Inhibition of Neuronal Mitochondrial Complex I or Lysosomal Glucocerebrosidase is associated with Increased Dopamine and Serotonin Turnover

Carmen de la Fuente [a], Derek Burke [b], Simon Eaton [c] and Simon J Heales [b,d,*]

[a] ICH Centre for Translational Omics, Genetic and Genomic Medicine Development Bio & Cancer Programme, UCL GOS Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK. Email address: c.barrign@ucl.ac.uk

[b] ICH Centre for Translational Omics, Genetic and Genomic Medicine Development Bio & Cancer Programme, UCL GOS Institute of Child Health and Chemical Pathology, Great Ormond Street Hospital, London, WC1N 3JH, UK. Email address: derek.burke@gosh.nhs.uk

[c] ICH Development Biology and Cancer Programme, UCL GOS Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK. Email address: s.eaton@ucl.ac.uk

[d] ICH Centre for Translational Omics, Genetic and Genomic Medicine Development Bio & Cancer Programme, UCL GOS Institute of Child Health and Chemical Pathology, Great Ormond Street Hospital, London, WC1N 3JH, UK.

Email address: s.heales@ucl.ac.uk

*Corresponding author: Prof Simon J R Heales

Current address: Department of Chemical Pathology, Great Ormond Street Hospital, Great Ormond Street London, WC1N 1EH. E-mail address: s.heales@ucl.ac.uk (S.J.R. Heales).

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder caused by loss of dopaminergic and serotoninergic signalling. A number of pathogenic mechanisms have been implicated including loss of mitochondrial function at the level of complex I, and lysosomal metabolism at the level of lysosomal glucocerebrosidase (GBA1). In order to investigate further the potential involvement of complex I and GBA1 in PD, we assessed the impact of loss of respective enzyme activities upon dopamine and serotonin turnover. Using SH-SY5Y cells, complex I deficiency was modelled by using rotenone whilst GBA1 deficiency was modelled by the use of conduritol B epoxide (CBE). Dopamine, its principal metabolites, and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the extracellular medium were quantified by HPLC. Inhibition of complex I significantly increased extracellular concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-HIAA. Comparable results were observed with CBE. These results suggest increased monoamine oxidase activity and provide evidence for involvement of impaired complex I or GBA1 activity in the dopamine/serotonin deficiency seen in PD. Use of extracellular media may also permit relatively rapid assessment of dopamine/serotonin metabolism and permit screening of novel therapeutic agents.

Keywords: Parkinson disease, complex I, glucocerebrosidase, dopamine metabolism, monoamine oxidase (maximum 6).

Chemical compounds: dopamine (PubChem CID: 681), DOPAC (PubChem CID: 547), HVA (PubChem CID: 1738), 5-HIAA (PubChem CID: 1826), 3-OMD (PubChem CID: 38853).

Abbreviations

3-MT, 3-methoxytyramine;

3-OMD, 3-*O*-methyldopa;

5-HIAA, 5-hydroxyindoleacetic acid;

5-HTP, 5-hydroxytryptophan;

AADC, aromatic amino acid decarboxylase;

ALDH, aldehyde dehydrogenase;

BH4, tetrahydrobiopterin;

CBE, conduritol B-epoxide;

COMT, catechol-*O*-methyl transferase;

DAT, dopamine transporter;

DOPAC, 3,4-dihydroxyphenylacetic acid;

ETC, electron transport chain;

GBA1, lysosomal glucocerebrosidase;

GSH, reduced glutathione;

HPLC, high performance liquid chromatography;

HVA, homovanillic acid;

L-DOPA, L-dihydroxyphenylalanine;

MAO, monoamine oxidase;

PD, Parkinson's disease;

PLP, pyridoxal phosphate;

TH, tyrosine hydroxylase;

TPH, tryptophan hydroxylase;

VMAT2, vesicular monoamine transporter 2;

1. Introduction

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder of dopaminergic neurons with characteristic symptoms that include tremor, rigidity and bradykinesia. Despite the considerable research that has been carried out in this field, the primary cause of PD is still unknown. Consequently, current therapies provide a temporary symptomatic relief by aiming to increase dopamine availability. These treatments vary depending on the stage of the disease, but gold standard treatment for PD is the dopamine precursor L-dihydroxyphenylalanine (L-DOPA) along with inhibitors of the dopamine degradation enzymes (Birkmayer et al., 1975).

Concerning putative mechanisms, loss of brain mitochondrial complex I activity has been reported in patients with PD (Schapira et al., 1990). Observations relating to the parkinsonian features associated with exposure to complex I inhibitors, such as rotenone and MPP+, provide credence for deficiency of complex I being a factor in PD. (Dauer and Przedborski, 2003). With regards to the consequences of reduced complex I activity, a number of mechanisms have been proposed, including reduced ATP formation and oxidative stress.

Neuronal complex I activity appears to exert particular control over mitochondrial ATP formation and losses of activity comparable to those seen in PD can be expected to lead to compromised brain energy metabolism (Davey and Clark, 1996). Furthermore, in the presence of oxidative stress, the magnitude of complex I loss required to inhibit ATP generation is significantly less (Davey et al., 1998). Oxidative stress occurs as a result of excessive generation of oxidising molecules, such as reactive oxygen and nitrogen species,

and/or loss of antioxidant capacity for scavengers such as alpha-tocopherol, ascorbate and reduced glutathione (GSH; Barker et al., 1996; Bolanos et al., 1995; Riederer et al., 1989). Inhibition of the mitochondrial respiratory chain is associated with increased generation of reactive oxygen species, a situation that appears to precede impairment of energy metabolism (Jacobson et al., 2005).

With regards to antioxidant status, decreased GSH levels have been reported in the brain of patients with PD (Perry et al., 1982; Sian et al., 1994). Furthermore, comparable losses have been reported in patients deemed to have pre-symptomatic PD (Jenner et al., 1992). Although GSH levels in the pre-PD patients are decreased to comparable level to those seen in PD, it is of note that complex I activity was not decreased (Jenner et al., 1992). This raises the possibility that GSH loss precedes and contributes to the loss of complex I activity, a hypothesis that is supported by a number of observations in both cellular and animal models (Barker et al., 1996; Bolanos et al., 1996; Heales et al., 1994; Heales et al., 2011).

Another potential mechanism implicated in PD pathogenesis is failure of lysosomal autophagy (Lynch-Day et al., 2012) leading to impaired protein processing, e.g. alpha-synuclein and formation of Lewy bodies that are characteristic of PD (Beyer, 2007). In post mitotic neurons, this failure of autophagy may also lead to the accumulation of defective mitochondria and provides a further potential mechanism for the compromised mitochondrial function in PD. Support for lysosomal involvement in PD comes from the study of patients with either homozygous mutations (Gaucher disease) or heterozygous mutations in lysosomal glucocerebrosidase (GBA1). Such individuals have a significantly increased risk of developing PD (Neumann et al., 2009). A number of mechanisms have been proposed to link the increased risk of PD with impairment of GBA1. Amongst these are

aberrant alpha-synuclein processing, oxidative stress and mitochondrial defects including loss of mitochondrial respiratory chain activity (Cleeter et al., 2013; Mazzulli et al., 2011; Osellame et al., 2013; Sidransky and Lopez, 2012). The link between PD and loss of GBA1 activity is further evidenced by reports of altered dopamine metabolites in CSF of patients with Gaucher/PD (Alonso-Canovas et al., 2010; Machaczka et al., 2012). In such studies, compromised serotonin metabolism was also implicated.

