therapy and iron supplementation reverses manganese accumulation and motor impairments		(130, 131)
	DBT mutation	Dysregulation of glutamate signalling	Deficits in evoked swimming response Absence of C-bending evoked swimming response (severe dystonia)	ND		(132)
	Matrine and sophocarpine	ND	Decrease in total distance moved and swimming velocity Decrease in locomotor activity	ND	Developmental defects	(133)
Chorea in Huntington's disease	HTT knockdown	Neuronal apoptosis Decreased brain-derived neurotrophic factor expression	ND	ND	Neurodevelopmental abnormalities	(138–140)
	HTT polyQ expanded fragments overexpression	Insoluble protein inclusions	ND	ND	Developmental defects Increased apoptosis	(147, 148)
	mHTT-ΔN17-97Q overexpression	Neuronal death Intranuclear protein aggregates	Abnormal swimming	ND		(149)
Stereotypies in Rett syndrome	MeCP2 mutation	ND	Increased duration and number of contractions in evoked swimming response Decrease in locomotor activity and swimming velocity	ND		(157)
	MeCP2 knockdown	Abnormal axonal branching and outgrowth	Decrease in locomotor activity Deficits in evoked swimming response	ND		(166, 167)
Essential tremor	TENM4 knockdown	Abnormal axonal branching and outgrowth	ND	ND		(171)
	Human mutated TENM4 overexpression	Abnormal axonal branching and outgrowth	ND	ND		(171)

ND, not determined.

homeostasis (136, 137), energy metabolism (136), and brain development (138–140). Its knockdown leads to diverse phenotypes, including hemoglobulin deficiency (136), neuronal apoptosis in the midbrain and hindbrain (138, 139), neurophysiologic abnormalities (138–140), decreased expression of brain-derived neurotrophic factor (BDNF) (138), deficient formation of neural tubes and cell adhesion (140), increased activity of metalloproteinases (ADAM10 and Ncadherin) (140), and severe reduction in cartilage biogenesis (**Table 3**) (137). Consistently, patients with HD exhibit deficits in iron homeostasis (141, 142), energy metabolism (143), BDNF expression (144), and metalloproteinases activity (145). As observed in *HTT* knockout mice, the complete abrogation of the expression of zebrafish HTT results in embryonic lethality (138). In fact, the mouse models of HD either lack overt phenotypes or exhibit premature death (146). This makes the HTT morphant zebrafish a valuable alternative model to study the cellular function of HTT and its role in the pathological mechanisms of HD. Nevertheless, morpholino-mediated knockdown may result in variable phenotypes and must be considered cautiously.

Zebrafish lines expressing normal and expanded polyglutamine (polyQ) fragments of HTT have been reported (147, 148). In these lines, the misfolding, oligomerization, aggregation, and toxicity of the polyQ fragments are length dependent, in a manner similar to that observed in other animal models and in patients. Zebrafish embryos overexpressing fragments with more than 35Q repeats (mutant form) display insoluble protein inclusions and increased apoptosis (148). This leads to abnormal morphology and development. Strikingly, apoptosis can be detected in cells with no visible inclusions, suggesting that the oligomeric forms of the HTT may be the toxic components. The ubiquitous expression of the polyQ proteins may contribute to the severe phenotype observed. In alternative, it would be interesting to observe the effects of polyQ expression restricted to the CNS. The toxic effects and aggregation of the mutant fragments can be suppressed either by the chaperones Hsp40 and Hsp70 or by the ubiquitin ligase C-terminal Hsp70-interacting protein, resembling other HD models.

Remarkably, the expression of a mutated polyQ fragment lacking the 17 amino acids of the HTT N-terminal tail (mHTT-ΔN17-97Q) elicits toxicity only in neuronal cells of a transgenic zebrafish line (149). Particularly in neurons, mHTT-ΔN17-97Q fragments rapidly form massive intranuclear aggregates. This demonstrates that the neuronal cells have lower capacity to maintain the proteostasis of the expanded polyQ fragments. Moreover, the mHTT-ΔN17-97Q fragments tend to aggregate more and induce a more severe phenotype than the polyQ fragments with an intact N17 terminal. In mice, the expression of the *htt-97Q* gene lacking the N17 causes dramatic accumulation of nuclear mutant HTT aggregates and a robust striatal neurodegeneration that leads to adult-onset movement disorder (150). These results suggest that the N17 portion of the HTT protein substantially prevents the translocation of mutant HTT into the nucleus and plays an important role in the molecular mechanisms of the pathogenesis of HD. mHTT-ΔN17-97Q transgenic zebrafish are the first to recapitulate one of the pathological hallmarks of HD.

Chemical Zebrafish Models of HD

The administration of quinolinic acid (QA) into the striatum of adult rodents has been used to induce brain injury that replicates HD (151). Interestingly, while inducing injury, this excitotoxin also stimulates the subventricular neurogenesis zone and neuroblast migration (152, 153). This observation encouraged Skaggs and colleagues (154) to lesion the telencephalon of adult zebrafish with QA and study its neuronal effects. The QA induces cell death and microglial infiltration in the zebrafish CNS (154). However, it also stimulates cell proliferation and neurogenesis that results in total repair of the damage. The authors suggested that this zebrafish model is a powerful tool to study neuronal regeneration in an adult vertebrate and to test potential disease-modifying therapies. Still, the neurogenesis process in the CNS of adult zebrafish is very different from mammals, which might render the translation of the observations puzzling.

In general, the zebrafish HTT shares several important functions with the mammalian ortholog. Zebrafish lines that overexpress mutant HTT are proving to be useful to model HD. Nevertheless, there is only a shallow description of the motor phenotypes, and the responsiveness to pharmacotherapies still needs to be tested on these models.

Stereotypies in Rett Syndrome

Rett syndrome is a non-neurodegenerative disorder, which affects 1 in 10,000 females by the age of 12 (155). Caused by either nonsense or missense mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene, RTT is characterized by hand stereotypies (**Table 1**). MeCP2 is a nuclear protein that recognizes DNA methylation to, presumably, regulate gene expression and activation. The patients with RTT, exhibit abnormally small and densely packed neurons, with reduced dendritic complexity and synaptic density. At the cellular level, alterations in different signaling and homeostatic pathways are reported, along with mitochondrial dysfunction and oxidative stress. The options available for the treatment of RTT are currently limited, but several compounds are under clinical trials. These include modulators of neurotransmitters or regulators of cellular metabolism and homeostasis (**Table 1**).

Genetic Zebrafish Models of RTT

In mice, null mutations in MeCP2 drastically reduce lifespan (156). By contrast, zebrafish carrying a null mutation in MeCP2 show normal viability and fertility (157). Instead, MeCP2-null zebrafish exhibit clear motor impairments at early developmental stage (**Table 3**). These include spontaneous and sensory-evoked motor anomalies, and defective anxiety-like behavior. This zebrafish line has a nonsense mutation in the methyl-CpG binding domain ($mecp2^{Q63X}$), crucial for protein function. The authors suggested that the modest phenotype observed may result from a compensatory mechanism triggered by other proteins belonging to the MeCP2 family or from gene duplication in zebrafish. On the other hand, the studies exploring thigmotaxis in mouse models of RTT have yielded confounding results, some of them contradictory to the ones observed in zebrafish. The authors advocated that the complexity of the neuronal circuitry in mice

may have hampered the interpretation of the results in behavioral tests. Later proteomic analysis in the MeCP2-null zebrafish line revealed changes in the expression of proteins critical for energy metabolism, balance of redox status and muscle function (158). This is in line with the reported in RTT patients and experimental mouse models (159–164).

Additional studies in zebrafish confirmed the essential role of MECP2 in neuronal differentiation (165), axonal branching of primary motor neurons (166), and peripheral innervation of sensory neurons (167). The studies further proved that MeCP2 regulates the expression of several cell differentiating factors (Id1–Her2 axis), BDNF and axonal guidance cues (such as Sema5b and Robo2). The indirect disruption of the expression of these genes is involved in RTT-like phenotypes. Accordingly, downregulation of MeCP2 induces a decrease in motor activity and impairments in the sensory function of zebrafish, as observed in mice with partial loss of MeCP2 (168).

All these studies strengthened the notion that the zebrafish MeCP2 is crucial for the regulation of gene expression and activation. Therefore, Mecp2-deficient zebrafish have several phenotypes, reminiscent of the phenomenology observed in mouse models of RTT and in patients with RTT. The normal lifespan in MeCP2-null zebrafish may enable a more profound characterization of the pathophysiological dynamics of RTT and the screening of new drugs.

Other Hyperkinetic Syndromes

Tremor is a rhythmic oscillation of a body part. Besides resting tremor in PD, this phenomenology is mostly common in the neurologic disorder essential tremor (ET) (120). Progressive action tremor is the classic feature of ET. The most effective drugs for the treatment of ET are propranolol and primidone (**Table 1**) (169). However, these drugs induce highly variable therapeutic effects and are associated with several adverse effects. The anticonvulsant, topimarate, the GABA agonist, gabapentine, and several benzodiazepines have also shown to improve tremor. The pathological mechanisms and etiology of ET are highly heterogeneous (170). In addition, these are difficult to identify, because ET is commonly a comorbidity. Several genetic and environmental risk factors have been suggested, but none was consistently confirmed in larger cohort studies. This renders the discovery of effective pharmacologic treatments particularly difficult.

Zebrafish may be a practical choice to unravel some of the pathological mechanisms and risk factors implicated in ET. This model has been used to explore the physiological role of *TENM4* and the pathological effects of mutations identified in the *TENM4* of families with ET. Morpholino knockdown of the *TENM4* zebrafish ortholog results in a modest reduction of myelination and aberrant extension, branching and architecture of small axons in the CNS of zebrafish (**Table 3**) (171). Zebrafish expressing mutated human *TENM4* mRNA show a similar phenotype. These observations are concordant with studies in other animal models. Since 2015, no other study reported the generation of a zebrafish model of tremor, possibly because of the limited knowledge about the nature of ET.

Perhaps for the same reason, at the time of this review, no zebrafish model of Tourette's syndrome has been reported. There is limited understanding of the etiology of this multifactorial syndrome (**Table 1**), because several genetic variants and mutations, and non-genetic determinants are implicated. Moreover, these are not exclusive of the disease. This heterogeneity results in a complex and variable neurological and clinical phenomenology. Tourette's syndrome is typically manifested by various motor and phonic chronic tics (172). The cortico–striato–thalamocortical circuitry is potentially impaired, but the specific neuronal pathway(s) involved remain unknown. Neuroleptic drugs and the monoamine depleting drug, tetrabenazine, are the most effective for tic suppression (**Table 1**). There exist different rodent models of tics, but their validity is debatable. The inability to assess several human symptomatic features, such as premonitory sensation, is an important limitation of these animals. While the same skepticism can apply to zebrafish, zebrafish models of Tourette's syndrome may help to elucidate the interplay between genetic and non-genetic risk factors. Ultimately, this may provide valuable clues about the neuropathology of this syndrome.

DISCUSSION

Zebrafish have been extensively used in the study of the CNS. More recently, the use of zebrafish as a model of human brain diseases and for drug discovery has increased (2, 173). Here, we review several zebrafish models of movement disorders and discuss their translational value. Overall, these models exhibit conserved biochemical and neurobehavioral features. In retrospect, many advantages can be named, but pitfalls must also be highlighted.

First, all studies used zebrafish during embryonic, larval or young adult stage. Practical reasons can justify the use of zebrafish at these developmental stages. For instance, at embryonic and larval stage zebrafish are more permeable, enabling the delivery of drugs through the water. In higher vertebrates, some of the neurotoxic agents used to model movement disorders have to be delivered directly in the brain (174). This approach increases variability between animals and is much more invasive, resulting in the death of some animals. Furthermore, the time lapse between drug administration and appearance of the first phenotypes is much longer in rodents than in zebrafish. Nevertheless, the use of zebrafish larvae is not as accurate as the use of adult zebrafish, where the BBB is fully functional and better mimics the mammalian physiology. The use of zebrafish at early developmental stages also allows to explore the function of specific genes during system development and maturation. However, it must be considered carefully when extrapolating the phenotypes observed to chronic and late-onset disorders. Moreover, the mutant and transgenic lines described here were characterized very early during development, and whether these lines display any pathology in adulthood was not reported. Inevitably, the brain of zebrafish is more complex at adulthood and may mimic more accurately the physiologic features of the mammalian brain.

In turn, morpholino-mediated knockdown is extensively used, because it is a practical tool to reveal the phenotypes induced by downregulation of a gene in zebrafish, but it also

has many pitfalls. The extension of abrogation of the expression of the gene can vary drastically, depending on the knockdown strategy and efficiency. Zebrafish possess duplicates of several genes, which can result in a differentiated regulation of gene expression and different phenotypes. In addition, morpholino-mediated knockdown has an acute and transient effect, which does not mimic the chronic effects of downregulation of a gene. On the other hand, non-specific and off-target effects are common, most of the studies lacked the right controls to rule out these effects and, therefore, could not exclude that other systems could be affected. Finally, the knockdown of a gene may not be suited to model some diseases. This is the case of HD, as loss of function of the HTT protein alone does not seem to lead to the disorder (175). The genetic manipulation of zebrafish is particularly easy and should be more exhaustively explored. The mutated or transgenic zebrafish lines develop rapidly, which is an advantage when compared with the time-consuming development of rodent models. When developing transgenic lines, neuronal promoters should be used, to prevent overt toxicity of the transgene. This has been observed for zebrafish lines that overexpress human α-synuclein or HTT polyQ fragments with non-specific promoters (75, 148). In rodents, neuronal promoters are commonly used to restrict the expression of the proteins to the CNS (176).

Another limitation of most of the studies is the restriction of the characterization of the zebrafish model to a single cellular biomarker or behavioral parameter. For instance, most of the studies in zebrafish models of PD determined the expression of TH to assess the integrity of the dopaminergic system, but DAT would be a more specific biomarker for this neuronal population. The zebrafish proteomics is conserved, which allows the use of commercially available biomarkers of other species. Several of these markers have already been tested on zebrafish targets, and many present similar reactivity. In turn, almost all studies limited the evaluation of behavioral changes to total locomotor activity and/or speed, which does not represent the multi symptomatic nature of movement disorders. The assessment of these parameters in zebrafish is a sound strategy, because they are related to the parameters used in rodents to depict bradykinesia. Nevertheless, zebrafish possess a diverse repertoire of behaviors with homology to humans, which have been cataloged and can be easily explored by experimenter-independent behavioral tracking systems (177). Several behavioral tests have been optimized to evaluate motor performance, motor coordination, balance, escape responses, exploratory behavior, reward/punishment-related behavior, learning, memory, social interaction, and aggressive or anxious behaviors (178–183). It is now crucial to overview the array of behavioral tests available for zebrafish, as it has been systematically done for rodents (184, 185).

Furthermore, similar to other models, a deeper characterization of zebrafish will certainly improve the validity of this model system. Regardless the differences between the zebrafish brain and mammalian brain, homologous functions have been attributed to different neuronal regions of each vertebrate. This is the case of the zebrafish diencephalic dopaminergic region (15). However, other neuronal components and physiologic events that modulate movement are still highly unknown. It would be relevant to understand the cerebral components of the zebrafish brain that correspond to the constituents of the mammalian basal ganglia–thalamocortical circuits and to investigate how they are interconnect. Is there a direct and indirect pathway-like system in zebrafish? How do zebrafish control movement features like velocity or direction? Perhaps, to dissect these processes in simpler brain circuitries, as the zebrafish ones, will help to understand more complex mechanisms in mammals. For instance, the small size of the zebrafish brain is useful for three-dimensional mapping of brain structures. With the up-to-date microscopic techniques, whole-brain neuronal connectivity can be easily performed in zebrafish and reveal anatomical relationships, that in larger brains may not be as facilitated. Another particularity that should be further explored is the regenerative capacity of the zebrafish CNS. Several neuronal proliferating sites were identified in the zebrafish brain as compared with two found in the mammalian brain (186). Notwithstanding, many of the molecular and cellular factors that drive regeneration in the brain of adult zebrafish are poorly understood and yet unknown. This may difficult the translation of disease-modifying drugs identified using zebrafish.

Finally, the distinctive and reproducible behaviors of zebrafish exposed to certain neuroactive drugs are a powerful evidence of the conserved functional properties of the neuronal circuitries in vertebrates (187–189). In addition, it highlights the translational value of zebrafish. To improve the validity of this model, it is now important to explore the mechanisms triggered by these drugs on the zebrafish targets. This will increase our understanding of the zebrafish neuronal modulation, and most importantly, enlighten the pharmacodynamic properties of the compounds in zebrafish. In fact, the factors that influence the pharmacodynamic and pharmacokinetic properties of drugs in zebrafish are poorly understood. For instance, previously, it has been assumed that zebrafish totally absorb and distribute through the system the small molecules present in the water (190). However, a recent review has suggested that the absorption of chemicals, as well as distribution through the BBB in zebrafish is comparable to mammals (191). Therefore, the chemical properties of the compounds should be considered when extrapolating concentrations between these two models. The metabolism and excretion of drugs in zebrafish are also difficult to predict. Despite zebrafish have important metabolic enzymes also found in mammals (192), these are not fully characterized. In addition, several differences in the metabolism of chemicals have been reported (193). The compounds used in the clinic are extensively characterized and are particularly adequate to investigate the differences of these compounds on fish and mammals. This would also create a basis to more precisely extrapolate doses between both models.

Probably, a key pitfall in the discovery of new drugs in zebrafish is the absence of general guidelines to calculate mammalian equivalent doses from zebrafish doses. While between mammalian models there exist established formulas (194, 195), the dose extrapolation from zebrafish to mammals is still empirical nowadays. Moreover, much of the existing literature

has omitted this rationale (196–199). The only way to understand this rational is by increasing the number of studies where the properties of drugs in zebrafish are translated to mammals. Until now, it seems that only a minority has reached this far. It is not clear dough, whether there is little interest on these drugs, or they actually did not show the same properties in rodents and were not reported. The translation of the discoveries will allow the elaboration of a meta-analysis where the effective doses in zebrafish and mammals can be compared. Ultimately, it would boost the generation of formulas that rule dose extrapolation from zebrafish to different mammalian models and increase the validity of zebrafish models.

CONCLUSION

This review underscores the strengths and limitations of the zebrafish models of movement disorders developed to date. Importantly, it raises awareness that zebrafish can mimic the phenomenology of different movement disorders but needs further characterization. To date, there are a substantial number of studies reporting the use of zebrafish as a model for PD. However, for other movement disorders, this number is still limited. Considering the pathological hallmarks, motor phenotypes, and responsiveness to pharmacotherapies, from the seven movement disorders reviewed here, zebrafish models have only been fully characterized in the context of PD. Notwithstanding, the use of zebrafish to model human disorders dates back to the beginning of the twenty-first century, which compared with the 500 years of use of rodent models, is at embryonic stage. This difference of half a century may explain the skepticism that still exists about the use of zebrafish as an animal model of human diseases. The number of studies reporting the use of zebrafish as an animal model is growing and, therefore, the analysis of the pros and cons of the use of this vertebrate model for drug discovery is important.

This study also provides a comprehensive assessment of the methodologies adopted and emphasizes that most of the limitations are inherent to it. Many techniques are available to surpass these limitations and generate consistent and well-characterized models of movement disorders. With the next-generation sequencing, to couple genomic approaches with *in vivo* studies will not only improve our ability to understand the pathogenic mechanisms of complex diseases, as movement disorders but also precipitate the discovery of novel drugs for these disorders. Zebrafish should be considered a practical and inexpensive tool for this approach, provided that, as with any other non-mammalian model, the potential molecules selected in it are further validated by studies in mammals. It is not expected of zebrafish models of movement disorders to fully recapitulate such complex human phenomenology. Even mammalian models have their flaws and do not precisely mimic the symptomatology evidenced in patients. Furthermore, several compounds selected using the traditional models have also failed to demonstrate therapeutic effects in humans. Finally, it is vital to create a comprehensive correlation between zebrafish and mammalian models and, ultimately, be able to translate the findings to humans.

Overall, while it is already evident that zebrafish models of movement disorders share many cellular and physiologic mechanisms with mammalian models and patients, this model is still showing its usefulness for drug discovery. It was not until 2011 that a positive hit from a zebrafish-based drug screening entered phase I clinical trials (200). The usefulness of zebrafish to model human diseases will only be unquestionable when a drug selected in this model proves efficacy in human patients. Meanwhile, the use of zebrafish to study movement disorders will certainly result in a better understanding of their mechanisms and, hopefully, in the discovery of better therapies.

AUTHOR CONTRIBUTIONS

RV and JF conceived and outlined the study. RV wrote the first draft. TO and JF did critical revision.

ACKNOWLEDGMENTS

The authors would like to thank Mariana Trigo Pereira, who kindly provided the pictures of zebrafish. RV was supported by a grant (SFRH/BD/78077/2011) from Fundação para e a Ciência e Tecnologia. TO is supported by the DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB).

REFERENCES

1. Fahn S. Classification of movement disorders. *Mov Disord* (2011) 26:947–57. doi:10.1002/mds.23759
2. Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol Sci* (2014) 35:63–75. doi:10.1016/j.tips.2013.12.002
3. Goldsmith P. Zebrafish as a pharmacological tool: the how, why and when. *Curr Opin Pharmacol* (2004) 4:504–12. doi:10.1016/j.coph.2004.04.005
4. Rennekamp AJ, Peterson RT. 15 Years of zebrafish chemical screening. *Curr Opin Chem Biol* (2015) 24:58–70. doi:10.1016/j.cbpa.2014.10.025
5. Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? *Br J Pharmacol* (2010) 160:204–16. doi:10.1111/j.1476-5381.2010.00725.x
6. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* (2007) 8:353–67. doi:10.1038/nrg2091
7. Williams CH, Hong CC. Multi-step usage of in vivo models during rational drug design and discovery. *Int J Mol Sci* (2011) 12:2262–74. doi:10.3390/ijms12042262
8. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* (2013) 496:498–503. doi:10.1038/nature12111
9. Jeong J, Kwon H, Ahn J, Kang D, Kwon S, Park JA, et al. Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain Res Bull* (2008) 75:619–28. doi:10.1016/j.brainresbull.2007.10.043
10. Xie J, Farage E, Sugimoto M, Anand-Apte B. A novel transgenic zebrafish model for blood-brain and blood-retinal barrier development. *BMC Dev Biol* (2010) 10:76. doi:10.1186/1471-213X-10-76
11. Kaslin J, Panula P. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *J Comp Neurol* (2001) 440:342–77. doi:10.1002/cne.1390
12. Candy J, Collet C. Two tyrosine hydroxylase genes in teleosts. *Biochim Biophys Acta* (2005) 1727:35–44. doi:10.1016/j.bbaexp.2004.11.005

13. Chen Y-C, Priyadarshini M, Panula P. Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. *Histochem Cell Biol* (2009) 132:375–81. doi:10.1007/s00418-009-0619-8
14. Holzschuh J, Ryu S, Aberger F, Driever W. Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. *Mech Dev* (2001) 101:237–43. doi:10.1016/S0925-4773(01)00287-8
15. Rink E, Wullimann MF. The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Res* (2001) 889:316–30. doi:10.1016/S0006-8993(00)03174-7
16. Sallinen V, Sundvik M, Reenilä I, Peitsaro N, Khrustalyov D, Anichtchik O, et al. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. *J Neurochem* (2009) 109:403–15. doi:10.1111/j.1471-4159.2009.05986.x
17. Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, et al. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiol Dis* (2010) 40:46–57. doi:10.1016/j.nbd.2010.05.010
18. Anichtchik O, Sallinen V, Peitsaro N, Panula P. Distinct structure and activity of monoamine oxidase in the brain of zebrafish (*Danio rerio*). *J Comp Neurol* (2006) 498:593–610. doi:10.1002/cne.21057
19. Ruuskanen JO, Laurila J, Xhaard H, Rantanen V-V, Vuoriluoto K, Wurster S, et al. Conserved structural, pharmacological and functional properties among the three human and five zebrafish alpha 2-adrenoceptors. *Br J Pharmacol* (2005) 144:165–77. doi:10.1038/sj.bjp.0706057
20. Rink E, Guo S. The too few mutant selectively affects subgroups of monoaminergic neurons in the zebrafish forebrain. *Neuroscience* (2004) 127:147–54. doi:10.1016/j.neuroscience.2004.05.004
21. Norton WHJ, Folchert A, Bally-Cuif L. Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. *J Comp Neurol* (2008) 511:521–42. doi:10.1002/cne.21831
22. Wang Y, Takai R, Yoshioka H, Shirabe K. Characterization and expression of serotonin transporter genes in zebrafish. *Tohoku J Exp Med* (2006) 208:267–74. doi:10.1620/tjem.208.267
23. Eriksson KS, Peitsaro N, Karlstedt K, Kaslin J, Panula P. Development of the histaminergic neurons and expression of histidine decarboxylase mRNA in the zebrafish brain in the absence of all peripheral histaminergic systems. *Eur J Neurosci* (1998) 10:3799–812. doi:10.1046/j.1460-9568.1998.00394.x
24. Peitsaro N, Sundvik M, Anichtchik OV, Kaslin J, Panula P. Identification of zebrafish histamine H1, H2 and H3 receptors and effects of histaminergic ligands on behavior. *Biochem Pharmacol* (2007) 73:1205–14. doi:10.1016/j.bcp.2007.01.014
25. Maximino C, Herculano AM. A review of monoaminergic neuropsychopharmacology in zebrafish. *Zebrafish* (2010) 7:359–78. doi:10.1089/zeb.2010.0669
26. Rico EP, Rosemberg DB, Seibt KJ, Capiotti KM, Da Silva RS, Bonan CD. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. *Neurotoxicol Teratol* (2011) 33:608–17. doi:10.1016/j.ntt.2011.07.007
27. Kalia LV, Lang AE. Parkinson's disease. *Lancet* (2015) 386:896–912. doi:10.1016/S0140-6736(14)61393-3
28. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* (1983) 219:979–80. doi:10.1126/science.6823561
29. Lam CS, Korzh V, Strahle U. Zebrafish embryos are susceptible to the dopaminergic neurotoxin MPTP. *Eur J Neurosci* (2005) 21:1758–62. doi:10.1111/j.1460-9568.2005.03988.x
30. McKinley ET, Baranowski TC, Blavo DO, Cato C, Doan TN, Rubinstein AL. Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. *Brain Res Mol Brain Res* (2005) 141:128–37. doi:10.1016/j.molbrainres.2005.08.014
31. Bretaud S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. *Neurotoxicol Teratol* (2004) 26:857–64. doi:10.1016/j.ntt.2004.06.014
32. Sallinen V, Torkko V, Sundvik M, Reenilä I, Khrustalyov D, Kaslin J, et al. MPTP and MPP+ target specific aminergic cell populations in larval zebrafish. *J Neurochem* (2009) 108:719–31. doi:10.1111/j.1471-4159.2008.05793.x
33. Wen L, Wei W, Gu W, Huang P, Ren X, Zhang Z, et al. Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish. *Dev Biol* (2008) 314:84–92. doi:10.1016/j.ydbio.2007.11.012
34. Bové J, Perier C. Neurotoxin-based models of Parkinson's disease. *Neuroscience* (2012) 211:51–76. doi:10.1016/j.neuroscience.2011.10.057
35. Dukes AA, Bai Q, Van Laar VS, Zhou Y, Ilin V, David CN, et al. Live imaging of mitochondrial dynamics in CNS dopaminergic neurons in vivo demonstrates early reversal of mitochondrial transport following MPP+ exposure. *Neurobiol Dis* (2016) 95:238–49. doi:10.1016/j.nbd.2016.07.020
36. Anichtchik OV, Kaslin J, Peitsaro N, Scheinin M, Panula P. Neurochemical and behavioural changes in zebrafish *Danio rerio* after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* (2004) 88:443–53. doi:10.1111/j.1471-4159.2004.02190.x
37. Babu NS, Murthy CLN, Kakara S, Sharma R, Swamy CVB, Idris MM. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced Parkinson's disease in zebrafish. *Proteomics* (2016) 16:1407–20. doi:10.1002/pmic.201500291
38. Serra PA, Sciola L, Delogu MR, Spano A, Monaco G, Miele E, et al. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induces apoptosis in mouse nigrostriatal glia. Relevance to nigral neuronal death and striatal neurochemical changes. *J Biol Chem* (2002) 277:34451–61. doi:10.1074/jbc.M202099200
39. Perry TL, Yong VW, Jones K, Wall RA, Clavier RM, Foulks JG, et al. Effects of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its metabolite, N-methyl-4-phenylpyridinium ion, on dopaminergic nigrostriatal neurons in the mouse. *Neurosci Lett* (1985) 58:321–6. doi:10.1016/0304-3940(85)90074-6
40. Vijayanathan Y, Lim FT, Lim SM, Long CM, Tan MP, Majeed ABA, et al. 6-OHDA-lesioned adult zebrafish as a useful Parkinson's disease model for dopaminergic neuroregeneration. *Neurotox Res* (2017) 32(3):496–508. doi:10.1007/s12640-017-9778-x
41. Höglinger GU, Alvarez-Fischer D, Arias-Carrión O, Djufri M, Windolph A, Keber U, et al. A new dopaminergic nigro-olfactory projection. *Acta Neuropathol* (2015) 130:333–48. doi:10.1007/s00401-015-1451-y
42. Zupanc GKH, Hinsch K, Gage FH. Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. *J Comp Neurol* (2005) 488:290–319. doi:10.1002/cne.20571
43. Feng C, Wen Z, Huang S, Hung H, Chen C, Yang S, et al. Effects of 6-hydroxydopamine exposure on motor activity and biochemical expression in zebrafish (*Danio rerio*) larvae. *Zebrafish* (2014) 11:227–39. doi:10.1089/zeb.2013.0950
44. Yamauchi R. Vitamin E: mechanisms of its antioxidant activity. *Food Sci Technol Int Tokyo* (1997) 3:301–9. doi:10.3136/fsti9596t9798.3.301
45. Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci U S A* (2001) 98:14669–74. doi:10.1073/pnas.251341998
46. Day BJ, Patel M, Calavetta L, Chang LY, Stamler JS. A mechanism of paraquat toxicity involving nitric oxide synthase. *Proc Natl Acad Sci U S A* (1999) 96:12760–5. doi:10.1073/pnas.96.22.12760
47. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. *Environ Health Perspect* (2011) 119:866–72. doi:10.1289/ehp.1002839
48. Bortolotto JW, Cognato GP, Christoff RR, Roesler LN, Leite CE, Kist LW, et al. Long-term exposure to paraquat alters behavioral parameters and dopamine levels in adult zebrafish (*Danio rerio*). *Zebrafish* (2014) 11:142–53. doi:10.1089/zeb.2013.0923
49. Nunes ME, Müller TE, Braga MM, Fontana BD, Quadros VA, Marins A, et al. Chronic treatment with paraquat induces brain injury, changes in antioxidant defenses system, and modulates behavioral functions in zebrafish. *Mol Neurobiol* (2017) 54:3925–34. doi:10.1007/s12035-016-9919-x
50. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Res* (1999) 823:1–10. doi:10.1016/S0006-8993(98)01192-5
51. Ren J, Zhao Y, Sun X. Toxic influence of chronic oral administration of paraquat on nigrostriatal dopaminergic neurons in C57BL/6 mice. *Chin Med J (Engl)* (2009) 122:2366–71.

52. Czerniczyniec A, Karadayian AG, Bustamante J, Cutrera RA, Lores-Arnaiz S. Paraquat induces behavioral changes and cortical and striatal mitochondrial dysfunction. *Free Radic Biol Med* (2011) 51:1428–36. doi:10.1016/j.freeradbiomed.2011.06.034
53. Songin M, Strosznajder JB, Fitał M, Kuter K, Kolasiewicz W, Nowak P, et al. Glycogen synthase kinase 3β and its phosphorylated form (Y216) in the paraquat-induced model of parkinsonism. *Neurotox Res* (2011) 19:162–71. doi:10.1007/s12640-010-9153-7
54. Breckenridge CB, Sturgess NC, Butt M, Wolf JC, Zadory D, Beck M, et al. Pharmacokinetic, neurochemical, stereological and neuropathological studies on the potential effects of paraquat in the substantia nigra pars compacta and striatum of male C57BL/6J mice. *Neurotoxicology* (2013) 37:1–14. doi:10.1016/j.neuro.2013.03.005
55. Nellore J, Nandita P. Paraquat exposure induces behavioral deficits in larval zebrafish during the window of dopamine neurogenesis. *Toxicol Rep* (2015) 2:950–6. doi:10.1016/j.toxrep.2015.06.007
56. Wang Q, Liu S, Hu D, Wang Z, Wang L, Wu T, et al. Identification of apoptosis and macrophage migration events in paraquat-induced oxidative stress using a zebrafish model. *Life Sci* (2016) 157:116–24. doi:10.1016/j.lfs.2016.06.009
57. Miller GW. Paraquat: the red herring of Parkinson's disease research. *Toxicol Sci* (2007) 100:1–2. doi:10.1093/toxsci/kfm223
58. Fleming SM, Zhu C, Fernagut P-O, Mehta A, DiCarlo CD, Seaman RL, et al. Behavioral and immunohistochemical effects of chronic intravenous and subcutaneous infusions of varying doses of rotenone. *Exp Neurol* (2004) 187:418–29. doi:10.1016/j.expneurol.2004.01.023
59. Wang Y, Liu W, Yang J, Wang F, Sima Y, Zhong ZM, et al. Parkinson's disease-like motor and non-motor symptoms in rotenone-treated zebrafish. *Neurotoxicology* (2017) 58:103–9. doi:10.1016/j.neuro.2016.11.006
60. Doty RL, Deems DA, Stellar S. Olfactory dysfunction in parkinsonism: a general deficit unrelated to neurologic signs, disease stage, or disease duration. *Neurology* (1988) 38:1237–44. doi:10.1212/WNL.38.8.1237
61. Ziemssen T, Reichmann H. Non-motor dysfunction in Parkinson's disease. *Parkinsonism Relat Disord* (2007) 13:323–32. doi:10.1016/j.parkreldis.2006.12.014
62. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* (2000) 3:1301–6. doi:10.1038/81834
63. Santiago RM, Barbieiro J, Lima MM, Dombrowski PA, Andreatini R, Vital MA. Depressive-like behaviors alterations induced by intranigral MPTP, 6-OHDA, LPS and rotenone models of Parkinson's disease are predominantly associated with serotonin and dopamine. *Prog Neuropsychopharmacol Biol Psychiatry* (2010) 34:1104–14. doi:10.1016/j.pnpbp.2010.06.004
64. Godoy R, Noble S, Yoon K, Anisman H, Ekker M. Chemogenetic ablation of dopaminergic neurons leads to transient locomotor impairments in zebrafish larvae. *J Neurochem* (2015) 135:249–60. doi:10.1111/jnc.13214
65. Hu Q, Guo F, Zhao F, Fu Z. Effects of titanium dioxide nanoparticles exposure on parkinsonism in zebrafish larvae and PC12. *Chemosphere* (2017) 173:373–9. doi:10.1016/j.chemosphere.2017.01.063
66. Toni M, Cioni C. Fish synucleins: an update. *Mar Drugs* (2015) 13:6665–86. doi:10.3390/md13116665
67. Kumar S, Meena R, Paulraj R. Role of macrophage (M1 and M2) in titanium-dioxide nanoparticle-induced oxidative stress and inflammatory response in rat. *Appl Biochem Biotechnol* (2016) 180:1257–75. doi:10.1007/s12010-016-2165-x
68. Sun Z, Gitler AD. Discovery and characterization of three novel synuclein genes in zebrafish. *Dev Dyn* (2008) 237:2490–5. doi:10.1002/dvdy.21569
69. Milanese C, Sager JJ, Bais Q, Farrells TC, Cannons JR, Greenamyre JT, et al. Hypokinesia and reduced dopamine levels in zebrafish lacking beta- and gamma1-synucleins. *J Biol Chem* (2012) 287:2971–83. doi:10.1074/jbc.M111.308312
70. Chandra S, Fornai F, Kwon HB, Yazdani U, Atasoy D, Liu X, et al. Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions. *Proc Natl Acad Sci U S A* (2004) 101:14966–71. doi:10.1073/pnas.0406283101
71. Greten-Harrison B, Polydoro M, Morimoto-Tomita M, Diao L, Williams AM, Nie EH, et al. Alpha beta gamma-synuclein triple knockout mice reveal age-dependent neuronal dysfunction. *Proc Natl Acad Sci U S A* (2010) 107:19573–8. doi:10.1073/pnas.1005005107
72. Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* (2010) 329:1663–7. doi:10.1126/science.1195227
73. Anwar S, Peters O, Millership S, Ninkina N, Doig N, Connor-Robson N, et al. Functional alterations to the nigrostriatal system in mice lacking all three members of the synuclein family. *J Neurosci* (2011) 31:7264–74. doi:10.1523/JNEUROSCI.6194-10.2011
74. Lulla A, Barnhill L, Bitan G, Ivanova MI, Nguyen B, O'Donnell K, et al. Neurotoxicity of the Parkinson's disease-associated pesticide ziram is synuclein-dependent in zebrafish embryos. *Environ Health Perspect* (2016) 124:1766–75. doi:10.1289/EHP141
75. Prabhudesai S, Sinha S, Attar A, Kotagiri A, Fitzmaurice AG, Lakshmanan R, et al. A novel "molecular tweezer" inhibitor of α-synuclein neurotoxicity in vitro and in vivo. *Neurotherapeutics* (2012) 9:464–76. doi:10.1007/s13311-012-0120-2
76. Anichtchik O, Diekmann H, Fleming A, Roach A, Goldsmith P, Rubinsztein DC. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. *J Neurosci* (2008) 28:8199–207. doi:10.1523/JNEUROSCI.0979-08.2008
77. Flinn LJ, Keatinge M, Bretaud S, Mortiboys H, Matsui H, De Felice E, et al. TigarB causes mitochondrial dysfunction and neuronal loss in PINK1 deficiency. *Ann Neurol* (2013) 74:837–47. doi:10.1002/ana.23999
78. Vives-Bauza C, Przedborski S. Mitophagy: the latest problem for Parkinson's disease. *Trends Mol Med* (2011) 17:158–65. doi:10.1016/j.molmed.2010.11.002
79. Priyadarshini M, Tuimala J, Chen YC, Panula P. A zebrafish model of PINK1 deficiency reveals key pathway dysfunction including HIF signaling. *Neurobiol Dis* (2013) 54:127–38. doi:10.1016/j.nbd.2013.02.002
80. Schroedl C, McClintock DS, Budinger GR, Chandel NS. Hypoxic but not anoxic stabilization of HIF-1α requires mitochondrial reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* (2002) 283:L922–31. doi:10.1152/ajplung.00014.2002
81. Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* (2005) 9:617–28. doi:10.1016/j.devcel.2005.09.010
82. Xi Y, Ryan J, Noble S, Yu M, Yilbas AE, Ekker M. Impaired dopaminergic neuron development and locomotor function in zebrafish with loss of pink1 function. *Eur J Neurosci* (2010) 31:623–33. doi:10.1111/j.1460-9568.2010.07091.x
83. Sallinen V, Kolehmainen J, Priyadarshini M, Toleikyte G, Chen YC, Panula P. Dopaminergic cell damage and vulnerability to MPTP in Pink1 knockdown zebrafish. *Neurobiol Dis* (2010) 40:93–101. doi:10.1016/j.nbd.2010.06.001
84. Priyadarshini M, Orosco LA, Panula PJ. Oxidative stress and regulation of pink1 in zebrafish (*Danio rerio*). *PLoS One* (2013) 8:e81851. doi:10.1371/journal.pone.0081851
85. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* (2004) 304:1158–60. doi:10.1126/science.1096284
86. Kitada T, Pisani A, Porter DR, Yamaguchi H, Tscherter A, Martella G, et al. Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. *Proc Natl Acad Sci U S A* (2007) 104:11441–6. doi:10.1073/pnas.0702717104
87. Flinn L, Mortiboys H, Volkmann K, Kster RW, Ingham PW, Bandmann O. Complex i deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (*Danio rerio*). *Brain* (2009) 132:1613–23. doi:10.1093/brain/awp108
88. Müftüoglu M, Elibol B, Dalmızrak Ö, Ercan A, Kulaksız G, Ögüs H, et al. Mitochondrial complex I and IV activities in leukocytes from patients with parkin mutations. *Mov Disord* (2004) 19:544–8. doi:10.1002/mds.10695
89. Mortiboys H, Thomas KJ, Koopman WJ, Klaffke S, Abou-Sleiman P, Olpin S, et al. Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. *Ann Neurol* (2008) 64:555–65. doi:10.1002/ana.21492
90. Perez FA, Palmiter RD. Parkin-deficient mice are not a robust model of parkinsonism. *Proc Natl Acad Sci U S A* (2005) 102:2174–9. doi:10.1073/pnas.0409598102
91. Thomas B, von Coelln R, Mandir AS, Trinkaus DB, Farah MH, Leong Lim K, et al. MPTP and DSP-4 susceptibility of substantia nigra and locus

coeruleus catecholaminergic neurons in mice is independent of parkin activity. *Neurobiol Dis* (2007) 26:312–22. doi:10.1016/j.nbd.2006.12.021
92. Fett ME, Pilsl A, Paquet D, van Bebber F, Haass C, Tatzelt J, et al. Parkin is protective against proteotoxic stress in a transgenic zebrafish model. *PLoS One* (2010) 5:e11783. doi:10.1371/journal.pone.0011783
93. Petrucelli L, O'Farrell C, Lockhart PJ, Baptista M, Kehoe K, Vink L, et al. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. *Neuron* (2002) 36:1007–19. doi:10.1016/S0896-6273(02)01125-X
94. Noble S, Ismail A, Godoy R, Xi Y, Ekker M. Zebrafish Parla- and Parlb-deficiency affects dopaminergic neuron patterning and embryonic survival. *J Neurochem* (2012) 122:196–207. doi:10.1111/j.1471-4159.2012.07758.x
95. Shi G, Lee JR, Grimes DA, Racacho L, Ye D, Yang H, et al. Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease. *Hum Mol Genet* (2011) 20:1966–74. doi:10.1093/hmg/ddr077
96. Bretaud S, Allen C, Ingham PW, Bandmann O. p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. *J Neurochem* (2007) 100:1626–35. doi:10.1111/j.1471-4159.2006.04291.x
97. Baulac S, Lu H, Strahle J, Yang T, Goldberg MS, Shen J, et al. Increased DJ-1 expression under oxidative stress and in Alzheimer's disease brains. *Mol Neurodegener* (2009) 4:1–12. doi:10.1186/1750-1326-4-12
98. Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP, Pownall S, et al. Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) and oxidative stress. *Proc Natl Acad Sci U S A* (2005) 102:5215–20. doi:10.1073/pnas.0501282102
99. de la Monte SM, Sohn YK, Ganju N, Wands JR. P53- and CD95-associated apoptosis in neurodegenerative diseases. *Lab Invest* (1998) 78:401–11.
100. Hartmann A, Michel PP, Troadec JD, Mouatt-Prigent A, Faucheux BA, Ruberg M, et al. Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? *J Neurochem* (2001) 76:1785–93. doi:10.1046/j.1471-4159.2001.00160.x
101. Sheng D, Qu D, Kwok KH, Ng SS, Lim AY, Aw SS, et al. Deletion of the WD40 domain of LRRK2 in zebrafish causes parkinsonism-like loss of neurons and locomotive defect. *PLoS Genet* (2010) 6:e1000914. doi:10.1371/journal.pgen.1000914
102. Prabhudesai S, Bensabeur FZ, Abdullah R, Basak I, Baez S, Alves G, et al. LRRK2 knockdown in zebrafish causes developmental defects, neuronal loss, and synuclein aggregation. *J Neurosci Res* (2016) 94:717–35. doi:10.1002/jnr.23754
103. Ren G, Xin S, Li S, Zhong H, Lin S. Disruption of lrrk2 does not cause specific loss of dopaminergic neurons in zebrafish. *PLoS One* (2011) 6:e20630. doi:10.1371/journal.pone.0020630
104. Tan EK, Zhao Y, Skipper L, Tan MG, Di Fonzo A, Sun L, et al. The LRRK2 Gly2385Arg variant is associated with Parkinson's disease: genetic and functional evidence. *Hum Genet* (2007) 120:857–63. doi:10.1007/s00439-006-0268-0
105. Wang L, Xie C, Greggio E, Parisiadou L, Shim H, Sun L, et al. The chaperone activity of heat shock protein 90 is critical for maintaining the stability of leucine-rich repeat kinase 2. *J Neurosci* (2008) 28:3384–91. doi:10.1523/JNEUROSCI.0185-08.2008
106. Andres-Mateos E, Mejias R, Sasaki M, Li X, Lin BM, Biskup S, et al. Unexpected lack of hypersensitivity in LRRK2 knock-out mice to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). *J Neurosci* (2009) 29:15846–50. doi:10.1523/JNEUROSCI.4357-09.2009
107. Fonzo AD, Dekker MC, Montagna P, Baruzzi A, Yonova EH, Guedes LC, et al. FBXO7 mutations cause autosomal recessive, early-onset parkinsonian-pyramidal syndrome. *Neurology* (2009) 72:240–5. doi:10.1212/01.wnl.0000338144.10967.2b
108. Zhao T, Zondervan-van der Linde H, Severijnen L-A, Oostra BA, Willemsen R, Bonifati V. Dopaminergic neuronal loss and dopamine-dependent locomotor defects in Fbxo7-deficient zebrafish. *PLoS One* (2012) 7:e48911. doi:10.1371/journal.pone.0048911
109. Lopes da Fonseca T, Correia A, Hasselaar W, van der Linde HC, Willemsen R, Outeiro TF. The zebrafish homologue of Parkinson's disease ATP13A2 is essential for embryonic survival. *Brain Res Bull* (2013) 90:118–26. doi:10.1016/j.brainresbull.2012.09.017
110. Weingarten L, Dave H, Li H, Crawford D. Developmental expression of P5 ATPase mRNA in the mouse. *Cell Mol Biol Lett* (2012) 17:153–70. doi:10.2478/s11658-011-0039-3
111. Farrell TC, Cario CL, Milanese C, Vogt A, Jeong J-H, Burton EA. Evaluation of spontaneous propulsive movement as a screening tool to detect rescue of Parkinsonism phenotypes in zebrafish models. *Neurobiol Dis* (2011) 44:9–18. doi:10.1016/j.nbd.2011.05.016
112. Irons TD, Kelly PE, Hunter DL, MacPhail RC, Padilla S. Acute administration of dopaminergic drugs has differential effects on locomotion in larval zebrafish. *Pharmacol Biochem Behav* (2013) 103:792–813. doi:10.1016/j.pbb.2012.12.010
113. Makhija DT, Jagtap AG. Studies on sensitivity of zebrafish as a model organism for Parkinson's disease: comparison with rat model. *J Pharmacol Pharmacother* (2014) 5:39–46. doi:10.4103/0976-500X.124422
114. Boxer AL, Yu J-T, Golbe LI, Litvan I, Lang AE, Höglinger GU. Advances in progressive supranuclear palsy: new diagnostic criteria, biomarkers, and therapeutic approaches. *Lancet Neurol* (2017) 16:552–63. doi:10.1016/S1474-4422(17)30157-6
115. Lopez A, Lee SE, Wojta K, Ramos EM, Klein E, Chen J, et al. A152T tau allele causes neurodegeneration that can be ameliorated in a zebrafish model by autophagy induction. *Brain* (2017) 140:1128–46. doi:10.1093/brain/awx005
116. Maeda S, Djukic B, Taneja P, Yu G-Q, Lo I, Davis A, et al. Expression of A152T human tau causes age-dependent neuronal dysfunction and loss in transgenic mice. *EMBO Rep* (2016) 17:530–51. doi:10.15252/embr.201541438
117. Decker JM, Krüger L, Sydow A, Dennissen FJ, Siskova Z, Mandelkow E, et al. The Tau/A152T mutation, a risk factor for frontotemporal-spectrum disorders, leads to NR2B receptor-mediated excitotoxicity. *EMBO Rep* (2016) 17:552–69. doi:10.15252/embr.201541439
118. Phukan J, Albanese A, Gasser T, Warner T. Primary dystonia and dystonia-plus syndromes: clinical characteristics, diagnosis, and pathogenesis. *Lancet Neurol* (2011) 10:1074–85. doi:10.1016/S1474-4422(11)70232-0
119. Hallett M. Neurophysiology of dystonia: the role of inhibition. *Neurobiol Dis* (2011) 42:177–84. doi:10.1016/j.nbd.2010.08.025
120. Jankovic J. Treatment of hyperkinetic movement disorders. *Lancet Neurol* (2009) 8:844–56. doi:10.1016/S1474-4422(09)70183-8
121. Ozelius LJ, Hewett JW, Page CE, Bressman SB, Kramer PL, Shalish C, et al. The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nat Genet* (1997) 17:40–8. doi:10.1038/ng0997-40
122. Sager JJ, Torres GE, Burton EA. The zebrafish homologue of the human DYT1 dystonia gene is widely expressed in CNS neurons but non-essential for early motor system development. *PLoS One* (2012) 7:e45175. doi:10.1371/journal.pone.0045175
123. Goodchild RE, Kim CE, Dauer WT. Loss of the dystonia-associated protein torsinA selectively disrupts the neuronal nuclear envelope. *Neuron* (2005) 48:923–32. doi:10.1016/j.neuron.2005.11.010
124. Kruer MC, Hiken M, Gregory A, Malandrini A, Clark D, Hogarth P, et al. Novel histopathologic findings in molecularly-confirmed pantothenate kinase-associated neurodegeneration. *Brain* (2011) 134:947–58. doi:10.1093/brain/awr042
125. Zizioli D, Tiso N, Guglielmi A, Saraceno C, Busolin G, Giuliani R, et al. Knock-down of pantothenate kinase 2 severely affects the development of the nervous and vascular system in zebrafish, providing new insights into PKAN disease. *Neurobiol Dis* (2016) 85:35–48. doi:10.1016/j.nbd.2015.10.010
126. Zech M, Lam DD, Francescatto L, Schormair B, Salminen AV, Jochim A, et al. Recessive mutations in the alpha-3 (VI) collagen gene COL6A3 cause early-onset isolated dystonia. *Am J Hum Genet* (2015) 96:1–11. doi:10.1016/j.ajhg.2015.04.010
127. Doganli C, Beck HC, Ribera AB, Oxvig C, Lykke-Hartmann K. α3Na+/K+-ATPase deficiency causes brain ventricle dilation and abrupt embryonic motility in zebrafish. *J Biol Chem* (2013) 288:8862–74. doi:10.1074/jbc.M112.421529
128. de Carvalho Aguiar P, Sweadner KJ, Penniston JT, Zaremba J, Liu L, Caton M, et al. Mutations in the Na+/K+-ATPase α3 gene ATP1A3 are associated with rapid-onset dystonia parkinsonism. *Neuron* (2004) 43:169–75. doi:10.1016/j.neuron.2004.06.028
129. Dobretsov M, Stimers JR. Neuronal function and alpha3 isoform of the Na/K-ATPase. *Front Biosci* (2005) 10:2373–96. doi:10.2741/1704
130. Tuschl K, Meyer E, Valdivia LE, Zhao N, Dadswell C, Abdul-Sada A, et al. Mutations in SLC39A14 disrupt manganese homeostasis and cause childhood-onset parkinsonism-dystonia. *Nat Commun* (2016) 7:1–16. doi:10.1038/ncomms11601