In view of the fact that loss of dopaminergic neurons is the ultimate consequence for all the possible mechanisms considered in PD, its metabolism will be briefly reviewed. Serotonin metabolism will also be considered, due to the possible involvement of this neurotransmitter in PD (Olivola et al., 2014) and the clear overlap between dopamine and serotonin metabolism.

Synthesis of dopamine commences with the transformation of the amino acid L-tyrosine into L-DOPA by the tetrahydrobiopterin (BH4)-dependent tyrosine hydroxylase (TH). This is then converted by aromatic amino acid decarboxylase (AADC), a pyridoxal phosphate (PLP) requiring enzyme, to dopamine **[Fig 1]**. L-DOPA that is not metabolised via AADC can be converted to 3-*O*-methyldopa (3-OMD) via catechol-*O*-methyl transferase (COMT) **[Fig 1]**. As dopamine is not stable at physiological pH, it is internalised and stored in synaptic vesicles by vesicular monoamine transporter 2 (VMAT2; Chaudhry et al., 2008). This transporter uses a proton gradient generated by the vacuolar-type ATPase proton pump. In these vesicles the pH is two units lower than in the cytosol, so dopamine does not spontaneously oxidise (Guillot and Miller, 2009). It has been proposed that TH, AADC and VMAT2 interact forming a complex to make dopamine internalisation in vesicles as efficient as possible (Cartier et al., 2010). When acting as a neurotransmitter, dopamine is released into the synaptic cleft and

binds to receptors. After its release, dopamine is removed from the synaptic cleft by the pre-synaptic neuronal terminal cells which capture the dopamine via the dopamine transporter (DAT; reviewed by Eriksen et al., 2010). The internalised dopamine can be either recycled or degraded. For recycling, dopamine is sequestered into synaptic vesicles by VMAT2. The non-recycled neurotransmitter is further metabolised via two parallel pathways. In the first route, monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH) metabolise dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC). Then, DOPAC is subsequently converted to homovanillic acid (HVA) by COMT. In the second route, COMT first transforms dopamine to 3-methoxytyramine (3-MT). HVA is then formed by the consecutive action of MAO and ALDH **[Fig 1]**.

Dopamine metabolism is closely related to serotonin synthesis and degradation, because both pathways share some enzymes **[Fig 1]**. Serotonin synthesis starts with the oxidation of L-tryptophan by tryptophan hydroxylase (TPH) and the cofactor BH4. This reaction is the rate limiting step in this pathway and produces 5-hydroxytryptophan (5-HTP). 5-HTP is decarboxylated by AADC with PLP as cofactor, synthesising serotonin. As with dopamine, serotonin has to be quickly degraded after its action. To accomplish this, MAO and ALDH transform serotonin into 5-hydroxyindoleacetic acid (5-HIAA), its final degradation metabolite.

In view of the clear link between PD and loss of mitochondrial and lysosomal function, in this study we have examined the effects of loss of complex I or GBA1 upon dopamine and serotonin metabolism. Extracellular media was evaluated to ascertain whether relatively rapid insight into the metabolism of these neurotransmitters could be achieved.

2. Materials and methods

2.1. Materials

All the chemicals were supplied by Sigma Aldrich (Poole, UK), Thermo Fisher Scientific UK Ltd (Loughborough, UK) and VWR International Ltd (Lutterworth, UK). C18HS column, 250 mm × 4.6 mm with a pore size of 100A and a particle size of 5um, was purchased from Kromatek (Dunmow, UK).

2.2. Tissue culture and L-DOPA treatment

Undifferentiated SH-SY5Y cells obtained from the European Collection of Cell Cultures (Public Health England, Salisbury, UK) were used in this study, as they have been widely used for PD research (Alberio et al., 2014; Hong-rong et al., 2010; Khwanraj et al., 2015; Lopes et al., 2010; Noelker et al., 2015). SH-SY5Y cells between passage 21 and 24 were seeded at a density of 6×10^3 cells/cm^2 with DMEM/F-12 supplemented with 10% FBS and 2 mM L-glutamine. Cells were grown at 37°C in a 5% CO_2 incubator. On day 6 after seeding, SH-SY5Y cells were treated with rotenone at concentration of 100 nM (Aylett et al., 2013). In parallel, SH-SY5Y cells were seeded in supplemented DMEM/F-12 media containing 100 µM conduritol B-epoxide (CBE), a selective and irreversible GBA1 inhibitor. On day 7 after seeding, the cells were exposed to L-DOPA, the immediate precursor of dopamine, to assess dopamine metabolism **[Fig 1]** (Woodard et al., 2014). SH-SY5Y cells were treated for 1 hour with 100 µM L-DOPA in DMEM/F-12 phenol red free media, supplemented with 10% of FBS. Media was collected and mixed with perchloric acid to a final concentration of 0.4 M. Samples were incubated for 10 min at 4°C in the dark and centrifuged at 12000×*g* for 5 min at 4°C. Supernatant was collected and analysed by high performance liquid chromatography

(HPLC) coupled to an electrochemical detector. The appearance of dopamine and its metabolites in the cell culture medium was considered as a reflection of cellular dopamine metabolism. To assess serotonin turnover, the MAO product 5-HIAA was also quantified in the cell culture medium.

In order to confirm that L-DOPA was being converted to dopamine through AADC, cells were incubated with 10 µM 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015) for 24 hours before L-DOPA treatment.

2.3. High performance liquid chromatography

Dopamine, 3-OMD, HVA, DOPAC and 5-HIAA were quantified using reverse-phase HPLC with an electrochemical detector, following the method of Allen et al. (2013) with some modifications for the contemporary detection of all five compounds. The stationary phase was maintained at 27°C. The mobile phase (flow rate of 1.5 ml/min) was aqueous with 16% methanol, 20 mM sodium acetate trihydrate (pH 3.45), 12.5 mM citric acid monohydrate, 0.1 mM EDTA sodium and 3.35 mM 1-octanesulfonic acid. 50 µl of each sample were injected into the system. At voltage of 450 mV for the detector electrode and 20 mV for the screening electrode, sample quantification was calculated against an external standard mixture of 500 nM of all five compounds made in ultrapure water with few drops of 12 M hydrochloric acid. Peak area from the electrochemical detector was quantified with EZChrom Elite™ chromatography data system software, version 3.1.7 (JASCO UK Ltd., Great Dunmow, UK).

2.4. GBA1 Activity

GBA1 activity was measured using the synthetic substrate 4-methylumbelliferone-b-glucopyranoside as previously described (Burke et al., 2013).