131. Xia Z, Wei J, Li Y, Wang J, Li W, Wang K, et al. Zebrafish slc30a10 deficiency revealed a novel compensatory mechanism of Atp2c1 in maintaining manganese homeostasis. *PLoS Genet* (2017) 13:e1006892. doi:10.1371/journal.pgen.1006892
132. Friedrich T, Lambert AM, Masino MA, Downes GB. Mutation of zebrafish dihydrolipoamide branched-chain transacylase E2 results in motor dysfunction and models maple syrup urine disease. *Dis Model Mech* (2012) 5:248–58. doi:10.1242/dmm.008383
133. Lu ZG, Li MH, Wang JS, Wei DD, Liu QW, Kong LY. Developmental toxicity and neurotoxicity of two matrine-type alkaloids, matrine and sophocarpine, in zebrafish (*Danio rerio*) embryos/larvae. *Reprod Toxicol* (2014) 47:33–41. doi:10.1016/j.reprotox.2014.05.015
134. Ross CA, Tabrizi SJ. Huntington's disease: from molecular pathogenesis to clinical treatment. *Lancet Neurol* (2011) 10:83–98. doi:10.1016/S1474-4422(10)70245-3
135. Karlovich CA, John RM, Ramirez L, Stainier DY, Myers RM. Characterization of the Huntington's disease (HD) gene homologue in the zebrafish *Danio rerio*. *Gene* (1998) 217:117–25. doi:10.1016/S0378-1119(98)00342-4
136. Lumsden AL, Henshall TL, Dayan S, Lardelli MT, Richards RI. Huntingtin-deficient zebrafish exhibit defects in iron utilization and development. *Hum Mol Genet* (2007) 16:1905–20. doi:10.1093/hmg/ddm138
137. Futter M, Diekmann H, Schoenmakers E, Sadiq O, Chatterjee K, Rubinsztein DC. Wild-type but not mutant huntingtin modulates the transcriptional activity of liver X receptors. *J Med Genet* (2009) 46:438–46. doi:10.1136/jmg.2009.066399
138. Diekmann H, Anichtchik O, Fleming A, Futter M, Goldsmith P, Roach A, et al. Decreased BDNF levels are a major contributor to the embryonic phenotype of huntingtin knockdown zebrafish. *J Neurosci* (2009) 29:1343–9. doi:10.1523/JNEUROSCI.6039-08.2009
139. Henshall TL, Tucker B, Lumsden AL, Nornes S, Lardelli MT, Richards RI. Selective neuronal requirement for huntingtin in the developing zebrafish. *Hum Mol Genet* (2009) 18:4830–42. doi:10.1093/hmg/ddp455
140. Lo Sardo V, Zuccato C, Gaudenzi G, Vitali B, Ramos C, Tartari M, et al. An evolutionary recent neuroepithelial cell adhesion function of huntingtin implicates ADAM10-Ncadherin. *Nat Neurosci* (2012) 15:713–21. doi:10.1038/nn.3080
141. Morrison PJ, Nevin NC. Serum iron, total iron binding capacity and ferritin in early Huntington disease patients. *Ir J Med Sci* (1994) 163:236–7. doi:10.1007/BF02943258
142. Bartzokis G, Cummings J, Perlman S, Hance DB, Mintz J. Increased basal ganglia iron levels in Huntington disease. *Arch Neurol* (1999) 56:569–74. doi:10.1001/archneur.56.5.569
143. Browne SE, Beal MF. The energetics of Huntington's disease. *Neurochem Res* (2004) 29:531–46. doi:10.1023/B:NERE.0000014824.04728.dd
144. Zuccato C, Marullo M, Conforti P, MacDonald ME, Tartari M, Cattaneo E. Systematic assessment of BDNF and its receptor levels in human cortices affected by Huntington's disease. *Brain Pathol* (2008) 18:225–38. doi:10.1111/j.1750-3639.2007.00111.x
145. Miller JP, Holcomb J, Al-Ramahi I, de Haro M, Gafni J, Zhang N, et al. Matrix metalloproteinases are modifiers of huntingtin proteolysis and toxicity in Huntington's disease. *Neuron* (2010) 67:199–212. doi:10.1016/j.neuron.2010.06.021
146. Nasir J, Floresco SB, O'Kusky JR, Diewert VM, Richman JM, Zeisler J, et al. Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell* (1995) 81:811–23. doi:10.1016/0092-8674(95)90542-1
147. Miller VM, Nelson RF, Gouvion CM, Williams A, Rodriguez-Lebron E, Harper SQ, et al. CHIP suppresses polyglutamine aggregation and toxicity in vitro and in vivo. *J Neurosci* (2005) 25:9152–61. doi:10.1523/JNEUROSCI.3001-05.2005
148. Schiffer NW, Broadley SA, Hirschberger T, Tavan P, Kretzschmar HA, Giese A, et al. Identification of anti-prion compounds as efficient inhibitors of polyglutamine protein aggregation in a zebrafish model. *J Biol Chem* (2007) 282:9195–203. doi:10.1074/jbc.M607865200
149. Veldman MB, Rios-Galdamez Y, Lu X-H, Gu X, Qin W, Li S, et al. The N17 domain mitigates nuclear toxicity in a novel zebrafish Huntington's disease model. *Mol Neurodegener* (2015) 10:67. doi:10.1186/s13024-015-0063-2
150. Gu X, Cantle JP, Greiner ER, Lee CYD, Barth AM, Gao F, et al. N17 modifies mutant huntingtin nuclear pathogenesis and severity of disease in HD BAC transgenic mice. *Neuron* (2015) 85:726–41. doi:10.1016/j.neuron.2015.01.008
151. Beal MF, Kowall NW, Ellison DW, Mazurek MF, Swartz KJ, Martin JB. Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* (1986) 321:168–71. doi:10.1038/321168a0
152. Tattersfield A, Croon R, Liu Y, Kells A, Faull RL, Connor B. Neurogenesis in the striatum of the quinolinic acid lesion model of Huntington's disease. *Neuroscience* (2004) 127:319–32. doi:10.1016/j.neuroscience.2004.04.061
153. Collin T, Arvidsson A, Kokaia Z, Lindvall O. Quantitative analysis of the generation of different striatal neuronal subtypes in the adult brain following excitotoxic injury. *Exp Neurol* (2005) 195:71–80. doi:10.1016/j.expneurol.2005.03.017
154. Skaggs K, Goldman D, Parent JM. Excitotoxic brain injury in adult zebrafish stimulates neurogenesis and long-distance neuronal integration. *Glia* (2014) 62:2061–79. doi:10.1002/glia.22726
155. Leonard H, Cobb S, Downs J. Clinical and biological progress over 50 years in Rett syndrome. *Nat Rev Neurol* (2016) 13:37–51. doi:10.1038/nrneurol.2016.186
156. Chen RZ, Akbarian S, Tudor M, Jaenisch R. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet* (2001) 27:327–31. doi:10.1038/85906
157. Pietri T, Roman A-C, Guyon N, Romano SA, Washbourne P, Moens CB, et al. The first mecp2-null zebrafish model shows altered motor behaviors. *Front Neural Circuits* (2013) 7:118. doi:10.3389/fncir.2013.00118
158. Cortelazzo A, Pietri T, De Felice C, Leoncini S, Guerranti R, Signorini C, et al. Proteomic analysis of the Rett syndrome experimental model mecp2Q63X mutant zebrafish. *J Proteomics* (2017) 154:128–33. doi:10.1016/j.jprot.2016.12.010
159. Conti V, Gandaglia A, Galli F, Tirone M, Bellini E, Campana L, et al. MeCP2 affects skeletal muscle growth and morphology through non cell-autonomous mechanisms. *PLoS One* (2015) 10:e0130183. doi:10.1371/journal.pone.0130183
160. Segawa M. Early motor disturbances in Rett syndrome and its pathophysiological importance. *Brain Dev* (2005) 27:S54–8. doi:10.1016/j.braindev.2004.11.010
161. Kriaucionis S, Paterson A, Curtis J, Guy J, Macleod N, Bird A. Gene expression analysis exposes mitochondrial abnormalities in a mouse model of Rett syndrome. *Mol Cell Biol* (2006) 26:5033–42. doi:10.1128/MCB.01665-05
162. Viola A, Saywell V, Villard L, Cozzone PJ, Lutz NW. Metabolic fingerprints of altered brain growth, osmoregulation and neurotransmission in a Rett syndrome model. *PLoS One* (2007) 2:e157. doi:10.1371/journal.pone.0000157
163. Jin L-W, Horiuchi M, Wulff H, Liu X-B, Cortopassi GA, Erickson JD, et al. Dysregulation of glutamine transporter SNAT1 in Rett syndrome microglia: a mechanism for mitochondrial dysfunction and neurotoxicity. *J Neurosci* (2015) 35:2516–29. doi:10.1523/JNEUROSCI.2778-14.2015
164. Justice MJ, Buchovecky CM, Kyle SM, Djukic A. A role for metabolism in Rett syndrome pathogenesis. *Rare Dis* (2013) 1:e27265. doi:10.4161/rdis.27265
165. Gao H, Bu Y, Wu Q, Wang X, Chang N, Lei L, et al. Mecp2 regulates neural cell differentiation by suppressing the Id1-Her2/Hes5 axis in zebrafish. *J Cell Sci* (2015) 128:2340–50. doi:10.1242/jcs.167874
166. Nozawa K, Lin Y, Kubodera R, Shimizu Y, Tanaka H, Ohshima T. Zebrafish Mecp2 is required for proper axonal elongation of motor neurons and synapse formation. *Dev Neurobiol* (2017) 77:1101–13. doi:10.1002/dneu.22498
167. Leong WY, Lim ZH, Korzh V, Pietri T, Goh ELK. Methyl-CpG binding protein 2 (Mecp2) regulates sensory function through Sema5b and Robo2. *Front Cell Neurosci* (2015) 9:481. doi:10.3389/fncel.2015.00481
168. Samaco RC, Fryer JD, Ren J, Fyffe S, Chao H-T, Sun Y, et al. A partial loss of function allele of methyl-CpG-binding protein 2 predicts a human neurodevelopmental syndrome. *Hum Mol Genet* (2008) 17:1718–27. doi:10.1093/hmg/ddn062
169. Louis ED. Essential tremor. *Lancet Neurol* (2005) 4:100–10. doi:10.1016/S1474-4422(05)00991-9

170. Elble RJ. Tremor disorders. *Curr Opin Neurol* (2013) 26:413–9. doi:10.1097/WCO.0b013e3283632f46
171. Hor H, Francescatto L, Bartesaghi L, Ortega-Cubero S, Kousi M, Lorenzo-Betancor O, et al. Missense mutations in TENM4, a regulator of axon guidance and central myelination, cause essential tremor. *Hum Mol Genet* (2015) 24:5677–86. doi:10.1093/hmg/ddv281
172. Robertson MM, Eapen V, Singer HS, Martino D, Scharf JM, Paschou P, et al. Gilles de la Tourette syndrome. *Nat Rev Dis Primers* (2017) 3:16097. doi:10.1038/nrdp.2016.97
173. Fontana BD, Mezzomo NJ, Kalueff AV, Rosemberg DB. The developing utility of zebrafish models of neurological and neuropsychiatric disorders: a critical review. *Exp Neurol* (2018) 299:157–71. doi:10.1016/j.expneurol.2017.10.004
174. Jagmag SA, Tripathi N, Shukla SD, Maiti S, Khurana S. Evaluation of models of Parkinson's disease. *Front Neurosci* (2016) 9:503. doi:10.3389/fnins.2015.00503
175. Duyao MP, Auerbach AB, Ryan A, Persichetti F, Barnes GT, McNeil SM, et al. Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science* (1995) 269:407–10. doi:10.1126/science.7618107
176. Visanji NP, Brotchie JM, Kalia LV, Koprich JB, Tandon A, Watts JC, et al. α-Synuclein-based animal models of Parkinson's disease: challenges and opportunities in a new era. *Trends Neurosci* (2016) 39:750–62. doi:10.1016/j.tins.2016.09.003
177. Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, et al. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* (2013) 10:70–86. doi:10.1089/zeb.2012.0861
178. Panula P, Sallinen V, Sundvik M, Kolehmainen J, Torkko V, Tiittula A, et al. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. *Zebrafish* (2006) 3:235–47. doi:10.1089/zeb.2006.3.235
179. Stewart AM, Braubach O, Spitsbergen J, Gerlai R, Kalueff AV. Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci* (2014) 37:264–78. doi:10.1016/j.tins.2014.02.011
180. Nicolson T, Rüsch A, Friedrich RW, Granato M, Ruppersberg JP, Nüsslein-Volhard C. Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron* (1998) 20:271–83. doi:10.1016/S0896-6273(00)80455-9
181. Feng Y, Xu Q. Pivotal role of hmx2 and hmx3 in zebrafish inner ear and lateral line development. *Dev Biol* (2010) 339:507–18. doi:10.1016/j.ydbio.2009.12.028
182. Blazina AR, Vianna MR, Lara DR. The spinning task: a new protocol to easily assess motor coordination and resistance in zebrafish. *Zebrafish* (2013) 10:480–5. doi:10.1089/zeb.2012.0860
183. Chatterjee P, Padmanarayana M, Abdullah N, Holman CL, LaDu J, Tanguay RL, et al. Otoferlin deficiency in zebrafish results in defects in balance and hearing: rescue of the balance and hearing phenotype with full-length and truncated forms of mouse otoferlin. *Mol Cell Biol* (2015) 35:1043–54. doi:10.1128/MCB.01439-14
184. Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome* (1997) 8:711–3. doi:10.1007/s003359900551
185. Brooks SP, Dunnett SB. Tests to assess motor phenotype in mice: a user's guide. *Nat Rev Neurosci* (2009) 10:519–29. doi:10.1038/nrn2652
186. Zupanc GK. Adult neurogenesis and neuronal regeneration in the brain of teleost fish. *J Physiol Paris* (2008) 102:357–73. doi:10.1016/j.jphysparis.2008.10.007
187. Kokel D, Bryan J, Laggner C, White R, Cheung CY, Mateus R, et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat Chem Biol* (2010) 6:231–7. doi:10.1038/nchembio.307
188. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science* (2010) 327:348–51. doi:10.1126/science.1183090
189. Bruni G, Lakhani P, Kokel D. Discovering novel neuroactive drugs through high-throughput behavior-based chemical screening in the zebrafish. *Front Pharmacol* (2014) 5:153. doi:10.3389/fphar.2014.00153
190. Mathias JR, Saxena MT, Mumm JS. Advances in zebrafish chemical screening technologies. *Future Med Chem* (2012) 4:1811–22. doi:10.4155/fmc.12.115
191. Nishimura Y, Murakami S, Ashikawa Y, Sasagawa S, Umemoto N, Shimada Y, et al. Zebrafish as a systems toxicology model for developmental neurotoxicity testing. *Congenit Anom (Kyoto)* (2015) 55:1–16. doi:10.1111/cga.12079
192. Goldstone JV, Mcarthur AG, Kubota A, Zanette J, Parente T, Jönsson ME, et al. Identification and developmental expression of the full complement of cytochrome P450 genes in zebrafish. *BMC Genomics* (2010) 11:643. doi:10.1186/1471-2164-11-643
193. Chng HT, Ho HK, Yap CW, Lam SH, Chan ECY. An investigation of the bioactivation potential and metabolism profile of zebrafish versus human. *J Biomol Screen* (2012) 17:974–86. doi:10.1177/1087057112447305
194. FDA Center for Drug Evaluation and Research. *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Food and Drug Administration (FDA) (2005). Available from: https://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf
195. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm* (2016) 7:27–31. doi:10.4103/0976-0105.177703
196. Parng C, Ton C, Lin Y-X, Roy NM, McGrath P. A zebrafish assay for identifying neuroprotectants in vivo. *Neurotoxicol Teratol* (2006) 28:509–16. doi:10.1016/j.ntt.2006.04.003
197. Buckley CE, Marguerie A, Roach AG, Goldsmith P, Fleming A, Alderton WK, et al. Drug reprofiling using zebrafish identifies novel compounds with potential pro-myelination effects. *Neuropharmacology* (2010) 59:149–59. doi:10.1016/j.neuropharm.2010.04.014
198. Li G, Zhang Z, Quan Q, Jiang R-W, Szeto SS, Yuan S, et al. Discovery, synthesis, and functional characterization of a novel neuroprotective natural product from the fruit of alpinia oxyphylla for use in Parkinson's disease through LC/MS-based multivariate data analysis-guided fractionation. *J Proteome Res* (2016) 15:2595–606. doi:10.1021/acs.jproteome.6b00152
199. Robertson AL, Ogryzko NV, Henry KM, Loynes CA, Foulkes MJ, Meloni MM, et al. Identification of benzopyrone as a common structural feature in compounds with anti-inflammatory activity in a zebrafish phenotypic screen. *Dis Model Mech* (2016) 9:621–32. doi:10.1242/dmm.024935
200. Goessling W, Allen RS, Guan X, Jin P, Uchida N, Dovey M, et al. Prostaglandin E2 enhances engraftment of human cord blood stem cells and shows long-term safety in preclinical non-human primate transplant models. *Cell Stem Cell* (2011) 8:445–58. doi:10.1016/j.stem.2011.02.003

Conflict of Interest Statement: RV was affiliated with TechnoPhage SA, under the scope of a PhD student scholarship financed by FCT. All other authors declare no conflicts of interest.

frontiers in Neurology

REVIEW
published: 19 June 2018
doi: 10.3389/fneur.2018.00455

On Cell Loss and Selective Vulnerability of Neuronal Populations in Parkinson's Disease

Nicolas Giguère[†], *Samuel Burke Nanni*[†] *and Louis-Eric Trudeau*[*]

CNS Research Group, Department of Pharmacology and Physiology, Department of Neurosciences, Faculty of Medicine, Université de Montréal, Montreal, QC, Canada

Significant advances have been made uncovering the factors that render neurons vulnerable in Parkinson's disease (PD). However, the critical pathogenic events leading to cell loss remain poorly understood, complicating the development of disease-modifying interventions. Given that the cardinal motor symptoms and pathology of PD involve the loss of dopamine (DA) neurons of the substantia nigra pars compacta (SNc), a majority of the work in the PD field has focused on this specific neuronal population. PD however, is not a disease of DA neurons exclusively: pathology, most notably in the form of Lewy bodies and neurites, has been reported in multiple regions of the central and peripheral nervous system, including for example the locus coeruleus, the dorsal raphe nucleus and the dorsal motor nucleus of the vagus. Cell and/or terminal loss of these additional nuclei is likely to contribute to some of the other symptoms of PD and, most notably to the non-motor features. However, exactly which regions show actual, well-documented, cell loss is presently unclear. In this review we will first examine the strength of the evidence describing the regions of cell loss in idiopathic PD, as well as the order in which this loss occurs. Secondly, we will discuss the neurochemical, morphological and physiological characteristics that render SNc DA neurons vulnerable, and will examine the evidence for these characteristics being shared across PD-affected neuronal populations. The insights raised by focusing on the underpinnings of the selective vulnerability of neurons in PD might be helpful to facilitate the development of new disease-modifying strategies and improve animal models of the disease.

Keywords: Parkinson, vulnerability, dopamine, cell death, neurodegeneration

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Paolo Gubellini, Centre National de la Recherche Scientifique (CNRS), France
Nicolas Xavier Tritsch, Langone Medical Center, New York University, United States

***Correspondence:**
Louis-Eric Trudeau
louis-eric.trudeau@umontreal.ca

[†]*Co-first authors.*

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *05 April 2018*
Accepted: *29 May 2018*
Published: *19 June 2018*

Citation:
Giguère N, Burke Nanni S and Trudeau L-E (2018) On Cell Loss and Selective Vulnerability of Neuronal Populations in Parkinson's Disease. Front. Neurol. 9:455. doi: 10.3389/fneur.2018.00455

INTRODUCTION

Parkinson's disease (PD) was first described two centuries ago in *An essay on the shaking palsy* (1). Since then, great strides have been made in understanding the disease basics. However—as with many other neurodegenerative disorders—there is still no disease modifying treatment for PD. Unfortunately, progress has been slow, and a thorough understanding of the pathological processes has been elusive.

PD as a clinical diagnosis is characterized by the detection of significant motor deficits (including bradykinesia, resting tremor, and rigidity) due, in large part, to a loss of dopamine (DA)-containing neurons of the substantia nigra pars compacta (SNc). The SNc is a neuronal population projecting to the caudate and putamen and is critical for regulation of basal ganglia circuitry. At clinical presentation, it has been estimated that 40–60% of SNc DA neurons have already degenerated (2, 3). The clinical features of the disease are diverse and include substantial non-motor features including, autonomic and

olfactory dysfunction, constipation, sleep disturbances, depression, and anxiety (4–6).

The diagnostic criteria for PD have been recently re-defined by the International Parkinson and Movement Disorder Society (MDS), with the MDS Clinical Diagnostic Criteria for Parkinson's disease [MDS-PD Criteria (7)]. A diagnosis is made when there is documented parkinsonism (defined as bradykinesia, with tremor at rest and/or rigidity), followed by the exclusion of other possible causes of parkinsonism, and with additional supporting criteria, including olfactory dysfunction or cardiac sympathetic denervation [see (7)]. The recent nature of this re-evaluation illustrates both the heterogeneity of PD expression, and the difficulties encountered in defining it.

In ≈70% of the 'clinically typical PD cases', the hallmark pathological finding is the presence of Lewy pathology (LP) in the SNc (4, 5)—however, LP is also found across the central, peripheral, and enteric nervous system (CNS, PNS, and ENS) (6). This includes both Lewy bodies and Lewy neurites: both similar cellular inclusions, formed predominantly of aggregated α-synuclein, but also including a large number of different molecules, proteins and organelles, such as ubiquitin, tubulin, neurofilaments, lipids, and mitochondria (8).

In considering the broad localization of LP and the origins of the various symptoms of PD, a critical point to consider is the dysfunction and loss of neurons in regions of the CNS and PNS, other than the SNc. There have been, indeed, many studies concluding that cholinergic neurons in the pedunculopontine nucleus (PPN), noradrenergic neurons of the locus coeruleus (LC), cholinergic neurons of the nucleus basalis of Meynert (NBM) and of the dorsal motor nucleus of the vagus (DMV), and serotonergic neurons of the raphe nuclei (RN) are lost in PD. The strength of the evidence for actual neuronal cell body loss in these regions is highly variable and is one of the questions addressed in the present review. The fact that the diagnostic criteria for PD have over time been refined adds another layer of complexity to the task of identifying the origin of the diverse symptoms of PD. Presently, PD is classified into either primary or secondary subtypes. Primary parkinsonism includes genetic and idiopathic forms of the disease and secondary parkinsonism includes forms induced by drugs, infections, toxins, vascular defects, brain trauma or tumors or metabolic dysfunctions. This second subtype of PD is also sometimes called atypical parkinsonism when concomitant to progressive supranuclear palsy, multiple system atrophy or corticobasal degeneration, for example.

Since pathology is likely to emerge through different processes depending of PD subtypes, and since modern classification was non-existent when a substantial part of the research literature was produced, attempting to reach clear general vision of various pathophysiological markers and their link to disease progression for each sub-type of PD presents a significant challenge. This review will primarily focus on idiopathic PD, since this category represents the large majority of cases and is likely to represent most of the subjects examined in studies where PD type was not provided.

Another main hurdle in PD research is that the chain of events that leads to the death of neurons is still not clear. The fact that pathology is thought to begin years/decades before the appearance of symptoms might, in part, explain this lack of progress.

PD has been considered to exist as either a strictly monogenetic or environmentally-triggered disease, as well as a mixture of the two. The pathological mechanisms at the core of each form have been proposed to converge in causing cellular stress secondary to mitochondrial dysfunction, perturbed proteostasis and elevated oxidative stress. A major conundrum is that at first glance, these factors alone fail to explain why PD pathology is restricted to very limited subsets of brain nuclei. Therefore, a key question is what do these PD sensitive neurons have in common and what is it about them that renders them more vulnerable compared to neurons from other brain regions?

A better understanding of the fundamental nature of cell loss and cellular dysfunction in the parkinsonian brain is required to develop critically needed, novel, therapeutic strategies. In this review, we aim to re-evaluate the evidence for cell loss in PD, then to highlight the common characteristics that could explain their selective vulnerability.

PHYSIOPATHOLOGY OF PARKINSON'S DISEASE

The focus on SNc DA neurons has brought significant advances in our understanding of PD pathophysiology, as well as of the signaling pathways that lead to DA neuron death. Studies using DA neuron selective toxins such as 6-OHDA and MPTP, as well as investigations of gene products mutated in familial forms of the disease (including α-synuclein, Parkin, Pink1, LRRK2, DJ-1, and GBA1), have been instrumental to better understand some of the key dysfunctional processes implicated in the disease. These include protein clearance (9–11), mitochondrial turnover (12–14), ROS management (15, 16), and inflammation (17, 18). Perturbations of these processes have been proposed to underlie distinct physiological dysfunctions in PD-vulnerable neurons (19). Nonetheless, since the first introduction of Levodopa in the 1950s and the development of deep-brain stimulation in the 1990s, increased understanding of PD pathophysiology has not yet permitted the discovery of disease-modifying therapies.

As stated previously, PD is more than just a disease of DA and the SNc. Non-motor symptoms—including a reduced sense of smell, constipation, orthostatic hypotension, sleep disturbances, depression, and anxiety—are likely to be due to impaired function and/or loss of non-DA neurons (20). There has thus been a growing interest in better understanding the implications of other regions of the CNS and PNS in the progression of PD pathology. In the early 2000s, pioneering work by Braak and colleagues defined stages in PD based on the appearance of LP in

Abbreviations: AD, Alzheimer Disease; ADLB, Alzheimer's Disease with Lewy bodies; ADNLB, Alzheimer's Disease with no Lewy bodies; ALS, Amyotrophic Lateral Sclerosis; CBS, corticobasal syndrome; CGS, central gray substance; CJD, Creutzfeldt-Jakob disease; ctrl, control; DLB, dementia with Lewy bodies; H&Y, Hoehn and Yahr scale; iPA, idiopathic paralysis agitans; LBD or iLBD, Lewy body disease or idiopathic Lewy body disease; LDB or iLDB, dementia with Lewy bodies or idiopathic dementia with Lewy bodies; LID, levodopa (L-dopa)–induced dyskinesias; MS, multiple sclerosis; MSA, multiple system atrophy; NPH, normal pressure hydrocephalus; PD or iPD, Parkinson's disease or idiopathic Parkinson's Disease; PSP, progressive supranuclear palsy; UPDRS, unified Parkinson disease rating scale.

various regions of the nervous system, correlating their findings to the symptomatic progression of the disease (21–23). Most notably, LP was detected in the dorsal IX/X motor nuclei, the intermediate reticular zone, the medulla oblongata, the pontine tegmentum, the caudal RN, the gigantocellular reticular nucleus, the coeruleus–subcoeruleus complex, the pars compacta of the substantia nigra, the basal prosencephalon, the mesocortex, and the neocortex. However, multiple lines of evidence suggest that LP is not systematically seen in the PD brain and LP is also documented in healthy individuals (24). Also, in some cases of PD, and most notably in early-onset genetic forms, loss of SNc DA neurons has been reported to occur in the absence of detectable LP (25–27).

Although the role of LP in the pathogenesis of PD has been the subject of much debate (28), the detection of LP has remained central in investigations of the key brain regions and circuits underlying PD pathophysiology. In this context, it may be useful to focus attention on brain and PNS regions that show documented cell death and/or axonal degeneration, irrespective of the presence or absence of LP. This could perhaps provide new perspectives on the actual, more proximate, causes of the major symptoms of the disease and their progression. Relevant to the present point, in their most recent and insightful work, Braak and Tredici write, "We ascribed the same weight to axonopathy and nerve cell dysfunction (presumably attributable, but not limited, to the presence of Lewy pathology) as to neuronal death because the development of pathology together with neurotransmitter loss, axonal, and somatodendritic dysfunction in multiple neuronal populations could prove to be more stressful for involved neurons over time than premature cell death within a select neuronal population" (6).

WHERE AND WHEN DOES NEURONAL LOSS APPEAR IN PD?

Loss of neurons in the brain is thought to occur in the context of normal aging. For example, there have been multiple publications reporting significant age-dependent decline in neuron number in the SNc (29–37), as well as in regions such as the PPN (38), and LC (39, 40). Above and beyond such cell loss associated with normal aging, a key question is where in the brain can one find substantial neuronal loss in PD?

Although numerous publications have referred to cell loss occurring in many CNS and PNS regions in the context of PD, we believed it germane to re-evaluate the published scientific literature addressing this question.

To do so, we took great care to find work concentrating on neuronal loss and not only denervation [as is common for the heart, for example (41–43)]. We found 90 primary research articles reporting PD-specific cell loss in the following regions (**Table 1**): the SNc, VTA, amygdala, cortex, DMV, hypothalamus, laterodorsal tegmental nucleus, LC, NBM, OB, oral pontine reticular nucleus, PPN, pre-supplementary motor cortex, RN, supraoptic nucleus, sympathetic/parasympathetic ganglia, and thalamus. These original articles span from 1953 to 2015. The techniques used to quantify cell loss varied, and we have classified them accordingly. Across all regions examined, 14 of the examinations were defined as *observational*, 39 as implicating *manual* counting, 18 used *computer-assisted* counting, and 26 used *stereological* counting methods. While informative, the value of observational studies can be considered limited given their lack of precision and the fact that they are greatly influenced by the observer. Lack of bias is also difficult to assure in studies involving manual counting. This technique is also unable to assure that a cell is not being counted twice if present in two subsequent sections. Other techniques such as computer-assisted counting were developed to improve on these aforementioned methods, however, these are also limited in that they often lack rigorous systematic sampling, are sensitive to tissue shrinkage, and are often unable to account for local tissue thickness, or for cells damaged on slice edges. These issues are systematically addressed using modern stereological counting techniques. Another issue to consider is that many of the studies included in this review, including those employing stereology, either did not use age-matched controls, or did not state whether counting was conducted blind to diagnosis. Yet another apparent feature of this literature is the diversity of method iterations used, the varying number of brain regions assessed in each study and, importantly, the stage or type of PD studied (and how this was defined). Here, we will discuss the evidence of cell loss (if not otherwise stated, relative to healthy control cases), ordering the regions in subsections according to the strength of the evidence (**Table 1**).

SUBSTANTIA NIGRA PARS COMPACTA

Loss of SNc DA neurons in PD is indisputable. Here we found 38 studies addressing this directly with a total of 612 brains. However, if we consider the methods used, we found that 10 of these studies were observational, 8 involved manual counting methods, 8 used computer-assisted methods, and 12 used stereology. Considering stereological methods as best practice for unbiased evaluation of cell number, 181 brains were quantified as such for SNc: still a large number. The average cell loss reported for studies involving stereological methods is ∼68%. The definition and clinical stage of PD in most studies

Methodology and Scales of PD Progression

We searched the scientific literature using the search engines and databases of PubMed, Google Scholar and Science Direct. The following search terms were used: "PD," and "cell loss," "cell death," or "reduced cell/neuron number." Furthermore, these terms were used in combination with brain structure keywords: "SNc," "VTA," "LC," "Raphe," "DMV," "PPN," "NBM," and "enteric system" ("ENS"), and "gut." Review and original article abstracts were screened, then, where appropriate, read. Where any direct or indirect claim for cell loss was found (rather than only the presence of LP), the claim was followed to its original source.

The Hoehn and Yahr scale (H&Y) is a widely used clinical rating scale, which defines broad categories of motor function in PD (where 1 is the least severe, and 5, most severe symptoms) (132).

Braak staging is a method of classifying the progression of PD pathology and symptoms based on the presence of Lewy pathology (where 1 represents initial pathology in the brain stem, and 6, severe pathology including the neocortex) (21).

TABLE 1 | List of 90 studies quantifying the loss of neurons in the brain in PD.

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
Substantia nigra pars compacta (SNc)	Greenfield and Bosanquet (44)	o	19 (22)	Some	–	Not stated	iPA, <1–20 years	LC	–
	Pakkenberg and Brody (45)	m	10 (10)	66	Healthy controls and two young controls	Not stated/Yes	iPA	–	–
	Bernheimer et al. (46)	o	69 (0)	Some	No healthy controls, compared to type of PD and Huntington's disease	Not stated/Yes	PD, H&Y, 1–47 years	–	–
	Rajput and Rozdilsky (47)	o	6 (1)	Some	–	Not stated	iPA, H&Y, 3–18 years	LC, DMV, Cortex, Hypothalamus, Intermediolateral spinal cord, sympathetic ganglia	–
	Gaspar and Gray (48)	o	32 (6)	Some	–	Yes/Yes	iPD, 2–23 years	LC, NBM	–
	Tagliavini et al. (49)	o	6 (5)	Some	–	Not stated/Yes	iPD, 5–13 years	NBM	–
	Chan-Palay (50)	o	9 (22)	Some	–	Yes/Not stated	PD	NBM	–
	Gibb and Lees (51)	m	34 (–)	–	No healthy controls, compared young and old onset	Not stated	PD, 1–34 years	–	–
	Hirsch et al. (52)	c	4 (3)	77	–	Not stated	PD	A10, A8, CGS	–
	German et al. (53)	c	5 (3)	61	–	Not stated/Yes	PD, 5–27 years	VTA	–
	Rinne et al. (54)	s	12 (18)	60	–	Not stated/Yes	iPD, H&Y II–V	–	Yes
	Zweig et al. (55)	o	6 (8)	Mild to severe	Not compared—estimation	Not stated/Yes	PD, 5–14 years	PPN, DR, NBM	–
	Gibb et al. (56)	m	6 (6)	75	–	Not stated	PD	–	–
	Halliday et al. (57)	c	4 (4)	68	–	Not stated/Yes	PD	SNc + LC, RN, PPN, DMV	Yes (dementia score)
	Fearnley and Lees (31)	m	20 (36)	20–90	–	Not stated/Yes	PD, 1.5–38 years	–	Yes (also in controls)
	Pakkenberg et al. (58)	s	7 (7)	66	–	Not stated/Yes	PD, 4–16 years	–	–
	Paulus and Jellinger (59)	m	39 (14)	59	–	Not stated/Yes	PD, H&Y III–V, 1–31 years	LC, DRN, NBM	–
	Xuereb et al. (60)	o	5 (5)	Some	–	Not stated/Yes	PD	Thalamus (multiple nuclei)	–
	Moller (61)	c	3 (3)	80	–	Not stated/Yes	PD	–	–
	Zweig et al. (62)	m	13 (14)	Some	–	Yes	PD, H&Y 4.5, 11 years	LC, VTA, NBM	–
	Mouatt-Prigent et al. (63)	c	4 (3)	76	–	Not stated/Yes	iPD	VTA	–
	Ma et al. (64)	s	4 (7)	70	–	Not stated	PD	–	–
	Halliday et al. (65)	s	11 (15)	37–75	–	Not stated/Yes	PD, 1–18 years	–	Yes
	Ma et al. (66)	c	20 (8)	76	–	Not stated/Yes	PD	–	–
	Ma et al. (67)	s	12 (12)	55	–	Not stated/Yes	PD, H&Y III–V, 3–17 years	–	Yes
	Damier et al. (68)	c	5 (5)	86	–	Not stated	iPD	VTA	Yes

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
	Henderson et al. (69)	c	9 (8)	69	–	Not stated/Yes	PD, H&Y II–V, 3–17 years	Centromedian–Parafascicular Complex, mediodorsal or anterior principal nucleus	–
	Zarrow et al. (70)	m	19 (13)	78	Healthy controls, AD	Not stated/Yes	iPD, 12.4 years	LC, NBM	
	Greffard et al. (71)	o	14 (5)	50	–	Not stated/Yes	iPD, UPDRS3 = 53, 8.5 years	–	Yes
	Rudow et al. (35)	s	8 (23)	∼80 vs. young, ∼75 vs. old controls	Young, middle aged and old healthy controls	Not stated/Yes	PD, 7–20 years	–.	Yes, in controls
	Beach et al. (72)	o	66 (87)	some	Healthy controls, ILDB, DLB, ADLB, ADNLB	Yes/Not stated	PD + DLB, UPDRS = 41, 10.6 years	–	–
	Karachi et al. (73)	s	12 (8)	69–88	–	Yes	PD, UPDRS	PPN	–
	Milber et al. (74)	s	13 (17)	70	Healthy controls, iLBD	Yes/Not stated	PD, Braak stage I–VI, 8.3 years.	–	Yes in iLBD
	Kordower et al. (75)	s	28 (9)	50–90	–	Yes	PD, 1–27 years	–	Yes
	Dijkstra et al. (76)	s	24 (12)	56	Healthy controls, iLBD	Yes	PD and iLBD, Braak stage 0–VI, H&Y, 13.6 years	–	Yes
	Kraemmer et al. (77)	m	4 (0)	–	No healthy controls, compare to AD, CJD, CBS, NPH	Yes/Not stated	PD and DLB, 2–4 years	–	–
	Cheshire et al. (78)	s	44 (17)	75	–	Yes	PD, LID severity, 14.8 years	RN	–
	Iacono et al. (79)	s	6 (6)	82	–	Yes	iPD and iLDB, Braak stage I–IV, H&Y 2–5,	–	–
Total	**38**	**o10, m8, c8, s12**	**612 (452)**						
Locus coeruleus (LC)	Rajput and Rozdilsky (47)	o	6 (1)	Some	–	Not stated	iPA H&Y, 3–18 years	SN, DMV, Cortex, Hypothalamus, Intermediolateral spinal cord, sympathetic ganglia	–
	Gaspar and Gray (48)	o	32 (6)	Some	–	Yes	iPD, 2–23 years	SNc, NBM	–
	Hirsch et al. (52)	c	4 (3)	55	–	Not stated	PD	SNc, A10, A8	–
	Chan-Palay and Asan (80)	c	6 (3)	31–94*	–	Not stated/Yes	PD	–	–
	Zweig et al. (55)	o	6 (8)	Mild to severe	Not compared—estimation	Not stated/Yes	PD, 5–14 years	PPN, SNc, DR, NBM	–
	Halliday et al. (57)	c	4 (4)	68	–	Not stated/Yes	PD	SNc + LC, RN, PPN, DMV	–
	Gai et al. (81)	c	6 (5)	74	–	Not stated/Yes	iPD, 5–30 years	PPN, LTN, OPN, RN	Yes

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
	Paulus and Jellinger (59)	m	37 (12)	63	–	Not stated/Yes	PD, H&Y III–V, 1–31 years	SNc, DRN, NBM	–
	German et al. (82)	c	6 (7)	21–93	Healthy controls, AD, down-syndrome	Not stated/Yes	PD, 5–16 years	–	–
	Patt and Gerhard (83)	o	8 (8)	Some	–	Not stated	PD	–	–
	Zweig et al. (62)	m	13 (14)	46–69	–	Yes/Yes	PD, H&Y 4.5, 11 years	SNc, VTA, NBM	–
	Hoogendijk et al. (84)	c	5 (5)	39 NS	Healthy controls, AD, ALS	Not stated/Yes	PD, 7 years	–	–
	Bertrand et al. (85)	c	11 (6)	58–78	–	Not stated	PD	–	Yes
	Zarrow et al. (70)	m	19 (13)	83	Healthy controls, AD	Not stated/Yes	iPD, 12.4 years	SNc, NBM	–
	Brunnstrom et al. (86)	m	25 (0)	Mild-severe	Healthy controls, AD	Yes/Not stated	DLB and PD dementia	–	–
	McMillan et al. (87)	m	7 (8)	71–88	Healthy controls, AD, DLB	Yes	PD, 7–25 years	–	–
	Dugger et al. (88)	c	21 (11)	Some	–	Not stated/Yes	LBD, 8.4 years	PPN	–
	Del Tredici and Braak (89)	o	5 (1)	Some	–	Not stated	PD, H&Y 3–5, 7–15 years	–	–
Total	**18**	**o5, m4, c9, s0**	**221 (115)**						–
*31 w/o dementia, 48 w/dementia, 94 if Non-responsive to L-dopa									
Nucleus basalis of meynert (NBM)	Arendt et al. (90)	m	5 (14)	70	–	Not stated/Yes	Postencephalitic PD	–	–
	Candy et al. (91)	m	5 (5)	Some	Healthy controls, AD	Not stated	PD	–	–
	Nakano and Hirano (92)	m	2 (5)	90	–	Not stated/Yes	PD-dementia complex of Guam, 4–5 years	–	–
	Whitehouse et al. (93)	m	12 (10)	45–71	–	Yes	iPD, 4–26 years	–	–
	Gaspar and Gray (48)	m	32 (6)	36	–	Yes	iPD, 2–23 years	SNc, LC	–
	Nakano and Hirano (94)	m	11 (13)	60	–	Not stated/Yes	PD, 1–17 years	–	–
	Tagliavini et al. (49)	m	6 (5)	46–69	–	Not stated/Yes	iPD, 5–13 years	SNc	–
	Perry et al. (95)	m	4 (8)	17–72	Healthy controls, AD	Not stated/Yes	PD	–	–
	Rogers et al. (96)	m	4 (5)	Some	Healthy controls, PSP, Creutzfeldt-Jakob disease, ALS, MS and AD (+ individual cases of other diseases)	Not stated/Yes	PD	–	–
	Chan-Palay (50)	m	9 (22)	~50	Healthy controls, AD	Yes/Not stated	PD	SNc	–
	Paulus and Jellinger (59)	m	40 (17)	Some	–	Not stated/Yes	PD, H&Y III–V, 1–31 years	SNc, LC, DRN	–
	Zweig et al. (62)	o	13 (14)	Some	–	Yes	PD, H&Y 4.5, 11 years	LC, SNc, VTA	–
	Zarrow et al. (70)	m	19 (13)	37	Healthy controls, AD	Not stated/Yes	iPD, 12.4 years	SNc, LC	–
Total	**13**	**o1, m12, c0, s0**	**162 (137)**						

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
Pedunculopontine nucleus (PPN)	Hirsch et al. (97)	c	6 (4)	57	Healthy controls, supranuclear palsy	Not stated	PD	–	–
	Jellinger (98)	m	14 (15)	53	–	Not stated/Yes	PD, 10 years	–	–
	Zweig et al. (55)	m	4 (8)	46–69	–	Not stated/Yes	PD, 10–14 years	–	–
	Halliday et al. (57)	c	4 (4)	57	–	Not stated/Yes	PD	SNc + LC, RN, DMV	–
	Gai et al. (81)	c	6 (5)	43	–	Not stated/Yes	iPD, 5–30 years	LTN, OPN, RN, LC	Yes
	Rinne et al. (99)	s	11 (9)	40	–	Not stated/Yes	PD, H&Y 2.5 and 5, 9.3 years	–	Yes
	Schmeichel et al. (100)	m	13 (11)	65	Healthy controls, MSA	Yes/Not stated	DLB, 3–16 years	Laterodorsal tegmental nucleus	–
	Karachi et al. (73)	s	12 (8)	31–38	–	Yes	PD, UPDRS 0–IV	SN	–
	Dugger et al. (88)	c	21 (11)	Some	–	Not stated/Yes	LBD, 8.4 years	LC	–
	Hepp et al. (101)	s	9 (9)	41	Healthy controls, DLB	Yes	PD, Braak stage IV–VI, H&Y IV–V, 8–26 years	–	–
	Pienaar et al. (102)	s	8 (5)	50	–	Yes	PD, Braak stage II–IV, 6–13 years	–	–
Total	**11**	**o0, m3, c4, s4**	**108 (89)**						
Hypothalamus	Rajput and Rozdilsky (47)	o	6 (1)	None	–	Not stated	iPA, H&Y, 3–18 years	SN, LC, DMV, Cortex, intermediolateral spinal cord, sympathetic ganglia	–
	Kremer (103)	m	8 (15)	None	–	Not stated	PD	–	–
	Kremer and Bots (104)	m	8 (7)	None	–	Not stated/Yes	iPD, 4–17 years	–	–
	Purba et al. (105)	m	6 (6)	20	–	Not stated/Yes	PD	–	–
	Nakamura et al. (106)	m	8 (6)	None	–	Not stated/Yes	iPD	–	–
	Ansorge et al. (107)	m	7 (8)	12–29	–	Not stated/Yes	PD, 18 years	–	–
	Hoogendijk et al. (108)	m	12 (6)	None	–	Yes	iPD	–	–
	Fronczek et al. (109)	c	9 (9)	45	–	Yes	PD, late-stage	–	–
	Thannickal et al. (110)	s	10 (5)	50	–	Not stated/Yes	PD, H&Y I–V, 4–23 years	–	Yes
Total	**9**	**o1, m6, c1, s1**	**74 (63)**						
Dorsal motor nucleus of the vagus nerve (DMV)	Eadie (111)	m	8 (5)	30	–	Not stated/Yes	PD	Hypoglossal nuclei, nucleus ambiguus	–
	Rajput and Rozdilsky (47)	o	6 (1)	Some	–	Not stated	iPA, H&Y, 3–18 years	SN, LC, Cortex, Hypothalamus, Intermediolateral spinal cord, sympathetic ganglia	–

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
	Halliday et al. (112)	c	4 (4)	77	–	Not stated	PD	RN	–
	Halliday et al. (57)	c	4 (4)	77	–	Not stated/Yes	PD	SNc + LC, RN, PPN	–
	Saper et al. (113)	m	5 (5)	60	–	Not stated	PD, 2–16 years	–	–
	Gai et al. (114)	s	8 (6)	55	–	Not stated/Yes	PD, 5–24 years	Hypoglossal nucleus	Yes
	Benarroch et al. (115)	o	14 (12)	50	–	Yes/Not stated	PD or LBD, 10 years	Nucleus ambiguus	–
Total:	**7**	**o2, m2, c2, s1**	**49 (37)**						
Raphe nuclei (RN)	Yamamoto and Hirano (116)	m	2 (1)	50–90	–	Not stated/Yes	iPD	–	–
	Halliday et al. (112)	c	4 (4)	0 dorsal-56 median	–	Not stated	PD	DMV	–
	Halliday et al. (57)	c	4 (4)	0 dorsal-44 obscurus-60 median	–	Not stated/Yes	PD	SNc + LC, PPN, DMV	–
	Gai et al. (81)	c	6 (5)	76	–	Not stated/Yes	iPD, 5–30 years	PPN, LTN, OPN, LC	–
	Paulus and Jellinger (59)	m	23 (6)	37	–	Not stated/Yes	PD, H&Y III–V, 1–31 years	SNc, LC, RN, NBM	–
	Benarroch et al. (117)	m	14 (12)	60–67	–	Yes	DLB, 5–20 years	–	–
	Cheshire et al. (78)	s	44 (17)	None	–	Yes	PD, LID severity, 14.8 years	SNc	–
Total:	**7**	**o0, m3, c3, s1**	**97 (49)**						
Ventral Tegmental Area (VTA)	Javoy-Agid et al. (118)	m	2 (2)	77	–	Not stated	PD	–	–
	Hirsch et al. (52)	c	4 (3)	48	–	Not stated	PD	SNc, A10, A8, CGS	–
	German et al. (53)	c	5 (3)	42	–	Not stated/Yes	PD, 5–27 years	SNc	–
	Zweig et al. (62)	m	13 (14)	Some	–	Yes	PD, H&Y 4.5, 11 years	LC, SNc, NBM	–
	Mouatt-Prigent et al. (63)	c	4 (3)	Some	–	Not stated/Yes	iPD	SNc	–
	Dymecki et al. (119)	m	7 (6)	41–62	–	Not stated/Yes	PD, long-term	–	–
	McRitchie et al. (120)	s	3 (3)	31	–	Not stated/Yes	iPD, 1–27 years	A8, A10	–
	Damier et al. (68)	c	5 (5)	46	–	Not stated	iPD	SNc	Yes
Total	**8**	**o0, m3, c4, s1**	**43 (39)**						
Olfactory bulb (OB)	Pearce et al. (121)	m	7 (7)	57	–	Not stated/Yes	PD, 8–19 years	–	–
	Huisman et al. (122)	s	10 (10)	Increase of 100	–	Not stated/Yes	PD, 4–23 years	–	–
	Huisman et al. (123)	s	20 (19)	Increase of 100 in female	–	Yes	iPD, 3–30 years	–	–

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
	Mundinano et al. (124)	s	6 (15)	Increase Of 100	–	Not stated/Yes	PD, Braak stage II–V	–	–
Total	**4**	**o0, m1, c0, s3**	**43 (51)**						
Thalamus	Xuereb et al. (60)	m	5 (5)	None	–	Not stated/Yes	PD	Thalamus (multiple nuclei)	–
	Henderson et al. (125)	c	9 (10)	40–55	–	Not stated/Yes	PD, H&Y II–V, 7.2 years	Caudal intralaminar nuclei, limbic thalamic nuclei	–
	Henderson et al. (69)	s	9 (8)	50–70	–	Not stated/Yes	PD, H&Y II–V, 3–17 years	SNc, Centromedian–parafascicular complex, mediodorsal or anterior principal nucleus	–
	Halliday et al. (126)	s	9 (9)	None	–	Not stated/Yes	PD, H&Y II–V, 9 years	Motor thalamus, Cortex	–
Total	**4**	**o0, m1, c1, s2**	**32 (32)**						
Sympathic/ parasympathic ganglia	Rajput and Rozdilsky (47)	o	6 (1)	Some	–	Not stated	iPA, H&Y, 3–18 years	SN, LC, DMV, Cortex, Hypothalamus	–
	Wakabayashi and Takahashi (127)	m	25 (25)	31–43	–	Not stated/Yes	PD	–	–
	Benarroch et al. (115)	o	14 (12)	None	–	Yes/Not stated	PD or LBD, 10 years	DMV, nucleus ambiguus	–
Total:	**3**	**o2, m1, c0, s0**	**45 (38)**						
Cortex	Rajput and Rozdilsky (47)	o	6 (1)	None	–	Not stated	iPA, H&Y, 3–18 years	SN, LC, DMV, Hypothalamus, Intermediolateral spinal cord, sympathetic ganglia	–
	Pedersen et al. (128)	s	10 (12)	None	–	Not stated/Yes	PD, 2–25 years	–	–
Total	**2**	**o1, m0, c0, s1**	**16 (13)**						
Pre-supplementary and premotor cortex	MacDonald and Halliday (129)	m	5 (5)	32–45	–	Yes	PD, 10–17 years	–	–
	Halliday et al. (126)	s	9 (9)	None	–	Not stated/Yes	PD, H&Y II–V, 9 years	Motor thalamus	–
Total	**2**	**o0, m1, c0, s1**	**14 (14)**						

(Continued)

TABLE 1 | Continued

Regions	Publications (reference #)	Technique	N (ctrl)	Loss of neurons (%)	Comparison group info (healthy controls unless stated otherwise)	Blinded/age matched	Stated diagnosis, scale of severity, disease duration (expressed in range or mean, when available)	Other regions counted	Correlations (with disease severity, duration or age)
Amygdala, corticomedial complex	Harding et al. (130)	s	**18 (16)**	30	–	Yes	PD, 13 years	–	–
Hippocampus	Joelving et al. (131)	s	**8 (8)**	None	–	Not stated/Yes	PD, 2–25 years	–	–
Laterodorsal tegmental nucleus (LTN)	Gai et al. (81)	c	**6 (5)**	41	–	Not stated/Yes	iPD, 5–30 years	PPN, OPN, RN, LC	Yes
Oral pontine reticular nucleus (OPN)	Gai et al. (81)	c	**6 (5)**	41	–	Not stated/Yes	iPD, 5–30 years	PPN, LTN, RN, LC	Yes

*Included in the table are the technique used for quantification (o, observation; m, manual c, computer assisted; s, stereological counting), the number of subjects and controls (ctrl) studied, the estimated % loss of neurons, any particularity in the comparison group, mention if studies were performed blind and with age-matched controls, the stated diagnosis, scale of severity and disease duration when mentioned and note on other regions counted. Where an average value of loss was not given by authors, this number was calculated from available data. Bold values indicates total numbers per region. *Indicates details which are given at the end of that section.*

varied greatly, especially in reporting. For example, for the 12 studies using stereological methods, three papers (74, 76, 79) staged each case according to the Braak staging (to be expected given that Braak staging only came about in the early 2000s). In the same 12 studies, the age "since disease onset" varied between 1 and 27 years when stated, the Hoehn and Yahr ratings (H&Y, used to describe the progression severity of PD symptoms) varied between 2 to 5 and the UPDSR score (that includes H&Y rating, symptoms and quality-of-life scores) was also on occasion provided. A correlation with disease duration/severity was found in 10 studies. It is relevant here to mention that some authors, including Gibb et al. (56) have discussed the selective vulnerability of restricted sub-regions within the SNc. These data are important and relevant to the progression of the field; however, we found this distinction absent in the majority of the work we examined.

PEDUNCULOPONTINE NUCLEUS AND LOCUS COERULEUS

The evidence for cell loss for both the PPN (11 studies), containing cholinergic neurons and the LC (18 studies), containing noradrenergic neurons, is also relatively strong.

For the PPN, four studies used stereological methods. In these four studies, the average loss of cholinergic PPN neurons was 41% and the range of PD stages amongst the subjects evaluated was broad. For example, in Rinne et al. (99), the PD cases ranged from a H&Y rating of 2.5 to 5; in Karachi et al. (73), UDPRS score was used, and in both Hepp et al. (101), and Pienaar et al. (102), the PD cases were between Braak stages 4 and 6 and between 2 and 4, respectively. Although sample sizes were relatively small in these two last studies, nine and eight, respectively, it is somewhat surprising that in the most advanced PD group, loss of cholinergic PPN neurons was not higher than for less advanced PD subjects, contrarily to the report by Rinne et al. (99).

Surprisingly, we found no study quantifying loss of LC neurons using stereological counting methods. For the LC, 221 brains were studied, with cell loss ranging from "some" to 94%. Five of the studies were based on observational quantifications, 4 on manual counting and 9 used computer-assisted counting. In these 18 papers, when stated, the H&Y score was between 3 and 5, and disease duration was between 1 and 31 years. A correlation of the extent of cell loss with disease duration was found in two of these studies (81, 85).

DORSAL MOTOR NUCLEUS OF THE VAGUS, RAPHE NUCLEI, NUCLEUS BASALIS OF MEYNERT AND VENTRAL TEGMENTAL AREA

Substantial cell loss has been documented in the DMV, containing cholinergic neurons, with 7 studies evaluating this loss in 49 cases. Of these, only one study (114) used stereology, where they reported 55% neuronal loss in eight PD cases, ranging from 5 to 24 years post-diagnosis and reported correlation with disease duration/severity.

The importance of re-evaluating cell loss is PD is apparent when considering the serotonergic RN. For these nuclei, which are considered by many authors to be lost in PD, we found 7 papers describing neuronal loss varying between 0 to 90%. Cheshire et al. however, using stereology in 44 late-stage PD subjects, found no cell loss in the dorsal raphe nucleus (78). In the NBM, containing cholinergic neurons, we found 13 papers, 12 using manual counting methods, and one observational, which estimated an average neuronal loss of between "some" to 72%. No correlation with disease duration was reported. The high prevalence of concomitant PD and Alzheimer's disease (AD) might explain why cell loss varied so much for this region. Surprisingly, only 8 studies directly evaluated neuronal loss in the VTA, a dopaminergic region often considered to be only modestly affected in PD. Of these, one study used stereology (120) to evaluate the loss of neurons in 3 cases of PD (or 6 including PD with a secondary diagnosis) that were between 1 and 27 years post-diagnosis and reported an average neuronal loss of 31%. One paper reported correlation of the extent of cell loss with disease duration (68).

THALAMUS, HYPOTHALAMUS, OLFACTORY BULB

Four studies reported neuronal loss in thalamic nuclei, with 2 using stereology (69, 126). In (69), 9 subjects with H&Y disease ratings between 2 and 5 statistically significant loss of 30–40% was reported in the centromedian-parafasicular complex. However, no loss was found in the motor thalamus in 9 subjects with similar H&Y disease ratings in the work of Halliday et al. (126). Neuronal loss has also sometimes been reported in the hypothalamus (9 studies), with one using stereology; Thannickal et al. (110) reported a 50% cell loss in 10 PD cases, with increased loss with disease severity. Olfactory dysfunction is now well established as an early symptom of PD. Four studies evaluating cell loss in the olfactory bulb were reported. One of these (121) described a 57% decrease in neuronal number (identified as cells with "a prominent nucleolus surrounded by Nissl substance"), while the others (122–124), using stereology, reported a 100% increase in the number of TH-positive neurons.

PERIPHERAL NERVOUS SYSTEM, SPINAL CORD AND OTHER BRAIN REGIONS

Though there is substantial evidence for LP occurring in the ENS (133), we did not find any study reporting direct—quantitative evidence—for neuronal loss in the gut. Though it has been inferred that ENS glial cell loss is occurring (134), there is evidence that neuronal loss in the gut is not associated with PD (135). Of note, a publication often cited in support of neuronal loss in the ENS (115) shows, in fact, neuronal loss in the DMV. With regards to the spinal cord, published evidence is also scarce; of the studies most relevant here, Wakabayashi et al. (127), using manual counting methods, described a loss of 31% and 43% respectively in the 2nd and 9th thoracic segments of the intermediolateral of the spinal cord. For the amygdala, the pre-supplementary motor cortex, several other cortical regions, the laterodorsal tegmental nucleus and the oral pontine reticular nucleus, we found only single studies supporting loss, with stereology used for the amygdala (30% loss) (130), and cortex (10% loss) (130) (see **Table 1**).

REGIONAL ORDER OF CELL LOSS?

In summary, it seems clear that there is some level of cell loss in PD in restricted regions including the SNc, LC, NBM, PPN, DMV, VTA, and probably the RN. However—because of the lack of data for some regions, the variety of techniques used to count neurons, potentially numerous unintentional sources of bias, and because of the inconsistency in criteria used for subject sampling—firm conclusions are somewhat limited. In particular, it is difficult to conclude on the relative extent and temporal order of cell loss in these different brain regions as a function of disease progression, information that would be critical to advance the field. Indeed, a direct comparison of the extent of neuronal loss in different regions examined in different studies is hazardous, even if stereological studies were to be selected. Interestingly, of the 38 studies we identified evaluating cell loss in the SNc, only 5 of these also looked at the VTA, and of these only 1 used stereology. Given the importance of the difference in vulnerability of these two nuclei, a systematic evaluation of the extent of loss of these neurons in PD would be very informative. But even if as a technique, stereology mitigates for most of the classic biases, it is still unable to account for the variation in subject sampling, i.e., variation in disease duration, sex and age, unless these criteria were considered in a similar way for each study. Unfortunately, this has not, thus far, been the case. In conclusion, it seems clear that stereological studies comparing multiple regions in the same subjects and these regions in subjects at different stages of PD are critically needed to advance the field.

WHAT ARE THE COMMON FEATURES SHARED BY NEURONS AFFECTED IN PD?

Although, as mentioned previously, the evidence for the extent of cell loss in regions other than the SNc in the PD brain is not always sufficiently documented, it is clear that some level of cell loss occurs in a limited subset of regions beyond the SNc (**Figure 1A**), or, to the least, that neuronal functions including neurotransmission are perturbed in multiple neuronal circuits. It is therefore of great interest to identify some of the biological features that distinguish neuronal subgroups in terms of their basal vulnerability to some of the cellular stresses that are invoked to trigger PD, including altered proteostasis (due to lysosomal and/or proteosomal impairment), mitochondrial dysfunction, and sustained oxidant stress (including from highly reactive DA metabolites).

Several groups have been tackling this question by interrogating the characteristics that render neurons, starting with those of the SNc, particularly vulnerable to degeneration / cell death (136–138). It is likely that some shared functional or

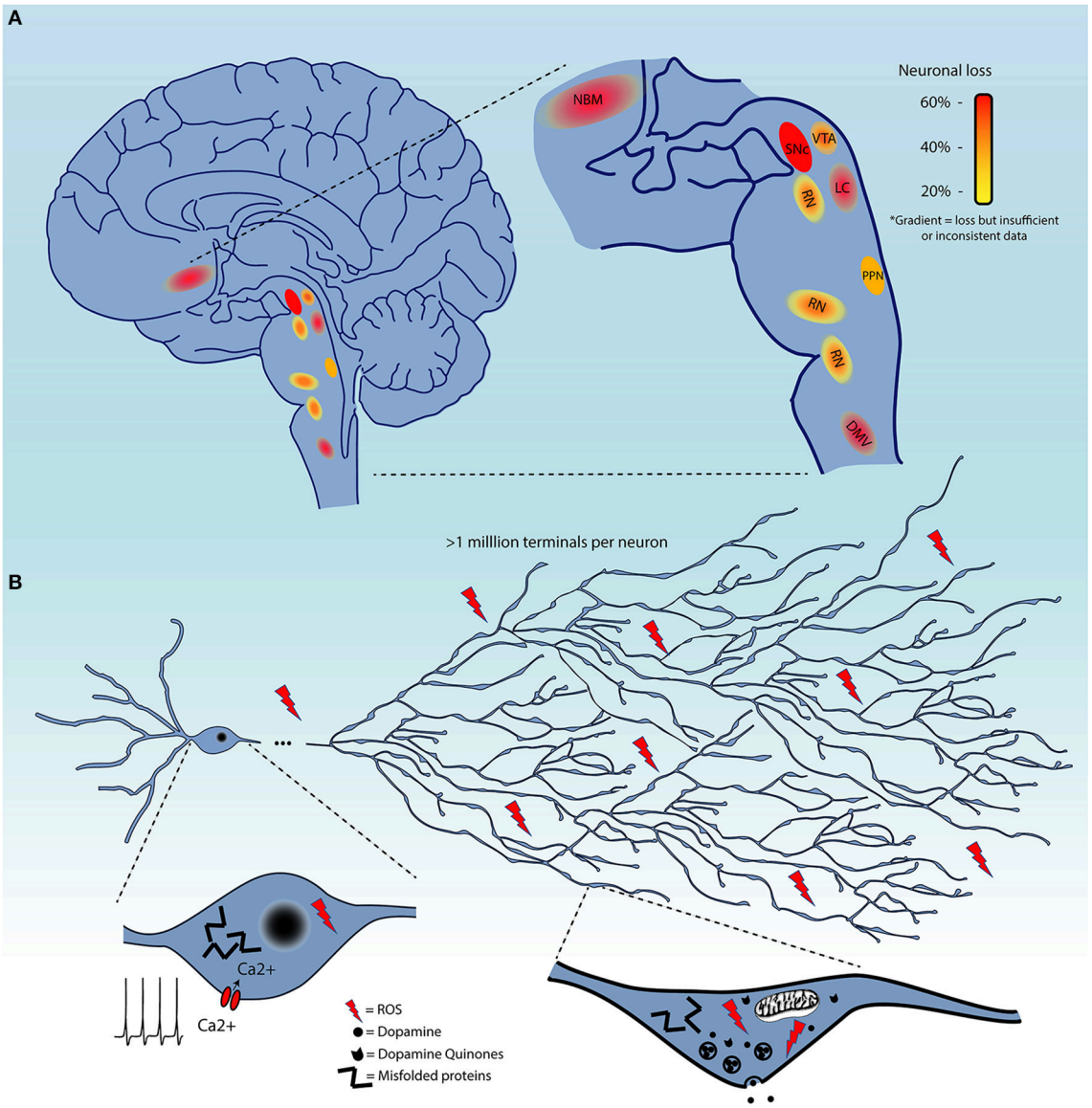


FIGURE 1 | (A) Schematic representation of brain regions demonstrating cell loss in Parkinson's disease. These are color-coded based on the evidence of cell loss. Red = 60%, orange = 40%, and yellow = 20%. Color gradients indicate uncertainty in the extent of this cell loss. **(B)** Summary of the converging hypotheses that may explain the origins of the selective vulnerability of neurons in Parkinson's disease. This includes the exceptionally large axonal arbor of PD-affected neurons, their electrophysiological properties, including calcium-dependent pacemaking, and high levels of oxidant stress in the somatodendritic and axonal domain, all thought to be contributing to cellular dysfunction and cell loss. Pathological protein aggregation and reactive dopamine quinones are considered as additional precipitating factors.

structural properties are responsible for selective vulnerability of affected nuclei, as opposed to features truly unique to SNc DA neurons. The causative characteristic(s) should be present in all affected neurons, but also be absent in neurons that do not degenerate or that degenerate much later in the disease. Four main converging hypotheses on selective vulnerability in PD have been gaining attention lately (**Figure 1B**), related to DA toxicity, iron-content, autonomous pacemaking and axonal arborization size. The next section will explore the likelihood that these hypotheses can explain why select neuronal populations are particularly vulnerable in PD.

DOPAMINE TOXICITY

Firstly, it has been suggested that DA neurons in general are most at risk because they produce DA as a neurotransmitter, a molecule that can be toxic in certain conditions through the generation of reactive quinones during its oxidation (139). This oxidation has been proposed to be implicated in the production of neuromelanin in SNc DA neurons. These DA quinones have been shown to interact with and negatively impact the function of mitochondrial protein complexes I, III, and V (140) and of other proteins such as tyrosine hydroxylase, the

DA transporter and α-synuclein (141, 142). Such reactive by-products can promote mitochondrial dysfunction, pathological aggregation of proteins such as α-synuclein and oxidative stress (143). Increasing the vesicular packaging of DA accordingly reduces the vulnerability of DA neurons, while down-regulating vesicular packaging has the opposite effect (144–147). Although highly relevant, this phenomenon alone does not readily explain the differential vulnerability of different dopaminergic neuron subgroups (such as SNc vs. VTA) and cannot contribute to the potential vulnerability of non-dopaminergic neurons in PD. Also, in the context of DA-induced toxicity, it is puzzling that levodopa therapy, acting to increase DA synthesis, does not appear to accelerate cell loss (148, 149). For these reasons, even if DA toxicity most certainly contributes to degeneration of SNc DA neurons, it is certainly not the sole factor driving neuronal death in PD.

IRON CONTENT

Secondly, iron content is thought to also be an important contributor to the selective vulnerability of SNc DA neurons. Iron is known to be able to generate ROS by the Fenton reaction and has been shown to accumulate with age in SNc (150–152). Since the mitochondrial electron transport chain relies on iron sulfur clusters for its function and since it is believed that SNc neurons have particularly high bioenergetic demands (136, 138, 153), elevated iron content could in part underlie elevated and sustained mitochondrial activity. Another interesting feature of iron in SNc DA neurons is that it can be chelated by neuromelanin, which renders it unavailable for mitochondrial function. Even if the affinity of iron for neuromelanin is much lower than for other iron binding proteins such as ferritin, it is possible that accumulation of neuromelanin and loss of ferritin concentration with age impacts gradually mitochondrial function, which could eventually promote cell death. However, data about potential iron content and iron-binding protein concentration changes in PD is still a matter of debate (154, 155). In addition, data is lacking on iron levels in other brain regions presenting cell death in PD. In fact, the only other region studied in this context has been the LC, which did not show high iron relative to the SNc (156–159).

AUTONOMOUS PACEMAKING

A third highly attractive hypothesis to explain the vulnerability of SNc DA neurons has its origins in the fact that these neurons demonstrate autonomous pacemaking. Many receptors/channels can potentially modulate the excitability and survival of DA neurons (160). The fact that pacemaking activity in SNc DA neurons is accompanied by slow oscillations in intracellular calcium concentrations, caused by the opening of voltage-dependent Cav1 plasma membrane calcium channels (Cav1.1 and 1.3) has recently renewed interest to this topic. In the Cav1 family, Cav1.3 has been suggested to be of particular interest because its voltage-sensitivity and inactivation properties allow a subset of the calcium channels to always stay open during pacemaking, causing extensive calcium entry (137). These oscillations have a positive contribution to cell physiology because they help maintain pacemaking and directly promote mitochondrial oxidative phosphorylation (OXPHOS) (161). However, by doing so, they have been proposed to also promote chronically high levels of ROS production (162, 163). Along with a reduction in mitochondrial function with age, chronically elevated oxidative stress has been proposed to be a causative factor in the decline of neuronal survival (164). Interestingly, CaV currents and autonomous pacemaking are also a feature of LC and DMV neurons (162, 163), and have been hypothesized to be involved in their vulnerability. The fact that other neuronal populations also expressing Cav1.3 such as hippocampal neurons (165) and striatal spiny projection neurons (166) do not degenerate in PD highlights the possibility that the particular vulnerability of SNc DA neurons is due to a combination of physiological phenotypes and not only intracellular calcium oscillations. Intriguingly, recent post-mortem studies showed that there was no decrease in Cav1.3 mRNA level in early or late stage PD in human SNc compared to controls (166, 167), despite significant loss of SNc neurons. Finally, in addition to CaV channels, ATP sensitive potassium channels (K-ATP) have also been reported to regulate the excitability and vulnerability of SNc DA neurons (168).

AXONAL ARBORIZATION SIZE

A fourth hypothesis proposes that neurons such as those of the SNc are particularly vulnerable because of the massive scale of their axonal arborization, leading to very high numbers of axon terminals, elevated energetic requirements, and chronically high oxidant stress. Indeed, it has been shown that SNc DA neurons have an exuberant and highly arborized axonal arborization with estimates upwards of a million neurotransmitter release sites per SNc DA neuron in humans (136, 169): this would make them some of the most highly arborized neurons in the nervous system. This characteristic has the potential to place a very large bioenergetic burden on these cells, leaving little margin for additional bioenergetic stress (136, 138, 153). Related to this, it has been calculated that the ATP requirement for propagation of one action potential grows exponentially with the level of branching (170). In a recent publication (138), we demonstrated *in vitro* that reducing the axonal arbor size of SNc DA neurons to a size more similar to that of VTA DA neurons using the axonal guidance factor Semaphorin 7A, was sufficient to greatly reduce basal OXPHOS and reduce their vulnerability to toxins including MPP+ and rotenone. Although as previously discussed, the extent of neuronal loss is still unclear for many neuronal populations, it does seem likely that most neuronal nuclei affected in PD include neurons that are relatively few in number, but all possess long and profuse unmyelinated axonal arbors and a large number of axonal terminals (171–176). However, comparative data evaluating axonal arbor size amongst these populations and in populations of neurons that do not degenerate in PD is presently lacking. An interesting possible exception to this hypothesis could be striatal cholinergic interneurons, which were previously estimated in rats to present 500,000 axonal varicosities (177, 178), but have not been reported to degenerate

in PD. This estimate was obtained by dividing the estimated number of terminals by the estimated number of cholinergic interneurons in the striatum, which was based on the total number of striatal neurons and the proportion of cholinergic interneurons. Considering recent stereological counting of the number of neurons in the rat striatum, it is possible that the total number of terminals estimated for striatal cholinergic neurons may have been overestimated by a factor of six (179). Based on this report, axonal arborization size of striatal cholinergic interneurons would be less than half of that of SNc neurons. Careful quantitative and comparative studies are clearly needed.

A GLOBAL BIOENERGETIC FAILURE HYPOTHESIS

One commonality between these four hypotheses is that they all suggest that vulnerable neurons are under intense mitochondrial/bioenergetic demand. This could alter the oxidative stress response by depleting antioxidants like glutathione (GSH), as previously suggested to occur in the PD brain (180–182). This stress could also, at a certain point, place the cells in a situation in which the rate of OXPHOS required to sustain neurotransmitter release and cellular excitability leaves too little of the cell's resources to sustain other key cellular functions such as degradation of damaged or misfolded proteins (137). This could lead to preferential dysregulation of axon terminals, triggering a dying back cascade culminating later in cell death (3, 183, 184). Approximately half of the oxygen consumed by mitochondria in SNc DA neurons appears to be used by activity-dependent cellular processes such as firing and neurotransmitter release (138). In this context, axon terminal degeneration seen early in the disease, prior to cell death, could be in part an attempt by stressed neurons to adapt to such excessively high metabolic needs. Such a dying back process could also lead to increased amounts of damaged axonal proteins to manage, potentially promoting their accumulation in intracellular inclusions. Since α-synuclein is highly concentrated in axon terminals, it is possible that retraction of axonal processes in a cell where protein degradation systems are overwhelmed, promotes creation of pathological aggregates of this protein, thus accelerating cell death. Interestingly, lysosomal defects secondary to GBA1 gene mutations are present in up to 10% of PD patients. This gene encodes a glucocerebrosidase responsible for breaking down lysosomal glucolipid. When GBA1 is mutated, the level of glucolipid and of misfolded proteins increases in neurons. This is likely to represent a particular challenge for highly arborized neurons such as those of the SNc, perhaps explaining why such mutations are now considered the greatest genetic risk factor for PD (185–191). Similarly, mutations in gene products implicated in mitophagy and mitochondrial antigen presentation (PARK2, PINK1) (192, 193), oxidative stress response (PARK7) (194, 195), or vesicular trafficking (LRRK2) (196, 197) are present in familial forms of PD and their detrimental impact on cellular functions could also represent larger challenges for highly arborized and energetically ambitious neurons.

TOWARD BETTER TREATMENTS OF PD

In the context of the hypotheses discussed here regarding the origin of the selective vulnerability of neurons in PD, novel strategies to promote survival and preservation of cellular functions amongst challenged neuronal populations could possibly come from approaches that aim to reduce mitochondrial burden by either reducing neuronal metabolic needs or optimizing mitochondrial function. As an example, the CaV1.3 channel inhibitor isradipine is presently in phase 3 clinical trial and could possibly reduce the calcium- and activity-related metabolic stress of SNc DA neurons leading to neuroprotection (198). Other promising molecules could come from the repurposing of drugs used to treat diabetes and other metabolic diseases. One example is exenatide, a glucagon-like-peptide-1 agonist that has the property to increase glucose-induced insulin secretion, to prevent the rise of ROS and prevent decreases of mitochondrial function in diet-induced obese mice (199). This agonist was found to reduce the loss of DA neurons in the MPTP mouse model (200) and a recent clinical trial has shown improved motor function after 60 days of administration to PD patients (201). Overexpression of the mitochondrial deacetylase SIRT3 has also recently been shown in two studies to reduce basal OXPHOS by DA neurons and to protect SNc neurons in rodent models of PD (202, 203). With further discoveries of the underlying causes of the intrinsic vulnerability of neurons in the PD brain and PNS, multiple other strategies may soon be devised to address some of the specific challenges faced by energetically challenged neurons.

In conclusion, although the presently available data strongly argue that multiple populations of neurons are affected in PD and degenerate to varying extents, new work is needed to provide a more systematic, comparative, and time-dependent quantification of neuronal loss in this disease. More comprehensive and convincing data on cell death and axon terminal dysfunction in PD will likely provide additional impetus for new work aiming to solve the long-awaited challenge of identifying disease-modifying therapeutic approaches for this incapacitating and ill-treated disorder.

AUTHOR CONTRIBUTIONS

NG and SB performed the litterature review. NG, SB, and L-ET wrote the manuscript. NG and SB contributed equally to this work.

ACKNOWLEDGMENTS

This work was supported by a grant from the Brain Canada and Krembil Foundations, as well as by the Canadian Institutes of Health Research. We would like to express our gratitude to Ms. Sarah Ouellet for her help preparing the illustrations and to Drs. Edward Fon, Michael Schlossmacher and Birgit Liss who graciously provided feedback on an earlier version of this manuscript. We also thank Dr. Heiko Braak for helping us identify some key litterature.

REFERENCES

1. Parkinson J. An essay on the shaking palsy. (1817). *J Neuropsychiatry Clin Neurosci*. (2002) 14:223–36; discussion: 2.doi: 10.1176/jnp.14.2.223
2. Cheng HC, Ulane CM, Burke RE. Clinical progression in Parkinson disease and the neurobiology of axons. *Ann Neurol.* (2010) 67:715–25. doi: 10.1002/ana.21995
3. Burke RE, O'Malley K. Axon degeneration in Parkinson's disease. *Exp Neurol.* (2013) 246:72–83. doi: 10.1016/j.expneurol.2012.01.011
4. Lewy FH. Paralysis agitans. In: *Handbuch der Neurologie, hsg. von Max Lewandowsky, Dritter Band, Spezielle Neurologie II. Für Später Vormerken* Vol. 3, ed M. Lewandowsky (Berlin: Springer), (1912). p. 920–958.
5. Tretiakoff C. *Contribution a l'Etude de l'Anatomiepathologique du Locus Niger de Soemmering Avec Quelques Deductions Relatives a la Pathologenie des Troubles Dutonus Musculaire de Lamaladiede Parkinson, thèse*, Paris (1919).
6. Braak H, Del Tredici K. Neuropathological staging of brain pathology in sporadic Parkinson's disease: Separating the wheat from the Chaff. *J Parkinsons Dis*. (2017) 7(s1):S71–S85. doi: 10.3233/JPD-179001
7. Postuma RB, Berg D, Stern M, Poewe W, Olanow CW, Oertel W, et al. MDS clinical diagnostic criteria for Parkinson's disease. *Mov Disord.* (2015) 30:1591–601. doi: 10.1002/mds.26424
8. Shults CW. Lewy bodies. *Proc Natl Acad Sci USA*. (2006) 103:1661–8. doi: 10.1073/pnas.0509567103
9. Vilchez D, Saez I, Dillin A. The role of protein clearance mechanisms in organismal ageing and age-related diseases. *Nat Commun*. (2014) 5:5659. doi: 10.1038/ncomms6659
10. Mercado G, Castillo V, Soto P, Sidhu A. ER stress and Parkinson's disease: pathological inputs that converge into the secretory pathway. *Brain Res.* (2016) 1648(Pt B):626–32. doi: 10.1016/j.brainres.2016.04.042
11. Cai Y, Arikkath J, Yang L, Guo ML, Periyasamy P, Buch S. Interplay of endoplasmic reticulum stress and autophagy in neurodegenerative disorders. *Autophagy.* (2016) 12:225–44. doi: 10.1080/15548627.2015.1121360
12. Truban D, Hou X, Caulfield TR, Fiesel FC, Springer W. PINK1, Parkin, and Mitochondrial Quality Control: What can we Learn about Parkinson's Disease Pathobiology? *J Parkinsons Dis.* (2017) 7:13–29. doi: 10.3233/JPD-160989
13. Giannoccaro MP, La Morgia C, Rizzo G, Carelli V. Mitochondrial DNA and primary mitochondrial dysfunction in Parkinson's disease. *Mov Disord.* (2017) 32:346–63. doi: 10.1002/mds.26966
14. Bose A, Beal MF. Mitochondrial dysfunction in Parkinson's disease. *J Neurochem.* (2016) 139(Suppl. 1):216–31. doi: 10.5607/en.2015.24.2.103
15. Hauser DN1, Hastings TG. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiol Dis.* (2013) 51:35–42. doi: 10.1016/j.nbd.2012.10.011
16. Jiang T, Sun Q, Chen S. Oxidative stress: A major pathogenesis and potential therapeutic target of antioxidative agents in Parkinson's disease and Alzheimer's disease. Prog. Neurobiol. (2016) 147:1–19. doi: 10.1016/j.pneurobio.2016.07.005
17. Tiwari PC, Pal R. The potential role of neuroinflammation and transcription factors in Parkinson disease. *Dialogues Clin Neurosci.* (2017) 19:71–80.
18. Joshi N, Singh S. Updates on immunity and inflammation in Parkinson disease pathology. *J Neurosci Res.* (2017) 96:379–90. doi: 10.1002/jnr.24185
19. Duda J, Potschke C, Liss B. Converging roles of ion channels, calcium, metabolic stress, and activity pattern of Substantia nigra dopaminergic neurons in health and Parkinson's disease. *J Neurochem*. (2016) 139(Suppl. 1):156–78. doi: 10.1111/jnc.13572
20. Pfeiffer RF. Non-motor symptoms in Parkinson's disease. *Parkinsonism Relat Disord.* (2016) 22(Suppl. 1):S119–22. doi: 10.1016/j.parkreldis.2015.09.004
21. Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* (2002) 24:197–211. doi: 10.1016/S0197-4580(02)00065-9
22. Braak H, Del Tredici K, Bratzke H, Hamm-Clement J, Sandmann-Keil D, Rüb U. Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J Neurol.* (2002) 249: iii1–5. doi: 10.1007/s00415-002-1301-4
23. Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K. Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res.* (2004) 318:121–34. doi: 10.1007/s00441-004-0956-9
24. Markesbery WR, Jicha GA, Liu H, Schmitt FA. Lewy body pathology in normal elderly subjects. *J Neuropathol Exp Neurol.* (2009) 68:816–22. doi: 10.1097/NEN.0b013e3181ac10a7
25. Sasaki S, Shirata A, Yamane K, Iwata M. Parkin-positive autosomal recessive juvenile Parkinsonism with α-synuclein-positive inclusions. *Neurology*. (2004) 63:678–82. doi: 10.1212/01.WNL.0000134657.25904.0B
26. Hayashi S, Wakabayashi K, Ishikawa A, Nagai H, Saito M, Maruyama M, et al. An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. *Mov Disord.* (2000) 15:884–8. doi: 10.1002/1531-8257(200009)15:5<884::AID-MDS1019>3.0.CO;2-8
27. Schneider SA, Alcalay RN. Neuropathology of genetic synucleinopathies with parkinsonism: Review of the literature. *Mov Disord*. (2017) 32:1504–23. doi: 10.1002/mds.27193
28. Surmeier DJ, Obeso JA, Halliday GM. Selective neuronal vulnerability in Parkinson disease. *Nat Rev Neurosci*. (2017) 18:101–13. doi: 10.1038/nrn.2016.178
29. Buchman AS, Shulman JM, Nag S, Leurgans SE, Arnold SE, Morris MC, et al. Nigral pathology and parkinsonian signs in elders without Parkinson disease. *Ann Neurol*. (2012) 71:258–66. doi: 10.1002/ana.22588
30. Cabello CR, Thune JJ, Pakkenberg H, Pakkenberg B. Ageing of substantia nigra in humans: cell loss may be compensated by hypertrophy. *Neuropathol Appl Neurobiol.* (2002) 28:283–91. doi: 10.1046/j.1365-2990.2002.00393.x
31. Fearnley JM, Lees AJ. Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* (1991) 114 (Pt 5):2283–301.
32. Fedorow H, Tribl F, Halliday G, Gerlach M, Riederer P, Double KL. Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. *Prog Neurobiol.* (2005) 75:109–24. doi: 10.1016/j.pneurobio.2005.02.001
33. Ma SY, Roytt M, Collan Y, Rinne JO. Unbiased morphometrical measurements show loss of pigmented nigral neurones with ageing. *Neuropathol Appl Neurobiol.* (1999) 25:394–9. doi: 10.1046/j.1365-2990.1999.00202.x
34. Naoi M, Maruyama W. Cell death of dopamine neurons in aging and Parkinson's disease. *Mech Ageing Dev*. (1999) 111:175–88.
35. Rudow G, O'Brien R, Savonenko AV, Resnick SM, Zonderman AB, Pletnikova O, et al. Morphometry of the human substantia nigra in ageing and Parkinson's disease. *Acta neuropathologica* (2008) 115:461–70. doi: 10.1007/s00401-008-0352-8
36. Stark AK, Pakkenberg B. Histological changes of the dopaminergic nigrostriatal system in aging. *Cell Tissue Res*. (2004) 318:81–92. doi: 10.1007/s00441-004-0972-9
37. Thiessen B, Rajput AH, Laverty W, Desai H. Age, environments, and the number of substantia nigra neurons. *Adv Neurol*. (1990) 53:201–6.
38. Ransmayr G, Faucheux B, Nowakowski C, Kubis N, Federspiel S, Kaufmann W, et al. Age-related changes of neuronal counts in the human pedunculopontine nucleus. *Neurosci Lett*. (2000) 288:195–8. doi: 10.1016/S0304-3940(00)01244-1
39. Lohr JB, Jeste DV. Locus ceruleus morphometry in aging and schizophrenia. *Acta Psychiatr Scand.* (1988) 77:689–97. doi: 10.1111/j.1600-0447.1988.tb05189.x
40. Shibata E, Sasaki M, Tohyama K, Kanbara Y, Otsuka K, Ehara S, et al. Age-related changes in locus ceruleus on neuromelanin magnetic resonance imaging at 3 Tesla. *Magn Reson Med Sci*. (2006) 5:197–200. doi: 10.2463/mrms.5.197
41. Goldstein DS. Cardiac denervation in patients with Parkinson disease. *Cleve Clin J Med*. (2007) 74(Suppl. 1):S91–4. doi: 10.3949/ccjm.76.s2.10
42. Wong KK, Raffel DM, Koeppe RA, Frey KA, Bohnen NI, Gilman S. Pattern of cardiac sympathetic denervation in idiopathic Parkinson disease studied with 11C hydroxyephedrine PET. *Radiology* (2012) 265:240–7. doi: 10.1148/radiol.12112723
43. Orimo S, Oka T, Miura H, Tsuchiya K, Mori F, Wakabayashi K, et al. Sympathetic cardiac denervation in Parkinson's disease and pure autonomic failure but not in multiple system atrophy. *J Neurol Neurosurg Psychiatry*. (2002) 73:776–7. doi: 10.1136/jnnp.73.6.776

44. Greenfield JG, Bosanquet FD. The brain-stem lesions in Parkinsonism. *J Neurol Neurosurg Psychiatry* (1953) 16:213–26. doi: 10.1136/jnnp.16.4.213
45. Pakkenberg H, Brody H. The number of nerve cells in the substantia nigra in paralysis agitans. *Acta Neuropathol.* (1965) 5:320–4. doi: 10.1007/BF00686528
46. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci*. (1973) 20:415–55. doi: 10.1016/0022-510X(73)90175-5
47. Rajput AH, Rozdilsky B. Dysautonomia in Parkinsonism: a clinicopathological study. *J Neurol Neurosurg Psychiatry* (1976) 39:1092–100. doi: 10.1136/jnnp.39.11.1092
48. Gaspar P, Gray F. Dementia in idiopathic Parkinson's disease. A neuropathological study of 32 cases. *Acta Neuropathol*. (1984) 64:43–52. doi: 10.1007/BF00695605
49. Tagliavini F, Pilleri G, Bouras C, Constantinidis J. The basal nucleus of Meynert in idiopathic Parkinson's disease. *Acta Neurol Scandinav*. (1984) 70:20–8. doi: 10.1111/j.1600-0404.1984.tb00798.x
50. Chan-Palay V. Galanin hyperinnervates surviving neurons of the human basal nucleus of Meynert in dementias of Alzheimer's and Parkinson's disease: a hypothesis for the role of galanin in accentuating cholinergic dysfunction in dementia. *J Comp Neurol*. (1988) 273:543–57. doi: 10.1002/cne.902730409
51. Gibb WR, Lees AJ. A comparison of clinical and pathological features of young- and old-onset *Parkinson's disease. Neurology* (1988) 38:1402–6. doi: 10.1212/WNL.38.9.1402
52. Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature* (1988) 334:345–8. doi: 10.1038/334345a0
53. German DC, Manaye K, Smith WK, Woodward DJ, Saper CB. Midbrain dopaminergic cell loss in Parkinson's disease: computer visualization. *Ann Neurol*. (1989) 26:507–14. doi: 10.1002/ana.410260403
54. Rinne JO, Rummukainen J, Paljarvi L, Rinne UK. Dementia in Parkinson's disease is related to neuronal loss in the medial substantia nigra. *Ann Neurol*. (1989) 26:47–50. doi: 10.1002/ana.410260107
55. Zweig RM, Jankel WR, Hedreen JC, Mayeux R, Price DL. The pedunculopontine nucleus in Parkinson's disease. *Ann Neurol*. (1989) 26:41–6. doi: 10.1002/ana.410260106
56. Gibb WR, Fearnley JM, Lees AJ. The anatomy and pigmentation of the human substantia nigra in relation to selective neuronal vulnerability. *Adv Neurol*. (1990) 53:31–4.
57. Halliday GM, Li YW, Blumbergs PC, Joh TH, Cotton RG, Howe PR, et al. Neuropathology of immunohistochemically identified brainstem neurons in Parkinson's disease. *Ann Neurol*. (1990) 27:373–85. doi: 10.1002/ana.410270405
58. Pakkenberg B, Moller A, Gundersen HJ, Mouritzen Dam A, Pakkenberg H. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. *J Neurol Neurosurg Psychiatry*. (1991) 54:30–3. doi: 10.1136/jnnp.54.1.30
59. Paulus W, Jellinger K. The neuropathologic basis of different clinical subgroups of Parkinson's disease. *J Neuropathol Exp Neurol*. (1991) 50:743–55. doi: 10.1097/00005072-199111000-00006
60. Xuereb JH, Perry RH, Candy JM, Perry EK, Marshall E, Bonham JR. Nerve cell loss in the thalamus in Alzheimer's disease and Parkinson's disease. *Brain* (1991) 114 (Pt 3):1363–79.
61. Moller A. Mean volume of pigmented neurons in the substantia nigra in Parkinson's disease. *Acta Neurol Scand Suppl*. (1992) 137:37–9. doi: 10.1111/j.1600-0404.1992.tb05036.x
62. Zweig RM, Cardillo JE, Cohen M, Giere S, Hedreen JC. The locus ceruleus and dementia in Parkinson's disease. *Neurology* (1993) 43:986–91. doi: 10.1212/WNL.43.5.986
63. Mouatt-Prigent A, Agid Y, Hirsch EC. Does the calcium binding protein calretinin protect dopaminergic neurons against degeneration in Parkinson's disease? *Brain Res*. (1994) 668:62–70. doi: 10.1016/0006-8993(94)90511-8
64. Ma SY, Collan Y, Roytta M, Rinne JO, Rinne UK. Cell counts in the substantia nigra: a comparison of single section counts and disector counts in patients with Parkinson's disease and in controls. *Neuropathol Appl Neurobiol*. (1995) 21:10–7. doi: 10.1111/j.1365-2990.1995.tb01023.x
65. Halliday GM, McRitchie DA, Cartwright H, Pamphlett R, Hely MA, Morris JG. Midbrain neuropathology in idiopathic Parkinson's disease and diffuse Lewy body disease. *J Clin Neurosci*. (1996) 3:52–60. doi: 10.1016/S0967-5868(96)90083-1
66. Ma SY, Rinne JO, Collan Y, Roytta M, Rinne UK. A quantitative morphometrical study of neuron degeneration in the substantia nigra in Parkinson's disease. *J Neurol Sci*. (1996) 140:40–5. doi: 10.1016/0022-510X(96)00069-X
67. Ma SY, Roytta M, Rinne JO, Collan Y, Rinne UK. Correlation between neuromorphometry in the substantia nigra and clinical features in Parkinson's disease using disector counts. *J Neurol Sci*. (1997) 151:83–7. doi: 10.1016/S0022-510X(97)00100-7
68. Damier P, Hirsch EC, Agid Y, Graybiel AM. The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain* (1999) 122 (Pt 8):1437–48. doi: 10.1093/brain/122.8.1437
69. Henderson JM, Carpenter K, Cartwright H, Halliday GM. Degeneration of the centre median-parafascicular complex in Parkinson's disease. *Ann Neurol*. (2000) 47:345–52. doi: 10.1002/1531–8249(200003)47:3<345::AID-ANA10>3.0.CO;2-V
70. Zarrow C, Lyness SA, Mortimer JA, Chui HC. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in alzheimer and Parkinson diseases. *Arch Neurol*. (2003) 60:337–41. doi: 10.1001/archneur.60.3.337
71. Greffard S, Verny M, Bonnet AM, Beinis JY, Gallinari C, Meaume S, et al. Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lewy body-associated neuronal loss in the substantia nigra. *Arch Neurol*. (2006) 63:584–8. doi: 10.1001/archneur.63.4.584
72. Beach TG, Adler CH, Lue L, Sue LI, Bachalakuri J, Henry-Watson J, et al. Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. *Acta Neuropathol*. (2009) 117:613–34. doi: 10.1007/s00401-009-0538-8
73. Karachi C, Grabli D, Bernard FA, Tande D, Wattiez N, Belaid H, et al. Cholinergic mesencephalic neurons are involved in gait and postural disorders in Parkinson disease. *J Clin Invest*. (2010) 120:2745–54. doi: 10.1172/JCI42642
74. Milber JM, Noorigian JV, Morley JF, Petrovitch H, White L, Ross GW, et al. Lewy pathology is not the first sign of degeneration in vulnerable neurons in Parkinson disease. *Neurology* (2012) 79:2307–14. doi: 10.1212/WNL.0b013e318278fe32
75. Kordower JH, Olanow CW, Dodiya HB, Chu Y, Beach TG, Adler CH, et al. Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain* (2013) 136(Pt 8):2419–31. doi: 10.1093/brain/awt192
76. Dijkstra AA, Voorn P, Berendse HW, Groenewegen HJ, Netherlands Brain B, Rozemuller AJ, et al. Stage-dependent nigral neuronal loss in incidental Lewy body and Parkinson's disease. *Mov Disord*. (2014) 29:1244–51. doi: 10.1002/mds.25952
77. Kraemmer J, Kovacs GG, Perju-Dumbrava L, Pirker S, Traub-Weidinger T, Pirker W. Correlation of striatal dopamine transporter imaging with post mortem substantia nigra cell counts. *Mov Disord* (2014) 29:1767–73. doi: 10.1002/mds.25975
78. Cheshire P, Ayton S, Bertram KL, Ling H, Li A, McLean C, et al. Serotonergic markers in Parkinson's disease and levodopa-induced dyskinesias. *Mov Disord*. (2015) 30:796–804. doi: 10.1002/mds.26144
79. Iacono D, Geraci-Erck M, Rabin ML, Adler CH, Serrano G, Beach TG, et al. Parkinson disease and incidental Lewy body disease: Just a question of time? *Neurology* (2015) 85:1670–9. doi: 10.1212/WNL.0000000000002102
80. Chan-Palay V, Asan E. Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. *J Comp Neurol*. (1989) 287:373–92. doi: 10.1002/cne.902870308
81. Gai WP, Halliday GM, Blumbergs PC, Geffen LB, Blessing WW. Substance P-containing neurons in the mesopontine tegmentum are severely affected in Parkinson's disease. *Brain* (1991) 114 (Pt 5):2253–67. doi: 10.1093/brain/114.5.2253

82. German DC, Manaye KF, White CL, 3rd, Woodward DJ, McIntire DD, Smith WK, et al. Disease-specific patterns of locus coeruleus cell loss. *Ann Neurol*. (1992) 32:667–76. doi: 10.1002/ana.410320510
83. Patt S, Gerhard L. A Golgi study of human locus coeruleus in normal brains and in Parkinson's disease. *Neuropathol Appl Neurobiol*. (1993) 19:519–23. doi: 10.1111/j.1365-2990.1993.tb00480.x
84. Hoogendijk WJ, Pool CW, Troost D, van Zwieten E, Swaab DF. Image analyser-assisted morphometry of the locus coeruleus in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. *Brain* (1995) 118 (Pt 1):131–43. doi: 10.1093/brain/118.1.131
85. Bertrand E, Lechowicz W, Szpak GM, Dymecki J. Qualitative and quantitative analysis of locus coeruleus neurons in Parkinson's disease. *Folia Neuropathol*. (1997) 35:80–6.
86. Brunnstrom H, Friberg N, Lindberg E, Englund E. Differential degeneration of the locus coeruleus in dementia subtypes. *Clin Neuropathol*. (2011) 30:104–10. doi: 10.5414/NPP30104
87. McMillan PJ, White SS, Franklin A, Greenup JL, Leverenz JB, Raskind MA, et al. Differential response of the central noradrenergic nervous system to the loss of locus coeruleus neurons in Parkinson's disease and Alzheimer's disease. *Brain Res*. (2011) 1373:240–52. doi: 10.1016/j.brainres.2010.12.015
88. Dugger BN, Murray ME, Boeve BF, Parisi JE, Benarroch EE, Ferman TJ, et al. Neuropathological analysis of brainstem cholinergic and catecholaminergic nuclei in relation to rapid eye movement (REM) sleep behaviour disorder. *Neuropathol Appl Neurobiol*. (2012) 38:142–52. doi: 10.1111/j.1365-2990.2011.01203.x
89. Del Tredici K, Braak H. Dysfunction of the locus coeruleus-norepinephrine system and related circuitry in Parkinson's disease-related dementia. *J Neurol Neurosurg Psychiatry* (2013) 84:774–83. doi: 10.1136/jnnp-2011-301817
90. Arendt T, Bigl V, Arendt A, Tennstedt A. Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's Disease. *Acta Neuropathol*. (1983) 61:101–8. doi: 10.1007/BF00697388
91. Candy JM, Perry RH, Perry EK, Irving D, Blessed G, Fairbairn AF, et al. Pathological changes in the nucleus of Meynert in Alzheimer's and Parkinson's diseases. *J Neurol Sci*. (1983) 59:277–89. doi: 10.1016/0022-510X(83)90045-X
92. Nakano I, Hirano A. Neuron loss in the nucleus basalis of Meynert in parkinsonism-dementia complex of Guam. *Ann Neurol*. (1983) 13:87–91. doi: 10.1002/ana.410130118
93. Whitehouse PJ, Hedreen JC, White CL, 3rd, Price DL. Basal forebrain neurons in the dementia of Parkinson disease. *Ann Neurol*. (1983) 13:243–8. doi: 10.1002/ana.410130304
94. Nakano I, Hirano A. Parkinson's disease: Neuron loss in the nucleus basalis withouth concomitant Alzheimer's Disease. *Ann Neurol*. (1984) 15:415–8. doi: 10.1002/ana.410150503
95. Perry EK, Curtis M, Dick DJ, Candy JM, Atack JR, Bloxham CA, et al. Cholinergic correlates of cognitive impairment in Parkinson's disease: comparisons with Alzheimer's disease. *J Neurol Neurosurg Psychiatry* (1985) 48:413–21. doi: 10.1136/jnnp.48.5.413
96. Rogers JD, Brogan D, Mirra SS. The nucleus basalis of Meynert in neurological disease: a quantitative morphological study. *Ann Neurol*. (1985) 17:163–70. doi: 10.1002/ana.410170210
97. Hirsch EC, Graybiel AM, Duyckaerts C, Javoy-Agid F. Neuronal loss in the pedunculopontine tegmental nucleus in Parkinson disease and in progressive supranuclear palsy. *Proc Natl Acad Sci USA*. (1987) 84:5976–80. doi: 10.1073/pnas.84.16.5976
98. Jellinger K. The pedunculopontine nucleus in Parkinson's disease, progressive supranuclear palsy and Alzheimer's disease. *J Neurol Neurosurg Psychiatry* (1988) 51:540–3. doi: 10.1136/jnnp.51.4.540
99. Rinne JO, Ma SY, Lee MS, Collan Y, Roytta M. Loss of cholinergic neurons in the pedunculopontine nucleus in Parkinson's disease is related to disability of the patients. *Parkinsonism Related Disord*. (2008) 14:553–7. doi: 10.1016/j.parkreldis.2008.01.006
100. Schmeichel AM, Buchhalter LC, Low PA, Parisi JE, Boeve BW, Sandroni P, et al. Mesopontine cholinergic neuron involvement in Lewy body dementia and multiple system atrophy. *Neurology* (2008) 70:368–73. doi: 10.1212/01.wnl.0000298691.71637.96
101. Hepp DH, Ruiter AM, Galis Y, Voorn P, Rozemuller AJM, Berendse HW, et al. Pedunculopontine cholinergic cell loss in hallucinating Parkinson disease patients but not in dementia with Lewy bodies patients. *J Neuropathol Exp Neurol*. (2013) 72:1162–70. doi: 10.1097/NEN.0000000000000014
102. Pienaar IS, Elson JL, Racca C, Nelson G, Turnbull DM, Morris CM. Mitochondrial abnormality associates with type-specific neuronal loss and cell morphology changes in the pedunculopontine nucleus in Parkinson disease. *Am J Pathol*. (2013) 183:1826–40. doi: 10.1016/j.ajpath.2013.09.002
103. Kremer HP. The hypothalamic lateral tuberal nucleus: normal anatomy and changes in neurological diseases. *Prog Brain Res*. (1992) 93:249–61. doi: 10.1016/S0079-6123(08)64576-8
104. Kremer HP, Bots GT. Lewy bodies in the lateral hypothalamus: do they imply neuronal loss? *Mov Disord*. (1993) 8:315–20.
105. Purba JS, Hofman MA, Swaab DF. Decreased number of oxytocin-immunoreactive neurons in the paraventricular nucleus of the hypothalamus in Parkinson's disease. *Neurology* (1994) 44:84–9. doi: 10.1212/WNL.44.1.84
106. Nakamura S, Ohnishi K, Nishimura M, Suenaga T, Akiguchi I, Kimura J, et al. Large neurons in the tuberomammillary nucleus in patients with Parkinson's disease and multiple system atrophy. *Neurology* (1996) 46:1693–6. doi: 10.1212/WNL.46.6.1693
107. Ansorge O, Daniel SE, Pearce RK. Neuronal loss and plasticity in the supraoptic nucleus in Parkinson's disease. *Neurology* (1997) 49:610–3. doi: 10.1212/WNL.49.2.610
108. Hoogendijk WJ, Purba JS, Hofman MA, de Vos RA, Jansen EN, Swaab DF. Depression in Parkinson's disease is not accompanied by more corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus. *Biol Psychiatry* (1998) 43:913–7. doi: 10.1016/S0006-3223(97)00338-7
109. Fronczek R, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, et al. Hypocretin (orexin) loss in Parkinson's disease. *Brain* (2007) 130(Pt 6):1577–85. doi: 10.1093/brain/awm090
110. Thannickal TC, Lai YY, Siegel JM. Hypocretin (orexin) cell loss in Parkinson's disease. *Brain* (2007) 130(Pt 6):1586–95. doi: 10.1093/brain/awm097
111. Eadie MJ. The pathology of certain medullary nuclei in parkinsonism. *Brain* (1963) 86:781–92. doi: 10.1093/brain/86.4.781
112. Halliday GM, Blumbergs PC, Cotton RG, Blessing WW, Geffen LB. Loss of brainstem serotonin- and substance P-containing neurons in Parkinson's disease. *Brain Res*. (1990) 510:104–7. doi: 10.1016/0006-8993(90)90733-R
113. Saper CB, Sorrentino DM, German DC, de Lacalle S. Medullary catecholaminergic neurons in the normal human brain and in Parkinson's disease. *Ann Neurol*. (1991) 29:577–84. doi: 10.1002/ana.410290602
114. Gai WP, Blumbergs PC, Geffen LB, Blessing WW. Age-related loss of dorsal vagal neurons in Parkinson's disease. *Neurology* (1992) 42:2106–11. doi: 10.1212/WNL.42.11.2106
115. Benarroch EE, Schmeichel AM, Sandroni P, Low PA, Parisi JE. Involvement of vagal autonomic nuclei in multiple system atrophy and Lewy body disease. *Neurology* (2006) 66:378–83. doi: 10.1212/01.wnl.0000196638.98781.bb
116. Yamamoto T, Hirano A. Nucleus raphe dorsalis in parkinsonism-dementia complex of Guam. *Acta Neuropathol*. (1985) 67:296–9. doi: 10.1007/BF00687815
117. Benarroch EE, Schmeichel AM, Sandroni P, Parisi JE, Low PA. Rostral raphe involvement in Lewy body dementia and multiple system atrophy. *Acta Neuropathol*. (2007) 114:213–20. doi: 10.1007/s00401-007-0260-3
118. Javoy-Agid F, Ruberg M, Taquet H, Bokobza B, Agid Y, Gaspar P, et al. Biochemical neuropathology of Parkinson's disease. *Adv Neurol*. (1984) 40:189–98.
119. Dymecki J, Lechowicz W, Bertrand E, Szpak GM. Changes in dopaminergic neurons of the mesocorticolimbic system in Parkinson's disease. *Folia Neuropathol*. (1996) 34:102–6.