2.5. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Between 5 and 16 independent cell culture preparations were utilised. Individual comparisons of means were made using unpaired Student's *t*-test and multiple comparisons of means was made using one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism software (GraphPad Software INC. San Diego, CA, USA). In all cases $p<0.05$ was considered to be significant.

3. Results

In the absence of L-DOPA treatment, it was not possible to detect dopamine and its metabolites in the cell culture medium (data not shown). However, treatment with L-DOPA for 1 hour permitted clear quantification **[Fig 2]**. NSD-1015 treatment (n=5) significantly decreased dopamine formation confirming L-DOPA conversion to dopamine via AADC (control mean 953±142.3 nmol/L vs. NSD-1015 treated mean 135±34.8 nmol/L; $p<0.01$). In contrast to dopamine metabolism, 5-HIAA was detectable in the cell culture medium and did not require addition of a precursor.

Rotenone was used to model of complex I deficiency. A concentration of 100 nM was used as this has recently been demonstrated by our group to create an approximate 50% loss of

complex I activity in SH-SY5Y cells (Aylett et al., 2013). Although 3-OMD and dopamine concentrations were unaffected by rotenone treatment **[Fig 2]**, a significant increase in the concentration of DOPAC was observed **[Fig 2]**. On the contrary, HVA was significantly lower when compared with the control cells **[Fig 2]**. Concerning serotonin, 5-HIAA was also significantly increased in the cell culture medium of the rotenone treated cells **[Fig 2]**.

Treatment of the cells with CBE resulted in abolition of GBA1 activity (control = 280 nmol/hr/mg of protein vs CBE treated = 2 nmol/hr/mg protein). GBA1 inhibition, similarly to rotenone, resulted in a significant decrease in HVA and a significant increase in 5-HIAA compared with control cells **[Fig 2]**. However, GBA1 inhibition led to a significantly lower concentration of 3-OMD compared with complex I inhibition and a marked elevation in DOPAC compared with both controls and complex I inhibition **[Fig 2].**

4. Discussion

PD is a neurodegenerative condition affecting dopaminergic and serotonergic systems (Dauer and Przedborski, 2003; Olivola et al., 2014). In view of this and the evidence for compromised mitochondrial and lysosomal function in PD, we studied the effects of loss of complex I or GBA1 activity on dopamine and serotonin turnover. To achieve this we analysed the cell culture medium bathing SH-SY5Y cells neural cell line derived from neuroblastoma that has been widely used in PD studies (Hong-rong et al., 2010; Khwanraj et al., 2015; Lopes et al., 2010). Cell culture medium was considered as a potential means for rapid assessment of cellular dopamine and serotonin turnover, i.e. analogous to the use of CSF to evaluate CNS dopamine and serotonin metabolism (Kurian et al., 2011).

Although SH-SY5Y cells have the potential to become dopaminergic, this cell line does not synthesise or store appreciable amounts of intracellular dopamine under basal conditions (Balasooriya and Wimalasena, 2007). This is also reflected here by the lack of detectable extracellular dopamine, DOPAC and HVA in cells not incubated with L-DOPA. However, as expected, provision of L-DOPA resulted in an AADC-dependent appearance of dopamine and its metabolites in the extracellular medium. In contrast, endogenous serotonin metabolism appeared able to proceed, as evidenced by the detection of 5-HIAA in the cell culture medium. This suggests that serotonin precursors are available under basal conditions and is consistent with the reported ability of these cells to synthesise serotonin (Islahudin et al., 2014).

In order to model the partial complex I deficiency reported in PD, cells were pre-treated with the established complex I inhibitor, rotenone. Using this approach, complex I inhibition had no effect on levels of dopamine or 3-OMD. This suggests that rotenone is not having generalised effects resulting, e.g. from loss of cell viability, in non-specific discharge of cellular contents into the extracellular medium. However, when considering dopamine metabolites, DOPAC was significantly increased and HVA decreased. Whilst the mechanism for this differential effect is not known, inspection of the metabolic pathway for dopamine **[Fig 1]** could point to an increase in MAO activity and/or a reduction in COMT activity. Further insight into the potential mechanism may come by considering the serotonin metabolite, 5-HIAA. In rotenone treated cells, this metabolite was significantly elevated. Formation of 5-HIAA also occurs via MAO activity. Although further work is clearly required to ascertain whether these findings are occurring as a result of increased MAO activity, this suggestion is supported by the report of increased MAO activity in post mortem PD brain

and intracellular studies following rotenone treatment (Birkmayer et al., 1975; Sai et al., 2008).

MAO is located in the outer mitochondrial membrane and exists in two isoforms, MAO-A and MAO-B (Youdim and Bakhle, 2006). Whilst the different isoforms have different abilities to act upon substrates, there is overlap (Youdim and Bakhle, 2006). When considering MAO activity, it is important to note that a by-product of its activity is formation of hydrogen peroxide. Generation of the latter, in the presence of transition metals, such as iron, can generate highly oxidising species such as the hydroxyl radical **[Fig 3]**. This is of interest as iron is also reported to accumulate within the brain of PD patients (Riederer et al., 1989). Thus increased MAO activity may contribute to the oxidative stress observed in PD. However, further research is required to explain why such an increase might occur and, as implied here, in response to loss of complex I activity.

Within neuronal cells, complex I activity may exert particular control over mitochondrial ATP generation (Davey et al., 1998). Thus the loss of complex I, as created here, may limit neuronal ATP availability. Since loading of monoamines, such as dopamine, into vesicles via VMAT2, is ATP dependent (Chaudhry et al., 2008), it is possible that this process could be compromised **[Fig 3]**. This is supported by reports of impaired intracellular VMAT2 activity in rotenone treated cells (Watabe and Nakaki, 2008). Whilst a scenario whereby dopamine is prevented from entering vesicles could explain increased DOPAC formation, the observed decreased HVA concentration requires explanation. Studies on the effects of complex I inhibition on COMT activity are therefore required, particularly as CSF HVA levels are reported to be decreased in PD and Gaucher patients (Hartikainen et al., 1992).

In view of the fact that assessment of extracellular media may provide reliable insight into dopamine and serotonin metabolism this approach was extended to the evaluation of a GBA1 deficiency. CBE has been extensively used to model Gaucher disease (Cleeter et al., 2013; Noelker et al., 2015; Ridley et al., 2013). Using this inhibitor, our findings again point to increased dopamine and serotonin catabolism. As for rotenone above, the lack of any significant effect upon dopamine release suggests that CBE is also not causing generalised release of intracellular contents. Concerning the mechanism for the observed increased dopamine and serotonin catabolism, this is not known. Loss of mitochondrial respiratory chain function has been reported in both CBE treated SH-SY5Y cells and in a GBA1 deficient animal model (Cleeter et al., 2013; Manning-Bog et al., 2009; Osellame et al., 2013). In view of these reports and the comparable results between rotenone and CBE treated cells, further work is required to ascertain whether the CBE effects are occurring via secondary alterations in mitochondrial function. The finding that DOPAC levels achieved in the CBE model were markedly greater and 3-OMD lower to those observed with rotenone could point to additional mechanisms. Concerning this point, it should be noted that whilst CBE is a documented inhibitor of GBA1, off target effects have been reported (Ridley et al., 2013). These will also need to be considered with regards to identifying the mechanism(s) responsible for the results reported here.