120. McRitchie DA, Cartwright HR, Halliday GM. Specific A10 dopaminergic nuclei in the midbrain degenerate in Parkinson's disease. *Exp Neurol*. (1997) 144:202–13. doi: 10.1006/exnr.1997.6418
121. Pearce RK, Hawkes CH, Daniel SE. The anterior olfactory nucleus in Parkinson's disease. *Mov Disord*. (1995) 10:283–7. doi: 10.1002/mds.870100309
122. Huisman E, Uylings HB, Hoogland PV. A 100% increase of dopaminergic cells in the olfactory bulb may explain hyposmia in Parkinson's disease. *Mov Disord*. (2004) 19:687–92. doi: 10.1002/mds.10713
123. Huisman E, Uylings HB, Hoogland PV. Gender-related changes in increase of dopaminergic neurons in the olfactory bulb of Parkinson's disease patients. *Mov Disord*. (2008) 23:1407–13. doi: 10.1002/mds.22009
124. Mundinano IC, Caballero MC, Ordonez C, Hernandez M, DiCaudo C, Marcilla I, et al. Increased dopaminergic cells and protein aggregates in the olfactory bulb of patients with neurodegenerative disorders. *Acta Neuropathol*. (2011) 122:61–74. doi: 10.1007/s00401-011-0830-2
125. Henderson JM, Carpenter K, Cartwright H, Halliday GM. Loss of thalamic intralaminar nuclei in progressive supranuclear palsy and Parkinson's disease: clinical and therapeutic implications. *Brain*. (2000) 123 (Pt 7):1410–21. doi: 10.1093/brain/123.7.1410
126. Halliday GM, Macdonald V, Henderson JM. A comparison of degeneration in motor thalamus and cortex between progressive supranuclear palsy and Parkinson's disease. *Brain* (2005) 128(Pt 10):2272–80. doi: 10.1093/brain/awh596
127. Wakabayashi K, Takahashi H. Neuropathology of autonomic nervous system in Parkinson's disease. *Eur Neurol*. (1997) 38(Suppl. 2):2–7. doi: 10.1159/000113469
128. Pedersen KM, Marner L, Pakkenberg H, Pakkenberg B. No global loss of neocortical neurons in Parkinson's disease: a quantitative stereological study. *Mov Disord*. (2005) 20:164–71. doi: 10.1002/mds.20289
129. MacDonald V, Halliday GM. Selective loss of pyramidal neurons in the pre-supplementary motor cortex in Parkinson's disease. *Mov Disord*. (2002) 17:1166–73. doi: 10.1002/mds.10258
130. Harding AJ, Stimson E, Henderson JM, Halliday GM. Clinical correlates of selective pathology in the amygdala of patients with Parkinson's disease. *Brain* (2002) 125(Pt 11):2431–45. doi: 10.1093/brain/awf251
131. Joelving FC, Billeskov R, Christensen JR, West M, Pakkenberg B. Hippocampal neuron and glial cell numbers in Parkinson's disease–a stereological study. *Hippocampus* (2006) 16:826–33. doi: 10.1002/hipo.20212
132. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. *Neurology* (1967) 17:427–42. doi: 10.1212/WNL.17.5.427
133. Klingelhoefer L, Reichmann H. Pathogenesis of Parkinson disease–the gut-brain axis and environmental factors. *Nat Rev Neurol*. (2015) 11:625–36. doi: 10.1038/nrneurol.2015.197
134. Singaram C, Ashraf W, Gaumnitz EA, Torbey C, Sengupta A, Pfeiffer R, et al. Dopaminergic defect of enteric nervous system in Parkinson's disease patients with chronic constipation. *Lancet* (1995) 346:861–4. doi: 10.1016/S0140-6736(95)92707-7
135. Annerino DM, Arshad S, Taylor GM, Adler CH, Beach TG, Greene JG. Parkinson's disease is not associated with gastrointestinal myenteric ganglion neuron loss. *Acta Neuropathol*. (2012) 124:665–80. doi: 10.1007/s00401-012-1040-2
136. Bolam JP, Pissadaki EK. Living on the edge with too many mouths to feed: Why dopamine neurons die. *Mov Disord*. (2012) 27:1478–83. doi: 10.1002/mds.25135
137. Surmeier DJ, Schumacker PT, Guzman JD, Ilijic E, Yang B, Zampese E. Calcium and Parkinson's disease. *Biochem Biophys Res Commun*. (2017) 483:1013–9. doi: 10.1016/j.bbrc.2016.08.168
138. Pacelli C, Giguere N, Bourque MJ, Levesque M, Slack RS, Trudeau LE. Elevated mitochondrial bioenergetics and axonal arborization size are key contributors to the vulnerability of dopamine neurons. *Curr Biol*. (2015) 25:2349–60. doi: 10.1016/j.cub.2015.07.050
139. Segura-Aguilar J, Paris I, Munoz P, Ferrari E, Zecca L, Zucca FA. Protective and toxic roles of dopamine in Parkinson's disease. *J Neurochem*. (2014) 129:898–915. doi: 10.1111/jnc.12686
140. Van Laar VS, Mishizen AJ, Cascio M, Hastings TG. Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. *Neurobiol Dis*. (2009) 34:487–500. doi: 10.1016/j.nbd.2009.03.004
141. Xu Y, Stokes AH, Roskoski R, Jr, Vrana KE. Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. *J Neurosci Res*. (1998) 54:691–7. doi: 10.1002/(SICI)1097-4547(19981201)54:5<691::AID-JNR14>3.0.CO;2-F
142. Whitehead RE, Ferrer JV, Javitch JA, Justice JB. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. *J Neurochem*. (2001) 76:1242–51. doi: 10.1046/j.1471-4159.2001.00125.x
143. Mosharov EV, Larsen KE, Kanter E, Phillips KA, Wilson K, Schmitz Y, et al. Interplay between cytosolic dopamine, calcium, and alpha-synuclein causes selective death of substantia nigra neurons. *Neuron* (2009) 62:218–29. doi: 10.1016/j.neuron.2009.01.033
144. Lohr KM, Bernstein AI, Stout KA, Dunn AR, Lazo CR, Alter SP, et al. Increased vesicular monoamine transporter enhances dopamine release and opposes Parkinson disease-related neurodegeneration *in vivo*. *Proc Natl Acad Sci USA*. (2014) 111:9977–82. doi: 10.1073/pnas.1402134111
145. Pifl C, Rajput A, Reither H, Blesa J, Cavada C, Obeso JA, et al. Is Parkinson's disease a vesicular dopamine storage disorder? Evidence from a study in isolated synaptic vesicles of human and nonhuman primate striatum. *J Neurosci*. (2014) 34:8210–8. doi: 10.1523/JNEUROSCI.5456-13.2014
146. Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, et al. Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *J Neurosci*. (2007) 27:8138–48. doi: 10.1523/JNEUROSCI.0319-07.2007
147. Guillot TS, Miller GW. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. *Mol Neurobiol*. (2009) 39:149–70. doi: 10.1007/s12035-009-8059-y
148. Fahn S. Does levodopa slow or hasten the rate of progression of Parkinson's disease? *J Neurol*. (2005) 252(Suppl. 4):iv37–42. doi: 10.1007/s00415-005-4008-5
149. Lipski J, Nistico R, Berretta N, Guatteo E, Bernardi G, Mercuri NB. L-DOPA: a scapegoat for accelerated neurodegeneration in Parkinson's disease? *Prog Neurobiol*. (2011) 94:389–407. doi: 10.1016/j.pneurobio.2011.06.005
150. Haacke EM, Miao Y, Liu M, Habib CA, Katkuri Y, Liu T, et al. Correlation of putative iron content as represented by changes in R2* and phase with age in deep gray matter of healthy adults. *J Magn Reson Imaging* (2010) 32:561–76. doi: 10.1002/jmri.22293
151. Daugherty A, Raz N. Age-related differences in iron content of subcortical nuclei observed *in vivo*: a meta-analysis. *Neuroimage* (2013) 70:113–21. doi: 10.1016/j.neuroimage.2012.12.040
152. Bilgic B, Pfefferbaum A, Rohlfing T, Sullivan EV, Adalsteinsson E. MRI estimates of brain iron concentration in normal aging using quantitative susceptibility mapping. *Neuroimage* (2012) 59:2625–35. doi: 10.1016/j.neuroimage.2011.08.077
153. Ren Y, Liu W, Jiang H, Jiang Q, Feng J. Selective vulnerability of dopaminergic neurons to microtubule depolymerization. *J Biol Chem*. (2005) 280:34105–12. doi: 10.1074/jbc.M503483200
154. Friedman A, Galazka-Friedman J, Bauminger ER. Iron as a trigger of neurodegeneration in Parkinson's disease*Handb Clin Neurol*. (2007) 83:493–505. doi: 10.1016/S0072-9752(07)83023-5
155. Sian-Hulsmann J, Mandel S, Youdim MB, Riederer P. The relevance of iron in the pathogenesis of Parkinson's disease. *J Neurochem*. (2011) 118:939–57. doi: 10.1111/j.1471-4159.2010.07132.x
156. Kosta P, Argyropoulou MI, Markoula S, Konitsiotis S. MRI evaluation of the basal ganglia size and iron content in patients with Parkinson's disease. *J Neurol*. (2006) 253:26–32. doi: 10.1007/s00415-005-0914-9
157. Zecca L, Shima T, Stroppolo A, Goj C, Battiston GA, Gerbasi R, et al. Interaction of neuromelanin and iron in substantia nigra and other areas of human brain. *Neuroscience* (1996) 73:407–15. doi: 10.1016/0306-4522(96)00047-4
158. Zucca FA, Bellei C, Giannelli S, Terreni MR, Gallorini M, Rizzio E, et al. Neuromelanin and iron in human locus coeruleus and substantia nigra during aging: consequences for neuronal vulnerability.

J Neural Transm. (2006) 113:757–67. doi: 10.1007/s00702-006-0453-2
159. Zecca L, Stroppolo A, Gatti A, Tampellini D, Toscani M, Gallorini M, et al. The role of iron and copper molecules in the neuronal vulnerability of locus coeruleus and substantia nigra during aging. *Proc Natl Acad Sci USA*. (2004) 101:9843–8. doi: 10.1073/pnas.0403495101
160. Michel PP, Alvarez-Fischer D, Guerreiro S, Hild A, Hartmann A, Hirsch EC. Role of activity-dependent mechanisms in the control of dopaminergic neuron survival. *J Neurochem*. (2007) 101:289–97. doi: 10.1111/j.1471-4159.2006.04424.x
161. Guzman JN, Sanchez-Padilla J, Wokosin D, Kondapalli J, Ilijic E, Schumacker PT, et al. Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1. *Nature* (2010) 468:696–700. doi: 10.1038/nature09536
162. Goldberg JA, Guzman JN, Estep CM, Ilijic E, Kondapalli J, Sanchez-Padilla J, et al. Calcium entry induces mitochondrial oxidant stress in vagal neurons at risk in Parkinson's disease. *Nat Neurosci*. (2012) 15:1414–21. doi: 10.1038/nn.3209
163. Sanchez-Padilla J, Guzman JN, Ilijic E, Kondapalli J, Galtieri DJ, Yang B, et al. Mitochondrial oxidant stress in locus coeruleus is regulated by activity and nitric oxide synthase. *Nat Neurosci* (2014) 17:832–40. doi: 10.1038/nn.3717
164. Reeve A, Simcox E, Turnbull D. Ageing and Parkinson's disease: Why is advancing age the biggest risk factor? *Ageing Res Rev*. (2014) 14:19–30. doi: 10.1016/j.arr.2014.01.004
165. Hell JW, Westenbroek RE, Warner C, Ahlijanian MK, Prystay W, Gilbert MM, et al. Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. *J Cell Biol*. (1993) 123:949–62. doi: 10.1083/jcb.123.4.949
166. Hurley MJ, Gentleman SM, Dexter DT. Calcium CaV1 channel subtype mRNA expression in Parkinson's disease examined by *in situ* hybridization. *J Mol Neurosci*. (2015) 55:715–24. doi: 10.1007/s12031-014-0410-8
167. Schiemann J, Schlaudraff F, Klose V, Bingmer M, Seino S, Magill PJ, et al. K-ATP channels in dopamine substantia nigra neurons control bursting and novelty-induced exploration. *Nat Neurosci*. (2012) 15:1272–80. doi: 10.1038/nn.3185
168. Liss B, Haeckel O, Wildmann J, Miki T, Seino S, Roeper J. K-ATP channels promote the differential degeneration of dopaminergic midbrain neurons. *Nat Neurosci*. (2005) 8:1742–51. doi: 10.1038/nn1570
169. Matsuda W, Furuta T, Nakamura KC, Hioki H, Fujiyama F, Arai R, et al. Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum*J. Neurosci*. (2009) 29:444–53. doi: 10.1523/JNEUROSCI.4029-08.2009
170. Pissadaki EK, Bolam JP. The energy cost of action potential propagation in dopamine neurons: clues to susceptibility in Parkinson's disease. *Front Comput Neurosci*. (2013) 7:13. doi: 10.3389/fncom.2013.00013
171. Takakusaki K, Shiroyama T, Yamamoto T, Kitai ST. Cholinergic and noncholinergic tegmental pedunculopontine projection neurons in rats revealed by intracellular labeling. *J Comp Neurol*. (1996) 371:345–61. doi: 10.1002/(SICI)1096-9861(19960729)371:3<345::AID-CNE1>3.0.CO;2-2
172. Gao K, Mason P. Somatodendritic and axonal anatomy of intracellularly labeled serotonergic neurons in the rat medulla. *J Comp Neurol*. (1997) 389:309–28. doi: 10.1002/(SICI)1096-9861(19971215)389:2<309::AID-CNE9>3.0.CO;2-5
173. Gao H, Glatzer NR, Williams KW, Derbenev AV, Liu D, Smith BN. Morphological and electrophysiological features of motor neurons and putative interneurons in the dorsal vagal complex of rats and mice. *Brain Res*. (2009) 1291:40–52. doi: 10.1016/j.brainres.2009.07.024
174. Gagnon D, Parent M. Distribution of VGLUT3 in highly collateralized axons from the rat dorsal raphe nucleus as revealed by single-neuron reconstructions. *PLoS ONE*. (2014) 9:e87709. doi: 10.1371/journal.pone.0087709
175. Chazal G, Ma W. An ultrastructural analysis of serotoninergic neurons in the nucleus raphe magnus of the rat. *Neuroscience*. (1989) 33:301–10. doi: 10.1016/0306-4522(89)90210-8
176. Jones BE, Yang TZ. The efferent projections from the reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat. *J Comp Neurol*. (1985) 242:56–92. doi: 10.1002/cne.902420105
177. Contant C, Umbriaco D, Garcia S, Watkins KC, Descarries L. Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum. *Neuroscience* (1996) 71:937–47. doi: 10.1016/0306-4522(95)00507-2
178. Zhou FM, Wilson CJ, Dani JA. Cholinergic interneuron characteristics and nicotinic properties in the striatum. *J Neurobiol*. (2002) 53:590–605. doi: 10.1002/neu.10150
179. Meitzen J, Pflepsen KR, Stern CM, Meisel RL, Mermelstein PG. Measurements of neuron soma size and density in rat dorsal striatum, nucleus accumbens core and nucleus accumbens shell: differences between striatal region and brain hemisphere, but not sex. *Neurosci Lett*. (2011) 487:177–81. doi: 10.1016/j.neulet.2010.10.017
180. Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann Neurol*. (1994) 36:348–55. doi: 10.1002/ana.410360305
181. Pearce RK, Owen A, Daniel S, Jenner P, Marsden CD. Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease. *J Neural Transm*. (1997) 104:661–77. doi: 10.1007/BF01291884
182. Sofic E, Lange KW, Jellinger K, Riederer P. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett*. (1992) 142:128–30. doi: 10.1016/0304-3940(92)90355-B
183. Tagliaferro P, Burke RE. Retrograde axonal degeneration in Parkinson disease. *J Parkinsons Dis*. (2016) 6:1–15. doi: 10.3233/JPD-150769
184. O'Malley KL. The role of axonopathy in Parkinson's disease. *Exp Neurobiol*. (2010) 19:115–9. doi: 10.5607/en.2010.19.3.115
185. Kinghorn KJ, Asghari AM, Castillo-Quan JI. The emerging role of autophagic-lysosomal dysfunction in Gaucher disease and Parkinson's disease. *Neural Regener Res*. (2017) 12:380–4. doi: 10.4103/1673-5374.202934
186. Wong YC, Krainc D. Lysosomal trafficking defects link Parkinson's disease with Gaucher's disease. *Mov Disord*. (2016) 31:1610–8. doi: 10.1002/mds.26802
187. Migdalska-Richards A, Schapira AH. The relationship between glucocerebrosidase mutations and Parkinson disease. *J Neurochem*. (2016) 139(Suppl. 1):77–90. doi: 10.1111/jnc.13385
188. Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med*. (2009) 361:1651–61. doi: 10.1056/NEJMoa0901281
189. Gegg ME, Burke D, Heales SJ, Cooper JM, Hardy J, Wood NW, et al. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. *Ann Neurol*. (2012) 72:455–63. doi: 10.1002/ana.23614
190. Murphy KE, Halliday GM. Glucocerebrosidase deficits in sporadic Parkinson disease. *Autophagy* (2014) 10:1350–1. doi: 10.4161/auto.29074
191. Dehay B, Martinez-Vicente M, Caldwell GA, Caldwell KA, Yue Z, Cookson MR, et al. Lysosomal impairment in Parkinson's disease. *Mov Disord*. (2013) 28:725–32. doi: 10.1002/mds.25462
192. Ashrafi G, Schwarz TL. PINK1- and PARK2-mediated local mitophagy in distal neuronal axons. *Autophagy* (2015) 11:187–9. doi: 10.1080/15548627.2014.996021
193. Matheoud D, Sugiura A, Bellemare-Pelletier A, Laplante A, Rondeau C, Chemali M, et al. Parkinson's disease-related proteins PINK1 and parkin repress mitochondrial antigen presentation. *Cell* (2016) 166:314–27. doi: 10.1016/j.cell.2016.05.039
194. Sheng C, Heng X, Zhang G, Xiong R, Li H, Zhang S, et al. DJ-1 deficiency perturbs microtubule dynamics and impairs striatal neurite outgrowth. *Neurobiol Aging* (2013) 34:489–98. doi: 10.1016/j.neurobiolaging.2012.04.008
195. Di Nottia M, Masciullo M, Verrigni D, Petrillo S, Modoni A, Rizzo V, et al. DJ-1 modulates mitochondrial response to oxidative stress:

clues from a novel diagnosis of PARK7. *Clin Genet*. (2017) 92:18–25. doi: 10.1111/cge.12841
196. MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* (2006) 52:587–93. doi: 10.1016/j.neuron.2006.10.008
197. Yue M, Hinkle KM, Davies P, Trushina E, Fiesel FC, Christenson TA, et al. Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2 G2019S knock-in mice. *Neurobiol Dis*. (2015) 78:172–95. doi: 10.1016/j.nbd.2015.02.031
198. Surmeier DJ, Halliday GM, Simuni T. Calcium, mitochondrial dysfunction and slowing the progression of Parkinson's disease. *Exp Neurol*. (2017) 298(Pt B):202–9. doi: 10.1016/j.expneurol.2017.08.001
199. Wang Z, Hou L, Huang L, Guo J, Zhou X. Exenatide improves liver mitochondrial dysfunction and insulin resistance by reducing oxidative stress in high fat diet-induced obese mice. *Biochem Biophys Res Commun*. (2017) 486:116–23. doi: 10.1016/j.bbrc.2017.03.010
200. Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, et al. GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. *Proc Natl Acad Sci USA*. (2009) 106:1285–90. doi: 10.1073/pnas.0806720106
201. Athauda D, Maclagan K, Skene SS, Bajwa-Joseph M, Letchford D, Chowdhury K, et al. Exenatide once weekly versus placebo in Parkinson's disease: a randomised, double-blind, placebo-controlled trial. *Lancet* (2017) 390:1664–75. doi: 10.1016/S0140-6736(17)31585-4
202. Gleave JA, Arathoon LR, Trinh D, Lizal KE, Giguere N, Barber JHM, et al. Sirtuin 3 rescues neurons through the stabilisation of mitochondrial biogenetics in the virally-expressing mutant α-synuclein rat model of parkinsonism. *Neurobiol Dis*. (2017) 106:133–46. doi: 10.1016/j.nbd.2017.06.009
203. Shi H, Deng HX, Gius D, Schumacker PT, Surmeier DJ, Ma YC. Sirt3 protects dopaminergic neurons from mitochondrial oxidative stress. *Hum Mol Genet*. (2017) 26:1915–26. doi: 10.1093/hmg/ddx100

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.

frontiers in Neurology

ORIGINAL RESEARCH
published: 20 June 2018
doi: 10.3389/fneur.2018.00457

Pre-α-pro-GDNF and Pre-β-pro-GDNF Isoforms Are Neuroprotective in the 6-hydroxydopamine Rat Model of Parkinson's Disease

*Anna-Maija Penttinen[1], Ilmari Parkkinen[1], Merja H. Voutilainen[1], Maryna Koskela[1], Susanne Bäck[2], Anna Their[1], Christopher T. Richie[3], Andrii Domanskyi[1], Brandon K. Harvey[3], Raimo K. Tuominen[2], Liina Nevalaita[1], Mart Saarma[1] and Mikko Airavaara[1]**

[1] HiLIFE Unit, Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [2] Division of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland, [3] National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, United States

OPEN ACCESS

Edited by:
Graziella Madeo,
National Institutes of Health (NIH),
United States

Reviewed by:
Tommaso Schirinzi,
Università degli Studi di Roma Tor Vergata, Italy
Alessandro Tozzi,
University of Perugia, Italy

***Correspondence:**
Mikko Airavaara
mikko.airavaara@helsinki.fi

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: 06 March 2018
Accepted: 29 May 2018
Published: 20 June 2018

Citation:
Penttinen A-M, Parkkinen I, Voutilainen MH, Koskela M, Bäck S, Their A, Richie CT, Domanskyi A, Harvey BK, Tuominen RK, Nevalaita L, Saarma M and Airavaara M (2018) Pre-α-pro-GDNF and Pre-β-pro-GDNF Isoforms Are Neuroprotective in the 6-hydroxydopamine Rat Model of Parkinson's Disease. Front. Neurol. 9:457. doi: 10.3389/fneur.2018.00457

Glial cell line-derived neurotrophic factor (GDNF) is one of the most studied neurotrophic factors. GDNF has two splice isoforms, full-length pre-α-pro-GDNF (α-GDNF) and pre-β-pro-GDNF (β-GDNF), which has a 26 amino acid deletion in the pro-region. Thus far, studies have focused solely on the α-GDNF isoform, and nothing is known about the *in vivo* effects of the shorter β-GDNF variant. Here we compare for the first time the effects of overexpressed α-GDNF and β-GDNF in non-lesioned rat striatum and the partial 6-hydroxydopamine lesion model of Parkinson's disease. GDNF isoforms were overexpressed with their native pre-pro-sequences in the striatum using an adeno-associated virus (AAV) vector, and the effects on motor performance and dopaminergic phenotype of the nigrostriatal pathway were assessed. In the non-lesioned striatum, both isoforms increased the density of dopamine transporter-positive fibers at 3 weeks after viral vector delivery. Although both isoforms increased the activity of the animals in cylinder assay, only α-GDNF enhanced the use of contralateral paw. Four weeks later, the striatal tyrosine hydroxylase (TH)-immunoreactivity was decreased in both α-GDNF and β-GDNF treated animals. In the neuroprotection assay, both GDNF splice isoforms increased the number of TH-immunoreactive cells in the substantia nigra but did not promote behavioral recovery based on amphetamine-induced rotation or cylinder assays. Thus, the shorter GDNF isoform, β-GDNF, and the full-length α-isoform have comparable neuroprotective efficacy on dopamine neurons of the nigrostriatal circuitry.

Keywords: neurotrophic factors, neurodegeneration, GDNF, splice variant, alternative splicing, tyrosine hydroxylase, dopamine

INTRODUCTION

Originally purified from a rat glioma cell line, glial cell-derived neurotrophic factor (GDNF) was shown to promote differentiation and survival of rat midbrain dopamine neurons, increase outgrowth of neurites and dopamine uptake *in vitro* (1). Moreover, GDNF stimulated the formation of new axon terminals in dopamine neurons (2). These findings led to increased interest in GDNF's therapeutic potential for Parkinson's disease (PD), in which the progressive degeneration of midbrain dopamine neurons in substantia nigra pars compacta (SNpc) and their projections to striatum (caudate nucleus and putamen) is causing major motor disturbances, such as tremor and postural instability (3). Indeed, in animal models of PD, GDNF has been shown to protect the dopaminergic nigrostriatal pathway from 6-OHDA or MPTP-induced degeneration when administered as a protein or gene therapy (4–7), and to restore the dopaminergic phenotype (i.e., striatal dopaminergic markers, such as tyrosine hydroxylase (TH) and the dopamine level) of the pathway after the degeneration in rodent and non-human primate models of PD (3, 8–11).

The human GDNF gene consists of six exons and the rodent GDNF gene of three exons (12–14) (**Figure 1**). The alternative splicing site in the third exon produces two conserved splice isoforms; full-length pre-α-pro-GDNF (α-GDNF) and the shorter pre-β-pro-GDNF (β-GDNF), which has a deletion of 26 amino acids (GKRPPEAPAEDRSLGRRRAPFALSSDS) in the pro-region (12–16) (**Figure 1**). The deletion does not interfere with the proteolytic cleavage site, and both isoforms are cleaved to mature GDNF. The pre-region is cleaved off in the endoplasmic reticulum and the pro-region mainly in the secretory vesicles (1, 16, 17). The pro-region has been suggested to play a role in the folding and secretion of GDNF (18). *In vitro,* both isoforms are secreted from the cells upon overexpression but in drastically different manner. α-GDNF and the corresponding mature GDNF are secreted constitutively while β-GDNF and its corresponding mature GDNF are secreted activity-dependently (17). Furthermore, the isoforms have different localization patterns inside the cells: α-GDNF is mainly localized in the Golgi complex, whereas β-GDNF is localized in secretogranin II (scgII)- and Rab3A-positive vesicles of the regulated secretory pathway (17). Despite these differences in localization and secretion, the two major splice isoforms, α-GDNF and β-GDNF, are expressed in the same tissues, but in varying proportions (14–16). Interestingly, β-GDNF mRNA expression is present at relatively high levels during brain development when neuronal contacts are formed (15).

GDNF is functional as a homodimer, stabilized by a disulfide-bond (19). It exerts its functions via binding first to a lipid raft-resident glycosylphosphoinositol-anchored GDNF receptor α (GFRα), followed by formation of a heterohexameric complex with two Ret (rearranged during transfection) receptors (20). Alternatively, the signaling is initiated by GDNF-GFRα via NCAM (21) or syndecan-3 (22). The exact pro-survival mechanism of GDNF is not known, but activation of Ret can initiate several signaling cascades, of which the mitogen activated protein (MAP) kinase and phosphoinositositide-3-kinase (PI3K) pathways have been suggested to play a role in the survival promoting actions (23).

Although GDNF is a widely studied trophic factor, and its potential as a therapeutic agent for neurodegenerative diseases is well established including human clinical trials for Parkinson's disease, there are only few studies about the biology of β-GDNF. All previous studies have focused on the effects and properties of α-GDNF, whereas the biological effects of the shorter β-isoform are still largely unknown. This is the first study to compare the effects of the two major GDNF isoforms in non-lesioned striatum as well as in the 6-hydroxydopamine (6-OHDA) rat model of PD. We report here the effects of β-GDNF to be comparable to the effects of α-GDNF on the dopaminergic phenotype of the nigrostriatal dopamine neurons. In non-lesioned striatum, both GDNF isoforms increased the density of dopamine transporter (DAT)-immunoreactive striatal fibers 3 weeks after viral vector delivery, but only α-GDNF increased the use of contralateral paw in the cylinder test at the same time point. Four weeks later, overexpression of both isoforms downregulated TH. However, the isoforms equally protected the TH-immunoreactive cell bodies in SNpc against 6-OHDA-induced degeneration.

MATERIALS AND METHODS

Generation of pscAAV-CMV-pre-α-pro-GDNF and pscAAV-CMV-pre-β-pro-GDNF Constructs

To produce the self-complementary AAV (scAAV) vectors expressing human pre-α-pro-GDNF and pre-β-pro-GDNF, the cDNA fragments encoding human pre-α-pro-GDNF and pre-β-pro-GDNF were produced by PCR using pAAV-pre-α-pro-GDNF and pAAV-pre-β-pro-GDNF (17) as a template accordingly. PCR was performed with Phusion Hot-Start polymerase (ThermoFisher Scientific, Waltham, MA). PCR products were purified and digested by BamHI and NotI restriction enzymes (ThermoFisher Scientific, Waltham, MA) and ligated into a pscAAV-CMV vector using T4 DNA ligase (ThermoFisher Scientific, Waltham, MA). The plasmid pscAAV-CMV was obtained by cutting out the eGFP insert from pscAAV-CMV-eGFP using BamHI and NotI restriction sites. Both cloned constructs were verified by DNA sequencing. Primers used for cloning of pre-α-pro-GDNF and pre-β-pro-GDNF into pscAAV-CMV were forward 5′-TAGGATCCATGA AGTTATGGGATGTCGTGG-3′ containing BamHI restriction site and reverse 5′-TAGCGGCCGCTCAGATACATCCACACC TTTTA-3′ containing NotI restriction site.

The self-complementary AAV vectors, scAAV-pre-α-pro-GDNF, scAAV-pre-β-pro-GDNF and scAAV-CMV-eGFP were packaged as serotype 1 (24), then purified and titered as described

Abbreviations: 6-OHDA, 6-hydroxydopamine; α-GDNF, pre-α-pro-GDNF; β-GDNF, pre-β-pro-GDNF; ANOVA, analysis of variance; DAT, dopamine transporter; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; GP, globus pallidus; IHC, immunohistochemistry; PD, Parkinson's disease; scAAV, self-complementary adeno-associated virus; scgII, secretogranin II; SEM, standard error of mean; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

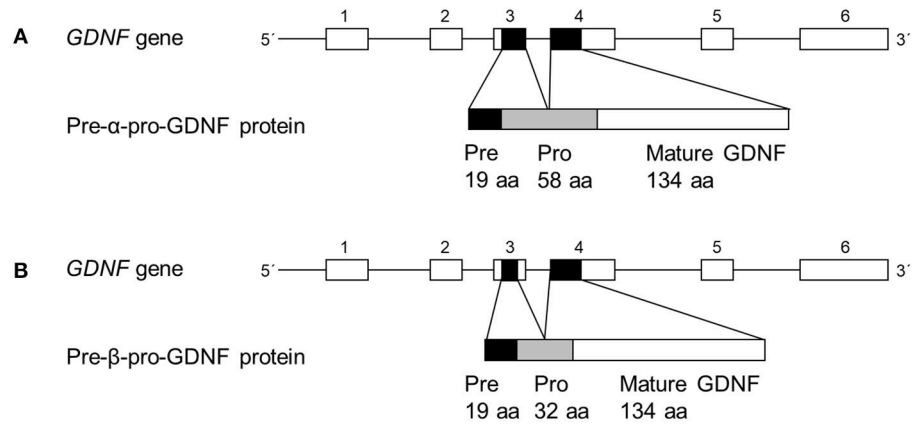


FIGURE 1 | Organization of main human GDNF splice isoforms. **(A,B)** In *GDNF* gene line represents introns and boxes represent exons (not in scale). Black boxes represent protein coding areas. Pre-α-pro-GDNF isoform has a full-length 58 amino acid pro-region, whereas the pre-β-pro-GDNF has shorter 32 amino acid pro-region.

previously (25). The titers for the vectors were scAAV1-CMV-eGFP 7.40×10^{13} vg/ml, scAAV1-CMV-pre-α-pro-GDNF 2.14×10^{12} vg/ml, and scAAV1-CMV-pre-β-pro-GDNF 1.73×10^{12} vg/ml, respectively. AAV vector work was conducted by the Optogenetics and Transgenic Technology Core, NIDA IRP, NIH, Baltimore MD, USA.

Animals

The experiments were carried out in accordance with the 3R principles of the EU directive 2010/63/EU on the care and use of experimental animals, and local laws and regulations [Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013) and Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)]. All animal procedures were reviewed and approved by the national Animal Experiment Board of Finland (License number ESAVI/7812/04.10.07/2015). A total of 123 adult male Wistar rats weighing 210-350 g (RRID: RGD_5508396, Harlan/Envigo, Horst, The Netherlands) were used in the experiments. The animals were group housed under standard laboratory conditions in 12 h light/dark cycle with free access to food and water. The well-being of the animals was observed on a regular basis.

Intrastriatal Administration of Viral Vectors and 6-OHDA

All stereotaxic surgeries were performed under isoflurane anesthesia (4% induction and 2.5% maintenance) and carprofen (5 mg/kg, s.c.) was used as post-operative analgesic as previously described (26). For the viral vector injections, animals were randomly allocated to treatment groups. 4.5 μl of scAAV1-pre-α-pro-GDNF, scAAV1-pre-β-pro-GDNF or scAAV1-eGFP was equally distributed to three sites in the right striatum. AAV injections were carried out as previously described (27). The injection coordinates according to bregma were (1) A/P +1.6 L/M −2.8 D/V −6.0 from skull, (2) A/P 0.0 L/M −4.1 D/V −5.5 from skull, and 3) A/P-1.2 L/M −4.5 D/V −5.5 from skull (28). Injections were done in a 10° angle at a rate of 0.5 μl/min. The microinjection needle was kept in place for additional 5 min to avoid backflow of the solution (26). In the neuroprotection experiment 3×2 μg of 6-OHDA (Sigma Aldrich, St. Louis, MO) was injected to the same sites as the viral vectors 3 weeks later (**Figure 4A**).

Tissue Levels of GDNF

To assess the tissue levels of GDNF, 15 animals received 3 μl of scAAV1-pre-α-pro-GDNF ($n = 5$), scAAV1-pre-β-pro-GDNF ($n = 5$) or scAAV1-eGFP ($n = 5$) distributed evenly to the three striatal injection sites as described above. Three weeks later, animals were deeply anesthetized with pentobarbital (90 mg/kg, i.p., MebunatVet, Orion Pharma, Espoo, Finland) and decapitated. Brains were snap frozen in cold isopentane and stored at −70°C. The striatal samples were collected from the frozen brain and mechanically homogenized in lysis buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1% Igepal, 10% glycerol, 1:25 Complete Mini EDTA-free (Roche, Basel, Switzerland) and centrifuged at 5,000 × g for 5 min at +4°C. The GDNF levels were determined from the supernatants by commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommendations (Promega, Madison, WI).

Behavioral Assays

Cylinder Test

Motor asymmetry was assessed with the cylinder test before viral vector administration and 3 and 7 weeks after the administration (**Figures 2A**, **3A**). In the neuroprotection experiment the cylinder test was conducted 3 and 7 weeks after virus injection (before and 4 weeks after 6-OHDA injection) (**Figure 4A**). Freely moving rats were monitored for 5 min in a plexiglass cylinder (diameter 20 cm) under red light, and the contacts between forepaws and the cylinder wall were counted by a blinded observer. Placement of the whole palm on the cylinder wall to support the body while exploring was considered as a touch.

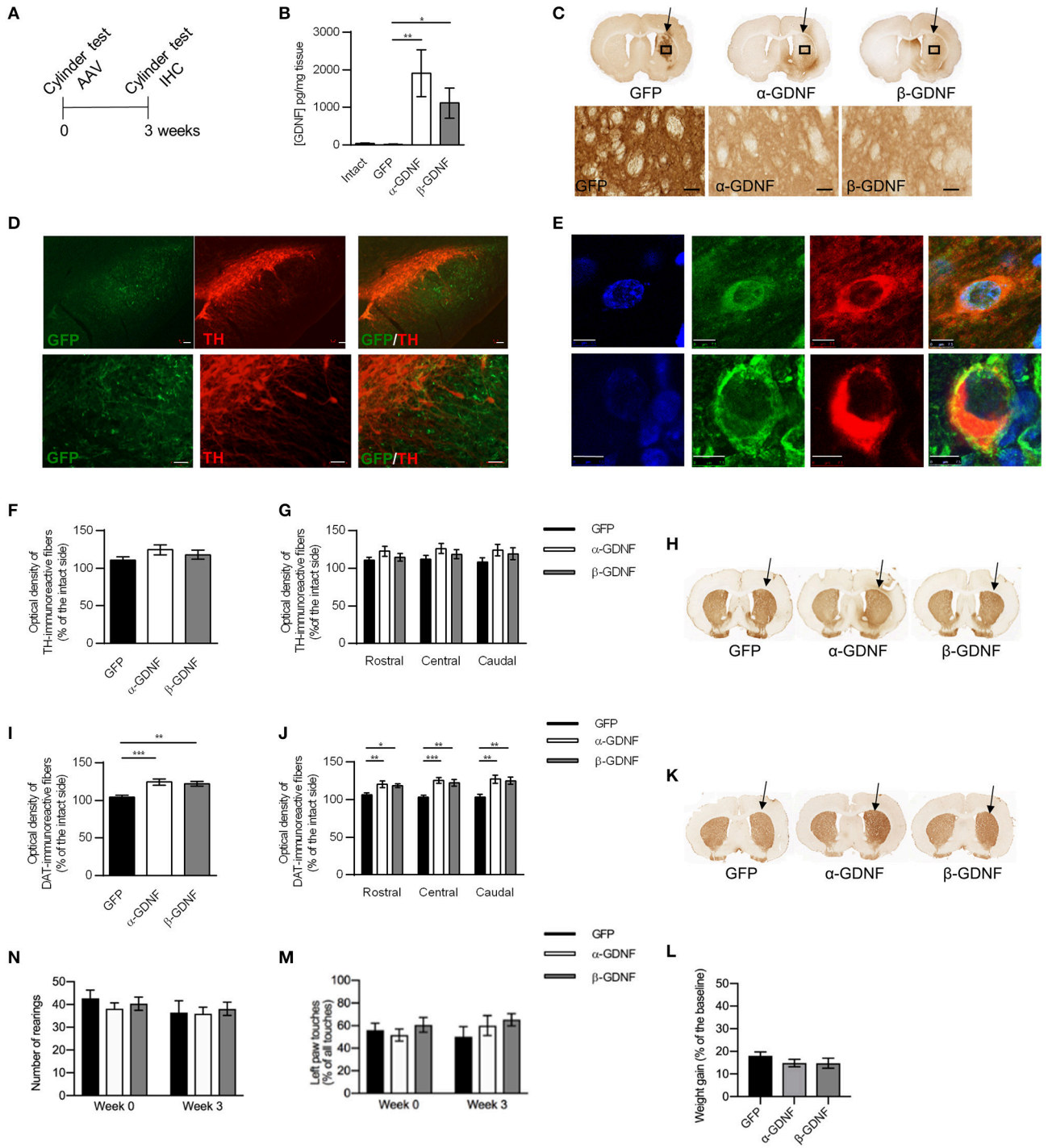


FIGURE 2 | Effects of GDNF isoform overexpression on dopaminergic markers in non-lesioned striatum 3 weeks after AAV-injection. **(A)** Experimental design. **(B)** Overexpression levels of GDNF isoforms were confirmed with ELISA [Kruskal-Wallis test $H(3) = 15.457$, $p = 0.001$, followed by Bonferroni corrected Mann-Whitney U *post-hoc* test, $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 5$ in each group]. **(C)** Representative images of GFP- and GDNF-stained striatal sections. Arrows point to the injected side. 40x magnification of the area is designated by the black box and scale bar is 50 μm. **(D)** GFP signal was observed in SN reticulata, but not in TH-immunoreactive cells in SNpc. Upper panels show 5x magnification with scale bar 100 μm, lower panels show 20x magnification with scale bar 50 μm. **(E)** Both GDNF isoforms co-localized with scgII-immunoreactive structures. Blue = dapi, green = scgII, red = GDNF (upper row alpha, lower row beta), scale bar 7.5 μm. **(F)** Optical density of striatal TH-immunoreactive fibers was similar in all treatment groups (GFP 111 ± 4%, α-GDNF 124 ± 7%, and β-GDNF 118 ± 6% of the intact side, $n = 8$–10 in each group) **(G)** Density of TH-immunoreactive fibers was at similar level in all treatment groups throughout the whole striatum ($n = 8$–10). **(H)** Representative images of TH-stained striatal sections. Arrows point to the injected side. **(I)** Overexpression of both GDNF isoforms increased the optical density of striatal DAT-immunoreactive fibers

(Continued)

FIGURE 2 | [one-way ANOVA $F_{(2,24)} = 11.336$, $p < 0.001$, Fisher's LSD *post-hoc* test α-GDNF vs. GFP $p < 0.001$ and β-GDNF vs. GFP $p = 0.002$, $^{***}p < 0.001$, $^{**}p < 0.01$, $n = 8$–10]. **(J)** The effects of GDNF isoforms were consistent throughout whole striatum [one-way ANOVA rostral $F_{(2,24)} = 5.315$, $p = 0.012$, Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p = 0.005$ and β-GDNF vs. GFP $p = 0.026$; central $F_{(2,24)} = 11.339$, $p < 0.0001$, Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p < 0.0001$ and β-GDNF vs. GFP $p = 0.002$; caudal: $F_{(2,24)} = 7.674$, $p = 0.003$ Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p = 0.001$ and β-GDNF vs. GFP $p = 0.006$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ $n = 8$–10]. **(K)** Representative images of DAT-stained striatal sections. Arrows point to the injected side. **(L,M)** Short-term overexpression of GDNF isoforms in non-lesioned striatum did not induce behavioral changes in the cylinder test, as measured by **(N)** vertical activity (baseline GFP 43 ± 4, α-GDNF 38 ± 3, and β-GDNF 40 ± 3 rearings, 3 weeks after scAAV GFP 37 ± 5, α-GDNF 36 ± 3, and β-GDNF 38±3 rearings) or **(M)** contralateral paw touches, ($n = 8$–10 in each group). **(L)** All animals gained weight in similar manner during the 3 weeks of the experiment ($n = 15$ in each group). Data is expressed as mean ± SEM.

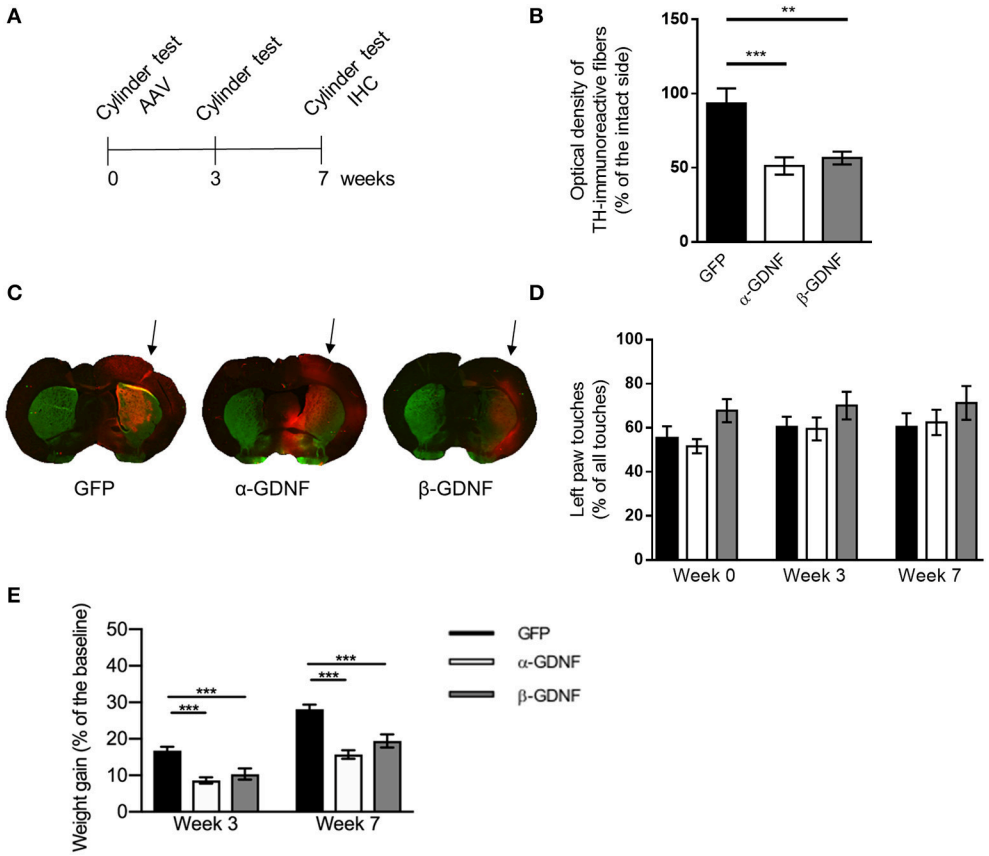


FIGURE 3 | Effects of GDNF isoform overexpression on dopaminergic markers in non-lesioned striatum 7 weeks after AAV-injection. **(A)** Experimental design. **(B)** Optical density of striatal TH-immunoreactive fibers was significantly lower in both isoform treated groups compared to GFP [one-way ANOVA, $F_{(2,28)} = 10.56$, $p = 0.0004$, followed by Bonferroni *post-hoc* test, $^{***}p < 0.001$, $^{**}p < 0.01$ $n = 10$–11]. **(C)** Representative images of TH- (green), GFP-, (red) and GDNF- (red) stained striatal sections from infrared analysis, arrows point to the injected side. **(D)** No significant changes in contralateral (left) paw touches were observed 3 or 7 weeks after injection of GDNF isoforms [one-way ANOVA $F_{(2,28)} = 0.7678$, $p = 0.4736$]. Data is expressed as mean ± SEM. **(E)** Animals treated with either α- or β-GDNF isoform gained weight significantly less compared to GFP-treated animals, both 3 and 7 weeks after AAV-injections [two-way ANOVA treatment effect $F_{(2,58)} = 33.044$, $p < 0.0001$; time effect $F_{(1,58)} = 5\,2{,}966$, $p < 0.0001$; treatment × time interaction $F_{(2,58)} = 0.358$, $p = 0.701$]. 3 week time point 1-way ANOVA $F_{(2,29)} = 13.040$, $p < 0.0001$, Fisher's LSD *post-hoc* test GFP vs. α-GDNF $p < 0.001$, GFP vs. β-GDNF vs. $p = 0.001$, and α-GDNF vs. β-GDNF $p = 0.294$. Seven week time point one-way ANOVA $F_{(2,29)} = 18.689$, $p < 0.0001$, Fisher's LSD *post-hoc* test GFP vs. α-GDNF $p < 0.0001$, GFP vs. β-GDNF vs. $p = 0.0001$, and α-GDNF vs. β-GDNF $p = 0.073$, $^{***}p < 0.001$, $n = 10$ in each group.

Rotation Assay

In the neuroprotection experiment, the motor asymmetry was also measured with the d-amphetamine-induced rotation assay. The rotation assay was performed as previously described (26). In brief, the rotational behavior was monitored for 120 min after administration of d-amphetamine sulfate (2.5 mg/kg, s.c., Sigma Aldrich, St. Louis, MO) in automated rotation bowls (Med Associates, Inc., Fairfax, VT). Full 360° ipsilateral turns were given positive value.

Tissue Processing and Immunohistochemistry

Three or seven weeks (neuroprotection experiment) after the virus injection, animals were anesthetized with pentobarbital

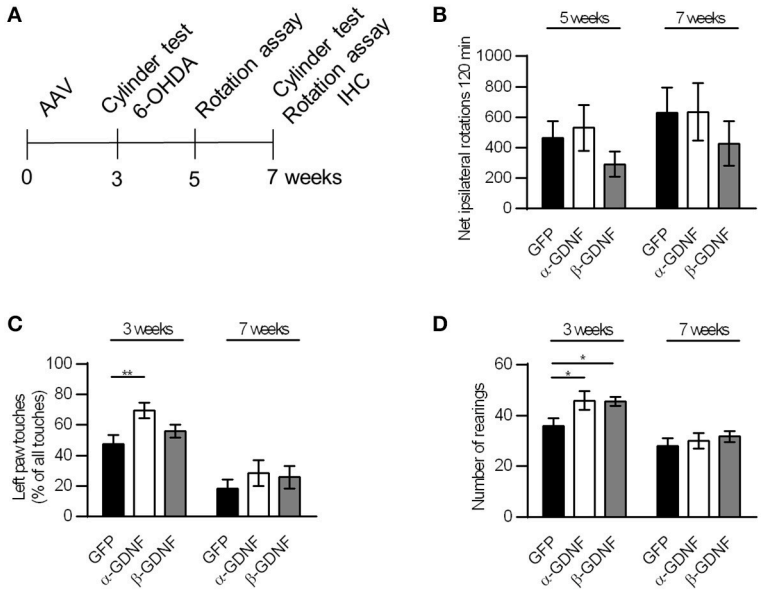


FIGURE 4 | Neither GDNF isoform displayed neuroprotective effects in the rotational assay or the drug-free cylinder test. **(A)** Experimental design. **(B)** The rotational behavior was at similar level in all treatment groups ($n = 15$–16). **(C)** α-GDNF increased the use of contralateral paw in the pre-lesion cylinder test on week three [one-way ANOVA $F_{(2, 43)} = 4.492$, $p = 0.017$, followed by Fisher's LSD *post-hoc* test $^{**}p < 0.01$, $n = 15$–16], but the effect was abolished after 6-OHDA administration. **(D)** Both GDNF isoforms increased the exploratory activity of the animals before the lesion in the cylinder test [one-way ANOVA $F_{(2, 43)} = 3.871$, $p = 0.028$, followed by Fisher's LSD *post-hoc* test $^{*}p < 0.05$, $n = 15$–16 in each group]. Data is expressed as mean ± SEM.

(90 mg/kg, i.p., MebunatVet, Orion Pharma, Espoo, Finland) and transcardially perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) solution. Brains were removed and post-fixed overnight in 4% PFA at +4°C and transferred to sucrose series of 20 and 30% sucrose.

The brains were cut in a freezing microtome in 40 μm thick sections in series of six. Free-floating sections were stained as previously described (26). In brief, the sections were washed and treated with 0.3% hydrogen peroxide solution. For DAT staining, the sections were incubated in 10 mM citrate buffer, pH 6.0, at 80°C for 30 min. After incubation in the blocking solution (4% bovine serum albumin and 0.1% Triton X-100 in PBS) the sections were incubated with the primary antibody overnight at +4°C. Primary antibodies and the dilutions used in the studies are designated in **Table 1**. Next, the sections were incubated with biotinylated secondary antibodies (anti-rat, anti-mouse, or anti-rabbit, Vector Laboratories, Burlingame, CA) and the staining was reinforced with avidin-biotin-complex (Vector Laboratories, Burlingame, CA) and visualized with 3′, 3′diaminobenzidine. The stained sections were scanned with automated microscope slide scanner (Pannoramic 250 Flash II, 3D Histech, Budapest, Hungary).

To detect scAAV1 transduction pattern in the SN, immunofluorescence staining was carried out for the sections. The sections were incubated in the blocking solution (4% bovine serum albumin and 0.1% Triton X-100 in PBS), followed by incubation with primary antibody (anti-TH, **Table 1**) overnight at +4°C. After washing, the sections were incubated with Alexa 568-conjugated goat-anti-mouse secondary antibody (1:300, ThermoFisher Scientific, Waltham, MA) and mounted on microscope slides. GFP signal was visible without immunofluorescence staining.

For the confocal microscopy, the striatal sections were incubated with blocking solution for 1 h followed by 1 h incubation with the first primary antibody (ScgII, **Table 1**) at RT. After this, the second primary antibody (anti-GDNF) was added and the sections were incubated at +4°C overnight. The following day, sections were incubated with Alexa 488-conjugated donkey-anti-mouse secondary antibody (1:500, ThermoFisher Scientific, Waltham, MA) antibody for 15 min and then for 1 h after the addition of Alexa 568-conjugated donkey-anti-goat secondary antibody (1:500, ThermoFisher Scientific, Waltham, MA) at RT. Sections were mounted in PBS, allowed to dry overnight, washed in ddH_2O, allowed to dry o/n and subsequently coverslipped using Vectashield HardSet Antifade Mounting Medium with DAPI (H-1500; Vector Labs, Burlingame, CA).

For infrared analysis, the sections were incubated with blocking solution for 1 h followed by 1 h incubation with the primary antibody for anti-TH at RT. After this, the second primary antibody (anti-GFP or anti-GDNF, **Table 1**) was added and the sections were incubated at +4°C overnight. Next day, sections were incubated in IRDye® 800CW secondary antibody for 15 min and then for 1 h after the addition of the other secondary antibody, anti-Goat or anti-Rabbit IRDye® 680RD (All secondary antibodies 1:2,000, LI-COR Biosciences, Lincoln, NE) at RT. Before mounting, the sections were rinsed with ddH_2O for 5 min at RT.

TABLE 1 | Antibodies and their dilutions used in immunohistochemistry.

Antigen	Host	Producer	Cat#	RRID	Dilution
Dopamine transporter (DAT)	Rat	Millipore	MAB369	AB_2190413	1:2,000
Glial cell line-derived neurotrophic factor (GDNF)	Goat	R & D systems	AF-212-NA	AB_2111398	1:3,000[a] 1:1,000[b]
Green fluorescent protein (GFP)	Rabbit	Life technologies	A11122	N/A	1:2,000
Tyrosine hydroxylase (TH)	Mouse	Millipore	MAB318	AB_2201528	1:2,000
Secretogranin II	Mouse	Abcam	ab20246	AB_445463	1:500

[a] *In expression pattern studies.*
[b] *Confocal microscopy and infrared analysis.*

Confocal Microscopy

Slides were imaged using a Leica TCS SP5 confocal microscope (CLSM; Leica Microsystems, Buffalo Grove, IL) through a 63× oil-immersion objective. The brightness/contrast of the image taken with Laser-405 (DAPI) was adjusted by ImageJ for optimal visual display.

Estimation of Optical Density of TH- and DAT-Immunoreactive Fibers in the Striatum

The density of TH- and DAT-immunoreactivity was measured from six adjacent sections with ImagePro software (Media Cybernetics, Inc., Rockville, MD) by a blinded observer. Corpus callosum was used as a background to correct the values. The data are presented as a percentage of the intact side.

In the infrared assay, the sections were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) with 42-micron resolution. The TH optical densities from the injected and non-injected (intact) side of four striatal sections per animal were measured using the Odyssey Infrared Imaging System software. Background optical density was measured from the cortex or corpus callosum depending on the integrity of the section. The density of TH-immunoreactive fibers was assessed by subtracting the background intensity values and normalizing the injected side to the optical density of the intact side. The data are presented as a percentage of the intact side.

Estimation of Number of TH-Immunoreactive Cells in the SNpc

The number of TH-immunoreactive cells in the SNpc was determined with Matlab (RRID: SCR_001622, MathWorks, Kista, Sweden) as previously described (26) by a blinded observer. Images taken with whole slide scanner (Pannoramic 250 Flash II, 3D Histech, Budapest, Hungary, with 20x objective) from six adjacent nigral sections were analyzed. The data are presented as a percentage of the intact side.

Statistics

Results are given as mean ± SEM. Statistical analysis was performed with SPSS (RRID: SCR_002865, IBM, Armonk, NY) or Prism version 6.01 (GraphPad Software, Inc., La Jolla, San Diego, CA). Differences between treatment groups were assessed with one-way analysis of variance (ANOVA) or two-way ANOVA and if significant, followed by Fisher's Least Significant Difference (LSD) or Bonferroni *post-hoc* analysis (7 week overexpression experiment). In cases of non-homogenous variances (ELISA assay), Kruskal-Wallis analysis of variance followed by Bonferroni corrected Mann-Whitney U *post-hoc* was conducted. A difference was considered to be significant at $p \leq 0.05$.

RESULTS

Overexpression of GDNF in the Non-lesioned Striatum

A scAAV-vector encoding pre-α-pro-GDNF (α-GDNF), pre-β-pro-GDNF (β-GDNF), or green fluorescent protein (GFP, as a control) was injected into three sites in the non-lesioned striatum. The level of GDNF overexpression was determined with ELISA 3 weeks after the gene delivery. The infusion of scAAVs produced a marked overexpression of GDNF in the striatum (**Figure 2B**). The level of GDNF in the intact (contralateral) side was 40 ± 8 pg/mg tissue, in scAAV-GFP-treated side 15 ± 6 pg/mg tissue, in scAAV-α-GDNF-treated side 1,906 ± 629 pg/mg tissue, and in scAAV-β-GDNF-treated side 1,115 ± 402 pg/mg tissue (GFP vs. α-GDNF $p = 0.005$, GFP vs. β-GDNF $p = 0.017$, and α-GDNF vs. β-GDNF $p = 0.465$, **Figure 2B**).

Although the ELISA results showed robust GDNF overexpression in the striatum, immunohistochemistry was also applied to explore the protein distribution along the nigrostriatal tract. Since GFP is retained inside the cells, it had more restricted staining pattern in the striatum (**Figure 2C**). In contrast, GDNF is a secretory protein (1) and the staining pattern was widely spread, covering most of the striatum of the injected side. Minimal immunoreactivity was observed on the contralateral, non-injected side. To determine whether scAAV1 transduces post-synaptic striatal neurons, pre-synaptic dopamine neurons, or both we carried out immunofluorescence staining for TH and compared it to GFP. GFP expression in the SN reticulata was not in TH-immunoreactive fibers or TH-immunoreactive cells of the SNpc (**Figure 2D**). This staining pattern suggests that striatal delivery of scAAV1 does not transduce nigrostriatal dopaminergic neurons, but nigral gene expression is due to transduction of striatal medium spiny projection neurons. *In vitro* β-GDNF has been shown to co-localize mostly with the scgII signal in the cells, unlike α-GDNF (17). In contrast, we found that overexpression with AAVs under the CMV promoter *in vivo* both α-GDNF and β-GDNF were found to be co-localized with the scgII-signal (**Figure 2E**).

Moreover, both isoforms were ubiquitously expressed in cell bodies, and no specific sub-localization patterns were observed.

Overexpression of GDNF Isoforms Do Not Alter the Density of TH-Immunoreactive Fibers but Increases the Density of DAT-Immunoreactive Fibers in the Non-lesioned Striatum 3 Weeks After scAAV Delivery

Since GDNF has been shown to regulate the markers for dopaminergic phenotype, we next studied the effects of α-GDNF or β-GDNF overexpression on the dopaminergic markers TH and dopamine transporter (DAT) in non-lesioned striatum 3 weeks after the scAAV administration. GDNF overexpression did not alter the striatal TH optical density. Thus, the optical densities of striatal TH-immunoreactive fibers were similar in all treatment groups (**Figures 2F,H**). We divided sections into three categories: rostral, central, and caudal, each containing two adjacent sections, to analyze the TH optical density along the rostrocaudal axis in the striatum. The density of TH-immunoreactive fibers was at the same level in all three striatal areas for all treatment groups (**Figure 2G**). In contrast, the density of DAT-immunoreactive fibers was increased in α- and in β-GDNF-treated animals compared to GFP-treated animals (α-GDNF vs. GFP p<0.001 and β-GDNF vs. GFP $p = 0.002$, **Figures 2I,K**). Furthermore, the effect of GDNF isoforms on DAT-immunoreactive fiber density was increased in all sections along the rostro-caudal axis in the striatum [two-way ANOVA treatment effect $F_{(2,72)} = 23.285$, $p < 0.0001$; site effect $F_{(2,72)} = 0.490$, $p = 0.615$; treatment × site effect $F_{(4,72)} = 0.588$, $p = 0.672$, **Figure 2J**).

Overexpression of GDNF isoforms did not change the behavior of the animals in the cylinder test. The vertical activity of the animals remained on the same level 3 weeks after scAAV-administration compared to baseline measured before the viral vector delivery (**Figure 2L**). The use of the contralateral paw was at similar level in all treatment groups both before viral vectors were administered and 3 weeks later (**Figure 2M**). Furthermore, animals gained weight comparably by the 3 week time point (**Figure 2N**).

Overexpression of GDNF Isoforms Decreases the Density of TH-Immunoreactive Fibers in the Non-lesioned Striatum 7 Weeks After Viral Vector Delivery

The effect of GDNF isoform overexpression on striatal TH-immunoreactivity was assessed also 7 weeks after viral vector delivery (**Figures 3A–C**). At this time point, there was a significant decrease in the density of TH-immunoreactive fibers in the non-lesioned striata of both α-GDNF and β-GDNF-treated animals (GFP vs. α-GDNF $p = 0.0006$ and GFP vs. β-GDNF $p = 0.0026$). There was no statistically significant difference between the GDNF isoform groups.

Unlike in the shorter (3 week) overexpression study, no differences in the use of contralateral paw was observed 7 weeks after injections in the cylinder test (**Figure 3D**). The use of the contralateral paw was at a similar level in all treatment groups, before viral vectors were administered, 3 weeks as well as 7 weeks after AAV injections.

Interestingly, non-lesioned GDNF-treated animals gained less weight than non-lesioned GFP-treated animals (**Figure 3E**). Three weeks after viral vector administration GFP-treated animals had gained weight 17 ± 1%, α-GDNF 9 ± 1% and β-GDNF 10 ± 1% compared to their initial weight (GFP vs. α-GDNF $p < 0.001$, GFP vs. β-GDNF vs. $p = 0.001$). Four weeks later, 7 weeks after the viral vector delivery, GFP-treated animals had gained weight 28 ± 1%, α-GDNF 16 ± 1%, and β-GDNF 19 ± 2% of their initial weight (GFP vs. α-GDNF $p < 0.0001$, GFP vs. β-GDNF vs. $p = 0.0001$, and α-GDNF vs. β-GDNF $p = 0.073$, **Figure 3E**).

GDNF Splice Isoforms Protect TH-Immunoreactive Cells in SNpc With no Behavioral Correlates

The neuroprotective effects of GDNF splice isoforms were tested in the 6-OHDA partial lesion model. scAAV encoding either α-GDNF, β-GDNF, or GFP was administered into three sites in the striatum, and 3 weeks later 6 μg of 6-OHDA was evenly distributed (3 × 2 μg) to the same sites as the viral vector. The effects were evaluated with the d-amphetamine-induced rotation assay 5 and 7 weeks after scAAV-injection (2 and 4 weeks after lesioning, respectively), as well as with the drug-free cylinder test 3 and 7 weeks after scAAV-injection (before and 4 weeks after lesioning, respectively, **Figure 4A**). Amphetamine-induced rotational behavior was at similar level in all treatment groups on week five and on week seven (**Figure 4B**). Two-way ANOVA did not show significant effects in rotational behavior [treatment effect $F_{(2,92)} = 1.333$, $p = 0.269$; time effect $F_{(1,92)} = 1.270$, $p = 0.263$; treatment × time $F_{(2,92)} = 0.020$, $p = 0.980$].

In the pre-lesion cylinder test on week 3, α-GDNF-treated animals showed increased use of contralateral (left) paw (GFP vs α-GDNF $p = 0.005$, **Figure 4C**). 6-OHDA injection reduced the use of the contralateral paw in all groups to the same level [two-way ANOVA treatment effect $F_{(2,86)} = 3.215$, $p = 0.045$; 6-OHDA effect $F_{(1,86)} = 41.803$, $p < 0.0001$; treatment × 6-OHDA interaction $F_{(2,86)} = 0.545$, $p = 0.582$). Even though only α-GDNF showed an effect in the spontaneous use of paws, both isoforms increased the exploratory activity of the animals on week three, seen as an increase in the amount of rearings (GFP vs α-GDNF $p = 0.019$ and GFP vs. β-GDNF $p = 0.024$, **Figure 4D**). Four weeks after 6-OHDA administration the exploratory activity was reduced to the same level in all treatment groups [two-way ANOVA treatment effect $F_{(2,86)} = 3.406$, $p = 0.038$; 6-OHDA effect $F_{(1,86)} = 29.071$, $p < 0.0001$; treatment × 6-OHDA interaction $F_{(2,86)} = 1.130$, $p = 0.328$].

The density of TH-immunoreactive fibers in the striatum was at the same level in all treatment groups (**Figures 5A,C**)

4 weeks after 6-OHDA administration. The density of TH-immunoreactive fibers was similar over the whole striatum in all groups (**Figure 5B**). The density of DAT-immunoreactive fibers was increased in GDNF-treated groups, but the difference did not reach statistical significance (**Figures 5D–F**). When the striatal DAT-immunoreactivity was analyzed in more detail throughout the striatum, two-way ANOVA revealed a significant treatment effect [treatment effect $F_{(2,85)} = 4.388$, $p = 0.015$; site effect $F_{(2,85)} = 0.272$, $p = 0.762$; treatment × site interaction $F_{(4,85)} = 0.130$, $p = 0.971$; **Figure 5E**].

In the SNpc both GDNF isoforms protected and rescued TH-immunoreactive cells (GFP vs. α-GDNF $p = 0.001$ and GFP vs. β-GDNF $p < 0.001$, **Figures 5G,I**). The difference between GDNF-treated animals and GFP-treated animals was consistent in all three analyzed areas [two-way ANOVA treatment effect $F_{(2,124)} = 21.493$, $p < 0.001$; site effect $F_{(2,124)} = 0.388$, $p = 0.679$; treatment × site interaction $F_{(4,124)} = 0.352$, $p = 0.842$, **Figure 5H**].

Striatal Overexpression of GDNF Isoforms Induces Sprouting of TH- and DAT-Immunoreactive Fibers in Globus Pallidus

Administration of exogenous GDNF has been shown to induce sprouting around the nigrostriatal pathway (29–32). In non-lesioned animals, sprouting was not observed 3 weeks after viral vector injection (**Figure 6**). Instead, the sprouting of TH- and DAT-immunoreactive fibers in the globus pallidus (GP) was detected 7 weeks after virus injection, 4 weeks after 6-OHDA injection, in both α- and β-GDNF-treated groups (**Figure 6**). In contrast, 6-OHDA injection cleared the TH- and DAT-immunoreactivity completely from the GP of GFP-treated animals.

DISCUSSION

Until now, very little has been known about the biology of the shorter β-GDNF isoform and its functions in the adult mammalian brain. We compared the effects of full-length α-GDNF and the shorter β-GDNF splice isoforms in non-lesioned animals and in the partial 6-OHDA rat model of PD. Both GDNF splice isoforms were overexpressed with their native pre-pro-sequences (pre-α-pro-GDNF and pre-β-pro-GDNF) in striatum using scAAV1 vectors. We found that in the non-lesioned striatum, both isoforms increased the density of DAT-immunoreactive fibers and decreased the density of TH-immunoreactive fibers. In the neuroprotection assay, both α-GDNF and β-GDNF overexpression increased the number of TH-immunoreactive cells after 6-OHDA-induced degeneration.

GDNF is produced as a precursor protein, pre-pro-GDNF, and proteolytically cleaved to mature GDNF in endoplasmic reticulum and secretory vesicles (1, 17). Although the pro-region is not necessarily needed for secretion of GDNF, it has been suggested to have a role in the protein folding and secretion (18, 33). In addition, the full-length pro-region of α-GDNF contains an 11 amino acids long peptide, dopamine neuron stimulating peptide-11 or brain excitatory peptide (14, 34, 35), which has both neurotrophic and neuroprotective properties *in vitro* and *in vivo* (35). GDNF produced in mammalian cells has been shown to be more stable than GDNF produced in *E. coli* (18), possibly due to posttranslational modifications. These findings support the consideration of pro-GDNF for future gene and protein-based therapies using GDNF.

Administration of AAVs encoding the GDNF isoforms to the striatum is in accordance with the target derived hypothesis of neurotrophic factors. This paradigm is also warranted by reports that the receptors for GDNF signaling, GFRα1 and Ret are expressed in the midbrain dopamine neurons (36, 37). The exact mechanism of GDNF's neuroprotective effects remains unknown, but the striatal delivery of GDNF might affect the neuronal targets of the nigrostriatal pathway, inducing axonal sprouting and re-innervation (7, 30). This results in functional recovery, despite only partially protecting nigral TH-immunoreactive cell bodies. However, protection of nigral TH-immunoreactive cells without beneficial effect on behavior has been reported (38). On the other hand, nigral administration of GDNF prior to 6-OHDA provides almost complete protection of TH-immunoreactive cell bodies without functional recovery (7, 30). This lack of functional recovery might be due to the lack of sufficient axonal growth response and re-innervation of the lesioned striatum at the time of analysis (6, 7, 30, 31). Recent work demonstrates the importance of Ret in mediating neuroprotective and neurorestorative effects of GDNF (39). In addition, although endogenous GDNF is not required for survival midbrain dopamine neurons (40), increasing concentrations of endogenous GDNF at its native locus is neuroprotective (41).

In our experiments the striatal delivery of the GDNF gene before 6-OHDA administration neither isoform was able to attenuate the acute effects of striatal 6-OHDA but protected the nigral TH-immunoreactive cells partially from degeneration. The 6-OHDA lesion used in the experiment produced rather severe, 67% loss of TH-immunoreactive cells in the SNpc and 69% loss of TH-immunoreactive fibers in the striatum. The robust lesion might partly explain the lack of behavioral recovery, the level of GDNF overexpression wasn't sufficient to protect the nerve terminals from degeneration. In the rotation assay, β-GDNF treatment showed a tendency for initial protective effect 2 weeks after 6-OHDA lesion. Whether this mild, albeit not significant effect was due to 6-OHDA and/or amphetamine-induced secretion of β-GDNF, remains to be elucidated. However, the lack of functional effects might also be due to short follow-up period, 4 weeks after 6-OHDA injection, since Kirik and colleagues (7) have shown the behavioral effects to be detectable at earliest 7 weeks post-lesion in the cylinder and rotation assays.

Previous studies have shown that long-term overexpression of GDNF can cause changes in behavior and dopamine phenotype, and long-term high-expression of GDNF may not provide optimal neuroprotective effect (11, 42). In the pre-lesion cylinder test, α-GDNF-treated animals used their contralateral paw more compared to GFP- or β-GDNF-treated animals. Additionally, both GDNF splice isoforms increased the activity of the animals

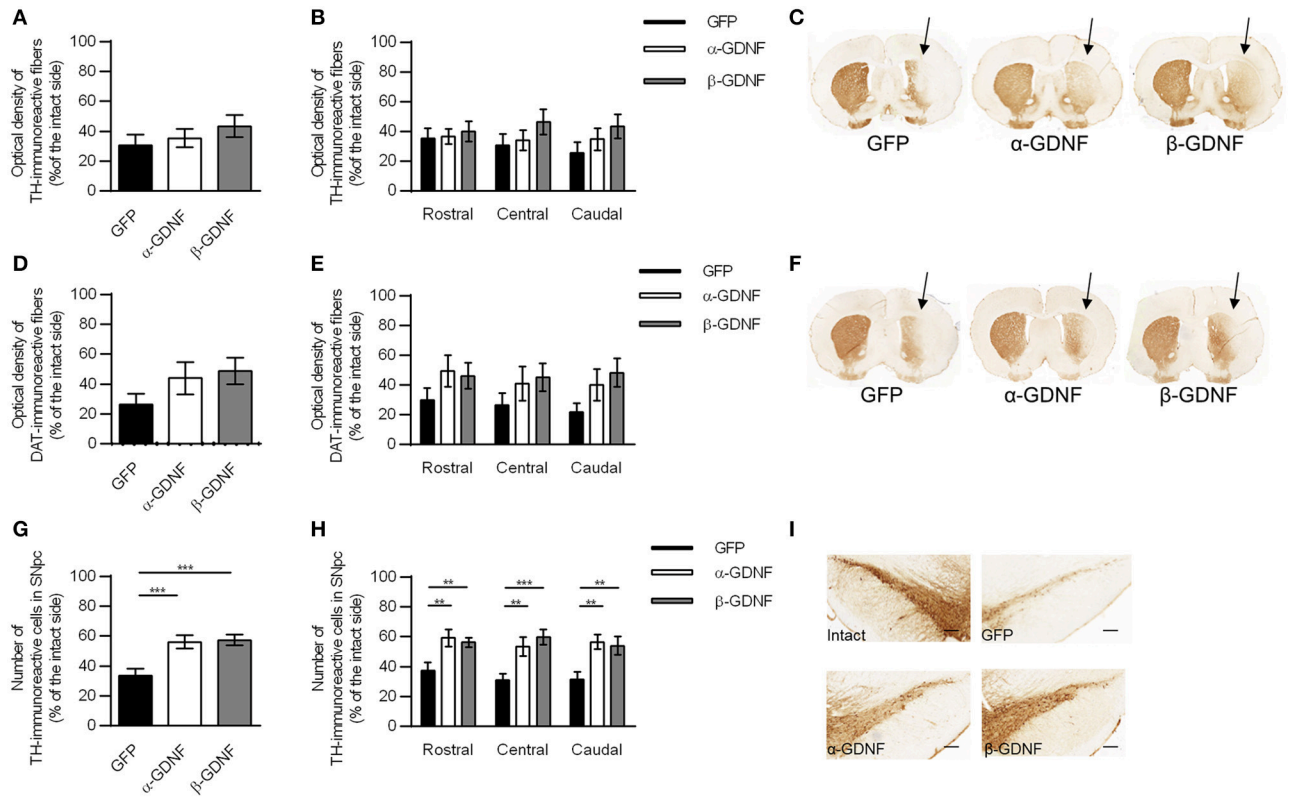


FIGURE 5 | Immunohistochemistry revealed both isoforms to protect TH-immunoreactive cells in the SNpc. **(A)** Optical density of striatal TH-immunoreactive fibers was decreased in all treatment groups 4 weeks after 6-OHDA administration ($n = 14–15$). **(B)** The density of TH-immunoreactive fibers was at the same level through the whole rostro-caudal axis of striatum ($n = 14–15$). **(C)** Representative images of striatal TH-immunoreactivity. **(D)** Optical density of striatal DAT-immunoreactive fibers [one-way ANOVA $F_{(2, 29)} = 1.815$, $p = 0.181$, $n = 10–11$]. **(E)** Optical density of DAT-immunoreactive fibers was increased in GDNF-treated animals throughout whole striatum [two-way ANOVA treatment effect $p = 0.015$, One-way ANOVA rostral $F_{(2, 28)} = 1.358$, $p = 0.274$; central: $F_{(2, 28)} = 1.045$, $p = 0.375$; caudal: $F_{(2, 29)} = 2.343$, $p = 0.114$, $n = 10–11$]. **(F)** Representative images of striatal DAT-immunoreactivity. **(G)** Both GDNF isoforms increased the number of TH-immunoreactive cells in the SNpc [one-way ANOVA $F_{(2, 42)} = 8.828$, $p < 0.001$, followed by Fisher's LSD analysis, $^{***}p < 0.001$, $n = 14–16$]. **(H)** The effect was consistent throughout the whole SNpc [One-way ANOVA rostral $F_{(2, 42)} = 6.004$, $p = 0.005$, Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p = 0.003$ and β-GDNF vs. GFP $p = 0.008$; central $F_{(2, 41)} = 8.784$, $p = 0.001$, Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p = 0.004$ and β-GDNF vs. GFP $p < 0.0001$; caudal $F_{(2, 41)} = 7.214$, $p = 0.002$ Fisher's LSD *post-hoc* analysis α-GDNF vs. GFP $p = 0.001$ and β-GDNF vs. GFP $p = 0.004$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 14–16$]. **(I)** Representative images of TH-immunoreactivity in the SN. Scale bar 200 μm. Data expressed as mean ± SEM.

in the pre-lesion cylinder test. This is in line with earlier studies, where GDNF increased the locomotor activity of the animals (7, 43, 44). Exogenous GDNF has been shown to initially increase TH expression (43) and activity (7, 45), as well as the level of dopamine (43, 44) and dopamine turnover (7, 43). On the other hand, long-lasting overexpression of GDNF has been documented to downregulate TH expression in both lesioned and non-lesioned rat striatum (32, 46–49). Our observations are in line with these previously published studies, as downregulation of striatal TH was observed after 7 weeks of overexpression, but not in earlier, 3 week time point. Time-dependent downregulation and associated decrease in enzymatic activity can be due to feedback regulation after long-term dopamine neuron activity (49–51). Interestingly, overexpression of both α- and β-GDNF increased the density of striatal DAT-immunoreactive fibers in non-lesioned striatum after 3 weeks. While the long-term effects of GDNF on striatal DAT expression are still unclear, there seems to be dose-dependence, where lower doses of GDNF do not affect DAT expression, but higher doses downregulate DAT expression (52). Moreover, GDNF has been suggested to regulate DAT activity by increasing dimerization and protein-protein interactions (41, 51, 53). Downregulation of TH might be a species-specific phenomenon, as it has not been detected in non-human primates treated with viral vectors encoding GDNF (10, 54–60). Instead, TH-immunoreactivity is increased in the putamen of naïve non-human primates after GDNF-treatment (54, 55, 58). Also, these changes on dopamine phenotypic markers can be one explanation why we did not observe robust neuroprotective effects on striatal fibers.

As reported here and previously by others (29–32, 61) striatal administration induces loss of GP-passing fibers and striatal administration of GDNF induces sprouting of dopaminergic fibers in rostral GP and entopeduncular nucleus. In the rostral GP TH-immunoreactive fibers can be roughly divided to two different categories, thick and thin fibers. The thick fibers are more likely to represent the dopaminergic projections from

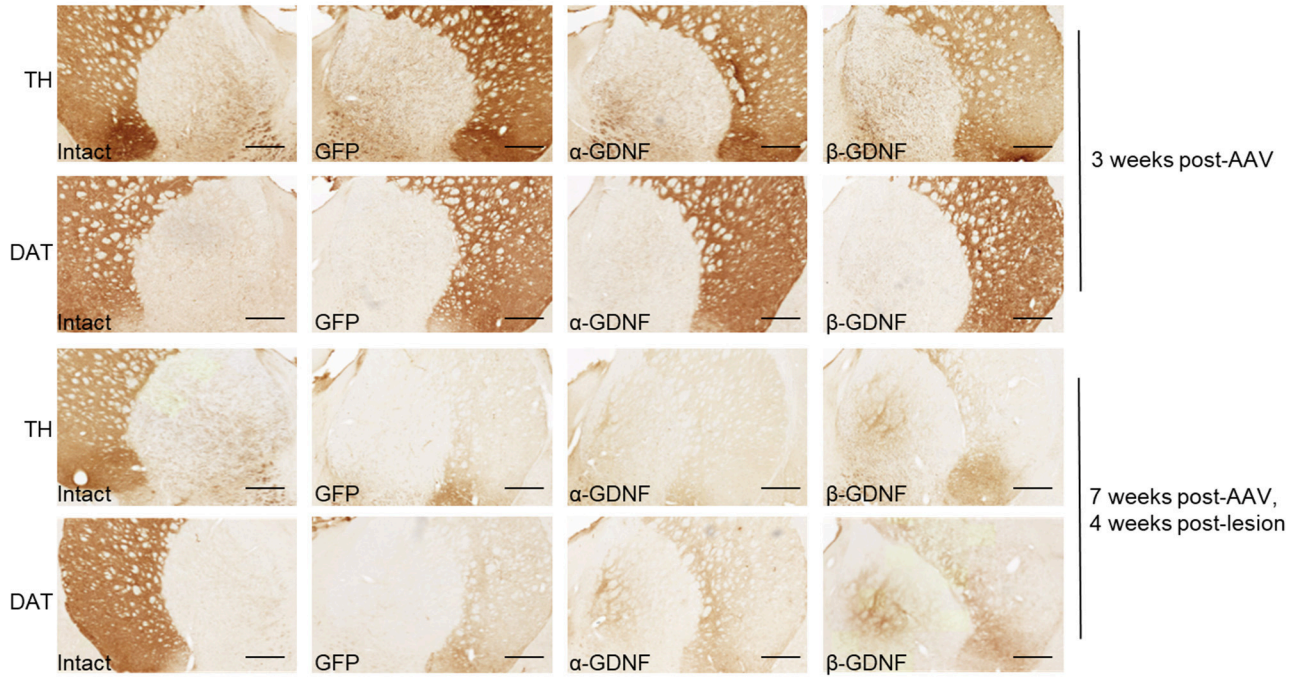


FIGURE 6 | Both GDNF isoforms induced sprouting of TH- and DAT-immunoreactive fibers in globus pallidus. Representative images from intact side, GFP, α-GDNF, and β-GDNF treated animals. Scale bar is 500 μm.

SNpc to striatum passing through GP and the thinner TH-immunoreactive fibers direct dopamine afferent projections to the GP (61, 62). Besides sprouting of TH-immunoreactive fibers, we also observed sprouting of DAT-immunoreactive fibers in GP, suggesting axonal sprouting toward the striatum. Whereas this sprouting is considered to be a more beneficial phenomenon, nigral administration of GDNF induces sprouting around SN and along the nigrostriatal tract, which can be detrimental to the animals and even mask the beneficial effects of GDNF (7, 31).

In addition to affecting the behavior and dopaminergic phenotype, GDNF overexpression has been reported to induce weight loss in rats (45, 63). Long-term overexpression of GDNF isoforms in non-lesioned striatum slowed down the weight gain of animals. Though in the initial 3 week treatment we did not observe differences in the weight gain, subsequent 7 week treatment experiment showed a significant reduction in the weight gain for GDNF treated group both at 3 and 7 weeks post-treatment. One possible explanation for this is the difference in the initial weight of the animals. The long-term overexpression experiment was started with animals with average weight 321 g, whereas the short-term experiment was started with animals weighing 281 g on average. The conclusion from this experiment is that there is no difference between the isoforms on the weight gain.

The amounts of GDNF protein used in the clinical trials have been suggested to be excessive (18). In our study, both isoforms were overexpressed in a level comparable to previously published *in vivo* studies using viral vectors (7, 32, 47). However, the level of α-GDNF protein was higher than the level of β-GDNF. A similar phenomenon was reported when the GDNF splice variants were overexpressed in the brain using DNA nanoparticles (64). Moreover, in human brain the expression level of α-GDNF mRNA is higher compared to β-GDNF mRNA (14).

Selection of the vector construct does not only affect the expression level of the transgene, but also the localization of the transgene expression. In contrast to differences in the intracellular localization of the isoforms *in vitro* (17), *in vivo* both isoforms seemed to co-localize with scgII-positive secretory vesicles but were also present in the scgII-negative areas. This discrepancy might be due to the used cytomegalovirus promoter in the vector construct. A more specific promoter should be chosen to mimic the endogenous expression and localization patterns. Furthermore, the titer should be optimized to target scgII-positive vesicles specifically and to avoid over-saturation of the vesicles. In addition, to mimic the expression pattern of endogenous GDNF in striatum, the expression should be targeted to parvalbumin-positive interneurons (65).

To summarize, we compared the effects of the major GDNF splice isoforms, α-GDNF and β-GDNF, in non-lesioned striatum and in a partial 6-OHDA lesion model of PD. Studies with β-GDNF are of interest, since many of the GDNF's aforementioned effects are suggested to be dose-dependent. The differentially regulated secretion yet similar neuroprotective effects of β-GDNF compared to α-GDNF make β-GDNF an interesting candidate for PD therapy. Further studies are first needed to establish optimal gene delivery and therapeutic efficacy of pre-pro-β-GDNF.

AUTHOR CONTRIBUTIONS

A-MP performed major components of the experiments, collected and analyzed the data and drafted the initial manuscript. IP conducted experiments, collected and analyzed data and edited the manuscript. AT, MK, MV, SB, and CR performed experiments and edited the manuscript. AD, BH, RT, LN, MS, and MA conceptualized the study and edited the manuscript. All authors have reviewed and approved the submitted version of the manuscript.

ACKNOWLEDGMENTS

Katrina Albert, Maria Haimi, Doug Howard, Antti Huovinen, Jaan Korpikoski, Aneta Petrušková, and Congjun Zheng, are acknowledged for technical assistance. This work was supported by The Finnish Parkinson Foundation, Finnish Cultural Foundation, and Instrumentarium Science Foundation; Academy of Finland grants #250275, #256398, #277910, and #281394; Biocentrum Helsinki; Sigrid Juselius Foundation; 3iRegeneration Tekes; and the Intramural Research Program at the National Institute on Drug Abuse, National Institutes of Health.

REFERENCES

1. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* (1993) 260:1130–2. doi: 10.1126/science.8493557
2. Bourque MJ, Trudeau LE. GDNF enhances the synaptic efficacy of dopaminergic neurons in culture. *Eur J Neurosci.* (2000) 12:3172–80. doi: 10.1046/j.1460-9568.2000.00219.x
3. D'anglemont De Tassigny X, Pascual A, Lopez-Barneo J. GDNF-based therapies, GDNF-producing interneurons, and trophic support of the dopaminergic nigrostriatal pathway. Implications for Parkinson's disease. *Front Neuroanat.* (2015) 9:10. doi: 10.3389/fnana.2015.00010
4. Kearns CM, Gash DM. GDNF protects nigral dopamine neurons against 6-hydroxydopamine *in vivo*. *Brain Res.* (1995) 672:104–11. doi: 10.1016/0006-8993(94)01366-P
5. Sauer H, Rosenblad C, Bjorklund A. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc Natl Acad Sci USA*. (1995) 92:8935–9. doi: 10.1073/pnas.92.19.8935
6. Winkler C, Sauer H, Lee CS, Bjorklund A. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. *J Neurosci.* (1996) 16:7206–15. doi: 10.1523/JNEUROSCI.16-22-07206.1996
7. Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J Neurosci.* (2000) 20:4686–700. doi: 10.1523/JNEUROSCI.20-12-04686.2000
8. Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, et al. Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons *in vivo*. *Neurosci Lett.* (1994) 182:107–11. doi: 10.1016/0304-3940(94)90218-6
9. Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* (1995) 373:335–9. doi: 10.1038/373335a0
10. Kells AP, Eberling J, Su X, Pivirotto P, Bringas J, Hadaczek P, et al. Regeneration of the MPTP-lesioned dopaminergic system after convection-enhanced delivery of AAV2-GDNF. *J Neurosci.* (2010) 30:9567–77. doi: 10.1523/JNEUROSCI.0942-10.2010
11. Tenenbaum L, Humbert-Claude M. Glial cell line-derived neurotrophic factor gene delivery in Parkinson's disease: a delicate balance between neuroprotection, trophic effects, and unwanted compensatory mechanisms. *Front Neuroanat.* (2017) 11:29. doi: 10.3389/fnana.2017.00029
12. Matsushita N, Fujita Y, Tanaka M, Nagatsu T, Kiuchi K. Cloning and structural organization of the gene encoding the mouse glial cell line-derived neurotrophic factor, GDNF. *Gene* (1997) 203:149–57. doi: 10.1016/S0378-1119(97)00506-4
13. Grimm L, Holinski-Feder E, Teodoridis J, Scheffer B, Schindelhauer D, Meitinger T, et al. Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3'-UTR and alternative splice products. *Hum Mol Genet.* (1998) 7:1873–86. doi: 10.1093/hmg/7.12.1873
14. Airavaara M, Pletnikova O, Doyle ME, Zhang YE, Troncoso JC, Liu QR. Identification of novel GDNF isoforms and cis-antisense GDNFOS gene and their regulation in human middle temporal gyrus of Alzheimer disease. *J Biol Chem.* (2011) 286:45093–102. doi: 10.1074/jbc.M111.310250
15. Suter-Crazzolara C, Unsicker K. GDNF is expressed in two forms in many tissues outside the CNS. *Neuroreport* (1994) 5:2486–8. doi: 10.1097/00001756-199412000-00020
16. Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, et al. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J Cell Biol.* (1995) 130:137–48. doi: 10.1083/jcb.130.1.137
17. Lonka-Nevalaita L, Lume M, Leppanen S, Jokitalo E, Peranen J, Saarma M. Characterization of the intracellular localization, processing, and secretion of two glial cell line-derived neurotrophic factor splice isoforms. *J Neurosci.* (2010) 30:11403–13. doi: 10.1523/JNEUROSCI.5888-09.2010
18. Piccinini E, Kalkkinen N, Saarma M, Runeberg-Roos P. Glial cell line-derived neurotrophic factor: characterization of mammalian posttranslational modifications. *Ann Med.* (2013) 45:66–73. doi: 10.3109/07853890.2012.663927
19. Butte MJ. Neurotrophic factor structures reveal clues to evolution, binding, specificity, and receptor activation. *Cell Mol Life Sci.* (2001) 58:1003–13. doi: 10.1007/PL00000915
20. Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, et al. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* (1996) 85:1113–24. doi: 10.1016/S0092-8674(00)81311-2
21. Paratcha G, Ledda F, Ibanez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* (2003) 113:867–79. doi: 10.1016/S0092-8674(03)00435-5
22. Bespalov MM, Sidorova YA, Tumova S, Ahonen-Bishopp A, Magalhaes AC, Kulesskiy E, et al. Heparan sulfate proteoglycan syndecan-3 is a novel receptor for GDNF, neurturin, and artemin. *J Cell Biol.* (2011) 192:153–69. doi: 10.1083/jcb.201009136
23. Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. *J Cell Sci.* (2003) 116:3855–62. doi: 10.1242/jcs.00786
24. Howard DB, Powers K, Wang Y, Harvey BK. Tropism and toxicity of adeno-associated viral vector serotypes 1:2, 5:6, 7:8, and 9 in rat neurons and glia *in vitro*. *Virology* (2008) 372:24–34. doi: 10.1016/j.virol.2007.10.007
25. Henderson MJ, Wires ES, Trychta KA, Richie CT, Harvey BK. SERCaMP: a carboxy-terminal protein modification that enables monitoring of ER calcium homeostasis. *Mol Biol Cell* (2014) 25:2828–39. doi: 10.1091/mbc.e14-06-1141
26. Penttinen AM, Suleymanova I, Albert K, Anttila J, Voutilainen MH, Airavaara M. Characterization of a new low-dose 6-hydroxydopamine model of Parkinson's disease in rat. *J Neurosci Res.* (2016) 94:318–28. doi: 10.1002/jnr.23708
27. Airavaara M, Chiocco MJ, Howard DB, Zuchowski KL, Peranen J, Liu C, et al. Widespread cortical expression of MANF by AAV serotype 7: localization and protection against ischemic brain

injury. *Exp Neurol.* (2010) 225:104–13. doi: 10.1016/j.expneurol.2010.05.020
28. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press (2005).
29. Rosenblad C, Kirik D, Devaux B, Moffat B, Phillips HS, Bjorklund A. Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *Eur J Neurosci.* (1999) 11:1554–66. doi: 10.1046/j.1460-9568.1999.00566.x
30. Kirik D, Rosenblad C, Bjorklund A. Preservation of a functional nigrostriatal dopamine pathway by GDNF in the intrastriatal 6-OHDA lesion model depends on the site of administration of the trophic factor. *Eur J Neurosci.* (2000) 12:3871–82. doi: 10.1046/j.1460-9568.2000.00274.x
31. Rosenblad C, Kirik D, Bjorklund A. Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. *Exp Neurol.* (2000) 161:503–16. doi: 10.1006/exnr.1999.7296
32. Georgievska B, Kirik D, Bjorklund A. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp Neurol.* (2002) 177:461–74. doi: 10.1006/exnr.2002.8006
33. Revishchin A, Moiseenko L, Kust N, Bazhenova N, Teslia P, Panteleev D, et al. Effects of striatal transplantation of cells transfected with GDNF gene without pre- and pro-regions in mouse model of Parkinson's disease. *BMC Neurosci.* (2016) 17:34. doi: 10.1186/s12868-016-0271-x
34. Immonen T, Alakuijala A, Hytonen M, Sainio K, Poteryaev D, Saarma M, et al. A proGDNF-related peptide BEP increases synaptic excitation in rat hippocampus. *Exp Neurol.* (2008) 210:793–6. doi: 10.1016/j.expneurol.2007.12.018
35. Bradley LH, Fuqua J, Richardson A, Turchan-Cholewo J, Ai Y, Kelps KA, et al. Dopamine neuron stimulating actions of a GDNF propeptide. *PLoS ONE* (2010) 5:e9752. doi: 10.1371/journal.pone.0009752
36. Treanor JJ, Goodman L, De Sauvage F, Stone DM, Poulsen KT, Beck CD, et al. Characterization of a multicomponent receptor for GDNF. *Nature* (1996) 382:80–3. doi: 10.1038/382080a0
37. Jain S, Golden JP, Wozniak D, Pehek E, Johnson EM Jr, Milbrandt J. RET is dispensable for maintenance of midbrain dopaminergic neurons in adult mice. *J Neurosci.* (2006) 26:11230–8. doi: 10.1523/JNEUROSCI.1876-06.2006
38. Georgievska B, Kirik D, Rosenblad C, Lundberg C, Bjorklund A. Neuroprotection in the rat Parkinson model by intrastriatal GDNF gene transfer using a lentiviral vector. *Neuroreport* (2002) 13:75–82. doi: 10.1097/00001756-200201210-00019
39. Drinkut A, Tillack K, Meka DP, Schulz JB, Kugler S, Kramer ER. Ret is essential to mediate GDNF's neuroprotective and neuroregenerative effect in a Parkinson disease mouse model. *Cell Death Dis.* (2016) 7:e2359. doi: 10.1038/cddis.2016.263
40. Kopra J, Vilenius C, Grealish S, Harma MA, Varendi K, Lindholm J, et al. GDNF is not required for catecholaminergic neuron survival *in vivo*. *Nat Neurosci.* (2015) 18:319–22. doi: 10.1038/nn.3941
41. Kumar A, Kopra J, Varendi K, Porokuokka LL, Panhelainen A, Kuure S, et al. GDNF overexpression from the native locus reveals its role in the nigrostriatal dopaminergic system function. *PLoS Genet.* (2015) 11:e1005710. doi: 10.1371/journal.pgen.1005710
42. Ibanez CF, Andressoo JO. Biology of GDNF and its receptors - relevance for disorders of the central nervous system. *Neurobiol Dis.* (2017) 97:80–9. doi: 10.1016/j.nbd.2016.01.021
43. Hudson J, Granholm AC, Gerhardt GA, Henry MA, Hoffman A, Biddle P, et al. Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits *in vivo*. *Brain Res Bull.* (1995) 36:425–32. doi: 10.1016/0361-9230(94)00224-O
44. Hebert MA, Gerhardt GA. Behavioral and neurochemical effects of intranigral administration of glial cell line-derived neurotrophic factor on aged Fischer 344 rats. *J Pharmacol Exp Ther.* (1997) 282:760–8.
45. Lapchak PA, Miller PJ, Jiao S. Glial cell line-derived neurotrophic factor induces the dopaminergic and cholinergic phenotype and increases locomotor activity in aged Fischer 344 rats. *Neuroscience* (1997) 77:745–52. doi: 10.1016/S0306-4522(96)00492-7
46. Rosenblad C, Georgievska B, Kirik D. Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system. *Eur J Neurosci.* (2003) 17:260–70. doi: 10.1046/j.1460-9568.2003.02456.x
47. Georgievska B, Jakobsson J, Persson E, Ericson C, Kirik D, Lundberg C. Regulated delivery of glial cell line-derived neurotrophic factor into rat striatum, using a tetracycline-dependent lentiviral vector. *Hum Gene Ther.* (2004) 15:934–44. doi: 10.1089/hum.2004.15.934
48. Georgievska B, Kirik D, Bjorklund A. Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. *J Neurosci.* (2004) 24:6437–45. doi: 10.1523/JNEUROSCI.1122-04.2004
49. Sajadi A, Bauer M, Thony B, Aebischer P. Long-term glial cell line-derived neurotrophic factor overexpression in the intact nigrostriatal system in rats leads to a decrease of dopamine and increase of tetrahydrobiopterin production. *J Neurochem.* (2005) 93:1482–6. doi: 10.1111/j.1471-4159.2005.03139.x
50. Iwata S, Nomoto M, Kaseda S, Tanoue S, Shimosaka M, Fukuda T. TH protein and mRNA in nigrostriatal dopaminergic neurons are down-regulated by continuous but not intermittent apomorphine. *Brain Res Mol Brain Res.* (2000) 82:133–6. doi: 10.1016/S0169-328X(00)00182-0
51. Barroso-Chinea P, Cruz-Muros I, Afonso-Oramas D, Castro-Hernandez J, Salas-Hernandez J, Chtarto A, et al. Long-term controlled GDNF over-expression reduces dopamine transporter activity without affecting tyrosine hydroxylase expression in the rat mesostriatal system. *Neurobiol Dis.* (2016) 88:44–54. doi: 10.1016/j.nbd.2016.01.002
52. Salvatore MF, Gerhardt GA, Dayton RD, Klein RL, Stanford JA. Bilateral effects of unilateral GDNF administration on dopamine- and GABA-regulating proteins in the rat nigrostriatal system. *Exp Neurol.* (2009) 219:197–207. doi: 10.1016/j.expneurol.2009.05.013
53. Kopra JJ, Panhelainen A, Af Bjerken S, Porokuokka LL, Varendi K, Olfat S, et al. Dampened amphetamine-stimulated behavior and altered dopamine transporter function in the absence of brain GDNF. *J Neurosci.* (2017) 37:1581–90. doi: 10.1523/JNEUROSCI.1673-16.2016
54. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* (2000) 290:767–73. doi: 10.1126/science.290.5492.767
55. Palfi S, Leventhal L, Chu Y, Ma SY, Emborg M, Bakay R, et al. Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration. *J Neurosci.* (2002) 22:4942–54. doi: 10.1523/JNEUROSCI.22-12-04942.2002
56. Eberling JL, Kells AP, Pivirotto P, Beyer J, Bringas J, Federoff HJ, et al. Functional effects of AAV2-GDNF on the dopaminergic nigrostriatal pathway in parkinsonian rhesus monkeys. *Hum Gene Ther.* (2009) 20:511–8. doi: 10.1089/hum.2008.201
57. Emborg ME, Moirano J, Raschke J, Bondarenko V, Zufferey R, Peng S, et al. Response of aged parkinsonian monkeys to *in vivo* gene transfer of GDNF. *Neurobiol Dis.* (2009) 36:303–11. doi: 10.1016/j.nbd.2009.07.022
58. Johnston LC, Eberling J, Pivirotto P, Hadaczek P, Federoff HJ, Forsayeth J, et al. Clinically relevant effects of convection-enhanced delivery of AAV2-GDNF on the dopaminergic nigrostriatal pathway in aged rhesus monkeys. *Hum Gene Ther.* (2009) 20:497–510. doi: 10.1089/hum.2008.137
59. Su X, Kells AP, Huang EJ, Lee HS, Hadaczek P, Beyer J, et al. Safety evaluation of AAV2-GDNF gene transfer into the dopaminergic nigrostriatal pathway in aged and parkinsonian rhesus monkeys. *Hum Gene Ther.* (2009) 20:1627–40. doi: 10.1089/hum.2009.103
60. Garea-Rodriguez E, Eesmaa A, Lindholm P, Schlumbohm C, Konig J, Meller B, et al. Comparative analysis of the effects of neurotrophic factors CDNF and GDNF in a nonhuman primate model of Parkinson's disease. *PLoS ONE* (2016) 11:e0149776. doi: 10.1371/journal.pone.0149776

61. Debeir T, Ginestet L, Francois C, Laurens S, Martel JC, Chopin P, et al. Effect of intrastriatal 6-OHDA lesion on dopaminergic innervation of the rat cortex and globus pallidus. *Exp Neurol.* (2005) 193:444–54. doi: 10.1016/j.expneurol.2005.01.007
62. Fuchs H, Hauber W. Dopaminergic innervation of the rat globus pallidus characterized by microdialysis and immunohistochemistry. *Exp Brain Res.* (2004) 154:66–75. doi: 10.1007/s00221-003-1638-7
63. Connor B, Kozlowski DA, Schallert T, Tillerson JL, Davidson BL, Bohn MC. Differential effects of glial cell line-derived neurotrophic factor (GDNF) in the striatum and substantia nigra of the aged Parkinsonian rat. *Gene Ther.* (1999) 6:1936–51. doi: 10.1038/sj.gt.3301033
64. Fletcher AM, Kowalczyk TH, Padegimas L, Cooper MJ, Yurek DM. Transgene expression in the striatum following intracerebral injections of DNA nanoparticles encoding for human glial cell line-derived neurotrophic factor. *Neuroscience* (2011) 194:220–6. doi: 10.1016/j.neuroscience.2011.07.072
65. Hidalgo-Figueroa M, Bonilla S, Gutierrez F, Pascual A, Lopez-Barneo J. GDNF is predominantly expressed in the PV+ neostriatal interneuronal ensemble in normal mouse and after injury of the nigrostriatal pathway. *J Neurosci.* (2012) 32:864–72. doi: 10.1523/JNEUROSCI.2693-11.2012

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.

frontiers in Neurology

REVIEW
published: 05 July 2018
doi: 10.3389/fneur.2018.00524

Opioidergic Modulation of Striatal Circuits, Implications in Parkinson's Disease and Levodopa Induced Dyskinesia

*Stefania Sgroi and Raffaella Tonini**

Neuromodulation of Cortical and Subcortical Circuits Laboratory, Neuroscience and Brain Technologies Department, Istituto Italiano di Tecnologia, Genoa, Italy

The functional organization of the dorsal striatum is complex, due to the diversity of neural inputs that converge in this structure and its subdivision into direct and indirect output pathways, striosomes and matrix compartments. Among the neurotransmitters that regulate the activity of striatal projection neurons (SPNs), opioid neuropeptides (enkephalin and dynorphin) play a neuromodulatory role in synaptic transmission and plasticity and affect striatal-based behaviors in both normal brain function and pathological states, including Parkinson's disease (PD). We review recent findings on the cell-type-specific effects of opioidergic neurotransmission in the dorsal striatum, focusing on the maladaptive synaptic neuroadaptations that occur in PD and levodopa-induced dyskinesia. Understanding the plethora of molecular and synaptic mechanisms underpinning the opioid-mediated modulation of striatal circuits is critical for the development of pharmacological treatments that can alleviate motor dysfunctions and hyperkinetic responses to dopaminergic stimulant drugs.

Keywords: opioids, dopamine, striatum, Parkinson's disease, signaling pathway, synaptic plasticity

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Gilberto Fisone, Karolinska Institutet (KI), Sweden
Giuseppina Martella, Università degli Studi di Roma Tor Vergata, Italy

***Correspondence:**
Raffaella Tonini
Raffaella.tonini@iit.it

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *30 March 2018*
Accepted: *13 June 2018*
Published: *05 July 2018*

Citation:
Sgroi S and Tonini R (2018) Opioidergic Modulation of Striatal Circuits, Implications in Parkinson's Disease and Levodopa Induced Dyskinesia. Front. Neurol. 9:524. doi: 10.3389/fneur.2018.00524

INTRODUCTION

Opioidergic signaling is involved in several functional aspects of the peripheral and central nervous system and due to the broad distribution of opioid receptors throughout the brain, its activation modulates different neural circuits. Opiate drugs are widely used as analgesic to induce antinociception and to treat pain disorders. However, edonic effects of opiates induce addictive behaviors that entail the involvement of opioidergic system in reward processes (1, 2). Opioid receptors and the endogenous opioid peptides Enkephalin (Enk) and Dynorphin (Dyn) are expressed at striatal circuits, where the opioid system modulates the activity of spiny projection neurons (SPNs) during movement control in both a healthy state and in motor disorders such as Parkinson's disease (PD). In PD, functional changes in striatal pathways are associated with a reorganization of molecular and synaptic mechanisms that counteract the loss of dopaminergic cells. However, aberrant neuroadaptations in the striatal circuit can be responsible for critical aspects of PD, as observed in levodopa-induced dyskinesia (LID). It is still unclear what role opioid transmission plays in striatal circuity and how this system affects neural reorganization, both in PD and in response to dopaminergic treatment. Here, we review recent findings on

the cell-type-specific effects of opioid transmission in the dorsal striatum, including the signaling pathways, synaptic and behavioral effects mediated by opioid ligands, as well as their interactions with dopaminergic transmission in both a PD state and in response to dopaminergic treatment with levodopa (L-DOPA).

ANATOMY AND PHYSIOLOGY OF THE BASAL GANGLIA

The basal ganglia (BG) comprise a distributed group of nuclei that include the striatum, which is composed by the caudate and putamen (CPu), the globus pallidus, with the pars externa (GPe) and interna (GPi), the subthalamic nucleus (STN); and the substantia nigra pars compacta (SNpc) and pars reticulata (SNpr). The Striatum and the STN represent the main input nuclei of the BG, while the GP and SNpr are the two output structures projecting to the thalamus and brainstem (3–7). The BG nuclei's connectivity to cortical regions provides a complex network of sensorimotor, limbic and associative information, conferring on the BG a pivotal role in the control of movement as well as in associative learning, emotion and reward-related behavior (8).

Nearly 95% of the striatum is composed of striatal projection neurons (SPNs), which are GABA (γ-aminobutyric acid)-ergic neurons that relay inhibitory efferent transmission and are rich in dopaminergic receptors (DR). These neurons are classified in two subtypes based on their projection targets, neuropeptides expression and DR subtypes (9). SPNs that express the neuropeptide Dyn and bear D1 excitatory receptors (D1Rs) (10) belong to what is termed the direct striatonigral pathway (dSPNs), projecting directly to the GPi/SNpr. On the other hand, SPNs expressing Enk and bearing D2 inhibitory receptors (D2Rs) project to the GPi/SNpr indirectly through the GPe, as part of the indirect striatopallidal pathway (iSPNs) (9, 11). In a healthy state (see **Figure 1A**), the activation of the direct pathway promotes movement execution by reducing the neural firing of the GPi/SNpr to the thalamus and boosting glutamatergic thalamocortical transmission. In parallel, activation of the indirect pathway reduces movement initiation, exciting GPi/SNpr transmission by inhibiting the GPe and activating the STN, ultimately leading to the inhibition of thalamocortical transmission (4, 12, 13). The concomitant activation of both striatofugal pathways maintains a balance between the direct and indirect pathways, activating specific and voluntary actions through the direct pathway and inhibiting involuntary movements through the indirect pathway (13, 14).

Excitatory corticostriatal transmission on SPNs is modulated by dopaminergic input from the SNpc through "diffusion-based volume transmission," where dopamine (DA) diffuses away from the synapse to reach extrasynaptic receptors and regulate excitability of SPNs (15). However, sparse release sites defined as active zone have been identified in the striatal DA axons to allow for a fast DA release and to generate a localized DA signal (16). Once released, DA exerts a dual effect on striatal neurons (17), exciting the direct pathway by binding to D1Rs and inhibiting the indirect pathway by binding to D2Rs. DA discharge from the dopaminergic neurons of the SNpc is crucial for the initiation and execution of motor sequences (14, 18).