5. Conclusion

We have created a model system to interrogate cellular dopamine and serotonin metabolism by assessment of extracellular media. Our results support previous intracellular findings around the use of rotenone to model PD and suggest the need to further consider

the potential role of MAO and serotonin in disease pathogenesis. Expanding the study to the potential link between GBA1 deficiency and PD, evidence has been provided to also implicate disruption of dopamine/serotonin and homeostasis. Finally, use of extracellular media could enable screening of potential therapeutic agents that are targeted to correct dopamine, serotonin, mitochondrial and lysosomal metabolism.

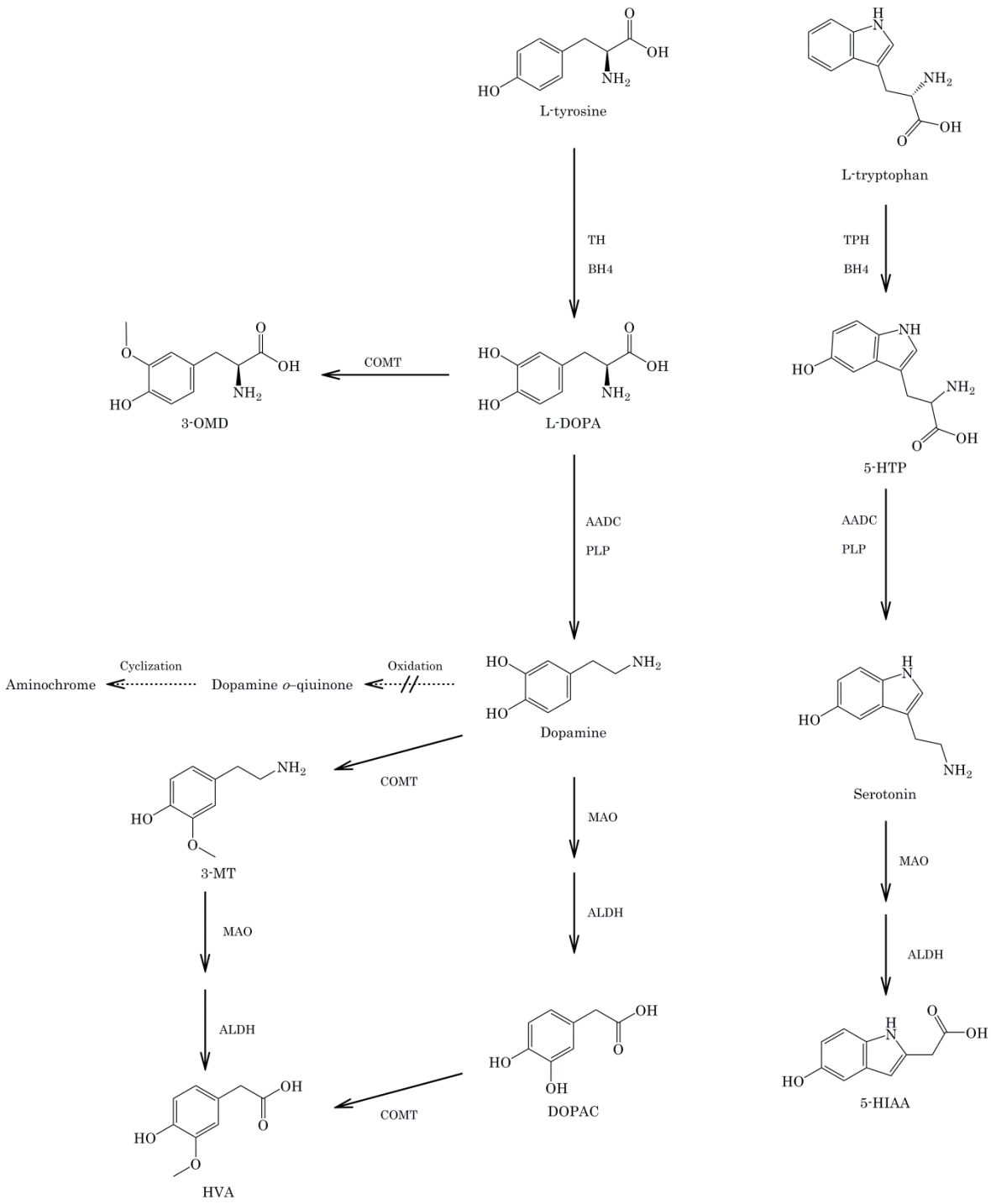
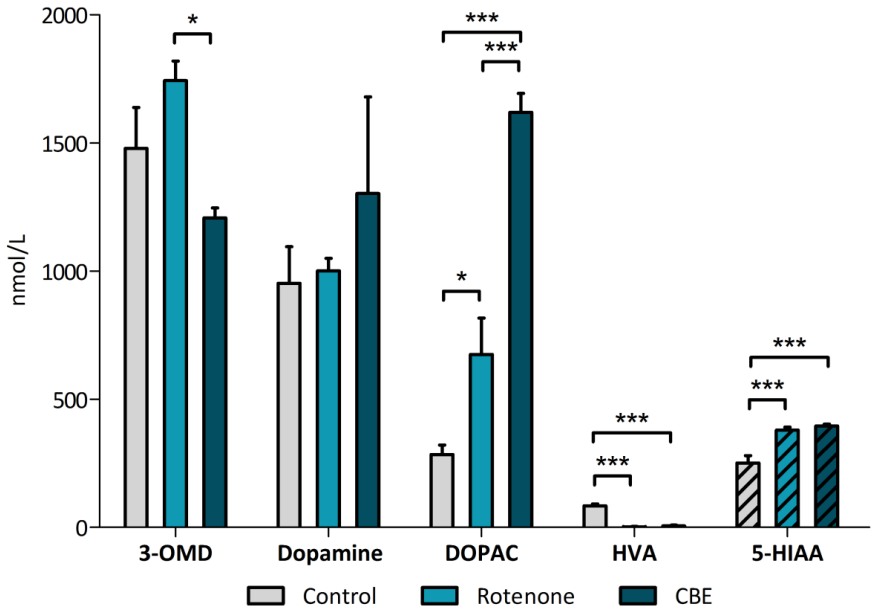


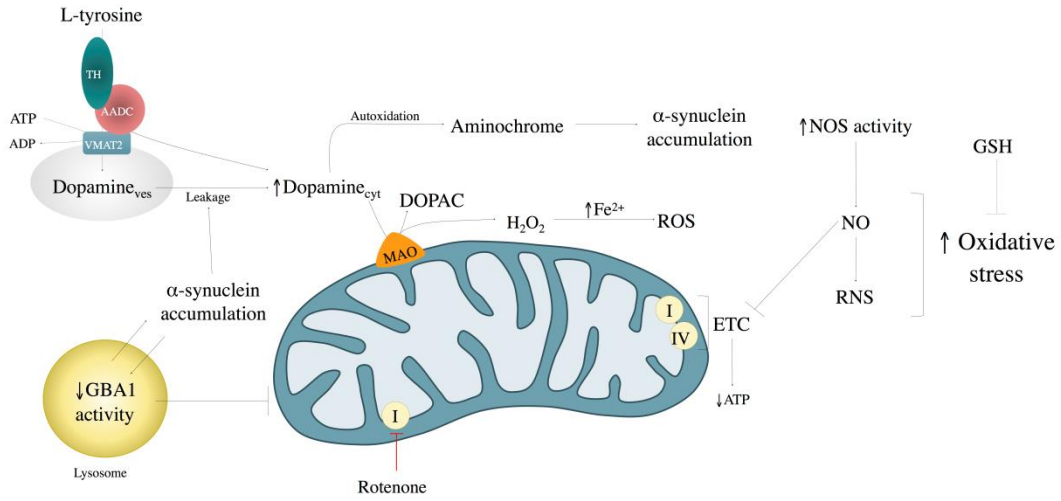
Figure 1: Dopamine and serotonin pathways. Synthesis of dopamine is catalysed by tyrosine hydroxylase (TH) and the cofactor tetrahydrobiopterin (BH4), producing L-dihydroxyphenylalanine (L-DOPA). L-DOPA is then transformed to dopamine by the aromatic L-amino acid decarboxylase (AADC) and its cofactor pyridoxal phosphate (PLP). Alternatively, L-DOPA can be transformed to 3-*O*-methyldopa (3-OMD) by catechol-*O*-methyl transferase

(COMT). Dopamine can be then catabolised by two pathways. In the first route, 3,4-dihydroxyphenylacetic acid (DOPAC) is produced by the serial action of monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH); to be further metabolised by COMT to produce the final product of the pathway, homovanillic acid (HVA). In the second route, dopamine is first metabolized by COMT, producing 3-methoxytyramine (3-MT). Then, 3-MT is transformed to HVA by MAO and ALDH. Serotonin pathway starts with the production of 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH), which also belongs to the BH4-dependent family. 5-HTP is transformed to serotonin by AADC and PLP. Finally, it is metabolised by MAO and ALDH producing 5-hydroxyindoleacetic acid (5-HIAA).



COLOR Figure 2: Release of dopamine and metabolites to the culture media by SH-SY5Y cells with impaired complex I and GBA1. Cells were treated with 100 nM rotenone for 24 hours to model complex I impairment (n=14). Separately, 100 µM CBE treatment was carried out for one week to model GBA1 deficiency (n=9). In both cases, along with control (n=16), cells

were treated with L-DOPA 100 µM for 1 hour on day 7 after seeding. Results are expressed as mean ± SEM; and statistics were determined by ANOVA followed by a Tukey's test (*p<0.05; **p<0.01; ***p<0.001).



COLOR Figure 3: Potential pathogenic mechanisms in PD. Impairment of the electron transport chain (ETC) might lead to an ATP deficient state and increased generation of ROS. Lower levels of ATP could result in dopamine accumulation in the cytosol, as dopamine internalisation into vesicles is an energy-dependent event (Chaudhry et al., 2008). This increase in cytosolic dopamine can then be then either catabolized (MAO) or autooxidised. The catabolism produces hydrogen peroxide (H_2O_2) that, in presence of metals, produces reactive oxygen species (ROS) that oxidize several cellular components (Aguirre et al., 2012). On the other hand, it has been described that autoxidation products, such as aminochrome, can damage complex I and stabilize alpha-synuclein (Aguirre et al., 2012; Munoz et al., 2012). Alpha-synuclein accumulation has been proposed to enhance dopamine leaking from vesicles, reinforcing the presence of dopamine in the cytosol (Beyer, 2007). As the most

common genetic risk factor in PD is lysosomal glucocerebrosidase (GBA1) mutation (Neumann et al., 2009), it has been proposed that the link between GBA1 and PD is alpha-synuclein. This is because decreased GBA1 activity reinforces alpha-synuclein accumulation (Mazzulli et al., 2011). Additionally loss of GBA1 may lead to a failure of mitophagy, further ROS production and contribute further to loss of mitochondrial function. It has also been described that enhanced nitric oxide synthase (NOS) activity, which produces nitric oxide (NO) along with other reactive nitrogen species (RNS), can ultimately inhibit the ETC (Bolanos et al., 1994), intensifying the ATP deficiency status. Both ROS and RNS increase the oxidative stress which, in healthy patients, is countered by antioxidant molecules such as reduced glutathione (GSH). However, GSH levels are decreased in PD brains (Sian et al., 1994). All this events would be jointly increasing oxidative stress leading to the dopaminergic neurodegeneration characteristic in PD.

Acknowledgments

Carmen de la Fuente has been funded by Marie Skłodowska-Curie Actions of the European Union's Seventh Framework Programme FP7 under REA grant agreement for “Training in Neurodegeneration, Therapeutics, Intervention and Neurorepair” project, n° 608381.

Simon Eaton is supported by Great Ormond Street Hospital NIHR Biomedical Research Centre and by Great Ormond Street Children’s Charity.

References

Aguirre, P., Urrutia, P., Tapia, V., Villa, M., Paris, I., Segura-Aguilar, J., Nunez, M.T., 2012. The dopamine metabolite aminochrome inhibits mitochondrial complex I and modifies the expression of iron transporters DMT1 and FPN1. Biometals 25, 795-803.

Alberio, T., Bondi, H., Colombo, F., Alloggio, I., Pieroni, L., Urbani, A., Fasano, M., 2014. Mitochondrial proteomics investigation of a cellular model of impaired dopamine homeostasis, an early step in Parkinson's disease pathogenesis. Mol Biosyst 10, 1332-1344.

Allen, G.F., Ullah, Y., Hargreaves, I.P., Land, J.M., Heales, S.J., 2013. Dopamine but not L-DOPA stimulates neural glutathione metabolism. Potential implications for Parkinson's and other dopamine deficiency states. Neurochem Int 62, 684-694.

Alonso-Canovas, A., Katschnig, P., Tucci, A., Carecchio, M., Wood, N.W., Edwards, M., Martinez Castrillo, J.C., Burke, D., Heales, S., Bhatia, K.P., 2010. Atypical parkinsonism with apraxia and supranuclear gaze abnormalities in type 1 Gaucher disease. Expanding the spectrum: case report and literature review. Mov Disord 25, 1506-1509.

Aylett, S.B., Neergheen, V., Hargreaves, I.P., Eaton, S., Land, J.M., Rahman, S., Heales, S.J., 2013. Levels of 5-methyltetrahydrofolate and ascorbic acid in cerebrospinal fluid are correlated: implications for the accelerated degradation of folate by reactive oxygen species. Neurochem Int 63, 750-755.