THE OPIOIDERGIC SYSTEM: PEPTIDES AND RECEPTORS

Enk, Dyn and β-endorphin belong to family of endogenous peptides produced through the proteolytic cleavage of protein precursors such as preproenkephalin-A (PPENK), which forms six copies of methionine-Enk (Met-Enk) and one copy of leucine-Enk; preproenkephalin-B (also known as preprodynorphin), which produces Dyn and endorphin; and finally, proopiomelanocortin, which produces β-endorphin. The endogenous peptides have different degrees of selectivity for the opioid receptors; Enk binds δ opioid receptors (DORs) and μ opioid receptors (MORs), Dyn is selective for κ-opioid receptors (KORs), and β-endorphin binds MORs (1).

Opioid receptors (ORs) are seven-transmembrane receptors and belong to a superfamily of G protein-coupled receptors (GPCRs) with inhibitory activity ($G\alpha_{i/o}$) on cellular excitability and synaptic transmission (1). OR activity promotes the activation of G-protein-coupled inwardly rectifying K^+ channels, inhibits Ca^+ channels and adenylyl cyclase (AC), and reduces neurotransmitter release and neural activity (19, 20). ORs are broadly distributed in the brain, with some structures exhibiting higher expression of a specific type of receptor, while others have three overlapping receptors that can interact locally with one another in synergistic or antagonistic ways (21).

A broad distribution of MORs has been observed in the thalamus, amygdala and locus coeruleus (1), and in the thalamic afferents to the striatum (22). MOR expression has also been observed in CPu striosomal compartments that project to the SNpc (23) (**Figure 2**). Specifically, MORs are expressed in striosomes both on dSPNs enriched in Dyn precursor and on iSPNs (24), where they colocalize with D2Rs in dendrites (25). MORs are also expressed on striatal cholinergic interneurons (26, 27).

DORs are abundant in layers II, III, IV and V of the cerebral cortex and in the striosomes and matrix compartments of the CPu, with a higher dorsolateral distribution than ventromedial (28). On a cellular level, DORs are expressed within the nucleus and in the somatodendritic area on iSPNs, but not on dSPNs (24, 29).

KORs are predominantly found in the medial sector of the CPu and in the nucleus accumbens and showed a higher coexpression with D1Rs (24). They are also localized presynaptically in the nigrostriatal afferents of the SNpc.

OPIOID RECEPTOR SIGNALING

The activation of opioidergic GPCRs by endogenous opioid peptides or exogenous agonists leads to the dissociation of Gα/βγ subunits that stimulate various intracellular effectors. The inhibitory activity of opioid receptors includes several processes that are selectively initiated by the Gα and Gβγ protein

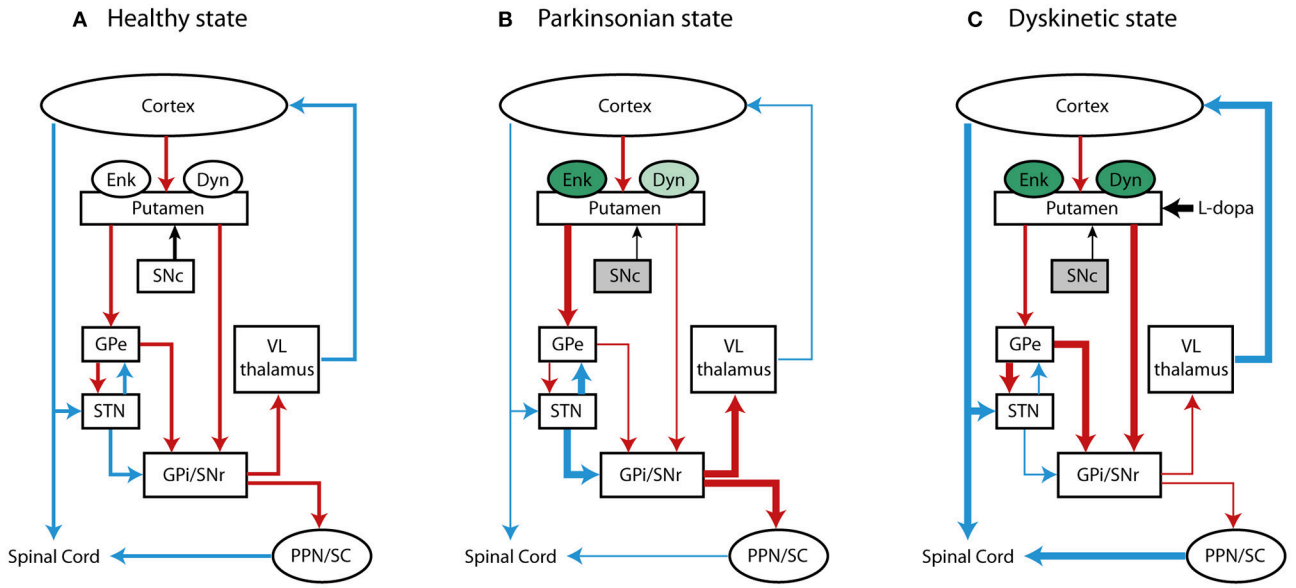


FIGURE 1 | Basal ganglia motor loop in **(A)** normal, **(B)** PD, and **(C)** LID condition. The schematic represents the direct, indirect and hyperdirect pathways projecting to the thalamus and spinal cord and the changes of expression of opiod peptides, Enk and Dyn. Red, blue and black lines indicate GABA-ergic, glutamatergic and dopaminergic projections, respectively. Changes in the rate of neural transmission are indicated with thick (increased activity) and thin (decreased activity) lines. Changes in the expression of Enk and Dyn are depicted in green (increased levels) and light green (decreased levels). The gray color of substantia nigra pars compact (SNc) is representative of PD state due to the loss of dopaminergic cells.

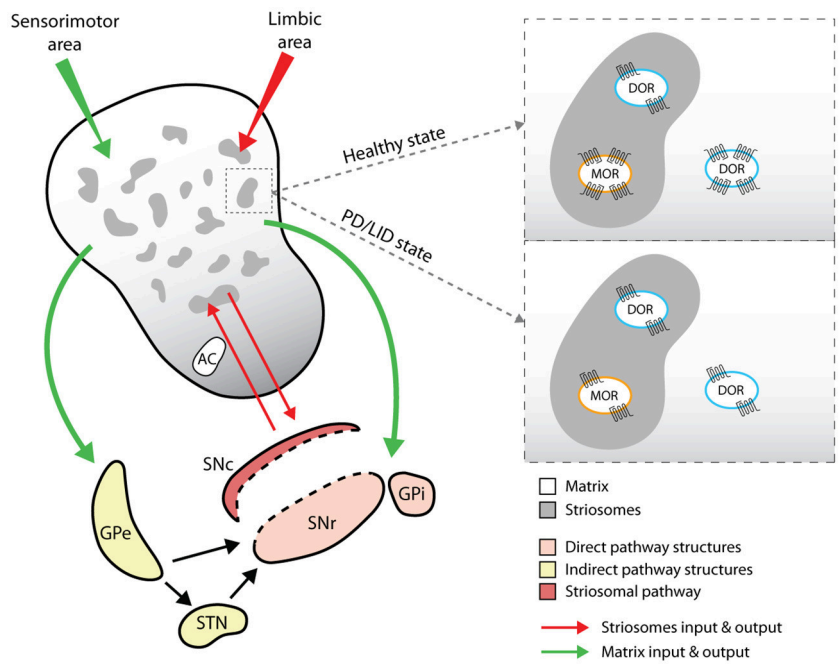


FIGURE 2 | Representative cartoon of opioid receptors distribution and expression in striosomes and matrix compartments of the dorsal striatum. The drawing shows the different input and output pathways related to the striosomes (red lines) and matrix compartments (green lines) and the changes in the expression levels of MOR and DOR. GPe (globus pallidus pars externa) and STN (subthalamic nucleus) indicate the indirect pathway targets; SNr (substantia nigra pars reticulata) and GPi (globus pallidus pars interna) indicate the direct pathway targets; SNc (substantia nigra pars compacta) is the target of the specific pathway arising from the striosomal compartments.

subunits. The **Gα** subunit inhibits AC by decreasing intracellular cyclic adenosine monophosphate (cAMP) levels and activates the inward-rectifier K^+ channel, leading to the hyperpolarization of the cellular membrane and the inhibition of neural activity (30). The inhibition of AC and cAMP by the Gα subunit can also lead to a reduction of Ca^{2+} conductance (31), although this reduction is predominately induced by the direct binding of **Gβγ** subunit to the channel, and the consequent decrease in neurotransmitter release. Indeed, activation of KORs on the nigrostriatal afferents of the SNpc reduces the release of DA and influences the kinetics of the DA transporter (32, 33). Intrastriatal injection of a MOR agonist alters extracellular DA levels in the shell and core of the nucleus accumbens and in the rostral and caudal subregions of the dorsal striatum, where the reduction is thought to be due to presynaptic activation of MORs on DA terminals (34, 35). Activation of MORs on striatal cholinergic interneurons reduces Ach release and decreases cholinergic interneurons excitability (26, 27).

Besides inhibiting the AC /cAMP, opioid receptors shape several other cellular responses. The interaction with different G proteins, β-arrestins or kinases, can promote the activation of different effectors or signaling pathways (36), or prompt the internalization and desensitization of receptor functional activity (19, 37), with significant changes in behavior (38). The direct activation of opioid receptors and the release of Gβγ subunits can promote the activation of mitogen-activated protein kinases (MAPKs) (19, 39). Notably, activation of MAPK can be also mediated by binding of DA to D1R. In the dorsal striatum of PD animal models, pulsatile replacement of DA, for example by L-DOPA treatment, leads to an overstimulation of the direct striatonigral pathway that promotes the activation of MAPK and its downstream effectors, such as extracellular signal-regulated kinases ERK1/2 or transcription factors (40–42). Increased levels of phosphorylated ERK (p-ERK) or immediate early genes are associated with aberrant cellular responses and dysfunctional behaviors in PD and LID state (43–45). Therefore, opioidergic and dopaminergic receptors could both activate postsynaptic signaling cascades that converge to ultimately promote an increase of proteins and transcriptional factors that affect striatal-based behaviors. However, it is still unclear whether alterations of the striatal motor function arise from a synergic activity of the dopaminergic and opioidergic system or if opioid transmission only modulates the molecular and synaptic mechanisms mediated by dopaminergic transmission.

COMPARTMENT-SPECIFICITY LOCALIZATION OF OPIOID RECEPTORS IN THE DORSAL STRIATUM

Beyond the classical division of the striatum into the direct and indirect pathways, this structure is also subdivided into striosomes (defined as striatal bodies) and matrix compartments (**Figure 2**), which are defined according to neurotransmitter and receptor segregation, afferent and efferent connections (46), signaling cascade activation (47) and neurophysiological features (48). Striosomes represent about 10–15% of the dorsal striatum and are mainly localized in the medial sector of the CPu (29, 49), where they are characterized by acetylcholinesterase (AchE)-poor zones and by immunoreactivity against Enk, substance P and GABA (50). The matrix compartment comprises 85% of the remaining striatum. It is rich in AchE, contains calcium-binding proteins such as parvoalbumin and calbindin, and is directly affiliated with the sensorimotor system (51). Both striosomes and matrix contain dSPNs and iSPNs, although dSPNs are more prevalent in the striosomal compartment and project predominantly to dopaminergic neurons in the SNpc (50, 52).

The matrix and striosomal compartments also receive inputs from different cortical areas; striosomes are related to the limbic area, whereas the matrix is associated with sensorimotor and associative areas (53) (see **Figure 2**). Overall, this complex striatal subdivision, together with a discrete distribution of neuromodulators between matrix and striosomes compartments, reflects that SPNs functional activity might differ in compartment-specific manner and affect different striatal-based behaviors (54).

While dSPNs and iSPNs are broadly distributed in both striosomes and matrix, opioid-mediated synaptic transmission seems to segregate (46), perhaps due to the different distributions of opioid receptors on dSPNs and iSPNs in these compartments. For example, application of MOR and DOR agonists reduces GABAergic synaptic responses in both dSPNs and iSPNs predominantly in the striosomal compartment, but not in the matrix (29, 48). Specifically, the binding of Enk to DORs located on iSPNs collaterals that synapse on dSPNs, suppresses the inhibition of dSPNs only in the striosomes, but not in matrix, leading to strengthened striosomal dSPNs responses to corticostriatal inputs (29). The behavioral implications of this connectivity might be relevant in PD, where changes in the levels of the endogenous opioid Enk might promote or reduce dSPNs response to cortical inputs, thereby affecting the release of DA through the striatonigral pathway (29).

OPIOID-MEDIATED NEUROTRANSMISSION AND SYNAPTIC PLASTICITY IN THE DORSAL STRIATUM

The first neurophysiological studies on the opioid-mediated neurostransmission at striatal circuits investigated the role of these neuropeptides in the modulation of glutamatergic inputs mainly arising from the cortex. These studies showed that MOR and DOR agonists inhibited glutamatergic inputs to the striatum (55) and more specifically, selective MOR agonists reduced the excitatory inputs at the corticostriatal level in both striosomes and matrix compartments (48, 56). In addition, the application of exogenous MOR and DOR agonists or the release of endogenous opioids induced long-term depression (LTD) on striatal SPNs in both the DLS and the dorsomedial striatum (DMS). Specifically, MOR activation inhibited thalamostriatal excitatory inputs, whereas the activation of DOR inhibited corticostriatal inputs; these results indicate the specificity of opioid-mediated synaptic plasticity in the dorsal striatum (22). Interestingly, applying an exogenous KOR agonist induced LTD more selectively in the

TABLE 1 | Summary of opioid-mediated neurotransmission and synaptic plasticity in the dorsal striatum.

Activation of opioid recepetors	Distribution	Signal
MOR	Thalamostriatal afferents	↓EPSCs (22)
	Striosomal dSPN and iSPN	↓IPSCs (29, 48)
	Cholinergic interneurons	↓ACh release (26, 27)
DOR	Nigrostriatal terminal	↓ DA release (34, 35)
	iSPN striosomal collaterals	↓ IPSCs (29)
	Corticostriatal afferents	↓ EPSCs (22)
KOR	Presynaptic nigrostriatal afferents	↓ DA release (33)
		LTP (57)
	Striatal SPNs in DLS	LTD (22)

According to the specific distribution of opioid receptors, changes in neurotransmitter release or synaptic plasticity are observed in response to exogenous and endogenous opioid agonists. ESPCs, excitatory postsynaptic currents; ISPCs, inhibitory postsynaptic currents; Ach, acetylcholine; DA, dopamine; LTP, long term potentiation; LTD, long term depression.

DLS than in the DMS, suggesting subregional specificity of KOR-mediated synaptic plasticity (22) (**Table 1**). This subregional difference between the DLS and DMS might be related to their distinct functional roles in motor control. Behavioral studies demonstrate that the DLS is more connected to the control of body movements rather than to more general control of locomotor activity (58, 59). In PD animals treated with L-DOPA, higher levels of Dyn precursor (PDYN) mRNA, selective for KOR binding, are expressed in the DLS than the DMS (60–62). Moreover, higher PDYN mRNA expression in the lateral striatal portion of the DA-denervated hemisphere correlates only with the severity of dyskinesia, instead of with locomotor variables that define animals' spontaneous motion (60, 62).

Opioids have been shown to regulate striatal LTD (22). In contrast, their effect on long-term potentiation (LTP) in the dorsal striatum remains unexplored. Most of the studies that have attempted to characterize the role of opioids in LTP have examined different functional areas, such as the ventral tegmental area (63), hippocampus or C-fiber of the spinal dorsal horn (64). A recent study investigated the effect of KOR activation on LTP in the corticostriatal pathway (57), and demonstrated that applying Dyn reduced the release of DA, as expected by binding to KOR on DA nigrostriatal terminals. Moreover, selective activation of the D1R-SPNs that promote the co-release of Dyn also led to impared corticostriatal LTP, likely due to the KOR-mediated reduction in DA release from the nigrostriatal pathway (57) (**Table 1**).

OPIOID NEURONTRANSMISSION IN PARKINSON'S DISEASE AND LEVODOPA-INDUCED DYSKINESIA

The broad distribution of opioid receptors in the striatum and their interplay with dopaminergic transmission point at critical role for opioidergic neuropeptides in modulating striatal activity and motor control, in particular, both in healthy and pathological states, such as in PD. This is a progressive neurodegenerative disorder characterized by the loss of dopaminergic cells in the SNpc, which results in motor deficits (i.e., bradykinesia, rest tremor, rigidity, and postural and gait impairment) (65, 66). PD patients develop these symptoms only after a significant depletion of striatal DA– by 60 to 80% (67) – likely because of compensatory DA production by surviving neurons or unknown compensatory mechanisms within or outside of the BG (68). Furthermore, the loss of dopaminergic neurons in the SNpc results in a functional imbalance in the two major output pathways of the striatum: hypoactivity in the direct circuit and hyperactivity in the indirect circuit. This imbalance leads to an overstimulation of the GPi/SNpr which decreases thalamic input to motor cortical areas, resulting in reduced movement and classical Parkinson's symptoms (**Figure 1B**).

Various animal models of PD are used to better understand the disease's pathophysiology, but none of them fully exhibit all PD symptoms, nor do they develop a neurodegenerative state similar to that in PD patients. For this reason, the most suitable animal model depends on the scientific question being investigated (69). Parkinsonian motor deficits due to DA depletion or DA neuronal death are usually recreated in animals through the injection of selective neurotoxins such as 6-hydroxydopamine (6-OHDA) in rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mouse or primate, while specific molecular events and protein aggregation are investigated using genetic models of PD-related mutations (70).

PD symptoms can be alleviated with dopaminergic treatments that aim to replace the DA deficiency in the nigrostriatal pathway. No curative treatments exist for PD patients, and currently available therapies are symptomatic. To date, L-DOPA remains the most effective drug for exogenous dopaminergic replacement and for counteracting PD symptoms. However, as the disease progresses and dosages of L-DOPA increase, many patients develop disabling complications, including severe fluctuations in motor function (on-off phenomena) and abnormal involuntary movements called L-DOPA-induced dyskinesia (LID) (71, 72). The pathophysiology of LID has been associated with aberrant activation of the direct striatal pathway and with increased levels of the endogenous opioid neuropeptides Enk and Dyn (**Figure 1C**). It is still unclear whether opioid transmission can affect the neural reorganization of striatal pathways, and if changes in opioid expression might have a compensatory or synergistic effect on striatal-based behaviors in PD and LID.

OPIOID PEPTIDE EXPRESSION IN PD AND LID

Several studies have been conducted in animal models to investigate changes in the expression of endogenous opioids in the dorsal striatum and their association with motor impairment and dyskinetic movements. Indeed, DA and its binding to D1Rs and D2Rs can modulate the striatal levels of mRNA expression of Dyn and Enk neuropeptide precursors (PDYN and PPENK). Changes in PDYN and PPENK mRNA levels have been observed in PD, where DA transmission is lost, and in LID, during the exogenous replacement of DA (**Figure 1**).

In the striatum of 6-OHDA and MPTP animal models (60, 62, 73, 74), as well as in PD patients (75), the levels of PPENK mRNA expression are increased, irrespective of L-DOPA treatment. The levels of PPENK mRNA remain highly expressed in PD animals also given chronic L-DOPA treatment (76) as well as in PD patients affected by dyskinesia (77), suggesting persistent adaptive changes in the Enk peptide (78).

In contrast, nigrostriatal DA denervation leads to a reduction in the levels of PDYN mRNA (60, 62, 79, 80) that increase under L-DOPA therapy compared to untreated or non-dyskinetic states, consistently across different study models (62, 76, 78, 81). These observations suggest that the expression of opioidergic neuropeptides involved in the modulation of BG output is strictly regulated by striatal DA levels, likely also through the activation of postsynaptic transcription factors that ultimately can promote the expression of multiple genes, including those for opioidergic peptides.

In addition, in dyskinetic PD rat model, high levels of both PPENK and PDYN are overall correlated with L-DOPA-induced locomotor alterations. While there is a more specific association between high levels of PDYN mRNA and dyskinetic movements (60), on the other hand, high expression of PPENK mRNA is also correlated with locomotor hyperactivity, beyond dyskinesia (62). These observations suggest that Enk and Dyn might play different roles in striatal-based behavioral effects and in locomotor alterations in response to dopaminergic treatment.

OPIOID RECEPTOR EXPRESSION IN PD AND LID

Along with different levels of opioidergic peptides expression, alterations in the levels of opioidergic receptor immunoreactivity have been observed in both PD patients and animal models. Piccini et al. (82) found reduced opioid receptor binding in the caudate of PD patients, and in the putamen and thalamus of dyskinetic PD patients compared to non-dyskinetic. Similar observations have been described in animal studies, although some differences were found across the various models.

Striatal levels of MOR binding and μ-immunoreactivity were reduced in PD rats (83) and in MPTP-lesioned macaques treated with L-DOPA (80), as well as in PD patients undergoing chronic L-DOPA therapy (84). Lower levels of DOR binding are expressed in the GP and striatal areas of 6-OHDA dyskinetic rats, while an increase of δ-immunoreactivity occurs in the motor and premotor cortex (83) (**Figure 2**). Consistent with these results, PD patients treated with L-DOPA have reduced levels of DOR binding compared with control patients (84). KOR binding levels are decreased in the striatal areas of dyskinetic PD rats and in the GP of PD rats with and without LID (83); low κ-immunoreactivity is observed only in the GP structure of MPTP-denervated macaques with and without dyskinesia (80).

Even though the exposure to L-DOPA treatment in PD animals and PD patients leads to a reduction in opioid receptor binding levels, Chen and colleagues (85) assessed G protein-coupled receptor signaling as a marker of MOR, DOR and KOR activity in MPTP-lesioned non-human primates. Interestingly, they found a hyperactive transduction signal mediated by all three opioid receptors in the striatum. This suggests that in the parkinsonian state under L-DOPA treatment, although the levels of receptor binding can be decreased, the response to activation of opioid receptors is in fact enhanced.

TABLE 2 | Summary of opioidergic drugs used as pharmacological intervention to counteract parkinsonian symptoms and dyskinetic movements in PD animal model.

Opioidergic drugs	Opioid receptor targets	Function	Effect
Cyprodine ADL5510	MOR	Antagonist	↓ LID (86, 87)
Naltrindole	DOR	Antagonist	↓ LID (86, 88) Akinesia (89)
SNC-80	DOR	Agonist	↑ Kinesia in PD state (90–92)
nor-BNI	KOR	Antagonist	No effect on LID (86)
U50,488	KOR	Agonist	↓ LID (93) ↑ Akinesia
Nalbuphine	KOR-MOR	Agonist-antagonist	↓ LID (94)
Naloxone	KOR-MOR-DOR	Antagonist	↓ LID (95, 96)

nor-BNI, nor-binaltorphimine; LID, levodopa-induced dyskinesia; PD, Parkinson disease.

PHARMACOLOGICAL IMPLICATIONS OF OPIOIDS IN MOTOR FUNCTION

Elucidating the role of opioidergic transmission in the molecular mechanisms that control motor function is complex, not only due to the striatum's neural heterogeneity, but also because of the broad distribution of opioid receptors throughout the brain. The activation of opioid-mediated postsynaptic signaling cascades likely depends on several factors, including opioid agonists and their response to ORs, the type of ORs activated, and whether receptor stimulation is acute or chronic. Systemic administration of opioidergic drugs might affect different neural circuits and modulate behavioral aspects beyond locomotor activity. Therefore, pharmacological approaches used to distinguish the neural pathways in the control and alteration of movement should be considered critically.

Considering the enhanced expression of endogenous opioid peptides in the striatum of PD animal models and in PD patients, selective agonists and antagonists to ORs have been used to counteract akinesia in PD and to reduce the development of dyskinesia in response to L-DOPA treatment (**Table 2**). MOR antagonists (cyprodine and ADL5510) alleviated LID in MPTP-lesioned non-human primates without interfering with the antiparkinsonian effects of L-DOPA (86, 87). A selective DOR antagonist (naltrindole) has a similar effect, reducing dyskinetic movements in MPTP-lesioned marmoset and 6-OHDA rats treated with L-DOPA (86, 88), although there is an akinetic effect on motor activity in a PD model without DA treatment (89). A selective DOR agonist (SNC-80) increased locomotor activity in naive and PD animals, but its potential therapeutic applications

are limited by its convulsive effects (90–92). In contrast, a κ-receptor antagonist (norBNI) did not induce any anti-dyskinetic effect in MPTP-lesioned macaques (86); yet a selective κ-receptor agonist (U50, 488) reduced LID in PD rats and monkeys, but impaired the anti-parkinsonian effects of L-DOPA treatment (93). In line with these effects, the synthetic opioid analgesic nalbuphine, acting as both a KOR agonist and a MOR antagonist, reduced LID in a non-human primate model of PD and decreased the levels of specific molecular markers associated with the development of dyskinesia (94). Also noteworthy is the effect of the non-selective antagonist naloxone, which reduced LID in 6-OHDA rats (95, 96), although results in MPTP-lesioned macaques and PD patients were inconclusive (97, 98).

The literature makes it clear that different pharmacological responses are expected across animal models and in human patients, likely due to the greater neural organization and connectivity in primates and humans. The lost of DA in PD and its exogenous replacement by L-DOPA lead to changes in the expression of opioid peptides and receptor immunoreactivity that reflect a strong interaction between dopaminergic and opioidergic systems in the BG motor circuit. However, it is still debated whether changes in the opioid transmission occur to compensate for DA denervation and L-DOPA treatment, or whether these changes interact with the molecular and synaptic mechanisms associated with altered neural responses in motor diseases.

CONCLUDING REMARKS

The recent advances in understanding the striatal functionality highlight the strong impact of opioidergic transmission to modulate synaptic plasticity and cellular responses of the SPNs. The studies here reviewed, demonstrate that opioid receptors have a regional (ventral vs. dorsal striatum), compartmental (striosomes vs. matrix) and cellular (dSPNs vs. iSPNs) specificity that affects the striatal activity in response to different inputs. Such specificity reflects the complexity of striatal organization and the efforts to find selective opioidergic treatments that can modulate specific neural pathways. Although the literature points out the inhibitory effect of opioid agonists on synaptic transmission and neurotransmitters release, it is still debated how opioid receptors interact with dopaminergic receptors and whether they share common mechanisms to activate postsynaptic signaling cascades and downstream effectors. The interaction between opioidergic and dopaminergic pathways becomes crucial in PD and LID where the high levels of endogenous opioids occurs in parallel with aberrant dopaminergic transmission, and are associated with altered striatal-based behaviors. Since the broad distribution of opioid receptors throughout the brain, pharmacological approaches should aim to selectively target defined receptor subtypes, in a cell-type- and input-specific manner. The use of chemogenetic or optogenetic approaches are therefore crucial to dissect opioidergic neurotransmission within the striatum and its interaction with dopaminergic system. This would be instrumental to develop specific pharmacological treatments able to restore maladaptive changes without interfering with other neuronal pathways.

AUTHOR CONTRIBUTIONS

SS wrote the manuscript. RT conceived the review contribution, supervised the writing and critically edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by Fondazione Istituto Italiano di Tecnologia. This review includes some sessions of PhD dissertation thesis - SS. (2015): Role of striatal opioidergic neuropeptides on locomotor hypersensitivity and dyskinesia in Parkinsonian rats (unpublished)-University of Bern and Laboratory for Biomedical Neurosciences (LBN), Switzerland.

REFERENCES

1. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev* (2009) 89:1379–412. doi: 10.1152/physrev.00005.2009
2. Lutz PE, Kieffer BL. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* (2013) 36:195–206. doi: 10.1016/j.tins.2012.11.002
3. Crossman AR. Neural mechanisms in disorders of movement. *Comp Biochem Physiol Comp Physiol.* (1989) 93:141–9.
4. DeLong MR. Primate models of movement disorders of basal ganglia origin. *Trends Neurosci* (1990) 13:281–5.
5. Redgrave P, Rodriguez M, Smith Y, Rodriguez-Oroz MC, Lehericy S, Bergman H, et al. Goal-directed and habitual control in the basal ganglia: implications for Parkinson's disease. *Nat Rev Neurosci.* (2010) 11:760–72. doi: 10.1038/nrn2915
6. Lei W, Jiao Y, Del Mar N, Reiner A. Evidence for differential cortical input to direct pathway versus indirect pathway striatal projection neurons in rats. *J Neurosci.* (2004) 24:8289–99. doi: 10.1523/JNEUROSCI.1990-04.2004
7. Barroso-Chinea P, Bezard E. Basal Ganglia circuits underlying the pathophysiology of levodopa-induced dyskinesia. *Front Neuroanat.* (2010) 4:131. doi: 10.3389/fnana.2010.00131
8. Obeso JA, Rodriguez-Oroz MC, Benitez-Temino B, Blesa FJ, Guridi J, Marin C, et al. Functional organization of the basal ganglia: therapeutic implications for Parkinson's disease. *Mov Disord.* (2008) 23 Suppl. 3:S548–59. doi: 10.1002/mds.22062
9. Gerfen CR, Young WS, III. Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study. *Brain Res* (1988) 460:161–7.
10. Fallon JH, Leslie FM, Cone RI. Dynorphin-containing pathways in the substantia nigra and ventral tegmentum: a double labeling study using combined immunofluorescence and retrograde tracing. *Neuropeptides* (1985) 5:457–60.
11. Cuello AC, Paxinos G. Evidence for a long Leu-enkephalin striopallidal pathway in rat brain. *Nature* (1978) 271:178–80.
12. Albin RL, Young AB, Penney JB. The functional anatomy of basal ganglia disorders. *Trends Neurosci.* (1989) 12:366–75.

13. Obeso JA, Marin C, Rodriguez-Oroz C, Blesa J, Benitez-Temino B, Mena-Segovia J, et al. The basal ganglia in Parkinson's disease: current concepts and unexplained observations. *Ann Neurol.* (2008) 64 Suppl. 2:S30–46. doi: 10.1002/ana.21481
14. Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM, et al. Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature* (2013) 494:238–42. doi: 10.1038/nature11846
15. Rice ME, Cragg SJ. Dopamine spillover after quantal release: rethinking dopamine transmission in the nigrostriatal pathway. *Brain Res Rev.* (2008) 58:303–13. doi: 10.1016/j.brainresrev.2008.02.004
16. Liu C, Kershberg L, Wang J, Schneeberger S, Kaeser PS. Dopamine secretion is mediated by sparse active zone-like release sites. *Cell* (2018) 172:706–18 e15. doi: 10.1016/j.cell.2018.01.008
17. West AR, Grace AA. Opposite influences of endogenous dopamine D1 and D2 receptor activation on activity states and electrophysiological properties of striatal neurons: studies combining *in vivo* intracellular recordings and reverse microdialysis. *J. Neurosci.* (2002) 22:294–304. doi: 10.1523/JNEUROSCI.22-01-00294.2002
18. da Silva JA, Tecuapetla F, Paixao V, Costa RM. Dopamine neuron activity before action initiation gates and invigorates future movements. *Nature* (2018) 554:244–8. doi: 10.1038/nature25457
19. Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev.* (2001) 81:299–343. doi: 10.1152/physrev.2001.81.1.299
20. Dacher M, Nugent FS. Opiates and plasticity. *Neuropharmacology* (2011) 61:1088–96. doi: 10.1016/j.neuropharm.2011.01.028
21. Smith AP, Lee NM. Opioid receptor interactions: local and nonlocal, symmetric and asymmetric, physical and functional. *Life Sci.* (2003) 73:1873–93. doi: 10.1016/S0024-3205(03)00549-6
22. Atwood BK, Kupferschmidt DA, Lovinger DM. Opioids induce dissociable forms of long-term depression of excitatory inputs to the dorsal striatum. *Nat Neurosci.* (2014) 17:540–8. doi: 10.1038/nn.3652
23. Tajima K, Fukuda T. Region-specific diversity of striosomes in the mouse striatum revealed by the differential immunoreactivities for mu-opioid receptor, substance P, and enkephalin. *Neuroscience* (2013) 241:215–28. doi: 10.1016/j.neuroscience.2013.03.012
24. Oude Ophuis RJ, Boender AJ, van Rozen AJ, Adan RA. Cannabinoid, melanocortin and opioid receptor expression on DRD1 and DRD2 subpopulations in rat striatum. *Front Neuroanat* (2014) 8:14. doi: 10.3389/fnana.2014.00014
25. Ambrose LM, Unterwald EM, Van Bockstaele EJ. Ultrastructural evidence for co-localization of dopamine D2 and micro-opioid receptors in the rat dorsolateral striatum. *Anat Record Part A Discov Mol Cell Evol Biol* (2004) 279:583–91. doi: 10.1002/ar.a.20054
26. Ponterio G, Tassone A, Sciamanna G, Riahi E, Vanni V, Bonsi P, et al. Powerful inhibitory action of mu opioid receptors (MOR) on cholinergic interneuron excitability in the dorsal striatum. *Neuropharmacology* (2013) 75:78–85. doi: 10.1016/j.neuropharm.2013.07.006
27. Jabourian M, Venance L, Bourgoin S, Ozon S, Perez S, Godeheu G, et al. Functional mu opioid receptors are expressed in cholinergic interneurons of the rat dorsal striatum: territorial specificity and diurnal variation. *Eur J Neurosci.* (2005) 21:3301–9. doi: 10.1111/j.1460-9568.2005.04154.x
28. Cahill CM, McClellan KA, Morinville A, Hoffert C, Hubatsch D, O'Donnell D, et al. Immunohistochemical distribution of delta opioid receptors in the rat central nervous system: evidence for somatodendritic labeling and antigen-specific cellular compartmentalization. *J Comp Neurol* (2001) 440:65–84. doi: 10.1002/cne.1370
29. Banghart MR, Neufeld SQ, Wong NC, Sabatini BL. Enkephalin disinhibits mu opioid receptor-rich striatal patches via delta opioid receptors. *Neuron* (2015) 88:1227–39. doi: 10.1016/j.neuron.2015.11.010
30. North RA, Williams JT, Surprenant A, Christie MJ. Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci USA.* (1987) 84:5487–91.
31. Al-Hasani R, Bruchas MR. Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* (2011) 115:1363–81. doi: 10.1097/ALN.0b013e318238bba6
32. Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* (1995) 18:22–9.
33. Kivell B, Uzelac Z, Sundaramurthy S, Rajamanickam J, Ewald A, Chefer V, et al. Salvinorin A regulates dopamine transporter function via a kappa opioid receptor and ERK1/2-dependent mechanism. *Neuropharmacology* (2014) 86:228–40. doi: 10.1016/j.neuropharm.2014.07.016
34. Hipolito L, Sanchez-Catalan MJ, Zanolini I, Polache A, Granero L. Shell/core differences in mu- and delta-opioid receptor modulation of dopamine efflux in nucleus accumbens. *Neuropharmacology* (2008) 55:183–9. doi: 10.1016/j.neuropharm.2008.05.012
35. Campos-Jurado Y, Marti-Prats L, Zornoza T, Polache A, Granero L, Cano-Cebrian MJ. Regional differences in mu-opioid receptor-dependent modulation of basal dopamine transmission in rat striatum. *Neurosci Lett.* (2017) 638:102–8. doi: 10.1016/j.neulet.2016.12.024
36. Costa-Neto CM, Parreiras ESLT, Bouvier M. A Pluridimensional View of Biased Agonism. *Mol Pharmacol.* (2016) 90:587–95. doi: 10.1124/mol.116.105940
37. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol.* (2012) 52:179–97. doi: 10.1146/annurev.pharmtox.010909.105800
38. Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, et al. *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE* (2009) 4:e5425. doi: 10.1371/journal.pone.0005425
39. Kramer HK, Onoprishvili I, Andria ML, Hanna K, Sheinkman K, Haddad LB, et al. Delta opioid activation of the mitogen-activated protein kinase cascade does not require transphosphorylation of receptor tyrosine kinases. *BMC Pharmacol.* (2002) 2:5. doi: 10.1186/1471-2210-2-5
40. Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, et al. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci USA.* (2005) 102:491–6. doi: 10.1073/pnas.0408305102
41. Belcheva MM, Clark AL, Haas PD, Serna JS, Hahn JW, Kiss A, et al. Mu and kappa opioid receptors activate ERK/MAPK via different protein kinase C isoforms and secondary messengers in astrocytes. *J Biol Chem.* (2005) 280:27662–9. doi: 10.1074/jbc.M502593200
42. Cenci MA, Lundblad M. Post- versus presynaptic plasticity in L-DOPA-induced dyskinesia. *J Neurochem.* (2006) 99:381–92. doi: 10.1111/j.1471-4159.2006.04124.x
43. Westin JE, Vercammen L, Strome EM, Konradi C, Cenci MA. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. *Biol Psychiatry* (2007) 62:800–10. doi: 10.1016/j.biopsych.2006.11.032
44. Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, et al. Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. *J Neurosci.* (2007) 27:6995–7005. doi: 10.1523/JNEUROSCI.0852-07.2007
45. Fasano S, Bezard E, D'Antoni A, Francardo V, Indrigo M, Qin L, et al. Inhibition of Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) signaling in the striatum reverts motor symptoms associated with L-dopa-induced dyskinesia. *Proc Natl Acad Sci USA.* (2010) 107:21824–9. doi: 10.1073/pnas.1012071107
46. Lopez-Huerta VG, Nakano Y, Bausenwein J, Jaidar O, Lazarus M, Cherassse Y, et al. Erratum to: The neostriatum: two entities, one structure? *Brain Struct Funct.* (2016) 221:1789. doi: 10.1007/s00429-015-1017-8
47. Graybiel AM. Neurotransmitters and neuromodulators in the basal ganglia. *Trends Neurosci.* (1990) 13:244–54.
48. Miura M, Saino-Saito S, Masuda M, Kobayashi K, Aosaki T. Compartment-specific modulation of GABAergic synaptic transmission by mu-opioid receptor in the mouse striatum with green fluorescent protein-expressing dopamine islands. *J Neurosci.* (2007) 27:9721–8. doi: 10.1523/JNEUROSCI.2993-07.2007
49. Graybiel AM, Ragsdale CW, Jr. Histochemically distinct compartments in the striatum of human, monkeys, and cat demonstrated by acetylthiocholinesterase staining. *Proc Natl Acad Sci USA.* (1978) 75:5723–6.
50. Gerfen CR. The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. *Nature* (1984) 311:461–4.

51. Gerfen CR, Baimbridge KG, Miller JJ. The neostriatal mosaic: compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. *Proc Natl Acad Sci USA.* (1985) 82:8780–4.
52. Fujiyama F, Sohn J, Nakano T, Furuta T, Nakamura KC, Matsuda W, et al. Exclusive and common targets of neostriatofugal projections of rat striosome neurons: a single neuron-tracing study using a viral vector. *Eur J Neurosci.* (2011) 33:668–77. doi: 10.1111/j.1460-9568.2010.07564.x
53. Crittenden JR, Graybiel AM. Basal Ganglia disorders associated with imbalances in the striatal striosome and matrix compartments. *Front Neuroanat.* (2011) 5:59. doi: 10.3389/fnana.2011.00059
54. Brimblecombe KR, Cragg SJ. The striosome and matrix compartments of the striatum: a path through the labyrinth from neurochemistry toward function. *ACS Chem Neurosci.* (2017) 8:235–42. doi: 10.1021/acschemneuro.6b00333
55. Jiang ZG, North RA. Pre- and postsynaptic inhibition by opioids in rat striatum. *J Neurosci.* (1992) 12:356–61.
56. Blomeley CP, Bracci E. Opioidergic interactions between striatal projection neurons. *J Neurosci.* (2011) 31:13346–56. doi: 10.1523/JNEUROSCI.1775-11.2011
57. Hawes SL, Salinas AG, Lovinger DM, Blackwell KT. Long-term plasticity of corticostriatal synapses is modulated by pathway-specific co-release of opioids through kappa-opioid receptors. *J Physiol.* (2017) 595:5637–52. doi: 10.1113/JP274190
58. Pisa M. Motor functions of the striatum in the rat: critical role of the lateral region in tongue and forelimb reaching. *Neuroscience* (1988) 24:453–63.
59. Andersson M, Hilbertson A, Cenci MA. Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol Dis.* (1999) 6:461–74. doi: 10.1006/nbdi.1999.0259
60. Cenci MA, Lee CS, Bjorklund A. L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. *Eur J Neurosci.* (1998) 10:2694–706.
61. Capper-Loup C, Frey CM, Rebell D, Kaelin-Lang A. Adaptive gene expression changes on the healthy side of parkinsonian rats. *Neuroscience* (2013) 233:157–65. doi: 10.1016/j.neuroscience.2012.12.027
62. Sgroi S, Capper-Loup C, Paganetti P, Kaelin-Lang A. Enkephalin and dynorphin neuropeptides are differently correlated with locomotor hypersensitivity and levodopa-induced dyskinesia in parkinsonian rats. *Exp Neurol.* (2016) 280:80–8. doi: 10.1016/j.expneurol.2016.03.024
63. Nugent FS, Penick EC, Kauer JA. Opioids block long-term potentiation of inhibitory synapses. *Nature* (2007) 446:1086–90. doi: 10.1038/nature05726
64. Drdla R, Gassner M, Gingl E, Sandkuhler J. Induction of synaptic long-term potentiation after opioid withdrawal. *Science* (2009) 325:207–10. doi: 10.1126/science.1171759
65. Massano J, Bhatia KP. Clinical approach to Parkinson's disease: features, diagnosis, and principles of management. *Cold Spring Harbor Perspect Med* (2012) 2:a008870. doi: 10.1101/cshperspect.a008870
66. Sgroi S, Kaelin-Lang A, Capper-Loup C. Spontaneous locomotor activity and L-DOPA-induced dyskinesia are not linked in 6-OHDA parkinsonian rats. *Front Behav Neurosci.* (2014) 8:331. doi: 10.3389/fnbeh.2014.00331
67. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci.* (1973) 20:415–55.
68. Bezard E, Gross CE, Brotchie JM. Presymptomatic compensation in Parkinson's disease is not dopamine-mediated. *Trends Neurosci* (2003) 26:215–21. doi: 10.1016/S0166-2236(03)00038-9
69. Cenci MA, Whishaw IQ, Schallert T. Animal models of neurological deficits: how relevant is the rat? *Nat Rev Neurosci.* (2002) 3:574–9. doi: 10.1038/nrn877
70. Blesa J, Phani S, Jackson-Lewis V, Przedborski S. Classic and new animal models of Parkinson's disease. *J Biomed Biotechnol.* (2012) 2012:845618. doi: 10.1155/2012/845618
71. Granerus AK. Factors influencing the occurrence of "on-off" symptoms during long-term treatment with L-dopa. *Acta Med Scand.* (1978) 203:75–85.
72. Fabbrini G, Brotchie JM, Grandas F, Nomoto M, Goetz CG. Levodopa-induced dyskinesias. *Mov Disord.* (2007) 22:1379–89. doi: 10.1002/mds.21475
73. Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ, Jr., et al. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* (1990) 250:1429–32.
74. Herrero MT, Augood SJ, Hirsch EC, Javoy-Agid F, Luquin MR, Agid Y, et al. Effects of L-DOPA on preproenkephalin and preprotachykinin gene expression in the MPTP-treated monkey striatum. *Neuroscience* (1995) 68:1189–98.
75. Nisbet AP, Foster OJ, Kingsbury A, Eve DJ, Daniel SE, Marsden CD, et al. Preproenkephalin and preprotachykinin messenger RNA expression in normal human basal ganglia and in Parkinson's disease. *Neuroscience* (1995) 66:361–76.
76. Westin JE, Andersson M, Lundblad M, Cenci MA. Persistent changes in striatal gene expression induced by long-term L-DOPA treatment in a rat model of Parkinson's disease. *Eur J Neurosci* (2001) 14:1171–6. doi: 10.1046/j.0953-816x.2001.01743.x
77. Calon F, Birdi S, Rajput AH, Hornykiewicz O, Bedard PJ, Di Paolo T. Increase of preproenkephalin mRNA levels in the putamen of Parkinson disease patients with levodopa-induced dyskinesias. *J Neuropathol Exp Neurol.* (2002) 61:186–96. doi: 10.1093/jnen/61.2.186
78. Tamim MK, Samadi P, Morissette M, Gregoire L, Ouattara B, Levesque D, et al. Effect of non-dopaminergic drug treatment on Levodopa induced dyskinesias in MPTP monkeys: common implication of striatal neuropeptides. *Neuropharmacology* (2010) 58:286–96. doi: 10.1016/j.neuropharm.2009.06.030
79. Tel BC, Zeng BY, Cannizzaro C, Pearce RK, Rose S, Jenner P. Alterations in striatal neuropeptide mRNA produced by repeated administration of L-DOPA, ropinirole or bromocriptine correlate with dyskinesia induction in MPTP-treated common marmosets. *Neuroscience* (2002) 115:1047–58. doi: 10.1016/S0306-4522(02)00535-3
80. Aubert I, Guigoni C, Li Q, Dovero S, Bioulac BH, Gross CE, et al. Enhanced preproenkephalin-B-derived opioid transmission in striatum and subthalamic nucleus converges upon globus pallidus internalis in L-3,4-dihydroxyphenylalanine-induced dyskinesia. *Biol Psychiatry* (2007) 61:836–44. doi: 10.1016/j.biopsych.2006.06.038
81. Bishop C, Krolewski DM, Eskow KL, Barnum CJ, Dupre KB, Deak T, et al. Contribution of the striatum to the effects of 5-HT1A receptor stimulation in L-DOPA-treated hemiparkinsonian rats. *J Neurosci Res.* (2009) 87:1645–58. doi: 10.1002/jnr.21978
82. Piccini P, Weeks RA, Brooks DJ. Alterations in opioid receptor binding in Parkinson's disease patients with levodopa-induced dyskinesias. *Ann Neurol.* (1997) 42:720–6. doi: 10.1002/ana.410420508
83. Johansson PA, Andersson M, Andersson KE, Cenci MA. Alterations in cortical and basal ganglia levels of opioid receptor binding in a rat model of l-DOPA-induced dyskinesia. *Neurobiol Dis.* (2001) 8:220–39. doi: 10.1006/nbdi.2000.0372
84. Fernandez A, de Ceballos ML, Jenner P, Marsden CD. Neurotensin, substance P, delta and mu opioid receptors are decreased in basal ganglia of Parkinson's disease patients. *Neuroscience* (1994) 61:73–9.
85. Chen L, Togasaki DM, Langston JW, Di Monte DA, Quik M. Enhanced striatal opioid receptor-mediated G-protein activation in L-DOPA-treated dyskinetic monkeys. *Neuroscience* (2005) 132:409–20. doi: 10.1016/j.neuroscience.2004.10.026
86. Henry B, Fox SH, Crossman AR, Brotchie JM. Mu- and delta-opioid receptor antagonists reduce levodopa-induced dyskinesia in the MPTP-lesioned primate model of Parkinson's disease. *Exp Neurol.* (2001) 171:139–46. doi: 10.1006/exnr.2001.7727
87. Koprich JB, Fox SH, Johnston TH, Goodman A, Le Bourdonnec B, Dolle RE, et al. The selective mu-opioid receptor antagonist ADL5510 reduces levodopa-induced dyskinesia without affecting antiparkinsonian action in MPTP-lesioned macaque model of Parkinson's disease. *Mov Disord.* (2011) 26:1225–33. doi: 10.1002/mds.23631
88. Billet F, Costentin J, Dourmap N. Influence of corticostriatal delta-opioid receptors on abnormal involuntary movements induced by L-DOPA in hemiparkinsonian rats. *Exp Neurol.* (2012) 236:339–50. doi: 10.1016/j.expneurol.2012.04.017
89. Mabrouk OS, Volta M, Marti M, Morari M. Stimulation of delta opioid receptors located in substantia nigra reticulata but not globus pallidus or striatum restores motor activity in 6-hydroxydopamine lesioned rats: new

insights into the role of delta receptors in parkinsonism. *J Neurochem.* (2008) 107:1647–59. doi: 10.1111/j.1471-4159.2008.05727.x
90. Spina L, Longoni R, Mulas A, Chang KJ, Di Chiara G. Dopamine-dependent behavioural stimulation by non-peptide delta opioids BW373U86 and SNC 80: 1. Locomotion, rearing and stereotypies in intact rats. *Behav Pharmacol.* (1998) 9:1–8.
91. Hudzik TJ, Howell A, Payza K, Cross AJ. Antiparkinson potential of delta-opioid receptor agonists. *Eur J Pharmacol.* (2000) 396:101–7. doi: 10.1016/S0014-2999(00)00209-0
92. Nozaki C, Le Bourdonnec B, Reiss D, Windh RT, Little PJ, Dolle RE, et al. delta-Opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion, and receptor internalization. *J Pharmacol Exp Ther.* (2012) 342:799–807. doi: 10.1124/jpet.111.188987
93. Cox H, Togasaki DM, Chen L, Langston JW, Di Monte DA, Quik M. The selective kappa-opioid receptor agonist U50,488 reduces L-dopa-induced dyskinesias but worsens parkinsonism in MPTP-treated primates. *Exp Neurol.* (2007) 205:101–7. doi: 10.1016/j.expneurol.2007.01.024
94. Potts LF, Park ES, Woo JM, Dyavar Shetty BL, Singh A, Braithwaite SP, et al. Dual kappa-agonist/mu-antagonist opioid receptor modulation reduces levodopa-induced dyskinesia and corrects dysregulated striatal changes in the nonhuman primate model of Parkinson disease. *Ann Neurol.* (2015) 77:930–41. doi: 10.1002/ana.24375
95. Lundblad M, Andersson M, Winkler C, Kirik D, Wierup N, Cenci MA. Pharmacological validation of behavioural measures of akinesia and dyskinesia in a rat model of Parkinson's disease. *Eur J Neurosci.* (2002) 15:120–32. doi: 10.1046/j.0953-816x.2001.01843.x
96. Klintenberg R, Svenningsson P, Gunne L, Andren PE. Naloxone reduces levodopa-induced dyskinesias and apomorphine-induced rotations in primate models of parkinsonism. *J Neural Trans.* (2002) 109:1295–307. doi: 10.1007/s00702-002-0715-6
97. Gomez-Mancilla B, Bedard PJ. Effect of nondopaminergic drugs on L-dopa-induced dyskinesias in MPTP-treated monkeys. *Clin Neuropharmacol.* (1993) 16:418–27.
98. Fox S, Silverdale M, Kellett M, Davies R, Steiger M, Fletcher N, et al. Non-subtype-selective opioid receptor antagonism in treatment of levodopa-induced motor complications in Parkinson's disease. *Mov Disord.* (2004) 19:554–60. doi: 10.1002/mds.10693

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.

The reviewer GF declared a past collaboration with one of the authors RT to the handling Editor.

frontiers in Neurology

ORIGINAL RESEARCH
published: 28 August 2018
doi: 10.3389/fneur.2018.00663

Oscillatory Activity in the Cortex, Motor Thalamus and Nucleus Reticularis Thalami in Acute TTX and Chronic 6-OHDA Dopamine-Depleted Animals

*Laura C. Grandi[1], Alain Kaelin-Lang[1,2,3], Gergely Orban[1], Wei Song[1], Agnese Salvadè[1], Alessandro Stefani[4], Giuseppe Di Giovanni[5,6] and Salvatore Galati[1]**

[1] Laboratory for Biomedical Neurosciences, Neurocenter of Southern Switzerland, Taverne, Switzerland, [2] Department of Neurology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland, [3] Faculty of Biomedical Sciences, Università della Svizzera Italiana, Lugano, Switzerland, [4] Department System Medicine, UOSD Parkinson, University of Rome Tor Vergata, Rome, Italy, [5] Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta, [6] Neuroscience Division, School of Biosciences, Cardiff University, Cardiff, United Kingdom

OPEN ACCESS

Edited by:
Graziella Madeo,
National Institutes of Health (NIH),
United States

Reviewed by:
Cinzia Costa,
University of Perugia, Italy
Davide Ferrazzoli,
Ospedale Moriggia Pelascini, Italy

***Correspondence:**
Salvatore Galati
salvatore.galati@eoc.ch

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: 30 March 2018
Accepted: 24 July 2018
Published: 28 August 2018

Citation:
Grandi LC, Kaelin-Lang A, Orban G, Song W, Salvadè A, Stefani A, Di Giovanni G and Galati S (2018) Oscillatory Activity in the Cortex, Motor Thalamus and Nucleus Reticularis Thalami in Acute TTX and Chronic 6-OHDA Dopamine-Depleted Animals. Front. Neurol. 9:663. doi: 10.3389/fneur.2018.00663

The motor thalamus (MTh) and the nucleus reticularis thalami (NRT) have been largely neglected in Parkinson's disease (PD) research, despite their key role as interface between basal ganglia (BG) and cortex (Cx). In the present study, we investigated the oscillatory activity within the Cx, MTh, and NRT, in normal and different dopamine (DA)-deficient states. We performed our experiments in both acute and chronic DA-denervated rats by injecting into the medial forebrain bundle (MFB) tetrodotoxin (TTX) or 6-hydroxydopamine (6-OHDA), respectively. Interestingly, almost all the electroencephalogram (EEG) frequency bands changed in acute and/or chronic DA depletion, suggesting alteration of all oscillatory activities and not of a specific band. Overall, δ (2–4 Hz) and θ (4–8 Hz) band decreased in NRT and Cx in acute and chronic state, whilst, α (8–13 Hz) band decreased in acute and chronic states in the MTh and NRT but not in the Cx. The β (13–40 Hz) and γ (60–90 Hz) bands were enhanced in the Cx. In the NRT the β bands decreased, except for high-β (Hβ, 25–30 Hz) that increased in acute state. In the MTh, Lβ and Hβ decreased in acute DA depletion state and γ decreased in both TTX and 6-OHDA-treated animals. These results confirm that abnormal cortical β band are present in the established DA deficiency and it might be considered a hallmark of PD. The abnormal oscillatory activity in frequency interval of other bands, in particular the dampening of low frequencies in thalamic stations, in both states of DA depletion might also underlie PD motor and non-motor symptoms. Our data highlighted the effects of acute depletion of DA and the strict interplay in the oscillatory activity between the MTh and NRT in both acute and chronic stage of DA depletion. Moreover, our findings emphasize early alterations in the NRT, a crucial station for thalamic information processing.

Keywords: motor thalamus, nucleus reticularis thalami, 6-hydroxydopamine, tetrodotoxin, Parkinson's disease, neuronal oscillations

INTRODUCTION

Recent evidence has suggested that abnormal oscillatory activity at specific frequencies within basal ganglia (BG) and cortex (Cx) represents a hallmark of Parkinson's disease (PD) (1–10). This abnormal oscillatory activity may reflect dysfunctions of cortico-BG-thalamo-cortical loop linked to Parkinsonian symptoms in both PD patients and/or animal models of this disease (8–11) and its recognition could provide possible biomarkers for the disease state.

Brain oscillatory activities are classically segmented into different frequency band intervals, i.e., 2–4 Hz (delta, δ), 4–8 Hz (theta, θ), 8–13 Hz (alpha, α), 13–40 Hz (beta, β), and 60–90 Hz (gamma, γ). Each band is associated with one or more specific physiological behavior and differently contribute to information processing (12). β oscillations are involved in motor control and are greatly enhanced at different sites within the BG circuit in both PD patients and animal models of PD (9, 11, 13, 14). In addition, elevated β band synchronization could be considered as an expression of bradykinesia (13, 15). As proof of its strong association with motor signs in PD, β activity is reduced by dopaminergic therapies (15, 16). Recent evidence (17) supports the idea of functional subdivision of this band in low-β (Lβ, 15–20 Hz) and high-β (Hβ, 25–30 Hz). Lβ in the subthalamic nucleus (STN) is tightly associated with Parkinsonian symptoms in patients that do not receive medications, whereas Hβ reflects the degree of coupling between cortical and STN activity (18, 19). Nevertheless, the exact role of Lβ and Hβ band in PD remains an unsolved question.

Opposite to β, γ band is supposed to be associated to dyskinesia (20) and more generally to modulation of movements (20). In particular, γ band is involved in voluntary movements (21, 22), but also in motor imagery (23), as well as in planning of movements (24).

In addition, γ band has also been related to sensory and cognitive processing (25), attention, long-term memory and language tasks (26, 27). In PD patients, a γ decrease has been shown during anti-Parkinsonian therapies (15, 28). In line, deep brain stimulation (DBS) of STN at γ frequencies facilitates movements (29) and it is powerfully expressed in both Cx and globus pallidus (GP) in levodopa-induced dyskinesia (LID) in 6-hydroxydopamine (6-OHDA)-lesioned rats (20, 30). Concerning STN DBS, a correlation between frequency of stimulation and improvement of symptoms has been recently shown (31). For example, during 5 Hz DBS, a worsening of bradykinesia has been shown, while both bradykinesia and tremor showed no improvement at frequencies below 50 Hz (32).

The θ band has been described in frontal and central cortical regions (33), as implicated in several functions different from the control of voluntary movements, such as sensory processing and memory in healthy people (34). The θ band increased in PD patients, selectively during a motor task (35), as well as in PD patients experiencing freezing of gait (36).

The δ band is instead associated with sleep functions (37) as well as with cognitive processes (38).

It has been shown the association between δ band disruption with PD (39–41). For instance, the administration of the Delta Sleep-inducing peptide into the SNc induces Parkinsonian syndrome in rat (42).

Moreover, Parker and colleagues (43) showed that δ expression on medial frontal cortex (MFC) is associated with cognitive dysfunctions in both PD patients and animal models and DA depletion in the MFC. In addition, sleep disturbances are common symptoms in PD (44) and often they arise before the onset of motor symptoms (45). Although, the DAergic treatment seems have no effect of sleep functions (41, 46), some might have positive effects on sleep quality (47).

Finally, α frequency, according to the inhibition-timing hypothesis (48), is negatively correlated with cortical excitability and its enhancement prevents task-irrelevant interference (49). The thalamic- and cortical-generated α activity has a role in attentive tasks in physiological conditions (50–52) and it is modulated by visual task performance in occipital lobe (53). In addition, α oscillation is modulated by visual stimuli (54), even if they are sub- and supraliminal stimuli (55). In line, correlation between the phase of α oscillatory activity and the saccadic reaction time in cognitive task responses has been reported (56). It has been hypothesized that changes in α band expression might underlie some cognitive and attentive difficulties observed in PD patients (57). Within the BG circuit, the sensory-motor thalamus (MTh) has critical role in motor information processing (58), but contrasting data exist concerning its neuronal activity in PD (59). According to the *searchlight hypothesis*, the nucleus reticularis thalami (NRT) has a fundamental role as the guardian of the thalamus, contributing to the encoding of thalamic information (60–68). In particular, the sensorimotor MTh is modulated by the NRT motor sector.

In spite of its importance, the oscillatory activity across multiple frequency bands within the MTh and the NRT is a neglected area in PD studies. Therefore, we monitored the electrocorticogram (ECoG) and the local field potentials (LFPs) of the MTh and the NRT in two dopamine (DA) depletion states in a PD animal model. We first performed our recordings in the standardized Parkinsonian animal model obtained with the injection of 6-OHDA, capable of causing a chronic DA depletion.

Additionally, since it has been shown that some PD symptoms, such as bradykinesia, are already associated with acute DA depletion state induced by tetrodotoxin (TTX), we performed a similar study in animal with acute DA depletion induced by TTX (69–74).

We hypothesized that the oscillatory activity within MTh-NRT might be different in acute DA depletion state from that recorded in chronic 6-OHDA-lesioned rats, due to the presence of adaptive mechanisms.

METHODS

Ethical Approval

All experimental electrophysiological and histological procedures were carried out in compliance with Switzerland laws on animal experimentation and approved by the Animal Research Committee and the Veterinary Office of the Canton

of Ticino, Switzerland (TI-08-2015). We analyzed 42 adult male Sprague Dawley rats weighing ∼300 g.

Pre-recording Surgery

Rats were anesthetized with urethane (1.4 g/kg, i.p.) (Sigma Chemical Co., St Louis, MO, USA) and mounted on a stereotaxic instrument (Stoelting Co., Wheat Lane, Wood Dale, IL, USA), maintaining the body temperature at 37–38°C with a heating pad (Stoelting Co., Wheat Lane, Wood Dale, IL, USA) placed beneath the animal. A midline scalp incision was made, the skull was drilled on the left side and the dura was then spread out to expose the cortical surface. All wound margins were infiltrated with a local anesthetic (lidocaine 0.5%). All electrophysiological recordings were performed in three categories of animals: in normal rats, in 6-OHDA-lesioned rats and in acutely DA-depleted animals (see **Table 1**).

Unilateral 6-OHDA Lesioning

Chronic DA depletion was induced by performing a unilateral 6-OHDA denervation in the left hemisphere with standard technique (75, 76). The animals were anesthetized with 1.5–2.5% isoflurane in oxygen, mounted on a stereotaxic instrument (Stoelting Co., Wheat Lane, Wood Dale, IL, USA) for the injection of the neurotoxin (8 μg/4 μl of saline solution containing 0.1% of ascorbic acid) in the medial forebrain bundle (MFB; stereotaxic coordinates: 2.56 mm posterior to the bregma, 2 mm lateral to the midline, and 8.6 mm below the cortical surface). The electrophysiological recordings were performed 21–29 days after the surgery.

Pharmacological Blockade of the Medial Forebrain Bundle

The pharmacological blockade of the MFB was performed according to previous publications (55–57). TTX was injected via inverse microdialysis by using a probe with 1 mm dialytic membrane (CMA/11 microdialysis probe, CMA Microdialysis, Stockholm, Sweden). TTX was perfused by using a syringe pump (CMA/400, CMA Microdialysis, Stockholm, Sweden) with a rate flow of 1 μl/min, for 10 min.

Electrophysiological Recordings

The ECoG was recorded through a screw electrode (Dentorama, Stockholm, Sweden, 8 mm of total length, 3 mm tip lenght) placed on the cortical surface above the right frontal Cx (3.0 mm anterior of bregma and 2.0 mm lateral to the midline) and referenced against an indifferent screw electrode placed above cerebellum. Raw ECoG was band-pass filtered (0.1–300 Hz) and amplified (×2000; Neurolog). The ECoG was on-line digitalized with a sample rate of 600 Hz through an analogical/digital interface (Micro1401 mk II, Cambridge Electronic Design, Cambridge, UK) and stored on a computer for the subsequent inspection. During cortical recordings, we collected LFPs from the left MTh or the NRT (from 1.2 mm to 1.8 posterior of bregma and from 2 to 2.6 mm lateral to the midline). The recordings were performed using tungsten electrodes (Word Precision Instrument, USA, TM33B01). At the end of the recordings, the animals were sacrificed. The recordings were carried out 21–29 days after the administration of 6-OHDA, while in TTX-treated animals, after TTX infusion.

TABLE 1 | Animals utilized in the study.

Animal groups for electrophysiology	Sacrificed	Analyzed
CTL rats	10	6
TTX-treated rats	34	27
6-OHDA-lesioned rats	15	9
Total	59	42

CTL, control; TTX, tetrodotoxin; 6-OHDA, 6-hydroxydopamine.

LFP and ECoG Analysis

The local field potentials were analyzed by Spike2 script (SUDSA22) to calculate the total power of δ band (δ, 2–4 Hz), θ band (θ, 4–8 Hz), α band (α, 8–13 Hz), low-β band (Lβ, 13–25 Hz), high-β band (Hβ, 25–40 Hz), and γ band (γ, 60–90 Hz) in the Cx, MTh, and NRT of control (CTL), acute and chronic DA-depleted rats. The analysis was performed with raw data in the first 6 min of recording using the fast Fourier transform (FFT) analysis (4096 points). **Figure 1** represents an example of recording, with smoothing signal.

Statistical Analysis

For the comparison of total power of analyzed bands, among CTL vs. TTX and 6-OHDA we performed the non-parametric Kruskal Wallis test followed by Mann Whitney U test for the comparisons CTL vs. TTX, CTL vs. 6-OHDA, and TTX vs. 6-OHDA. For each statistical analysis a value of $p < 0.05$, corrected per number of comparisons ($n = 3$), therefore $p = 0.016$, was considered statistically significant. For each condition, we calculated the mean of each of the 6 min and then compared the 6 min among conditions in the MTh and the NRT. The cortical bands were calculated as ECoG recorded during MTh and during NRT neurons. Therefore, the comparisons were made on the mean of 6 min ($n = 6$) for each structures. The results are expressed as mean ± SEM. For exact p value, please refer to Results section.

The ECoG and the LFP from the MTh and NRT was divided into the six different frequency bands. The total power of each band was calculated and compared among CTL, acute, and chronic DA depletion states (**Supplementary Tables 1–3**). Then the percentage of change in comparison to CTL was calculated for each band in the Cx, the MTh and the NRT (**Supplementary Tables 4A–C**).

We recorded from a total of 59 rats (CTL $n = 10$, TTX-treated $n = 34$, and 6-OHDA-denervated rats $n = 15$) and analyzed from a total of 42 animals (CTL $n = 6$; TTX-treated $n = 27$ and 6-OHDA-denervated rats $n = 9$). In detail, in CTL rats we analyzed a total of 7 LFP recordings from the NRT and 15 LFP recordings from the MTh, recorded parallel to EEG (total of $n = 22$); in TTX-treated rats we analyzed 11 LFP recordings from the NRT and 16 LFP recordings from the MTh, recorded parallel to EEG (total of $n = 27$); in 6-OHDA rats we analyzed 5 LFP recordings from the

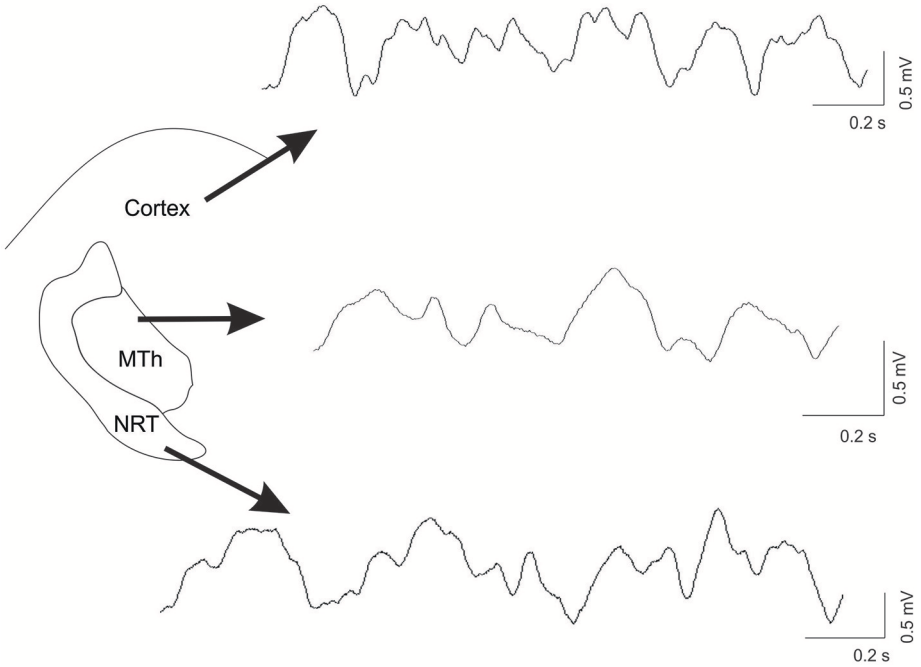


FIGURE 1 | Example of recordings, from the ECoG trace, the MTh and the NRT in control condition. The raw data was processing with smoothing.

NRT and 15 LFP recordings from the MTh, recorded parallel to EEG ($n = 20$).

RESULTS

Overall, after DA depletion the magnitude of changes of oscillatory activity in all analyzed frequency ranges within the NRT was more marked than that within the MTh and the Cx. **Figure 1** shows an example of recordings in control condition in the Cx, the MTh and the NRT.

Effects of DA-Depletion on Cortical Oscillatory Activities

The cortical activity changed after both chronic and acute DA depletion, with exception of α band (CTL: 0.0065 ± 0.0004; acute state: 0.0069 ± 0.00047; chronic state: 0.0064 ± 0.0003).

The δ band decreased of 20.5% in chronic DA depletion state (δ: 0.0439 ± 0.0009 in CTL, 0.026 ± 0.0052 in acute state and 0.0349 ± 0.0011 in chronic state; CTL vs. acute state $p = 0.021$, CTL vs. chronic state $p = 0.000$, acute vs. chronic state $p = 1$).

The θ band decreased in acute (32.3%) and chronic (17.2%) DA depletion states (θ: 0.0254 ± 0.0005 in CTL, 0.017 ± 0.002 in acute state, and 0.021 ± 0.0006 in chronic state; CTL vs. acute state $p = 0.000$, CTL vs. chronic state $p = 0.000$, acute vs. chronic state $p = 0.299$).

The Lβ, Hβ, and γ band frequencies increased in both acute and chronic DA depletion state (Lβ: 0.0049 ± 0.0003 in CTL, 0.0066 ± 0.0001 in acute state and 0.0077 ± 0.00007 in chronic state; CTL vs. acute state $p = 0.000$, CTL vs. chronic state $p = 0.000$. acute vs. chronic state $p = 0.000$. Hβ: 0.0024 ± 0.0002 in CTL, 0.0038 ± 0.00018 in acute state and 0.00398 ± 0.00009 in chronic state; CTL vs. acute state $p = 0.000$, CTL vs. chronic state $p = 0.000$, acute vs. chronic state $p = 0.686$. γ: 0.0004 ± 0.00002 in CTL, 0.0052 ± 0.00024 in acute state and 0.0014 ± 0.00004 in chronic state; CTL vs. acute state $p = 0.000$, CTL vs. chronic state $p = 0.000$, acute vs. chronic state $p = 0.000$). The Lβ increased by 34.9 and 55.6% in acute and chronic DA depletion state, respectively, the Hβ increased by 62% in acute state and of 67.9% in chronic DA depletion state, whilst the γ band increased by 1258.4% and of 261.6% in chronic state, in acute and chronic depletion states, respectively (**Figures 2A**, **3A**; **Supplementary Tables 1A,B, 4A**).

These results underlid that the cortical oscillatory activity in low frequencies range seems to be negatively affected by DA-depletion states, with exception of α band, that instead did not change in any conditions. On the contrary, the DA-depletion states tend to increase the oscillations in high frequencies ranges (Lβ, Hβ, and γ band). In addition, the results show that the cortical activity seems to be affected not just in chronic DAergic denervation but also in acute state, induced by TTX.

Effects of DA-Depletion on MTh Oscillatory Activities

In the MTh (**Supplementary Tables 2A,B**; **Figures 2B**, **3B**), the acute and chronic DA depletion affected differently the oscillatory activity. In particular, the δ (0.0745 ± 0.0024 in CTL, 0.0601 ± 0.0027 in acute state and 0.062 ± 0.0047

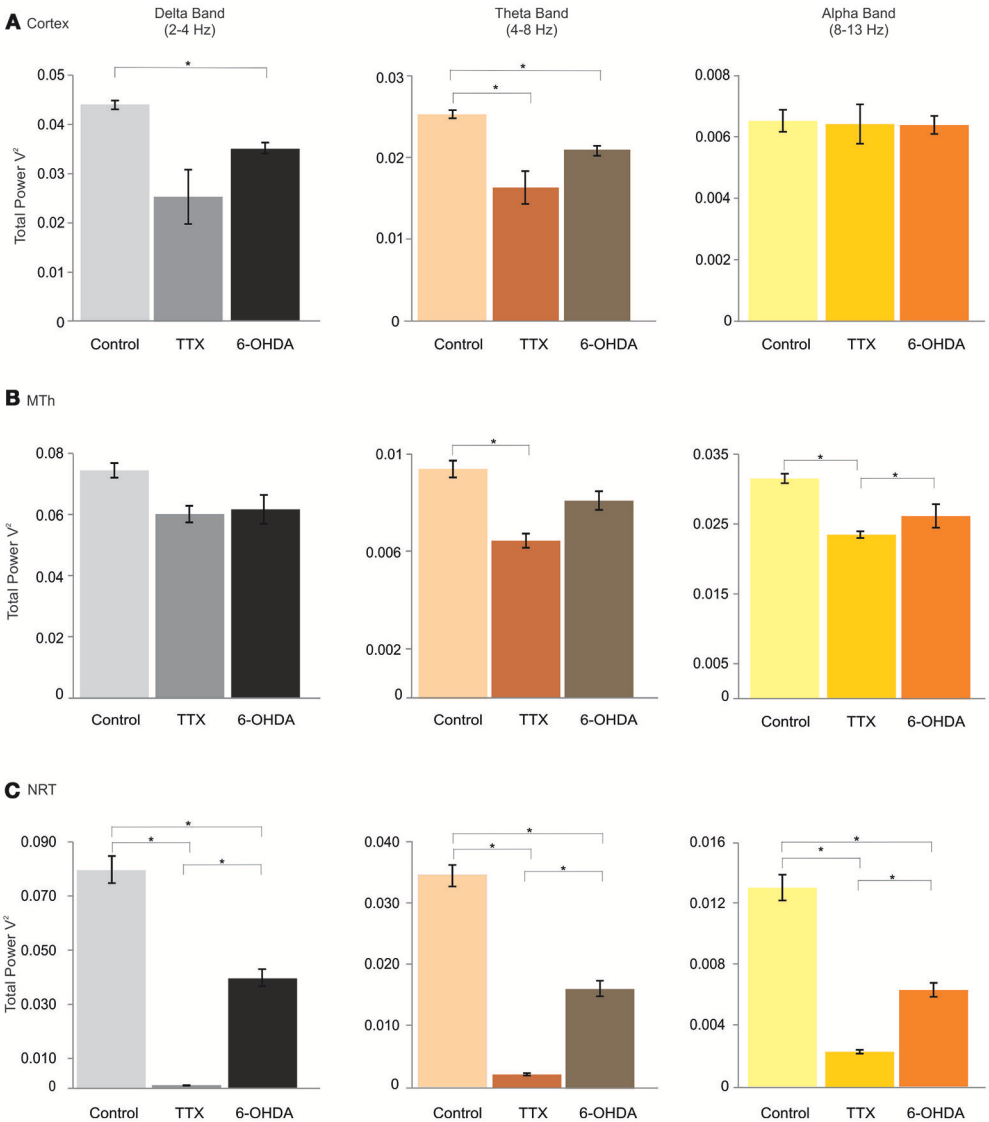


FIGURE 2 | δ (delta), θ (theta), and α (alpha) bands of the cortex (Cx), the MTh and the NRT (from top), in control, acute (TTX-infused rats) and chronic (6-OHDA-denervated rats) DA-depletion states. *$p < 0.016$, Mann Whitney Test.

in chronic state) did not change in DA depletion states in comparison to CTL. The θ and α bands decreased in acute state (θ: 0.0315 ± 0.0007 in CTL, 0.0235 ± 0.0005 in acute state and 0.0262 ± 0.0017 in chronic state; CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.037$, acute vs. chronic state $p = 0.337$. α: 0.0094 ± 0.0003 in CTL, 0.0064 ± 0.0003 in acute state and 0.0081 ± 0.0004 in chronic state; CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.025$, acute vs. chronic state $p = 0.01$). In particular, θ band decreased of 25.5% in acute state, whilst α band decreased of 31.7% in acute state (**Supplementary Table 4B**). Similarly, the Lβ and Hβ bands decreased just in acute DA depletion of 34.6 and 35.7%, respectively (**Supplementary Table 4B**), respectively (Lβ: 0.0052 ± 0.0002 in CTL, 0.0034 ± 0.00009 in acute state and 0.0053 ± 0.0002 in chronic state; CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 1$, acute vs. chronic state $p = 0.004$. Hβ: 0.0019 ± 0.00004 in CTL, 0.0012 ± 0.00003 in acute state and 0.0019 ± 0.00004 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.262$, acute vs. chronic state $p = 0.004$).

The γB decreased in both acute and chronic DA depletion state of 71.8 and 30.8%, respectively (0.0011 ± 0.00004 in CTL, 0.0003 ± 0.00002 in acute state and 0.0008 ± 0.00001 in chronic state; CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$).

Interestingly, these results show that the MTh oscillatory activity is strongly and mainly affected by acute DA depletion state. Indeed, TTX, but not 6-OHDA, with exception of δ and γ bands, induced the decrease of all analyzed bands.

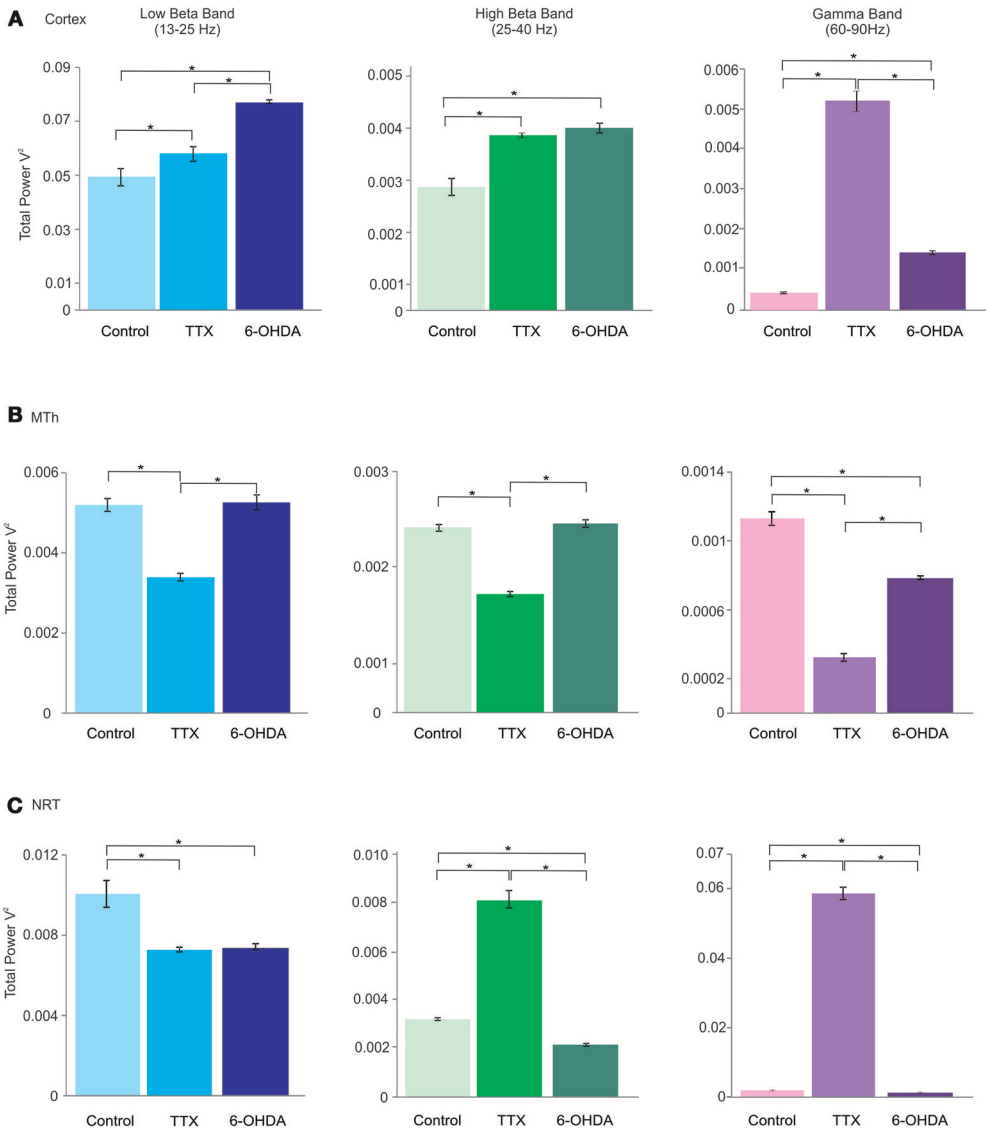


FIGURE 3 | Lβ (low beta), Hβ (high beta) and γ (gamma) bands of the cortex (Cx), the MTh and the NRT (from top), in control, acute (TTX-infused rats) and chronic (6-OHDA-denervated rats) DA-depletion states. *$p < 0.016$, Mann Whitney test.

Effects of DA-Depletion on the NRT Oscillatory Activities

In the NRT (**Supplementary Tables 3A,B**; **Figures 2C**, **3C**), the acute and chronic DA depletion states changed all the analyzed bands. In particular, δ (0.0806 ± 0.005 in CTL, 0.0010 ± 0.00007 in acute state and 0.0408 ± 0.0032 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$), θ (0.0347 ± 0.0018 in CTL, 0.002 ± 0.0002 in acute state and 0.0163 ± 0.0013 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$), α (0.013 ± 0.0008 in CTL, 0.002 ± 0.0001 in acute state and 0.0063 ± 0.0005 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$) and Lβ (0.0101 ± 0.0007 in CTL, 0.0073 ± 0.0001 in acute state and 0.0074 ± 0.0001 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.522$) bands decreased in both acute and chronic DA depletion state. Hβ (0.0032 ± 0.00005 in CTL, 0.0083 ± 0.0003 in acute state and 0.0022 ± 0.000043 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$) and γ (0.0014 ± 0.00003 in CTL, 0.059 ± 0.0017 in acute state and 0.00097 ± 0.00003 in chronic state, CTL vs. acute state $p = 0.004$, CTL vs. chronic state $p = 0.004$, acute vs. chronic state $p = 0.004$) increased in acute state and decreased in chronic state.

The δ, θ, α, and Lβ bands decreased by 98.8, 94.3, 83.4, and 27.8% in acute state, respectively, and by 49.4, 53, 51.4, and 27.2% in chronic state. The Hβ and γ bands increased by 154.8

and 4055.1% in acute state and decreased by 32.1 and 31.6% in chronic state (**Supplementary Table 4C**).

The results show that the NRT is strongly affected by both acute and chronic DA depletion states, differently from MTh. Indeed, the δ, θ, α, and Lβ bands, decreased in both DA depletion states. Interestingly, in the high frequencies range (Hβ and γ bands), the activity increased in acute DA depletion state and instead decreased in chronic state.

DISCUSSION

Compelling evidence shows that abnormal oscillatory activity within the Cx and BG circuit mainly in the β range, but not only, contributes to motor impairments in PD (13). On the other hand, the effects of DA depletion in crucial structures of the cortico-subcortical loop such as the MTh and its principal modulator, i.e., the NRT have been poorly investigated. In order to shed more light on this important field, we investigated band oscillations in the cortical and subcortical MTh-NRT loop in rats in both acute and chronic DA-depleted states.

Cortical and NRT δ Band Is Affected by Acute and Chronic DA Depletion

In line with the observation of a δ decrease in cognitively normal PD patients (39), we found a reduction of δ wave power at cortical level in chronic DA-depleted state. In addition, we found that δ wave also decrease after early acute DA-depletion NRT. The δ band is associated with sleep modulation and disruption of this activity reflect sleep-disorders (77). Interestingly, one of the most common symptom in early stage of PD concerns sleep deficits (44). In addition, frequencies around δ power intervals are associated with PD tremor and are detected in the STN in decision conflict situations (78). Our results showed a decrement of oscillations in δ frequency in both thalamic nuclei and Cx.

Cortical and Thalamic θ Band Decreases in Both Acute and Chronic Depletion State

Contrary to previous reports (79, 80), we observed a decrease of θ activity in both acute and chronic DA-depleted states in the three investigated areas. In the NRT we found an increase in chronic DA depletion state in comparison to acute state, without nevertheless reach the baseline level. Cavanagh and colleagues demonstrated that in PD patients the θ power in the MPC and the STN is associated with decision conflict situations and that STN-DBS alters this coupling (78). Therefore, θ power increases in frontal Cx, associated with PD in a specific task conditions, while it decreases in our anesthetized PD animal model.

Thalamic α Band Decreases in Acute and Chronic DA Depletion States

According to previous report, decrease of cortical α power correlates with dementia (57). Whilst we failed to find any changes in cortical α band, it decreased in the MTh and the NRT. In particular, NRT-α power decreased in both acute and chronic DA depletion in comparison to control, whereas in the MTh it decreased just in acute state. The power of α frequency was higher in chronic than acute DA depletion conditions. Consistently, the thalamus is supposed to be the α band rhythms generator (79), as postulated by the inhibition-timing hypothesis of α oscillations (48, 81).

Cortical β Bands Increase in DA Depletion State, Whilst It Decreases in MTh in Acute State

The β band is one of the most studied oscillatory activity critically involved in PD (8, 11, 13, 82). In physiological conditions it is suppressed by motions (83), whilst its impairment leads to deficits in complex sensorimotor processes such as repetitive movements (84, 85) and it is pathophysiological relevant to bradykinesia (10, 15). More precisely, it has been reported a correlation between rigidity and bradykinesia and the β band (86). Moreover, Lβ band (12–30 Hz) shows a decrease in power in response to dopaminergic treatment (87). Hβ power in STN is enhanced in patients with freezing of gait in comparison to patients without this common PD characteristic (88). In addition, the Lβ band is prominent in inattentive state, whilst it has been observed a shift to Hβ band during walking in the substantia nigra pars reticulata (SNr) of chronically 6-OHDA-denervated rats (17).

Here, we found an increment of cortical Lβ and Hβ band in both acute and chronic DA depletion state. Compared to the Cx, the thalamic β activity is differently affected by DA depletion. In MTh, β band power is decreased in acute state. In NRT, the Lβ band is decreased in both acute and chronic state, whilst the Hβ band is increased in acute state.

In addition, our results support the idea that β band has cortical and not thalamic origins (89). In particular, we observed that the cortical β band increased in both acute and chronic DA depletion states whilst MTh and NRT bands are differently modulated. The MTh Lβ and Hβ decreased just in acute state, whilst NRT Hβ increased in acute state and decreased in chronic state. The NRT Lβ decreased instead in both acute and chronic DA depletion states. Interestingly, in chronic state the β band in the MTh did not change in comparison to control and this may be due to the fact the MTh is affected by opposite influence by the Cx and the NRT.

γ Band Is Affected by Both Acute and Chronic DA Depletion

As it has been previously reported (90, 91), TTX-treated and 6-OHDA-lesioned rats showed an increment of the oscillatory activity in the Cx in the γ frequency. Similarly, NRT activity increased, whilst MTh γ activity is decreased. This increase of cortical and NRT γ band could be considered as a basis for developing of dyskinesia during levo-dihydroxyphenylalanine (L-DOPA) treatment. The cortical γ activity is coupled with thalamic α oscillations (92). We found that cortical γ and thalamic α bands showed opposite behaviors, indeed the DA depletion states determined the increment of cortical γ power and decrement of the thalamic α band.

CONCLUSION

Taking together, the evidence from literature and the present results reveal an evident complex oscillatory pattern of neuronal activity in PD, at the level of different nuclei of BG-thalamic-cortical network. Furthering our understanding of these aberrant oscillations will likely contribute to the advance of early diagnosis based on non-invasive investigation of brain activity.

Our results support the idea that there is not a unique band responsible of the PD pathological mechanisms, instead all bands could contribute to the pathological complexity of the oscillatory activity. Importantly, since the chronic DA depletion state did not drastically affect the thalamic oscillatory activity, our data raise the possibility that some aspects of these oscillatory activity in PD may be promoted by the acute DA loss (69, 70, 93), and the involvement of the NRT. The injection of TTX in MFB is accompanied by increase of cortical β and γ bands, as typically recorded in chronic DA denervation and in PD patients (13, 15, 20). The MTh oscillations change occurs preferentially in acute DA depletion state, while not in chronic state due to the fact that it may be compensated by the NRT activity. In the acute DA depletion state, the changes in different BG circuit sites, such as SN and GP (69, 70, 93) might instead result in the observed changes of thalamic activity.

This result could be considered an important starting point in order to shed some light on the role of the NRT, a structure usually neglected in PD pathophysiology, in a hypothetical widely Cx-BG network. Therefore, the thalamic information is processed in the NRT, and may enhance or suppress thalamic responsiveness, depending on the relative timing of afferent inputs and NRT activation (94). The NRT is implicated in a variety of functions, such as motor, arousal, sleep modulation, sensory, and associative stimuli coding (95), and each NRT sector encodes the relative specific information. Nevertheless, since it is a small and deep brain structure, it is difficult to investigate it *in vivo*, and elucidate its specific role in modulating larger-scale brain activity. Early models of the NRT functions posit that thalamocortical and NRT neurons are reciprocally innervated (96), determining the oscillatory phenomena (97, 98). However, computational models support the idea that an open-loop could explain the thalamic-NRT circuit. Accordingly, low-threshold bursting in an open-loop circuit could be consider a mechanism by which the NRT may paradoxically enhance thalamocortical activation, depending on the relative timing of the NRT and thalamocortical neurons (95). This dynamic NRT-thalamic-cortical loop could explain the hypothetical role of the NRT for thalamocortical modulation (95).

In pathological conditions, the strong changes of the NRT oscillatory activities in acute DA depletion state could explain the absence of acute cortical and the thalamic change and the later cortical and thalamic changes in chronic DA deficiency condition.

Our results are in line with the evidence of a strong influence of the NRT in cortical and thalamic firing mode modulation in physiological and pathological conditions involving dysfunctions of acetylcholine, nicotine and DA systems (99). Overall, the strong impairments of the NRT oscillatory activity in all analyzed frequencies in both acute and chronic DA depletion states may suggest a possible critical role of the NRT in both PD motor and non-motor symptoms, in early and late stages.

Our study has some caveats. Firstly, we have to consider that findings in PD animal models cannot totally be translated to human disease state. Moreover, we have to consider that (i) the dopaminergic depletion is not the unique feature of PD; (ii) the 6-OHDA lesion does not reflect totally the PD symptoms, and (iii) the electrophysiological recordings were performed under urethane anesthesia, rending impossible to explore if oscillatory activity depends on the motions and/or cognitive tasks, impaired in PD. In spite of these limitations, we think that our results represent an important starting point in order to better understand the changes of thalamo-cortical oscillations induced by dopaminergic denervation in PD.

AUTHOR CONTRIBUTIONS

SG, GD, AlS, and AK-L conception and design. AgS, AlS, LG, GD, GO, and WS acquisition of data analysis and interpretation of data. AlS, LG, GD, and SG drafting the article and revising it.

ACKNOWLEDGMENTS

We thank the *Parkinson Svizzera* and the *Fondazione per lo studio delle malattie neurodegenerative delle persone adulte e dell'anziano del Ticino* for financial support to SG. This work was partially supported by an IBRO-Swiss National Science Foundation grant to WS and AK-L. GD was supported by an international short visit grant (number IZK0Z3_163508) by Swiss National Science Foundation (SNSF).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00663/full#supplementary-material

REFERENCES

1. Brown P, Oliviero A, Mazzone P, Insola A, Tonali P, Di Lazzaro V. Dopamine dependency of oscillations between subthalamic nucleus and pallidum in Parkinson's disease. *J Neurosci* (2001) 21:1033–38. doi: 10.1523/JNEUROSCI.21-03-01033.2001
2. Lalo E, Thobois S, Sharott A, Polo G, Mertens P, Pogosyan A, et al. Patterns of bidirectional communication between cortex and basal ganglia during movement in patients with Parkinson disease. *J Neurosci.* (2008) 28:3008–16. doi: 10.1523/JNEUROSCI.5295-07.2008
3. Narabayashi Y, Oshima T. Parkinsonian thalamic activity represents pathophysiology underlying progression of motor symptoms. *Neurol Clin Neurosci.* (2015) 3:215–22. doi: 10.1111/ncn3.12014
4. Boraud T, Bezard E, Bioulac B, Gross CE. From single extracellular unit recording in experimental and human Parkinsonism to the development of

a functional concept of the role played by the basal ganglia in motor control. *Prog Neurobiol.* (2002) 66:265–83. doi: 10.1016/S0301-0082(01)00033-8
5. Bergman H, Wichmann T, Karmon B, DeLong MR. The primate subthalamic nucleus. II. Neuronal activity in the MPTP model of parkinsonism. *J Neurophysiol*. (1994) 72:507–20.
6. Nini A, Feingold A, Slovin H, Bergman H. Neurons in the globus pallidus do not show correlated activity in the normal monkey, but phase-locked oscillations appear in the MPTP model of parkinsonism. *J Neurophysiol.* (1995) 74:1800–5. doi: 10.1152/jn.1995.74.4.1800
7. Vorobyov VV, Schibaev NV, Morelli M, Carta AR. EEG modifications in the cortex and striatum after dopaminergic priming in the 6-hydroxydopamine rat model of Parkinson's disease. *Brain Res*. (2003) 972:177–85. doi: 10.1016/S0006-8993(03)02528-9
8. Mallet N, Pogosyan A, Sharott A, Csicsvari J, Bolam JP, Brown P, et al. Disrupted dopamine transmission and the emergence of exaggerated beta oscillations in subthalamic nucleus and cerebral cortex. *J Neurosci.* (2008) 28:4795–806. doi: 10.1523/JNEUROSCI.0123-08.2008
9. Alavi M, Dostrovsky JO, Hodaie M, Lozano AM, Hutchison WD. Spatial extent of β oscillatory activity in and between the subthalamic nucleus and substantia nigra pars reticulata of Parkinson's disease patients. *Exp Neurol*. (2013) 245:60–71. doi: 10.1016/j.expneurol.2012.09.021
10. Brown P. Bad oscillations in Parkinson's disease. *J Neural Transm.* (2006) 70:27–30. doi: 10.1007/978-3-211-45295-0_6
11. Eusebio A, Brown P. Synchronisation in the beta frequency band-the bad boy of parkinsonism or an innocent bystander? *Exp Neurol.* (2009) 217:1–3. doi: 10.1016/j.expneurol.2009.02.003
12. Pevzner A, Izadi A, Lee DJ, Shahlaie K, Gurkoff GG. Making waves in the brain: what are oscillations, and why modulating them makes sense for brain injury. *Front Syst Neurosci*. (2016) 10:30. doi: 10.3389/fnsys.2016.00030
13. Little S, Brown P. The functional role of beta oscillations in Parkinson's disease. *Parkinson Relat Dis.* (2014) S44–8. doi: 10.1016/S1353-8020(13)70013-0
14. van Wijk BMC, Pogosyan A, Hariz MI, Akram H, Foltynie T, Limousin P et al. Localization of beta and high-frequency oscillations within the subthalamic nucleus region. *NeuroImage* (2017) 16:175–83. doi: 10.1016/j.nicl.2017.07.018
15. Brown P. The oscillatory nature of human basal ganglia activity: relationship to the pathophysiology of Parkinson's disease. *Mov Disord.* (2003) 18:357–63. doi: 10.1002/mds.10358
16. George JS, Strunk J, Mak-McCully R, Houser M, Poizner H, Aron AR. Dopaminergic therapy in Parkinson's disease decreases cortical beta band coherence in the resting state and increases cortical beta band power during executive control. *NeuroImage* (2013) 3:261–270. doi: 10.1016/j.nicl.2013.07.013
17. Avila I, Parr-Brownlie LC, Brazhnik E, Castañeda E, Bergstrom DA, Walters JR. Beta frequency synchronization in basal ganglia output during rest and walk in a hemiparkinsonian rat. *Exp Neurol.* (2010) 221:307–19. doi: 10.1016/j.expneurol.2009.11.016
18. Litvak V, Jha A, Eusebio A, Oostenveld R, Foltynie T, Limousin P, et al. Resting oscillatory cortico-subthalamic connectivity in patients with Parkinson's disease. *Brain* (2011) 134:359–74. doi: 10.1093/brain/awq332
19. Oswal, P. Brown, V. Litvak. Synchronized neural oscillations and the pathophysiology of Parkinson's disease. *Cur Opin Neurol*. (2013) 26:662–70. doi: 10.1097/WCO.0000000000000034
20. Salvadè A, D'Angelo V, Di Giovanni G, Tinkhauser G, Sancesario G, Städler C, et al. Distinct roles of cortical and pallidal β and γ frequencies in hemiparkinsonian and dyskinetic rats. *Exp Neurol.* (2016) 275:199–208. doi: 10.1016/j.expneurol.2015.11.005
21. Miller KJ, Leuthardt EC, Schalk G, Rao RP, Anderson NR, Moran DW, et al. Spectral changes in cortical surface potentials during motor movement. *J Neurosci.* (2007) 27:2424–32. doi: 10.1523/JNEUROSCI.3886-06.2007
22. Kuhn AA, Kupsch A, Schneider GH, Brown P. Reduction in subthalamic 8–35 Hz oscillatory activity correlates with clinical improvement in Parkinson's disease. *Eur J Neurosci.* (2006) 23:1956–60. doi: 10.1111/j.1460-9568.2006.04717.x
23. Smith MM, Weaver KE, Grabowski TJ, Rao RPN, Darvas F. Non-invasive detection of high gamma band activity during motor imagery. *Front Hum Neurosci*. (2014) 8:817. doi: 10.3389/fnhum.2014.00817
24. Thürer B, Stockinger C, Focke A, Putze F, Schultz T, Stein T. Increased gamma band power during movement planning coincides with motor memory retrieval. *Neuroimage* (2016) 125:172–181. doi: 10.1016/j.neuroimage.2015.10.008
25. Başar E, Güntekin B. A review of brain oscillations in cognitive disorders and the role of neurotransmitters. *Brain Res.* (2008) 1235:172–93. doi: 10.1016/j.brainres.2008.06.103
26. Paller KA, Kutas M, Mayes AR. Neural correlates of encoding in an incidental learning paradigm. Electroencephalogr. *Clin Neurophysiol.* (1987) 67:360–71. doi: 10.1016/0013-4694(87)90124-6
27. Jerbi K, Ossandon T, Hamame CM, Senova S, Dalal SS, Jung J, et al. Task-related gamma-band dynamics from an intracerebral perspective: review and implications for surface EEG and MEG. Hum. *Brain Mapp.* (2009) 30:1758–71. doi: 10.1002/hbm.20750
28. Litvak V, Eusebio A, Jha A, Oostenveld R, Barnes G, Foltynie T, et al. Movement-related changes in local and long-range synchronization in Parkinson's disease revealed by simultaneous magnetoencephalography and intracranial recordings. *J. Neurosci.* (2012) 32:10541–53. doi: 10.1523/JNEUROSCI.0767-12.2012
29. Joundi RA, Jenkinson N, Brittain JS, Aziz TZ, Brown P. Driving oscillatory activity in the human cortex enhances motor performance. *Curr Biol*. (2012) 22:403–7. doi: 10.1016/j.cub.2012.01.024
30. Halje P, Tamtè M, Richter U, Mohammed M, Cenci MA, Petersson P. Levodopa-induced dyskinesia is strongly associated with resonant cortical oscillations. *J Neurosci* (2012) 32:16541–51. doi: 10.1523/JNEUROSCI.3047-12.2012
31. Huang H, Watts RL, Montgomery EB Jr. Effects of deep brain stimulation frequency on bradykinesia of Parkinson's disease. *Mov Disord.* (2014) 29:203–6. doi: 10.1002/mds.25773
32. Moro E, Esselink RJA, Xie J, Hommel M, Benabid AL, Pollak P. The impact on Parkinson's disease of electrical parameter settings in STN stimulation. *Neurology* (2002) 59:706–13. doi: 10.1212/WNL.59.5.706
33. Wiecki TV, Frank MJ. Neurocomputational models of motor and cognitive deficits in Parkinson's disease. *Prog Brain Res.* (2010) 183:275–97. doi: 10.1016/S0079-6123(10)83014-6
34. Ekstrom AD, Caplan JB, Ho E, Shattuck K, Fried I, Kahana MJ. Human hippocampal theta activity during virtual navigation. *Hippocampus* (2005) 15:881–9. doi: 10.1002/hipo.20109
35. Tan H, Pogosyan A, Anzak A, Bogdanovic M, Green AL, Aziz T, et al. Complementary roles of different oscillatory activities in the subthalamic nucleus in coding motor effort in Parkinsonism. *Exp Neurol.* (2013) 248:187–95. doi: 10.1016/j.expneurol.2013.06.010
36. Shine JM, Handojoseno AM, Nguyen TN, Tran Y, Naismith SL, Nguyen H et al. Abnormal patterns of theta frequency oscillations during the temporal evolution of freezing of gait in Parkinson's disease. *Clin Neurophysiol.* (2014) 125:569–76. doi: 10.1016/j.clinph.2013.09.006
37. Achermann P, Borbely AA. Low-frequency (<1 Hz) oscillations in the human sleep electroencephalogram. *Neuroscience* (1997) 81:213–22.
38. Güntekin B, Başar E. Review of evoked and event-related delta responses in the human brain. *Int J Psychophysiol.* (2016) 1031:43–52. doi: 10.1016/j.ijpsycho.2015.02.001
39. Emek-Savaş DD, Özmüş G, Güntekin B, Çolakoglu BD, Çakmur R, Başar E, et al. Decrease of delta oscillatory responses in cognitively normal Parkinson's Disease. *Clin EEG Neurosci.* (2016) 48:355–64. doi: 10.1177/1550059416666718
40. Güntekin B, Hanoglu L, Güner D, Yilmaz NH, Çadirci F, Mantar N, et al. Cognitive impairment in parkinson's disease is reflected with gradual decrease of EEG delta responses during auditory discrimination. *Front Psychol*. (2018) 9:170. doi: 10.3389/fpsyg.2018.00170
41. Brunner H, Wetter TC, Hogl B, Yassouridis A, Trenkwalder C, Friess E. Microstructure of the non-rapid eye movement sleep electroencephalogram in patients with newly diagnosed Parkinson's disease: effects of dopaminergic treatment. *Mov Disord*. (2002) 17:928–33. doi: 10.1002/mds.10242
42. Kryzhanovskii GN, Shandra AA, Godlevskii LS, Mikhaleva II. Appearance of parkinsonian syndrome after administration of delta sleep-inducing peptide into the rat substantia nigra. *Bull Exp Biol Med*. (1990) 109:119–21. doi: 10.1007/BF00841651