Balasooriya, I.S., Wimalasena, K., 2007. Are SH-SY5Y and MN9D Cell Lines Truly Dopaminergic? FASEB J 21.

Barker, J.E., Bolanos, J.P., Land, J.M., Clark, J.B., Heales, S.J., 1996. Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: implications for neuronal/astrocytic trafficking and neurodegeneration. Dev Neurosci 18, 391-396.

Beyer, K., 2007. Mechanistic aspects of Parkinson's disease: alpha-synuclein and the biomembrane. Cell Biochem Biophys 47, 285-299.

Birkmayer, W., Riederer, P., Youdim, M.B., Linauer, W., 1975. The potentiation of the anti akinetic effect after L-dopa treatment by an inhibitor of MAO-B, Deprenil. J Neural Transm 36, 303-326.

Bolanos, J.P., Heales, S.J., Land, J.M., Clark, J.B., 1995. Effect of Peroxynitrite on the Mitochondrial Respiratory Chain: Differential Susceptibility of Neurones and Astrocytes in Primary Cultures. Journal of Neurochemistry 64, 8.

Bolanos, J.P., Heales, S.J., Peuchen, S., Barker, J.E., Land, J.M., Clark, J.B., 1996. Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. Free Radic Biol Med 21, 995-1001.

Bolanos, J.P., Peuchen, S., Heales, S.J., Land, J.M., Clark, J.B., 1994. Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. J Neurochem 63, 910-916.

Burke, D.G., Rahim, A.A., Waddington, S.N., Karlsson, S., Enquist, I., Bhatia, K., Mehta, A., Vellodi, A., Heales, S., 2013. Increased glucocerebrosidase (GBA) 2 activity in GBA1 deficient mice brains and in Gaucher leucocytes. J Inherit Metab Dis 36, 869-872.

Cartier, E.A., Parra, L.A., Baust, T.B., Quiroz, M., Salazar, G., Faundez, V., Egana, L., Torres, G.E., 2010. A biochemical and functional protein complex involving dopamine synthesis and transport into synaptic vesicles. J Biol Chem 285, 1957-1966.

Chaudhry, F.A., Edwards, R.H., Fonnum, F., 2008. Vesicular neurotransmitter transporters as targets for endogenous and exogenous toxic substances. Annu Rev Pharmacol Toxicol 48, 277-301.

Cleeter, M.W., Chau, K.Y., Gluck, C., Mehta, A., Hughes, D.A., Duchen, M., Wood, N.W., Hardy, J., Mark Cooper, J., Schapira, A.H., 2013. Glucocerebrosidase inhibition causes mitochondrial dysfunction and free radical damage. Neurochem Int 62, 1-7.

Dauer, W., Przedborski, S., 2003. Parkinson's disease: mechanisms and models. Neuron 39, 889-909.

Davey, G.P., Clark, J.B., 1996. Threshold effects and control of oxidative phosphorylation in nonsynaptic rat brain mitochondria. J Neurochem 66, 1617-1624.

Davey, G.P., Peuchen, S., Clark, J.B., 1998. Energy thresholds in brain mitochondria. Potential involvement in neurodegeneration. J Biol Chem 273, 12753-12757.
Eriksen, J., Jorgensen, T.N., Gether, U., 2010. Regulation of dopamine transporter function by protein-protein interactions: new discoveries and methodological challenges. J Neurochem 113, 27-41.
Guillot, T.S., Miller, G.W., 2009. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. Mol Neurobiol 39, 149-170.
Hartikainen, P., Reinikainen, K.J., Soininen, H., Sirvio, J., Soikkeli, R., Riekkinen, P.J., 1992. Neurochemical markers in the cerebrospinal fluid of patients with Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis and normal controls. J Neural Transm Park Dis Dement Sect 4, 53-68.
Heales, S.J., Bolanos, J.P., Land, J.M., Clark, J.B., 1994. Trolox protects mitochondrial complex IV from nitric oxide-mediated damage in astrocytes. Brain Res 668, 243-245.
Heales, S.J., Menzes, A., Davey, G.P., 2011. Depletion of glutathione does not affect electron transport chain complex activity in brain mitochondria: Implications for Parkinson disease and postmortem studies. Free Radic Biol Med 50, 899-902.
Hong-rong, X., Lin-sen, H., Guo-yi, L., 2010. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J 123.
Islahudin, F., Tindall, S.M., Mellor, I.R., Swift, K., Christensen, H.E., Fone, K.C., Pleass, R.J., Ting, K.N., Avery, S.V., 2014. The antimalarial drug quinine interferes with serotonin biosynthesis and action. Sci Rep 4, 3618.
Jacobson, J., Duchen, M.R., Hothersall, J., Clark, J.B., Heales, S.J., 2005. Induction of mitochondrial oxidative stress in astrocytes by nitric oxide precedes disruption of energy metabolism. J Neurochem 95, 388-395.
Jenner, P., Dexter, D., Sian, J., Schapira, A.H., Marsden, C.D., 1992. Oxidative Stress as a Cause of Nigral Cell Death in Parkinson's Disease and Incidental Lewy Body Disease. Ann Neurol 32, 6.
Khwanraj, K., Phruksaniyom, C., Madlah, S., Dharmasaroja, P., 2015. Differential Expression of Tyrosine Hydroxylase Protein and Apoptosis-Related Genes in Differentiated and Undifferentiated SH-SY5Y Neuroblastoma Cells Treated with MPP(.). Neurol Res Int 2015, 734703.
Kurian, M.A., Gissen, P., Smith, M., Heales, S., Jr., Clayton, P.T., 2011. The monoamine neurotransmitter disorders: an expanding range of neurological syndromes. Lancet Neurol 10, 721-733.
Lopes, F.M., Schroder, R., da Frota, M.L., Jr., Zanotto-Filho, A., Muller, C.B., Pires, A.S., Meurer, R.T., Colpo, G.D., Gelain, D.P., Kapczinski, F., Moreira, J.C., Fernandes Mda, C., Klamt, F., 2010. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337, 85-94.
Lynch-Day, M.A., Mao, K., Wang, K., Zhao, M., Klionsky, D.J., 2012. The role of autophagy in Parkinson's disease. Cold Spring Harb Perspect Med 2, a009357.
Machaczka, M., Arce, M.P., Rucinska, M., Yoshitake, T., Kehr, J., Jurczak, W., Skotnicki, A.B., Mansson, J.E., Tylki-Szymanska, A., Svenningsson, P., 2012. A twelve-year follow-up study on a case of early-onset parkinsonism preceding clinical manifestation of Gaucher disease. JIMD Rep 3, 53-57.
Manning-Bog, A.B., Schule, B., Langston, J.W., 2009. Alpha-synuclein-glucocerebrosidase interactions in pharmacological Gaucher models: a biological link between Gaucher disease and parkinsonism. Neurotoxicology 30, 1127-1132.
Mazzulli, J.R., Xu, Y.H., Sun, Y., Knight, A.L., McLean, P.J., Caldwell, G.A., Sidransky, E., Grabowski, G.A., Krainc, D., 2011. Gaucher disease glucocerebrosidase and alpha-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell 146, 37-52.
Munoz, P., Huenchuguala, S., Paris, I., Segura-Aguilar, J., 2012. Dopamine oxidation and autophagy. Parkinsons Dis 2012, 920953.
Neumann, J., Bras, J., Deas, E., O'Sullivan, S.S., Parkkinen, L., Lachmann, R.H., Li, A., Holton, J., Guerreiro, R., Paudel, R., Segarane, B., Singleton, A., Lees, A., Hardy, J., Houlden, H., Revesz, T.,

Wood, N.W., 2009. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain 132, 1783-1794.
Noelker, C., Lu, L., Hollerhage, M., Vulinovic, F., Sturn, A., Roscher, R., Hoglinger, G.U., Hirsch, E.C., Oertel, W.H., Alvarez-Fischer, D., Andreas, H., 2015. Glucocerebrosidase deficiency and mitochondrial impairment in experimental Parkinson disease. J Neurol Sci 356, 129-136.
Olivola, E., Pierantozzi, M., Imbriani, P., Liguori, C., Stampanoni Bassi, M., Conti, M., D'Angelo, V., Mercuri, N.B., Stefani, A., 2014. Serotonin impairment in CSF of PD patients, without an apparent clinical counterpart. PLoS One 9, e101763.
Osellame, L.D., Rahim, A.A., Hargreaves, I.P., Gegg, M.E., Richard-Londt, A., Brandner, S., Waddington, S.N., Schapira, A.H., Duchen, M.R., 2013. Mitochondria and quality control defects in a mouse model of Gaucher disease--links to Parkinson's disease. Cell Metab 17, 941-953.
Perry, T.L., Godin, D.V., Hansen, S., 1982. Parkinson's Disease: A Disorder Due to Nigral Glutathione Deficiency? Neurosci Lett 33, 6.
Ridley, C.M., Thur, K.E., Shanahan, J., Thillaiappan, N.B., Shen, A., Uhl, K., Walden, C.M., Rahim, A.A., Waddington, S.N., Platt, F.M., van der Spoel, A.C., 2013. beta-Glucosidase 2 (GBA2) activity and imino sugar pharmacology. J Biol Chem 288, 26052-26066.
Riederer, P., Sofic, E., Rausch, W.D., Schmidt, B., Reynolds, G.P., Jellinger, K., Youdim, M.B., 1989. Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. J Neurochem 52, 515-520.
Sai, Y., Wu, Q., Le, W., Ye, F., Li, Y., Dong, Z., 2008. Rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism. Toxicol In Vitro 22, 1461-1468.
Schapira, A.H., Cooper, J.M., Dexter, D., Clark, J.B., Jenner, P., Marsden, C.D., 1990. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54, 823-827.
Sian, J., Dexter, D., Lees, A., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., Marsden, C.D., 1994. Alterations in Glutathione Lveles in Parkinson's Disease and Other Neurodegenerative Disorders Affecting Basal Ganglia. Ann Neurol 36, 8.
Sidransky, E., Lopez, G., 2012. The link between the GBA gene and parkinsonism. Lancet Neurol 11, 986-998.
Watabe, M., Nakaki, T., 2008. Mitochondrial complex I inhibitor rotenone inhibits and redistributes vesicular monoamine transporter 2 via nitration in human dopaminergic SH-SY5Y cells. Mol Pharmacol 74, 933-940.
Woodard, C.M., Campos, B.A., Kuo, S.H., Nirenberg, M.J., Nestor, M.W., Zimmer, M., Mosharov, E.V., Sulzer, D., Zhou, H., Paull, D., Clark, L., Schadt, E.E., Sardi, S.P., Rubin, L., Eggan, K., Brock, M., Lipnick, S., Rao, M., Chang, S., Li, A., Noggle, S.A., 2014. iPSC-derived dopamine neurons reveal differences between monozygotic twins discordant for Parkinson's disease. Cell Rep 9, 1173-1182.
Youdim, M.B., Bakhle, Y.S., 2006. Monoamine oxidase: isoforms and inhibitors in Parkinson's disease and depressive illness. Br J Pharmacol 147 Suppl 1, S287-296.

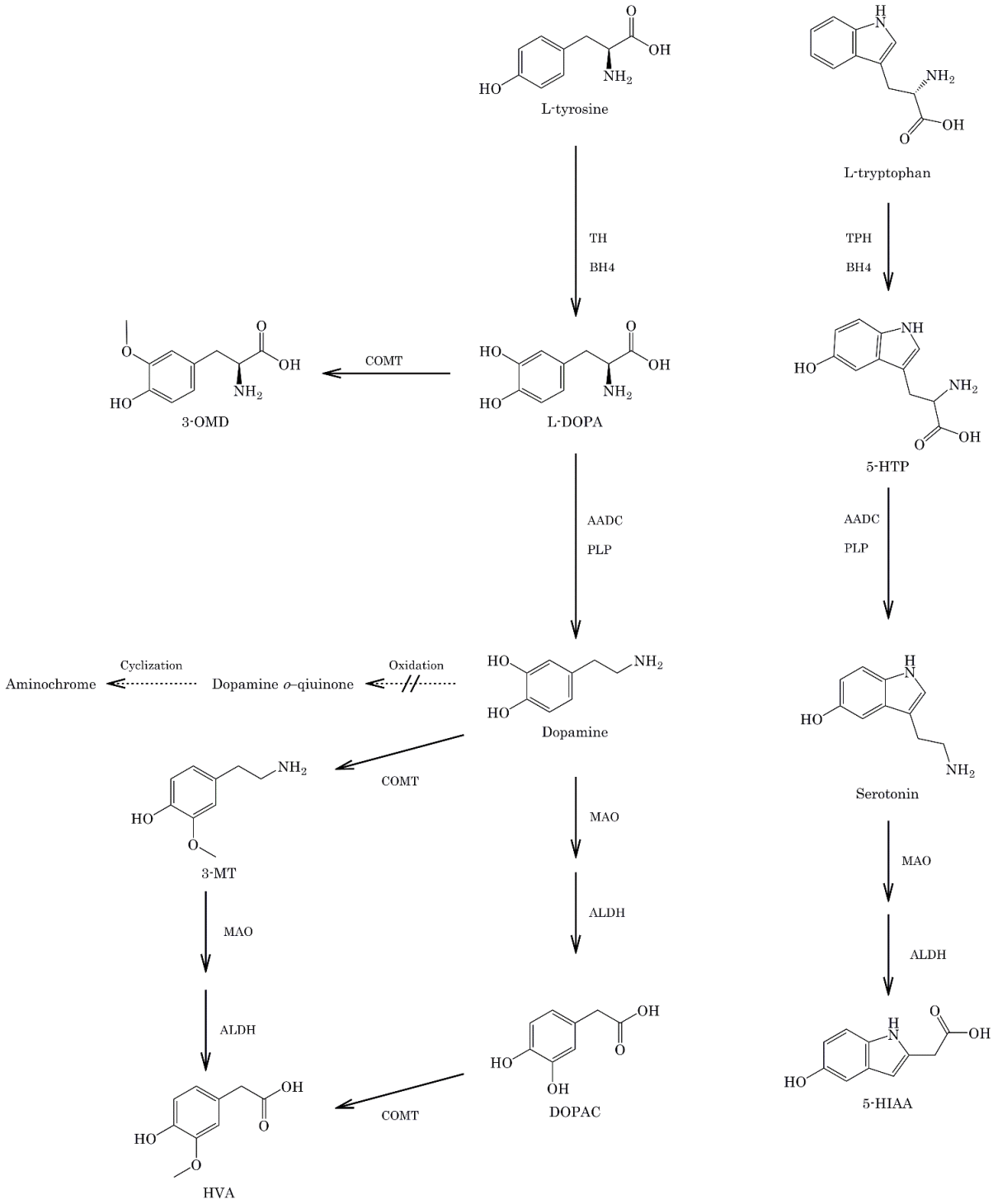

L-tyrosine
L-tryptophan
TH
BH4
TPH
BH4
3-OMD
COMT
L-DOPA
5-HTP
AADC
PLP
AADC
PLP
Cyclization
Aminochrome
Dopamine o-qiuinone
Oxidation
Dopamine
Serotonin
COMT
MAO
MAO
3-MT
ALDH
ALDH
MAO
ALDH
DOPAC
5-HIAA
COMT
HVA

Figure 1

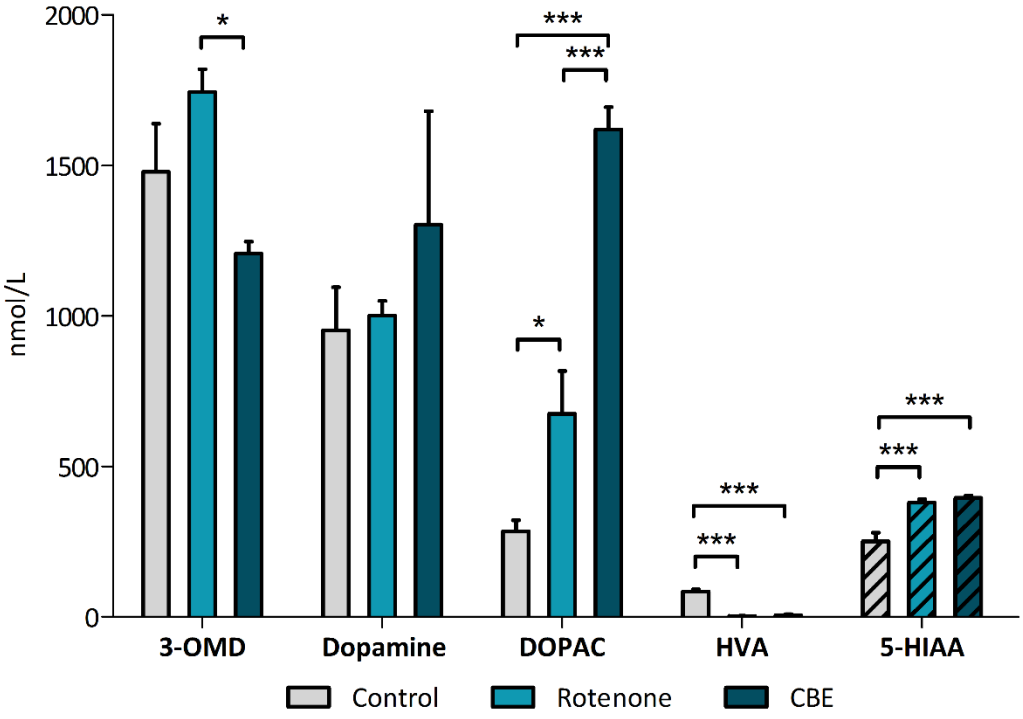

2000
1500
1000
500
0
nmol/L
*
***
***
*
***
***
***
***
3-OMD
Dopamine
DOPAC
HVA
5-HIAA
Control
Rotenone
CBE

Figure 2

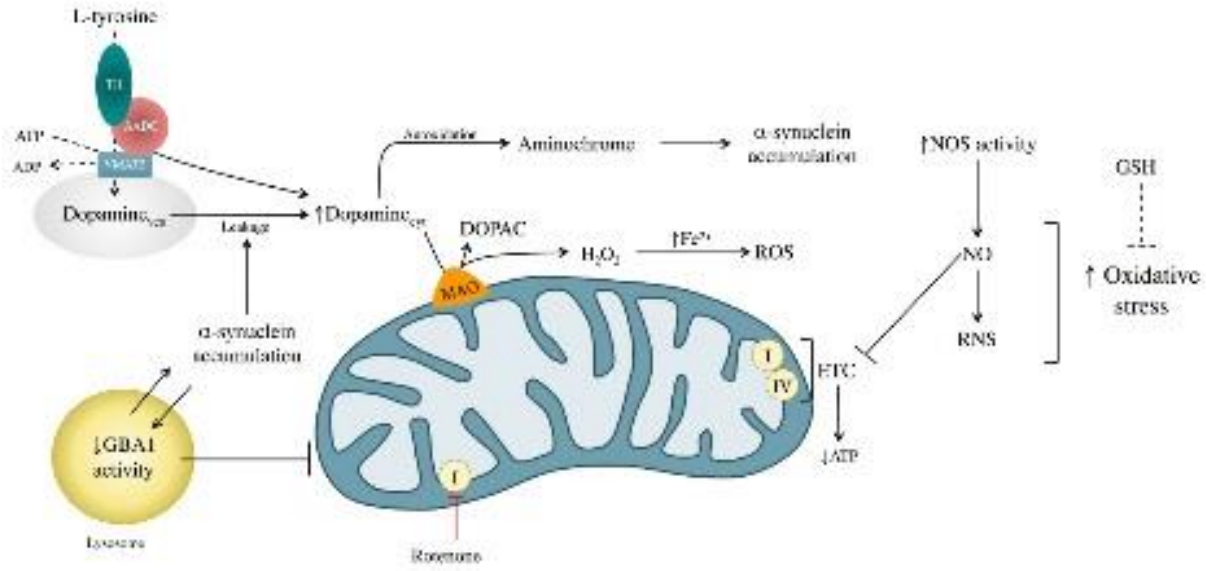

L-tyrosine
TH
AADC
ATP
ADP
VMAT2
Dopamine
Leakage
↑Dopamine
Accumulation
Aminochrome
α-synuclein accumulation
DOPAC
MAO
H_2O_2
↑Fe^{2+}
ROS
↑NOS activity
NO
RNS
GSH
↑ Oxidative stress
α-synuclein accumulation
↓GBA1 activity
ETC
I
IV
↓ATP
Rotenone

Figure 3