43. Parker KL, Chen K-H, Kingyon JR, Cavanagh JF, Narayanan NS. Medial frontal ~4-Hz activity in humans and rodents is attenuated in PD patients and in rodents with cortical dopamine depletion. *J Neurophysiol*. (2015) 114:1310–20. doi: 10.1152/jn.00412.2015
44. Menza M, Dobkin RD, Marin H, Bienfait K. Sleep disturbances in Parkinson's Disease. *Movem Dis*. (2010) 25(Suppl. 1):S117–22. doi: 10.1002/mds.22788
45. Barone P, Antonini A, Colosimo C, Marconi R, Morgante L, Avarello TP. et al. The PRIAMO study: a multicenter assessment of nonmotor symptoms and their impact on quality of life in Parkinson's disease. *Mov Disord*. (2009) 24:1641–9. doi: 10.1002/mds.22643.y
46. Diederich NJ, Paolini V, Vaillant M. Slow wave sleep and dopaminergic treatment in Parkinson's disease: a polysomnographic study. *Acta Neurol Scand*. (2009) 120:308–13 doi: 10.1111/j.1600-0404.2009.01167.x
47. Pierantozzi M, Placidi F, Liguori C, Albanese M, Imbriani P, Marciani MG et al. Rotigotine may improve sleep architecture in Parkinson's disease: a double-blind, randomized, placebo-controlled polysomnographic study. *Sleep Med*. (2016) 21:140–4. doi: 10.1016/j.sleep.2016.01.016
48. Klimesch W, Sauseng P, Hanslmayr S. EEG alpha oscillations: the inhibition-timing hypothesis. *Brain Res Rev*. (2007) 53:63–88. doi: 10.1016/j.brainresrev.2006.06.003
49. Jensen O, Gelfand J, Kounios J, Lisman JE. Oscillations in the alpha band (9-12 Hz) increase with memory load during retention in a short-term memory task. *Cereb Cortex* (2002) 12:877–82. doi: 10.1093/cercor/12.8.877
50. Lopes da Silva FH, van Lierop TH, Schrijer CF, van Leeuwen WS. Essential differences between alpha rhythms and barbiturate spindles: spectra and thalamo-cortical coherences. *Electroencephalogr Clin Neurophysiol*. (1973) 35:641–5. doi: 10.1016/0013-4694(73)90216-2
51. Rougeul-Buser A, Buser P. Rhythms in the alpha band in cats and their behavioural correlates. *Int J Psychophysiol*. (1997) 26:191–203. doi: 10.1016/S0167-8760(97)00764-2
52. Foxe JJ, Snyder AC. The role of alpha-band brain oscillations as a sensory suppression mechanism during selective attention. *Front Psychol*. (2011) 2:154. doi: 10.3389/fpsyg.2011.00154
53. De Graaf TA, Gross J, Paterson G, Rusch T, Sack AT, Thut G. Alpha-band rhythms in visual task performance: phase-locking by rhythmic sensory stimulation. *PLoS ONE* (2013) 8(3):e60035. doi: 10.1371/journal.pone.0060035
54. Mulholland TB. Occurrence of the electroencephalographic alpha rhythm with eyes open. *Nature* (1965) 206:746. doi: 10.1038/206746a0
55. Bareither I, Chaumon M, Bernasconi F, Villringer A, Busch N. Invisible visual stimuli elicit increases in alpha-band power. *J Neurophysiol*. (2014) 112:1082–1090. doi: 10.1152/jn.00550.2013
56. Jan Drewes, Rufin VanRullen. This is the rhythm of your eyes: the phase of ongoing electroencephalogram oscillations modulates saccadic reaction time. *J Neurosci*. 31:4698–708. doi: 10.1523/JNEUROSCI.4795-10.2011
57. Cozac VV, Gschwandtner U, Hatz F, Hardmeier M, Rüegg S, Fuhr P. Quantitative EEG and cognitive decline in Parkinson's Disease. *Parkinson's Disease* (2016) 2016:9060649. doi: 10.1155/2016/9060649
58. Nambu A, Tokuno H, Hamada I, Kita H, Imanishi M, Akazawa T. et al. Excitatory cortical inputs to pallidal neurons via the subthalamic nucleus in the monkey. *J Neurophysiol*. (2000) 84:289–300. doi: 10.1152/jn.2000.84.1.289
59. Bosch-Bouju C, Hyland BI, Parr-Brownlie LC. Motor thalamus integration of cortical, cerebellar and basal ganglia information: implications for normal and parkinsonian conditions. *Front Comput Neurosci*. (2013) 7:163. doi: 10.3389/fncom.2013.00163
60. Pinault D. The thalamic reticular nucleus: structure, function and concept. *Brain Res Rev*. (2004) 46:1–31. doi: 10.1016/j.brainresrev.2004.04.008
61. Ferrarelli F, Tononi G. The thalamic reticular nucleus and schizophrenia. *Schizophrenia Bulletin*. (2011) 37:306–15. doi: 10.1093/schbul/sbq142
62. Pratt JA, Morris BJ. The thalamic reticular nucleus: a functional hub for thalamocortical network dysfunction in schizophrenia and a target for drug discovery. *J Psychopharmacol*. (2015) 29:127–37. doi: 10.1177/0269881114565805
63. Crick F. Function of the thalamic reticular complex: the searchlight hypothesis. *Proc Nat Acad Sci USA*. (1984) 81:4586–90. doi: 10.1073/pnas.81.14.4586
64. Gandia JA, De Las Heras S, García M, Giménez-Amaya JM. Afferent projections to the reticular thalamic nucleus from the globus pallidus and the substantia nigra in the rat. *Brain Res Bull*. (1993) 32:351–8.
65. Graybiel AM, Aosaki T, Flaherty AW, Kimura M. The basal ganglia and adaptive motor control. *Science* (1994) 5180:1826–31.
66. Lam YW, Sherman SM. Functional topographic organization of the motor reticulothalamic pathway. *J Neurophysiol*. (2015) 113:3090–7. doi: 10.1152/jn.00847.2014
67. Takakusaki K, Oohinata-Sugimoto J, Saitoh K, Habaguchi T. Role of basal ganglia-brainstem systems in the control of postural muscle tone and locomotion. *Prog Brain Res*. (2004) 143:231–7. doi: 10.1016/S0079-6123(03)43023-9
68. Singh A. Oscillatory activity in the cortico-basal ganglia-thalamic neural circuits in Parkinson's disease. *Eur J Neurosci*. (2018). doi: 10.1111/ejn.13853. [Epub ahead of print].
69. Galati S, D'Angelo V, Olivola E, Marzetti F, Di Giovanni G, Stanzione P, Stefani, A. Acute inactivation of the medial forebrain bundle imposes oscillations in the SNr: a challenge for the 6-OHDA model? *Exp Neurol*. (2010) 225:294–301. doi: 10.1016/j.expneurol.2010.06.020
70. Prosperetti C, Di Giovanni G, Stefani A, Moller JC, Galati S. Acute nigro-striatal blockade alters cortico-striatal encoding: an *in vivo* electrophysiological study. *Exp Neurol*. (2013) 247:730–6. doi: 10.1016/j.expneurol.2013.03.018
71. Keefe KA, Sved AF, Zigmond MJ, Abercrombie ED. Stress-induced dopamine release in the neostriatum: evaluation of the role of action potentials in nigrostriatal dopamine neurons or local initiation by endogenous excitatory amino acids. *J Neurochem*. (1993) 61:1943–52.
72. Keefe KA, Zigmond MJ, Abercrombie ED. *In vivo* regulation of extracellular dopamine in the neostriatum: influence of impulse activity and local excitatory amino acids. *J Neural Transm Gen Sect*. (1993) 91:223–40.
73. Keefe KA, Zigmond MJ, Abercrombie ED. Extracellular dopamine in striatum: Influence of nerve impulse activity in medial forebrain bundle and local glutamatergic input. *Neuroscience* (1992) 47:325–32. doi: 10.1016/0306-4522(92)90248-Z
74. Commissiong JW, Slimovitch C, Toffano G. Regulation of the synthesis and metabolism of striatal dopamine after disruption of nerve conduction in the medial forebrain bundle. *Br J Pharmacol*. (1990) 99:741–9. doi: 10.1111/j.1476-5381.1990.tb13000.x
75. Galati S, Song W, Orban G, Luft A-R, Kaelin-Lang A. Cortical slow wave activity correlates with striatal synaptic strength in normal but not in Parkinsonian rats. *Exp Neurol*. (2017) 301:50–8. doi: 10.1016/j.expneurol.2017.12.004
76. Rubin JE, Mcintyre CC, Turner RS, Wichmann T. Basal ganglia activity patterns in parkinsonism and computational modeling of their downstream effects. *Eur J Neurosci*. (2012). 36:2213–28. doi: 10.1111/j.1460-9568.2012.08108.x
77. Weiner OM, Dang-Vu TT. Spindle oscillations in sleep disorders: a systematic review. *Neural Plasticity* (2016) 2016:7328725. doi: 10.1155/2016/7328725
78. Cavanagh JF, Zambrano-Vazquez L, Allen JJ. Theta lingua franca: a common mid-frontal substrate for action monitoring processes. *Psychophysiology* (2012) 49:220–38. doi: 10.1111/j.1469-8986.2011.01293.x
79. Sarnthein J, Morel A, von Stein1 A, Jeanmonod D. Thalamic theta field potentials and EEG: high thalamocortical coherence in patients with neurogenic pain, epilepsy and movement disorders. *Thalam Relat Syst* (2003) 2:231–8. doi: 10.1523/JNEUROSCI.2411-06.2007
80. Gulberti A, Moll CKE, Hamel W, Buhmann C, Koeppen JA, Boelmans K, et al. Predictive timing functions of cortical beta oscillations are impaired in Parkinson's disease and influenced by L-DOPA and deep brain stimulation of the subthalamic nucleus. *NeuroImage* (2015) 9:436–49. doi: 10.1016/j.nicl.2015.09.013
81. Jensen O, Mazaheri A. Shaping functional architecture by oscillatory alpha activity: gating by inhibition. *Front Hum Neurosci*. (2010) 4:186. doi: 10.3389/fnhum.2010.00186
82. Foffani G, Ardolino G, Egidi M, Caputo E, Bossi B, Priori A. Subthalamic oscillatory activities at beta or higher frequency do not change after high-frequency DBS in Parkinson's disease. *Brain Res Bull*. (2006) 69:123–30. doi: 10.1016/j.brainresbull.2005.11.012

83. Doyle LM, Kühn AA, Hariz M, Kupsch A, Schneider GH, Brown P. Levodopa-induced modulation of subthalamic beta oscillations during self-paced movements in patients with Parkinson's disease. *Eur J Neurosci.* (2005) 21:1403–12. doi: 10.1111/j.1460-9568.2005.03969.x
84. Hebb AO, Darvas F, Miller KJ. Transient and state modulation of beta power in human subthalamic nucleus during speech production and finger movement. *Neuroscience* (2012) 202:218–33. doi: 10.1016/j.neuroscience.2011.11.072
85. Joundi RA, Brittain JS, Green AL, Aziz TZ, Brown P, Jenkinson N. Persistent suppression of subthalamic beta-band activity during rhythmic finger tapping in Parkinson's disease. *Clin Neurophysiol.* (2013) 124:565–73. doi: 10.1016/j.clinph.2012.07.029
86. Kühn AA, Tsui A, Aziz T, Ray N, Brücke C, Kupsch A, Schneider G.-H, Brown P. Pathological synchronisation in the subthalamic nucleus of patients with Parkinson's disease relates to both bradykinesia and rigidity. *Exp Neurol.* (2009) 215:380–7. doi: 10.1016/j.expneurol.2008.11.008
87. Lopez-Azcarate J, Tainta M, Rodríguez-Oroz MC, Valencia M, González R, Guridi J, et al. Coupling between beta and high-frequency activity in the human subthalamic nucleus may be a pathophysiological mechanism in Parkinson's disease. *J Neurosci.* (2010) 30:6667–77. doi: 10.1523/JNEUROSCI.5459-09.2010
88. Toledo JB, López-Azcárate J, Garcia-Garcia D, Guridi J, Valencia M, Artieda J, et al. High beta activity in the subthalamic nucleus and freezing of gait in Parkinson's disease. *Neurobiol Dis.* (2014) 64:60–5. doi: 10.1016/j.nbd.2013.12.005
89. Hammond C, Bergman H, Brown P. Pathological synchronization in Parkinson's disease: networks, models and treatments. *Tr Neurosci.* (2007) 30:357–64. doi: 10.1016/j.tins.2007.05.004
90. Valencia M, Chavez M, Artieda J, Bolam JP, Mena-Segovia J. Abnormal functional connectivity between motor cortex and pedunculopontine nucleus following chronic dopamine depletion. *J Neurophysiol.* (2014) 111:434–40. doi: 10.1152/jn.00555.2013
91. Brazhnik E, Cruz AV, Avila I, Wahba MI, Novikov N, Ilieva NM, et al. State-dependent spike and local field synchronization between motor cortex and substantia nigra in hemiparkinsonian rats. *J Neurosci.* (2012) 32: 7869–80. doi: 10.1523/JNEUROSCI.0943-12.2012
92. Roux F, Wibral M, Singer W, Aru J, Uhlhaas PJ. The phase of thalamic alpha activity modulates cortical gamma-band activity: evidence from resting-state MEG recordings. *J Neurosci.* (2013) 33:17827–35. doi: 10.1523/JNEUROSCI.5778-12.2013
93. Galati S, Stanzione P, D'Angelo V, Fedele E, Marzetti F, Sancesario G, Procopio T. The pharmacological blockade of medial forebrain bundle induces an acute pathological synchronization of the cortico-subthalamic nucleus-globus pallidus pathway. *J Physiol.* (2009) 587:4405–23. doi: 10.1113/jphysiol.2009.172759
94. Parent M, Descarries L. Acetylcholine innervation of the adult rat thalamus: distribution and ultrastructural features in dorsolateral geniculate, parafascicular, and reticular thalamic nuclei. *J Comp Neurol.* (2008) 511:678–91. doi: 10.1002/cne.21868
95. Willis AM, Slater BJ, Gribkova ED, Llano DA. Open-loop organization of thalamic reticular nucleus and dorsal thalamus: a computational model. *J Neurophysiol.* (2015) 114:2353–67. doi: 10.1152/jn.00926.2014
96. Steriade M, McCormick D, Sejnowski T. Thalamocortical oscillations in the sleeping and aroused brain. *Science* (1993) 262:679–85. doi: 10.1126/science.8235588
97. Destexhe A, McCormick DA, Sejnowski TJ. Thalamic and thalamocortical mechanisms underlying 3 Hz spike-and-wave discharges. *Prog Brain Res.* (1999) 21:289–307. doi: 10.1016/S0079-6123(08)63080-0
98. Huguenard JR. Neuronal circuitry of thalamocortical epilepsy and mechanisms of antiabsence drug action. *Adv Neurol.* (1998) 79:991–9.
99. Behrendt RP. Dysregulation of thalamic sensory transmission in schizophrenia: neurochemical vulnerability to hallucinations. *J Psychopharmacol.* (2006) 20:356. doi: 10.1177/0269881105057696

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.

frontiers in Neurology

MINI REVIEW
published: 02 October 2018
doi: 10.3389/fneur.2018.00809

Tau Pathology in Parkinson's Disease

Xue Zhang, Fei Gao, Dongdong Wang, Chao Li, Yi Fu, Wei He and Jianmin Zhang**

Department of Immunology, Research Center on Pediatric Development and Diseases, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College, State Key Laboratory of Medical Molecular Biology, Beijing, China

Tau protein—a member of the microtubule-associated protein family—is a key protein involved in many neurodegenerative diseases. Tau pathology in neurodegenerative diseases is characterized by pathological tau aggregation in neurofibrillary tangles (NFTs). Diseases with this typical pathological feature are called tauopathies. Parkinson's disease (PD) was not initially considered to be a typical tauopathy. However, recent studies have demonstrated increasing evidence of tau pathology in PD. A genome-wide association (GWA) study indicated a potential association between tauopathy and sporadic PD. The aggregation and deposition of tau were also observed in ∼50% of PD brains, and it seems to be transported from neuron to neuron. The aggregation of NFTs, the abnormal hyperphosphorylation of tau protein, and the interaction between tau and alpha-synuclein may all contribute to the cell death and poor axonal transport observed in PD and Parkinsonism.

Keywords: tauopathy, Parkinson's disease (PD), hyperphosphorylation, alpha-synuclien, tau protein

OPEN ACCESS

Edited by:
Graziella Madeo, National Institutes of Health (NIH), United States

Reviewed by:
Giuseppe Sciamanna, Università degli Studi di Roma Tor Vergata, Italy
Francisco José Pan-Montojo, Ludwig-Maximilians-Universität München, Germany

****Correspondence:***
Wei He
heweingd@126.com
Jianmin Zhang
jzhang42@163.com

Specialty section:
This article was submitted to Movement Disorders, a section of the journal Frontiers in Neurology

Received: *27 November 2017*
Accepted: *07 September 2018*
Published: *02 October 2018*

Citation:
Zhang X, Gao F, Wang D, Li C, Fu Y, He W and Zhang J (2018) Tau Pathology in Parkinson's Disease. Front. Neurol. 9:809. doi: 10.3389/fneur.2018.00809

INTRODUCTION

Tau protein is produced from a single human gene named microtubule-associated protein tau (MAPT), which is located on chromosome 17 and encodes a cytoskeletal protein that stabilizes microtubules (1). Although tau is widely distributed in neurons of the central nervous system (CNS), its levels in CNS astrocytes and oligodendrocytes are low (2). Tau proteins play a role in stabilizing microtubules, binding to membrane, and regulating axonal transport (3–5). Under physiological conditions, tau is highly soluble and unfolded. However, with changes in isoforms or phosphorylation patterns in pathological states, tau proteins become insoluble and misfolded, causing damage to neurons and axonal transport (6, 7). Protein misfolding, accumulation, and aggregation have been observed in many neurodegenerative diseases (8), which may contribute to neuron damage and neurological disorders. The pathological aggregation of tau or neurofibrillary tangles are known as tauopathy, an important hallmark of many human neurodegenerative disease, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (9, 10).

Parkinson's disease, named after Dr. James Parkinson, is a major neurodegenerative disease that primarily affects motor systems but can also be accompanied by cognitive and behavioral problems (11). There is a widespread neuron degeneration in PD brains, affecting up to 70% of dopaminergic neurons in the substantia nigra (SN) by the time of death (12, 13). The neuropathological hallmarks of PD include Lewy bodies (LBs) in the SN, brainstem, and rostral and forebrain regions and the

selective deletion of dopaminergic neurons in the SN (14, 15). Cell-death induced damage in SN may be the source of patient movement disorders. Although the causes of this cell death are generally unclear, researchers have observed an enrichment of tau protein and alpha-synuclein in neuronal Lewy bodies, which may be related to tauopathy in PD (16). Immunohistochemistry with anti-tau antibodies showed high level of NFTs in the substantia nigra from post-mortem human brain tissue (17). Researchers have also reported that tauopathies in PD and PD with dementia (PDD) were only observed in DA neurons of the nigrostriatal region, which contrasts with the wide-spread expression pattern of tau throughout the entire brain in AD (18).

Although tau pathology in AD and other tau-associated neurodegenerative diseases have been previously described, the importance of tau pathology in PD has been undervalued. Therefore, we reviewed the tau pathologies that might be involved in PD (**Figure 1**), seeking to identify tau as a potential therapeutic target.

ASSEMBLED TAU IN PD

Structure of Normal TAU

There are six different isoforms of tau protein in the human brain, with the differences among them resulting from alternative mRNA splicing of a single gene located on chromosome 17 (19). The microtubule-binding domains of the protein consist of adjoining sequence and repeat sequences. The six isoforms are divided into two categories based on the number of these repeats, namely, 3R and 4R. The 3R tau isoform has three repeats, while 4R tau has four repeats (20). Each of the repeats is able to bind to microtubules, and the more repeats the protein has, the stronger affinity it will have with them (21). Therefore, when compared with 3R tau, 4R tau is considerably easier to bundle with and polymerize microtubules. In different tauopathies, the pathological tau protein has different isoforms and conformations. Progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) are both associated with Parkinson's disease and are associated with 4R tau deposits in neurons and microglia (22). A study of a multigenerational family suffering from X-linked parkinsonian syndrome also showed a strong 4R tauopathy in the striatum (23).

Assembled TAU in PD and Other Neurodegenerative Diseases

Tau protein is soluble and unfolded under physiological conditions; however, in many neurodegenerative diseases, tau appears to be insoluble and assembled (24). The most probable mechanism of tau assembly involves mutations of the microtubule-associated protein tau (MAPT). MAPT is a single gene located on chromosome 17q21, containing 16 exons (25). A genome-wide association (GWA) study for sporadic PD cases in Europe confirmed that MAPT is closely linked to sporadic PD (26). MAPT is divided into two haplogroups called H1 and H2 based on whether the gene is in the inverted orientation (27). A meta-analysis in Caucasian populations reported that the H2 haplotype is more relevant to PD than the H1 haplotype, as the risk of suffering from PD is lower in Caucasians with the H2 haplotype (28). Certain FTDP-17 mutations, including missense mutations, deletions in the coding region, and intronic mutations, result in tau aggregation. This aggregation can cause dominantly inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (29). Most missense mutations in the coding region tend to affect how well tau can associate with microtubules (30). However, studies also show that some missense mutations such as G272V, P301L, and P301S may play an important role in filament assembly because they markedly facilitated the propensity for tau to assemble (31). If the mutation is in the repeat region or if changes in the relative amounts of 3R tau or 4R tau could lead to overproduction of 4R tau, the filament morphology tends to be incorrectly over-folded (32). Tau deposition and assembled filaments are observed in many neurodegenerative diseases and are considered a typical neuropathological hallmark. Significantly lower levels of soluble tau and a lower 3R-tau to 4R-tau ratio has been shown in the SN of patients with PD (33), indicating tauopathy similarity between PD and AD. PSP and CBD are subtypes of Parkinson's disease known as Parkinson-plus syndromes, and both are associated with the formation of tau deposits. Filamentous tau deposits can be observed in neurons and microglia in these diseases (34).

Prion-Like Pathological TAU Spreading in Animal Models and Patients With PD

Increasing evidence shows that tau aggregation and deposition contribute to PD pathology. Thus, to best understand the mechanisms underlying PD pathogenesis, early diagnosis, and treatment, determining how tau aggregation spreads to other areas is imperative. Researchers have observed Lewy bodies in grafted neurons that patients with PD received as transplants (35). The assumption was that neurofibrillary lesions spread along the neuronal pathways in the brain. Recent evidence shows that misfolded tau can move from cell to cell, similar to prion disease (36). Clavaguera and other researchers inserted a mutant human tau transgene into mice to show that human tau can be transported from neuron to neuron (37). Another study has shown that the spreading of the tau inclusions depended on the initial injection site of synthetic tau fibrils. The pathological tau were more likely to spread through functionally connected neuroanatomical pathways rather than through adjacent anatomical locations (38).

After showing prion-like transmission and spreading of tauopathy by injecting pathological tau from the human brain into transgenic ALZ17 mice, researchers assessed the role that different tau strains play in this pathological process. By separately injecting human brain homogenates from patients who suffered from argyrophilic grain disease (AGD), PSP, and CBD into different ALZ17 mice, researchers demonstrated that the different tau isoforms may induce different tauopathies. Mice receiving CBD or AGD tau differentially displayed silver-positive or silver-negative astrocytic plaques that matched the injection patterns and that were highly similar to the types of tau-related pathological damage typically found in the brains of patients suffering from the respective diseases (39). Furthermore, 12 months after injecting ALZ17 mice with brain homogenate

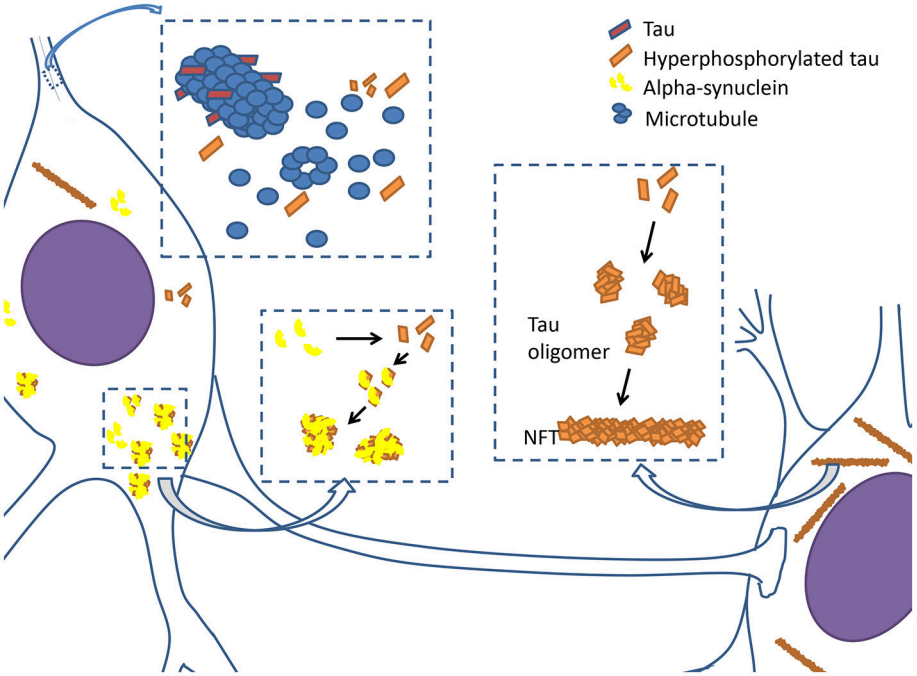


FIGURE 1 | Tauopathy in PD. Tau proteins are integrated with microtubules and stabilize microtubules in physiological condition. However, when tau proteins are hyperphosphorylated, they begin to disintegrate from microtubules, causing neuronal dysfunction. Hyperphosphorylated tau proteins prone to assemble together to form oligomers and develop into filamentous neurofibrillary tangles (NFTs). Hyperphosphorylated tau proteins also interact with α-synuclein to promote aggregation and fibrilization each other, and subsequently cause the formation of lewy bodies and axonal transport dysfunction.

from mice that had been injected with the human tau P301S transgene 18 months earlier, the ALZ17 mice showed fewer tau inclusions than those of mice that had been injected with AGD brain extracts (40). Similar phenomena were observed in mice injected with filament 4R-tau strains formed in HEK293T cells (41). Further, in patients with PD who received cell-replacement therapy to repair brain damage, hyperphosphorylated tau such as phospho-tau Ser202 and Thr205 were found in grafted neurons years after transplantation (42). Taken together, these findings demonstrate tau strain-specific prion-like transmission and spreading in the disease state, including in PD. Additionally, the specific strain plays an important role in causing distinct pathologies.

Abnormal Hyperphosphorylation of the TAU Protein

Hyperphosphorylation of the tau protein is another mechanism through which tau might accumulate and form filaments, which might also influence the ability of tau to bind to microtubules, possibly limiting how microtubules can be combined and resulting in their aggregation into NFTs (43). In this way, the microtubules might disintegrate, eventually leading to the impaired transport capacity of axon microtubules. Tau protein appears to be easily phosphorylated because of its 85 potential phosphorylation sites, and researchers have characterized over 20 kinases that may be related to the phosphorylation of tau protein after its transcription (44). With respect to the healthy human brain, there are only two or three phosphorylated amino acid residues in tau protein, while there may be considerably more in brains exhibiting tauopathy (45). Additional research indicates that the most likely mechanism underlying the hyperphosphorylation is either upregulated protein-kinase activity or downregulated protein-phosphatase activity (46). Among protein kinases, GSK-3β (a proline-directed protein kinases) and CDK5 (a non-PDPK non-proline-directed protein kinase) are probably the two most important kinases in tauopathy. Using neuronal stem/progenitor cells and transgenic mice, researchers have demonstrated a pivotal role for GSK-3β in the interaction between DA neurons and astrocytes during damage and recovery (47), which might be related to the death of DA neurons in PD. Further, the application of CDK5 in cortex suffering from Lewy body disease was reported in 2000, indicating that CDK5 may participate in the formation of Lewy bodies (48). In contrast, unlike GSK-3β and CDK5, a series of protein phosphatases (PP-2A, PP-2B, and PP-1) can dephosphorylate protein tau *in vitro* and *in vivo*, which may act to protect against tauopathy (49). Reduced activity levels of PP-2A in the brains of patients with PD and AD indirectly confirmed this inference (50, 51).

Hyperphosphorylation of tau protein is an important step in tau aggregation and the formation of neurofibrillary tangles (52). Antibodies targeting p-taus were able to detect tau isoforms in brain tissue suffering from sporadic PD or dementia with Lewy bodies, indicating the existence of hyperphosphorylated tau protein in NFTs (53). Tau aggregation more easily begins from the C-terminus of the protein (54). Therefore, kinases

that phosphorylate at the C-terminus might be crucial for the formation of tau filaments and aggregates. For example, an *in vitro* study indicated that the GSK-3β associated with the phosphorylation of tau at the C-terminal had an ability to promote the fibrillation of the protein, while the level of microtubule assembly stayed low due to DYRK1A (55). A study on the structure and dynamics of phosphorylated tau filaments using computer simulations indicated that the all the masses and charges had changed because of phosphorylation at regions associated with microtubules, resulting in further aggregation of tau (56).

Phosphorylated tau is also proved to be related to the N-methyl-D-aspartic receptor (NMDAR) at postsynaptic sites (57), which suggests that the toxic pathology of tau phosphorylation is associated with the synapse. Meanwhile, the FTDP-17 tau mutant, which is known to be associated with PDD, was shown to interfere with synaptic vesicles in presynaptic terminals, causing the dysfunction of vesicle traffic and presynaptic activity (58). There are also a number of studies showing that the hyperphosphorylation of tau protein may depolymerize microtubules, causing their dysfunction, impaired axonal transport, and ultimately cell death (59). Okadaic acid, an inhibitor of tau phosphorylation, was used to investigate the synaptic structure of neurons in rats (60). This study highlights the potential relationship between phosphorylated tau and the loss of synaptic function. Similar results have been shown in animal models. Transgenic mice expressing human tau P301L show Parkinsonism as early as 6.5 months (61), while a similar phenotype occurs in the K3 mouse model that expresses human tau with the K396I mutation. In this latter case, Parkinsonism symptoms can be improved using L-dopa (62).

P-TAU Associated With Alpha-Synuclein Leads to Toxic Injury in PD

One of the key proteins involved in PD pathology is alpha-synuclein, a highly soluble neuronal cytoplasmic protein that is localized to presynaptic elements in the CNS (63). Under certain conditions, such as missense mutations, post-translational modifications (e.g., phosphorylation and C-terminal truncation), and peroxynitrite stimulation, alpha-synuclein is prone to being fibrillated and to residing in Lewy bodies with other proteins (64), which is a feature of PD that occurs along with Lewy neurites.

Researchers found that tau protein, especially phosphorylated tau, existed in Lewy bodies along with alpha-synuclein and that neurofibrillary tangles could be observed around Lewy bodies (53, 65, 66). This phenomenon led researchers to hypothesize a positive interaction between tau and alpha-synuclein. A transgenic mouse model of PD showed increased levels of p-tau and the co-localization and overexpression of alpha-synuclein and p-tau, which were deposited in large inclusion bodies that are considered similar to Lewy bodies in PD (67). A series of experiments *in vitro* indicate that tau incubated with synthesized alpha-synuclein oligomers can induce all forms of tau, including the assembly of toxic tau forms (68). Furthermore, studies using QBI293 cells demonstrated that alpha-synuclein induces tangles of tau and promotes phosphorylation of tau in cells (69). Reports also indicate that the nucleus of neurons were surrounded by alpha-synuclein and human tau with the P301L mutation, which may be to blame for the loss of neuronal function (70). Researchers have been able to successfully detect the aggregation of the two proteins in brains of patients with PD using two novel antibodies specific to oligomeric tau and alpha-synuclein (71). Similar results were shown in a transgenic mouse model that was inoculated with alpha-synuclein supplied from preformed fibrils, indicating that the existence of both alpha-synuclein and tau promotes fibrillation, and this phenomenon is also confirmed in human brain (72).

Studies on the genetics of brain tissue indicate that an interaction between tau and alpha-synuclein in (PDD) with Lewy bodies (73).

As mentioned above, specific protein kinases may hyperphosphorylate tau protein at certain sites, causing toxic isoforms of tau. Among these kinases, protein kinases A can be stimulated by alpha-synuclein, resulting in tau phosphorylation at Ser262/356 (74). Studies focused on GSK-3β, which is associated with the toxic p-tau isoform in AD, also indicate that there may be an interaction between alpha-synuclein and accumulated p-tau (75). Another study using transgenic mice that overexpressed or lacked alpha-synuclein demonstrated that alpha-synuclein is indispensable for the activation of GSK-3β. A co-IP experiment in SH-SY5Y cells also demonstrated the existence of an alpha-synuclein, p-tau, and p-GSK-3β (76) complex. Furthermore, researchers have developed a mouse model with a S9A-point mutation of human GSK-3β to investigate the relationship between alpha-synuclein and p-tau, showing a positive association between the two proteins *in vitro*, as well as in behavioral, and biochemical experiments (77, 78). Additionally, p-GSK-3β-Y216, the kinase-active form of GSK-3β, is co-localized with both p-tau and alpha-synuclein and is broadly expressed in the whole brain, while p-tau, and alpha-synuclein are expressed in TH+ DA neurons of the midbrain (78).

After showing the positive relationship between p-tau and alpha-synuclein, researchers are still investigating the mechanism underlying the toxic interaction between the two proteins. Several studies indicate that neurotoxic MPP+ induces the abnormal hyperphosphorylation of tau along with alpha-synuclein *in vitro* (79) and *in vivo* (80). Studies in a drosophila model demonstrated that tau interacting with alpha-synuclein ruined the organization of the cytoskeleton, leaving low-functioning axonal transport and structural abnormalities in neuronal synapses that resulted in PD-related cell death (81). However, the precise relationship between tau and alpha-synuclein and the molecular mechanisms responsible for PD are still unclear. There might be a cascade reaction in which the accumulation of alpha-synuclein in synapses recruits tau (82) and induces damage; the resulting low-functioning axonal transport will further promote the accumulation of tau and alpha-synuclein, and therefore, more fibrillation will be present in neurons, which will eventually lead to cell death.

CONCLUSIONS

Tau is a key protein in many neurodegenerative diseases; however, its importance has been underestimated preoperatively in PD and PDD. Soluble, unfolded tau, after being phosphorylated or mutated, becomes insoluble and misfolded, resulting in conformational changes in microtubules and the aggregation of NFTs. The mobility of abnormal tau through brain tissue in PD is similar to prion-like diseases. The accumulation of hyperphosphorylated tau also affects axonal transport and appears to work with alpha-synuclein to contribute to tauopathy in PD and AD.

Although there is no effective treatment or drug therapy for PD and other similar neurodegenerative diseases, understanding the structure, function, and mechanism of tau and tau pathology might be helpful for early diagnosis and treatment of PD in the future.

AUTHOR CONTRIBUTIONS

XZ and FG wrote the manuscript. DW, CL, and YF helped to edit the manuscript. JZ and WH edited the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (31471016), the CAMS Initiative for Innovative Medicine (2016-I2 M-1-008), and the National Key Research and Development Program of China (2016YFA0101001).

REFERENCES

1. Shaw-Smith C, Pittman A, Willatt L, Martin H, Rickman L, Gribble S, et al. Microdeletion encompassing MAPT at chromosome 17q21.3 is associated with developmental delay and learning disability. *Nat Genet.* (2006) 38:1032–7. doi: 10.1038/ng1858
2. Shin RW, Iwaki T, Kitamoto T, Tateishi J. Hydrated autoclave pretreatment enhances tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. *Lab Invest.* (1991) 64:693–702.
3. Gauthierkemper A, Weissmann C, Golovyashkina N, Sebölemke Z, Drewes G, Gerke V, et al. The frontotemporal dementia mutation R406W blocks tau's interaction with the membrane in an annexin A2–dependent manner. *J Cell Biol.* (2011) 192:647–61. doi: 10.1083/jcb.201007161
4. Chen J, Kanai Y, Cowan NJ, Hirokawa N. Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. *Nature* (1992) 360:674–7. doi: 10.1038/360674a0
5. Brandt R, Léger J, Lee G. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *J Cell Biol.* (1995) 131:1327–40. doi: 10.1083/jcb.131.5.1327
6. Zaidi T, Novak M, Grundke-Iqbal I, Iqbal K. Hyperphosphorylation induces self-assembly of τ into tangles of paired helical filaments/straight filaments. *Proc. Natl Acad Sci USA.* (2001) 98:6923–8. doi: 10.1073/pnas.121119298
7. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del TK. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol.* (2006) 112:389–404. doi: 10.1007/s00401-006-0127-z
8. Jones DR, Moussaud S, McLean P. Targeting heat shock proteins to modulate α-synuclein toxicity. *TherAdv Neurol Disord.* (2014) 7:33. doi: 10.1177/1756285613493469
9. Arai T, Ikeda K, Akiyama H, Shikamoto Y, Tsuchiya K, Yagishita S, et al. Distinct isoforms of tau aggregated in neurons and glial cells in brains of patients with Pick's disease, corticobasal degeneration and progressive supranuclear palsy. *Acta Neuropathol.* (2001) 101:167–73.
10. Armstrong RA. Cairns NJ. Spatial patterns of the tau pathology in progressive supranuclear palsy. *Neurol Sci.* (2013) 34:337–44. doi: 10.1007/s10072-012-1006-0
11. Jenner P, Morris HR, Robbins TW, Goedert M, Hardy J, Benshlomo Y, et al. Parkinson's disease – the debate on the clinical phenomenology, aetiology, pathology and pathogenesis. *J Parkinsons Dis.* (2013) 3:1. doi: 10.3233/JPD-130175
12. Cookson MR. THE BIOCHEMISTRY OF PARKINSON'S DISEASE*. *Ann Rev Biochem.* (2005) 74:29–52. doi: 10.1146/annurev.biochem.74.082803.133400
13. Davie CA. A review of Parkinson's disease. *Br Med Bull.* (2008) 86:109–27. doi: 10.1093/bmb/ldn013
14. Braak, H. Braak E. Pathoanatomy of Parkinson's disease. *J Neurol.* (2000) 247:II3–II10. doi: 10.1007/PL00007758
15. Wakabayashi K, Matsumoto K, Takayama K, Yoshimoto M, Takahashi H. NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. *Neurosci Lett.* (1997) 239:45–8. doi: 10.1016/S0304-3940(97)00891-4
16. Ishizawa T, Mattila P, Davies P, Wang D, Dickson DW. Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. *J Neuropathol Exp Neurol.* (2003) 62:389–97. doi: 10.1093/jnen/62.4.389
17. Schneider JA, Li JL, Li Y, Wilson RS, Kordower JH, Bennett DA. Substantia nigra tangles are related to gait impairment in older persons. *Digest World Core Med J.* (2006) 59:166–73. doi: 10.1002/ana.20723
18. Wills J, Jones J, Haggerty T, Duka V, Joyce JN, Sidhu A. Elevated tauopathy and alpha-synuclein pathology in postmortem Parkinson's disease brains with and without dementia. *Exp Neurol.* (2010) 225:210–8. doi: 10.1016/j.expneurol.2010.06.017
19. Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA. Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron.* (1989) 3:519.
20. Hogg M, Grujic ZM, Baker M, Demirci S, Guillozet AL, Sweet AP, et al. The L266V tau mutation is associated with frontotemporal dementia and Pick-like 3R and 4R tauopathy. *Acta Neuropathol.* (2003) 106:323–36. doi: 10.1007/s00401-003-0734-x
21. Kumaran R, Kingsbury A, Coulter I, Lashley T, Williams D, de Silva R, et al. DJ-1 (PARK7) is associated with 3R and 4R tau neuronal and glial inclusions in neurodegenerative disorders. *Neurobiol Dis.* (2007) 28:122. doi: 10.1016/j.nbd.2007.07.012
22. Flament S, Delacourte A, Verny M, Hauw JJ, Javoyagid F. Abnormal Tau proteins in progressive supranuclear palsy. Similarities and differences with the neurofibrillary degeneration of the Alzheimer type. *Acta Neuropathol.* (1991) 81:591–6. doi: 10.1007/BF00296367
23. Poorkaj P, Raskind WH, Leverenz JB, Matsushita M, Zabetian CP, Samii A, et al. A novel X-linked 4-repeat tauopathy with parkinsonism and spasticity. *Move Disord..* (2010) 25:1409–17. doi: 10.1002/mds.23085.
24. King ME, Gamblin TC, Kuret J, Binder LI. Differential assembly of human tau isoforms in the presence of arachidonic acid. *J Neurochem.* (2000) 74:1749–57. doi: 10.1046/j.1471–4159.2000.0741749.x
25. Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A. Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. *Proc Natl Acad Sci USA.* (1988) 85:4051–5. doi: 10.1073/pnas.85.11.4051
26. Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat Genet.* (2014) 46:989–93. doi: 10.1038/ng.3043
27. Almos PZ, Horváth S, Czibula A, Raskó I, Sipos B, Bihari P, et al. H1 tau haplotype-related genomic variation at 17q21.3 as an Asian heritage of the European Gypsy population. *Heredity* (2008) 101:416–9. doi: 10.1038/hdy.2008.70

28. Zhang CC, Zhu JX, Wan Y, Tan L, Wang HF, Yu JT, et al. Meta-analysis of the association between variants in MAPT and neurodegenerative diseases. *Oncotarget* (2017) 8:44994–5007. doi: 10.18632/oncotarget.16690
29. Goedert, M. Spillantini MG. Tau mutations in frontotemporal dementia FTDP-17 and their relevance for Alzheimer's disease. *Biochimica Et Biophysica Acta* (2000) 1502:110–21.
30. Lee VM, Goedert M, Trojanowski JQ. Neurodegenerative tauopathies. *Ann Rev Neurosci.* (2001) 24:1121–59. doi: 10.1146/annurev.neuro.24.1.1121
31. Nacharaju P, Lewis J, Easson C, Yen S, Hackett J, Hutton M, et al. Accelerated filament formation from tau protein with specific FTDP-17 missense mutations. *Febs Lett.* (1999) 447:195.
32. Peck A, Sargin ME, Lapointe NE, Rose K, Manjunath BS, Feinstein SC, et al. Tau isoform-specific modulation of kinesin-driven microtubule gliding rates and trajectories as determined with tau-stabilized microtubules. *Cytoskeleton* (2011) 68:44–55. doi: 10.1002/cm.20494
33. Lei P, Ayton S, Finkelstein DI, Spoerri L, Ciccotosto GD, Wright DK, et al. Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export. *Nat Med.* (2012) 18:291–5. doi: 10.1038/nm.2613
34. Ouchi H, Toyoshima Y, Tada M, Oyake M, Aida I, Tomita I, et al. Pathology and sensitivity of current clinical criteria in corticobasal syndrome. *Move Disord.* (2014) 29:238–44. doi: 10.1002/mds.25746
35. Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat Med.* (2008) 14:501–3. doi: 10.1038/nm1746
36. Clavaguera F, Tolnay M, Goedert M. The prion-like behavior of assembled Tau in transgenic mice. *Cold Spring Harb Perspect Med.* (2017) 7:a024372. doi: 10.1101/cshperspect.a024372
37. Lasagnareeves CA, Castillocarranza DL, Sengupta U, Guerreromunoz MJ, Kiritoshi T, Neugebauer V, et al. Alzheimer brain-derived tau oligomers propagate pathology from endogenous tau. *Sci Rep.* (2012) 2:700. doi: 10.1038/srep00700
38. Stancu IC, Vasconcelos B, Ris L, Wang P, Villers A, Peeraer E, et al. Templated misfolding of Tau by prion-like seeding along neuronal connections impairs neuronal network function and associated behavioral outcomes in Tau transgenic mice. *Acta Neuropathol.* (2015) 129:875–94. doi: 10.1007/s00401-015-1413-4
39. Tolnay, M. Clavaguera F. Argyrophilic grain disease: a late-onset dementia with distinctive features among tauopathies. *Neuropathology* (2004) 24:269–83. doi: 10.1111/j.1440-1789.2004.00591.x
40. Clavaguera, F, Akatsu, H, Fraser G, Crowther RA, Frank S, Hench J, et al. Brain homogenates from human tauopathies induce tau inclusions in mouse brain. *Proc Natl Acad Sci USA.* (2013) 110:9535–40. doi: 10.1073/pnas.1301175110
41. Sanders DW, Kaufman SK, Devos SL, Sharma AM, Mirbaha H, A. Li, et al. Distinct tau prion strains propagate in cells and mice and define different tauopathies. *Neuron* (2014) 82:1271–88. doi: 10.1016/j.neuron.2014.04.047
42. Cisbani G, Maxan A, Kordower JH, Planel E, Freeman TB, Cicchetti F. Presence of tau pathology within foetal neural allografts in patients with Huntington's and Parkinson's disease. *Brain A J Neurol.* (2017) 140:2982–92. doi: 10.1093/brain/awx255
43. Chohan MO, Haque N, Alonso A, Elakkad E, Grundkeiqbal I, Grover A, et al. Hyperphosphorylation-induced self assembly of murine tau: a comparison with human tau. *J Neural Trans.* (2005) 112:1035–47. doi: 10.1007/s00702-004-0241-9
44. Duka V, Lee JH, Credle J, Wills J, Oaks A, Smolinsky C, et al. Identification of the sites of tau hyperphosphorylation and activation of tau kinases in synucleinopathies and Alzheimer's diseases. *PLoS ONE* (2013) 8:e75025. doi: 10.1371/journal.pone.0075025
45. Hanger DP, Anderton BH, Noble W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends Mol Med.* (2009) 15:112–9. doi: 10.1016/j.molmed.2009.01.003
46. Noble DW, Pooler AM, Hanger DP. Advances in tau-based drug discovery. *Expert Opin Drug Discovery* (2011) 6:797–810. doi: 10.1517/17460441.2011.586690
47. L'Episcopo F, Tirolo C, Testa N, Caniglia S, Morale MC, Serapide MF, et al. Wnt/beta-catenin signaling is required to rescue midbrain dopaminergic progenitors and promote neurorepair in ageing mouse model of Parkinson's disease. *Stem Cells* (2014) 32:2147–63. doi: 10.1002/stem.1708
48. Takahashi M, Iseki E, Kosaka K. Cyclin-dependent kinase 5 (Cdk5) associated with Lewy bodies in diffuse Lewy body disease. *Brain Res.* (2000) 862:253–6.
49. Sun XY, Wei YP, Xiong Y, Wang XC, Xie AJ, Wang XL, et al. Synaptic released zinc promotes tau hyperphosphorylation by inhibition of protein phosphatase 2A (PP2A). *J Biol Chem.* (2012) 287:11174–82. doi: 10.1074/jbc.M111.309070
50. Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur J Neurosci.* (2005) 22:1942–5. doi: 10.1111/j.1460-9568.2005.04391.x
51. Park HJ, Lee KW, Park ES, S. Oh, Yan R, Zhang J, et al. Dysregulation of protein phosphatase 2A in parkinson disease and dementia with lewy bodies. *Ann Clin Transl Neurol.* (2016) 3:769–80. doi: 10.1002/acn3.337
52. Alonso AC, Grundkeiqbal I, Iqbal K. Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nat Med.* (1996) 2:783–7.
53. Arima K, Hirai S, Sunohara N, Aoto K, Izumiyama Y, Uéda K, et al. Cellular co-localization of phosphorylated tau- and NACP/alpha-synuclein-epitopes in lewy bodies in sporadic Parkinson's disease and in dementia with Lewy bodies. *Brain Res.* (1999) 843:53–61.
54. Yanagawa H, Chung SH, Ogawa Y, Sato K, Shibata-Seki T, Masai J, et al. Protein anatomy: C-tail region of human tau protein as a crucial structural element in Alzheimer's paired helical filament formation *in vitro*. *Biochemistry* (1998) 37:1979–88. doi: 10.1021/bi9724265
55. Liu F, B. Li, Tung EJ, Grundkeiqbal I, Iqbal K, Gong CX. Site-specific effects of tau phosphorylation on its microtubule assembly activity and self-aggregation. *Eur J Neurosci* (2007) 26:3429–36.doi: 10.1111/j.1460-9568.2007.05955.x
56. Xu L, Zheng J, Margittai M, Nussinov R, Ma B. How does hyperphopsphorylation promote tau aggregation and modulate filament structure and stability? *Acs Chem Neurosci.* (2016) 7:565–75. doi: 10.1021/acschemneuro.5b00294
57. Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, Van EJ, et al. Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. *Cell* (2010) 142:387–97. doi: 10.1016/j.cell.2010.06.036
58. Zhou L, McInnes J, Wierda K, Holt M, Herrmann AG, Jackson RJ, et al. Tau association with synaptic vesicles causes presynaptic dysfunction. *Nat Commun.* (2017) 8:1–13. doi: 10.1038/ncomms15295
59. Jaworski T, Kügler S, Leuven FV. Modeling of tau-mediated synaptic and neuronal degeneration in alzheimer's disease. *Int J Alzheimer's Dis.* (2010) 2010:573138. doi: 10.4061/2010/573138
60. Malchiodi-Albedi F, Petrucci TC, Picconi B, Iosi F, Falchi M. Protein phosphatase inhibitors induce modification of synapse structure and tau hyperphosphorylation in cultured rat hippocampal neurons. *J Neurosci Res.* (2015) 48:425–38. doi: 10.1002/(SICI)1097-4547(19970601)48:5<425::AID-JNR4>3.0.CO;2-G
61. Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van SM, et al. Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat Genet.* (2000) 25:402–5. doi: 10.1038/78078
62. Ittner LM, Fath T, Ke YD, Bi M, Eersel JV, Li KM, et al. Parkinsonism and impaired axonal transport in a mouse model of frontotemporal dementia. *Proc Natl Acad Sci USA.* (2008) 105:15997–6002. doi: 10.1073/pnas.0808084105
63. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. Alpha-Synuclein locus triplication causes Parkinson's disease. *Science* (2003) 302:841.doi: 10.1126/science.1090278
64. Engelender S. Ubiquitination of alpha-synuclein and autophagy in Parkinson's disease. *Autophagy* (2008) 4:372–4.
65. Yamaguchi K, Cochran EJ, Murrell JR, Polymeropoulos MH, Shannon KM, Crowther RA, et al. Abundant neuritic inclusions and microvacuolar changes in a case of diffuse Lewy body disease with the A53T mutation in the alpha-synuclein gene. *Acta Neuropathol.* (2005) 110:298–305. doi: 10.1007/s00401-005-1042-4
66. Duda JE, Giasson BI, Mabon ME, Miller DC, Golbe LI, Lee VM, et al. Concurrence of alpha-synuclein and tau brain pathology in the Contursi kindred. *Acta Neuropathol.* (2002) 104:7–11. doi: 10.1007/s00401-002-0563-3
67. Haggerty T, Credle J, Rodriguez O, Wills J, Oaks AW, Masliah E, et al. Hyperphosphorylated Tau in an α-synuclein-overexpressing transgenic

model of Parkinson's disease. *Eur J Neurosci.* (2011) 33:1598–610. doi: 10.1111/j.1460-9568.2011.07660.x
68. Lasagnareeves CA, Castillocarranza DL, Guerreromuñoz MJ, Jackson GR, Kayed R. Preparation and characterization of neurotoxic tau oligomers. *Biochemistry* (2010) 49:10039–41. doi: 10.1021/bi1016233
69. Waxman EA, Giasson BI. Induction of intracellular tau aggregation is promoted by alpha-synuclein seeds, and provides novel insights into the hyperphosphorylation of tau. *J Neurosci Offic J Soc Neurosci.* (2011) 31:7604–18. doi: 10.1523/JNEUROSCI.0297-11.2011
70. Benussi L, Ghidoni R, Paterlini A, Nicosia F, Alberici AC, Signorini S, et al. Interaction between tau and alpha-synuclein proteins is impaired in the presence of P301L tau mutation. *Exp Cell Res.* (2005) 308:78–84. doi: 10.1016/j.yexcr.2005.04.021
71. Sengupta U, Guerrero-Muñoz MJ, Castillo-Carranza DL, Lasagna-Reeves CA, Gerson JE, Paulucci-Holthauzen AA, et al. Pathological interface between oligomeric alpha-synuclein and tau in synucleinopathies. *Biol Psychiatry* (2015) 78:672–83. doi: 10.1016/j.biopsych.2014.12.019
72. Guo JL, Covell DJ, Daniels JP, Iba M, Stieber A, Zhang B, et al. Distinct alpha-synuclein strains differentially promote tau inclusions in neurons. *Cell* (2013) 154:103–17. doi: 10.1016/j.cell.2013.05.057
73. Simónsánchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, et al. Genome-Wide Association Study reveals genetic risk underlying Parkinson's disease. *Nat Genet.* (2009) 41:1308–12. doi: 10.1038/ng.487
74. Jensen PH, Hager H, Nielsen MS, Hojrup P, Gliemann J, Jakes R. Alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. *J Biol Chem.* (1999) 274:25481–9.
75. Wills J, Credle J, Haggerty T, Lee JH, Oaks AW, Sidhu A. Tauopathic changes in the striatum of A53T α-synuclein mutant mouse model of Parkinson's Disease. *PLoS ONE* (2011) 6:e17953. doi: 10.1371/journal.pone.0017953
76. Duka T, Duka V, Joyce JN, Sidhu A. Alpha-Synuclein contributes to GSK-3beta-catalyzed Tau phosphorylation in Parkinson's disease models. *FASEB J.* (2009) 23:2820–30. doi: 10.1096/fj.08-120410
77. Li B, Ryder J, Su Y, Moore SA Jr, Liu F, Solenberg P, et al. Overexpression of GSK3betaS9A resulted in tau hyperphosphorylationand morphology reminiscent of pretangle-like neurons in the brainof PDGSK3beta transgenic mice. *Transgen Res.* (2004) 13:385–96. doi: 10.1023/B:TRAG.0000040039.44899.6f
78. Credle JJ, George JL, Wills J, Duka V, Shah K, Lee YC, et al. GSK-3beta dysregulation contributes to parkinson's-like pathophysiology with associated region-specific phosphorylation and accumulation of tau and alpha-synuclein. *Cell Death Differentiat.* (2015) 22:838–51. doi: 10.1038/cdd.2014.179
79. Duka, T. and Sidhu A. The neurotoxin, MPP +, induces hyperphosphorylation of Tau, in the presence of alpha- Synuclein, in SH-SY5Y neuroblastoma cells. *Neurotoxicity Res.* (2006) 10:1–10. doi: 10.1007/BF03033329
80. Duka T, Rusnak M, Drolet RE, Duka V, Wersinger C, Goudreau JL, et al. Alpha-synuclein induces hyperphosphorylation of Tau in the MPTP model of parkinsonism. *FASEB J.* (2006) 20:2302–12. doi: 10.1096/fj.06-6092com
81. Roy, B. Jackson GR. Interactions between Tau and alpha-synuclein augment neurotoxicity in a Drosophila model of Parkinson's disease. *Hum Mol Genet.* (2014) 23:3008–23. doi: 10.1093/hmg/ddu011
82. Martinez A. Phosphorylation of tau and alpha-synuclein in synaptic-enriched fractions of the frontal cortex in Alzheimer's disease, and in Parkinson's disease and related alpha-synucleinopathies. *Neuroscience* (2008) 152:913–23. doi: 10.1016/j.neuroscience.2008.01.030

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership