Neurotoxicity Research (2019) 36:27–38
https://doi.org/10.1007/s12640-019-00036-8

ORIGINAL ARTICLE

Divergent Expression Patterns of Drp1 and HSD10 in the Nigro-Striatum of Two Mice Strains Based on their MPTP Susceptibility

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Received: 17 December 2018 / Revised: 26 March 2019 / Accepted: 29 March 2019 / Published online: 16 April 2019

Abstract

Alterations in the basal ganglia circuitry are critical events in the pathophysiology of Parkinson's disease (PD). We earlier compared MPTP-susceptible C57BL/6J and MPTP-resistant CD-1 mice to understand the differential prevalence of PD in different ethnic populations like Caucasians and Asian-Indians. The MPTP-resistant CD-1 mice had 33% more nigral neurons and lost only 15–17% of them following MPTP administration. In addition to other cytomorphological features, their basal ganglia neurons had higher calcium-buffering protein levels. During disease pathogenesis as well as in MPTP-induced parkinsonian models, the loss of nigral neurons is associated with reduction in mitochondrial complex-1. Under these conditions, mitochondria respond by undergoing fusion or fission. 17β-hydroxysteroid type 10, i.e., hydroxysteroid dehydrogenase10 (HSD10) and dynamin-related peptide1 (Drp1) are proteins involved in mitochondrial hyperfusion and fission, respectively. Each plays an important role in mitochondrial structure and homeostasis. Their role in determining susceptibility to the neurotoxin MPTP in basal ganglia is however unclear. We studied their expression using immunohistochemistry and Western blotting in the dorsolateral striatum, ventral tegmental area, and substantia nigra pars compacta (SNpc) of C57BL/6J and CD-1 mice. In the SNpc, which exhibits more neuron loss following MPTP, C57BL/6J had higher baseline Drp1 levels; suggesting persistence of fission under normal conditions. Whereas, HSD10 levels increased in CD-1 following MPTP administration. This suggests mitochondrial hyperfusion, as an attempt towards neuroprotection. Thus, the baseline differences in HSD10 and DRP1 levels as well as their contrasting MPTP-responses may be critical determinants of the magnitude of neuronal loss/survival. Similar differences may determine the variable susceptibility to PD in humans.

Keywords Hydroxysteroid dehydrogenase10 and dynamin-related peptide1 · Mitochondrial fission · Mitochondrial hyperfusion · MPTP · Substantia nigra pars compacta · Neurodegeneration

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the geriatric population worldwide (Tysnes and Storstein 2017). PD affects the basal ganglia and meso-striatal circuitry that are primarily associated with movement, among many other non-motor functions (Alexander 1994).

PD involves degeneration of multiple pathways over the course of its pathogenesis. Among the several factors that significantly affect the prevalence of PD globally, ethnicity is an interesting aspect. Caucasian populations across continents show higher prevalence of PD (329/100,000; Strickland and Bertoni 2004; Van Den Eeden et al. 2003; Wirdefeldt et al. 2011) than Asian-Indians (52.85/100,000; Das et al. 2010) and other non-whites (Schoenberg et al. 1988). The Asian-Indian population is also significantly resistant to age-related loss of the dopaminergic neurons of substantia nigra pars compacta (SNpc) in comparison to Caucasians (Alladi et al. 2009). It was also found that the GDNF receptors, i.e., GFRα-1 and RET, were preserved (Alladi et al. 2010a) whereas there

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was a simple non-logarithmic increase in α-synuclein expression (Alladi et al. 2010b). The synaptic proteins synaptophysin and synaptotagmin-11 were preserved while the dendritic complexity of nigral neurons showed a mild age-related decline (Naskar et al. 2019). Both astrocytes and microglia showed morphological transformation during aging, while the oligodendroglial proteins were fairly unaffected (Jyothi et al. 2015). Due to the unavailability of specimens across ethnic populations; detailed investigations to delineate the molecular differences that determine the vulnerability are being conducted using animal models.

The N-MPTP model was developed to study the cellular mechanisms of PD, which caused selective lesioning of the meso-striatal pathway leading to the loss of dopaminergic neurons of the SNpc (Burns et al. 1983). Several studies report that based on the regimen, route of injection, and duration of the exposure, different effects or responses are observed (Jackson-Lewis and Przedborski 2007; Gibrat et al. 2009). The glial MAO-B converts MPTP to its metabolite, 1-methyl-4- phenylpyridinium ion (MPP^+) in the striatum, which is taken up by the terminals of the nigral DA neurons. The toxicity is brought about by the accumulation of MPP^+ within the mitochondria of the dopaminergic neurons (Chiba et al. 1984; Irwin and William Langston 1985). Within the mitochondria, MPP^+ inhibits complex-1 of electron transport chain, through steric interactions (Vyas et al. 1986). Thus, the MPTP model fulfills three aspects of cellular dysfunction associated with PD. These include oxidative stress, alteration in the core mitochondrial functions, and disrupted protein homeostasis within the striatum and SNpc (Browne and Flint Beal 2002; Nass and Przedborski 2008).

Earlier reports using MPTP provided evidence that different rodent strains have differential susceptibility to MPTP. For example, among the same strains obtained from different suppliers, differential susceptibility to MPTP was observed (Giovanni et al. 1991). C57BL/6J is the standard mouse model used in the studies related to aging and PD, owing to its lower nigral volume, lower detoxification through MAO-B and other enzymes in the liver, etc. (Meredith and Rademacher 2011). These factors make it highly "sensitive," i.e., with > 50% SNpc neuron loss in response to MPTP in comparison to other strains (Hamre et al. 1999). CD-1 on the other hand has higher nigral volume, moderate MAO-B activity, and is relatively resistant (i.e., < 25% SNpc neuron loss) to the toxic effects of MPTP (Muthane et al. 1994). Our recent studies confirmed that in comparison to C57BL/6J, the main attributes of the CD-1 mice nigra were higher number of nigral dopaminergic neurons, lower caspase and higher calbindin-D28K levels in nigral neurons and a balance in striatal interneuronal proteins, enhanced expression of striatal GDNF, as well as retention of higher number of neurons following MPTP administration. These mice also showed better performance on the rotarod and had better grip strength (Vidyadhara et al. 2016, 2017, 2019; Bhaduri et al. 2018). Hence, the differential vulnerability of C57BL/6J and CD-1 towards MPTP was extrapolated to explain the differences in the nigra of the Caucasians and Asian-Indians, respectively.

The differences in behavioral attributes may be a reflection of differences in mitochondrial bioenergetics and dynamics. Several compelling evidences implicate mitochondria and their derangement as cellular integrators in the genetic and sporadic, as well as primary and secondary PD. Observations including development of motor impairments following exposure to mitochondrial toxins (Ayala et al. 2007; Sato and Hattori 2011), higher percentage of mutated mt-DNA in the regions susceptible to aging and PD (Ikebe et al. 1990; Corral-Debrinski et al. 1992; Naydenov et al. 2010), and imbalance in mitochondrial dynamics (Stephen and Archer 2013; Willems et al. 2015; Gao et al. 2017) have linked mitochondria to PD. There are additional aspects in the context of mitochondria as well (Arduíno et al. 2011).

Many functions of mitochondria have been unraveled, in parallel, with advances in microscopy. Live-cell imaging presented mitochondria as mobile structures that constantly rearrange their networks in response to metabolic demands (Bereiter-Hahn 1990). Three processes are reported that modulate the rearrangements, which include division of existing mitochondria by fission and exchange of their contents through mitochondrial fusion as well as "mitophagy" as a measure of quality control.

Our study focuses on two proteins with complementary roles in the mitochondrial dynamics. Drp1 regulates mitochondrial fission in mammals and controls the distribution, separation, and clearance of mitochondria. It controls apoptosis and undergoes multiple post-translational modifications that control several processes in vitro and in vivo (Hu et al. 2017). Its expression increases with age (Jiang et al. 2014). MPP^+ induces mitochondrial fission in a Drp1-dependent manner (Barsoum et al. 2006; Wang et al. 2011). Controlled translocation and activity of Drp1 through genetic and pharmacological means have been shown to be neuroprotective and to counterbalance MPTP-mediated damage in cellular and animal models (Grohm et al. 2012; Rappold et al. 2014; Filichia et al. 2016).

17β-hydroxysteroid type 10 (HSD10) is a multi-functional mitochondrial enzyme associated with X-linked mental retardation (mutation), AD (overexpression), and PD (reduced expression) in different capacities(Zschocke et al. 2000). Its predominant function is in steroid metabolism, and among its non-enzymatic functions, it controls the cell survival (Rauschenberger et al. 2010) as well as the structural and functional integrity of mitochondria, through hyperfusion (Bertolin et al. 2015). Overexpression of HSD10 mitigates MPTP-mediated energy deficits in mice and confers a protective phenotype when compared to controls treated with MPTP (Tieu et al. 2004).

In the present study, we studied the expression of Drp1 and HSD10 in the dorsolateral striatum as well as within the dopaminergic neurons of the substantia nigra of C57BL/6J and CD-1 mice in response to a systemic dose of MPTP. Drp1 acted as a marker for fission and HSD10, for a form of fusion termed "hyperfusion." We evaluated the changes in localization and expression of these proteins in response to MPTP and investigated if the differences correlated with the differential susceptibility of the two strains towards MPTP.

Materials and Methods

Experimental Animals

All animal protocols were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH). We chose two strains of mice, i.e., C57BL/6J (MPTP-sensitive mice) and CD-1 (MPTP-resistant mice) aged between 15 and 17 weeks with six male animals ($n = 6$) studied in each study group. The animals were housed in polypropylene cages and maintained under standard laboratory conditions, temperature 25 ± 2 °C, 12/12-h light/dark cycle, and 50 ± 5% relative humidity. Food and water were provided ad libitum.

MPTP-HCl Treatment

We gave intraperitoneal, saline-diluted doses of MPTP-HCl to the mice in order to create an "acute" model of MPTP administration. The animals received 4 doses (15 mg/kg, i.p.) of MPTP-HCl at 2-h intervals between injections as per our established protocol (Vidyadhara et al. 2017). Seven days after the first MPTP injection, brain tissues were harvested for Western blot analysis following flash freezing in liquid nitrogen, sans perfusion. A separate set of mice were perfused intracardially with saline containing heparin sodium followed by 4% buffered paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4,for immunohistochemistry.

Tissue Processing

The brains of perfused mice were dissected and post-fixed in 4% PFA overnight and sequentially transferred to sucrose grades (15 and 30%) in 0.1 M PB. Forty-micrometer-thick serial, coronal sections of midbrains were sliced on a cryostat and collected on gelatin-coated slides. One series of every sixth section was either labeled with Drp1 or HSD10. Tyrosine hydroxylase (TH) was used as a co-label with both the proteins. Heat-induced antigen retrieval was performed using 0.1 M citrate buffer (pH 6.0) for HSD10 and Tris-EDTA buffer (pH 9.0) for Drp1, respectively, at 85 °C for 30 min in a hot air oven. All the specimens belonging to a group were stained together, under similar conditions.

Immunostaining

Separate series of cryosections containing the midbrain and striatum were stained for Drp1 and HSD10.The sections were equilibrated in 0.1 M PBS (pH 7.4) for 10 min and blocked for 3 h in 3% bovine serum albumin (BSA). Subsequently, the midbrain and striatal sections were incubated in rabbit anti-Drp1 (ab184247; 1:300 dilution factor, Abcam, UK) or rabbit anti-HSD10 (ab167410; 1:300 dilution factor, Abcam, UK) antibody for 48 h at 4 °C with intermittent incubation at RT for up to 2 h per day. This was followed by incubation with diluted secondary antibody in 0.1 M PBS buffer at 4 °C, overnight, i.e., donkey anti-rabbit Alexa-fluor 555 (1:500, Abcam) for Drp1 and goat anti-rabbit Alexa-fluor 488 (1:500, Abcam) for HSD10. The sections were thereafter co-labeled with mouse anti-TH primary antibody (1:800, Santa Cruz), followed by donkey anti-mouse Cy5 tagged secondary antibody (1:500, Sigma). TH immunostaining was done for the purpose of the identification of dopaminergic neurons. This was to facilitate the quantification of Drp1 and HSD10 within the dopaminergic neurons exclusively. The sections were rinsed with 0.1 PBST and 0.1 M PBS to remove excess antibody and cover-slipped using 80% glycerol.

Laser Confocal-Based Image Analysis

The fluorescently labeled images were acquired using a laser scanning confocal microscope (TCS-SL, Leica Microsystems, Germany) under the sequential scanning mode. For striatal sections, the fluorescence intensity was computed from three independent regions of interest (ROI) comprising of the dorsolateral striatum, striosome, and matrix compartments (Morigaki and Goto 2016). The dorsolateral striatum exhibits the most significant loss of TH-stained fibers since it receives terminal projections from SNpc (Song and Haber 2000). Since not all dopaminergic cell groups are equally vulnerable to the degenerative process (Hirsch et al. 1988), fluorescence intensities from midbrain sections were quantified from three subregions that are differentially susceptible to MPTP, i.e., the ventral tegmental area (fairly resistant), medial SNpc (moderately susceptible), and lateral SNpc (highly susceptible) (Varastet et al. 1994). An average of fluorescence intensities from the entire SNpc, excluding the VTA, was separately quantified to study the response in all of SN. The background staining was subtracted to minimize the effects of non-specific fluorescence, if any. At least 10 TH positive neurons were analyzed from each image captured from each field. For each region, approximately 10 images were captured.

Western Blotting

Flash-frozen brain samples were clamped to the cryostat stub without the embedding medium. Guided by the stereotaxic atlas, the entire striatum was sectioned at 5 μm thickness. Similarly, for the nigral sections, the medial midbrain, including VTA and non-nigral regions, were dissected out and sectioned at 5 μm to include SN alone. The striatal and nigral sections were separately solubilized in ice-cold RIPA buffer (Sigma, USA) containing protease inhibitor cocktail (Sigma, USA) at 4 °C. The solutions were sonicated and then centrifuged at 12,000 rpm for 25 min. Briefly, protein determination of the supernatant was performed by Bradford's method. Thirty micrograms of protein was loaded with Laemmli buffer, electrophoresed, and transferred onto PVDF membranes. The membranes were blocked using 5% BSA prepared in 1× PBS with Tween-20. The antigens were detected by probing the membrane for either Drp1 (1:1000, Abcam) or HSD10 (1:10000, Abcam). The bands were detected using chemiluminescent substrate for HRP (Advansta, USA) and were then quantitated using the NIH Image-J software. β-actin was used as the loading control.

Statistics

Statistical analysis of the raw data was performed using one-way ANOVA with multiple comparisons and Tukey's post host analysis. A p value ≤ 0.05 was considered to be significant. The results were represented as mean with standard error of mean. GraphPad Prism, version 7, was used for all statistical analyses.

Results

DRP1 and HSD10 Proteins Were Localized in the Perinuclear Space of the Cytoplasm

Co-labeling with TH confirmed that both the proteins were localized to the cytoplasm of the dopaminergic neurons, more prominently in the perinuclear region (Fig. 1). DRP1 showed granular or punctate staining (Fig. 1a, c) while TH expression was pan-cytoplasmic (Fig. 1b, e) as reported earlier (Vidyadhara et al. 2017). HSD10 expression was prominently punctate (Fig. 1d, f) and was found to localize in the cell body, as well as in the neuropil. Both dopaminergic (arrows; Fig. 1f) as well as non-dopaminergic neurons (arrowheads; Fig. 1f) expressed the protein.

Differences Exist in the Basal Expression Pattern of Drp1 between the Mice Strains

Qualitative analysis revealed higher expression of Drp1 in the VTA and nigra compared to the striatum. The striatum is seen on the ventromedial aspect of the corpus callosum (CC, Fig. 2a–d, dotted line). The striasomes showed slightly faint staining compared to the matrix. Quantitative analysis showed no significant differences in the basal expression of Drp1 in both the strains (Fig. 2 compare a and b with c and d; histogram e). Similarly, no differences were noted between the striosome and matrix components in either of the strains (Fig. 2f). However Western blot-based quantitation showed significant reduction in DRP1 expression

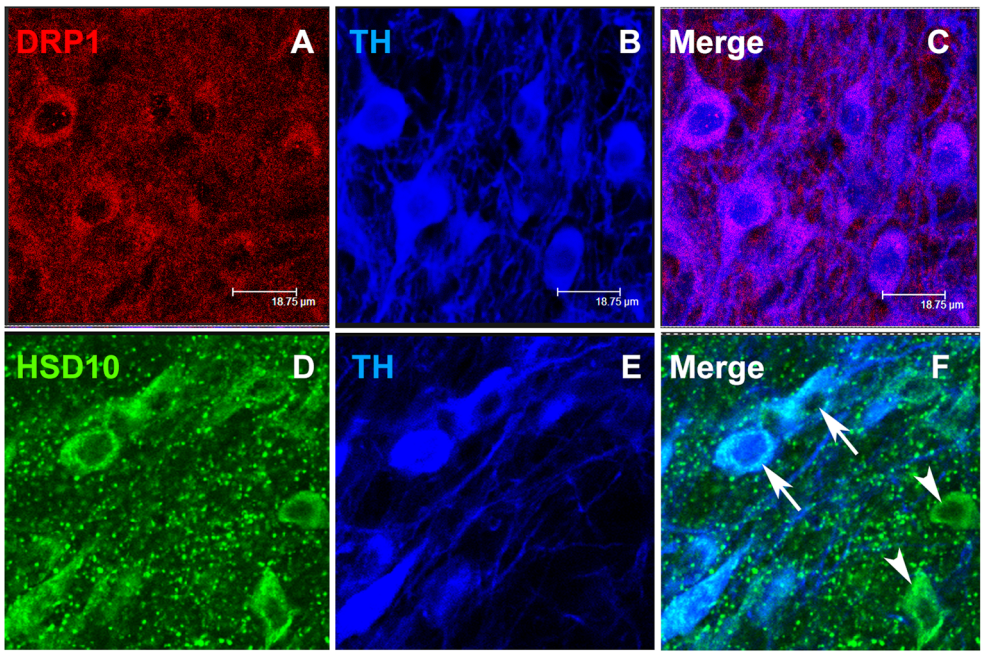

Fig. 1 Localization of DRP1 and HSD10 in dopaminergic neurons. High magnification confocal photomicrographs showing DRP1 (**a**, red) and TH (**b**, blue) and co-labeling of both proteins (**c**, pink). Note granular staining of DRP1 (**a** and **c**) as well as pan-cytoplasmic expression of TH (**b** and **e**). The second panel shows HSD10 expression (**d**, green) and TH (**e**, blue) and co-labeling of both proteins (**f**, cyan green). Note the punctate staining of HSD10 (**d** and **f**) in both dopaminergic (**f**, arrows, cyan green) and non-dopaminergic neurons (**f**, arrowheads, green) as well as in the neuropil

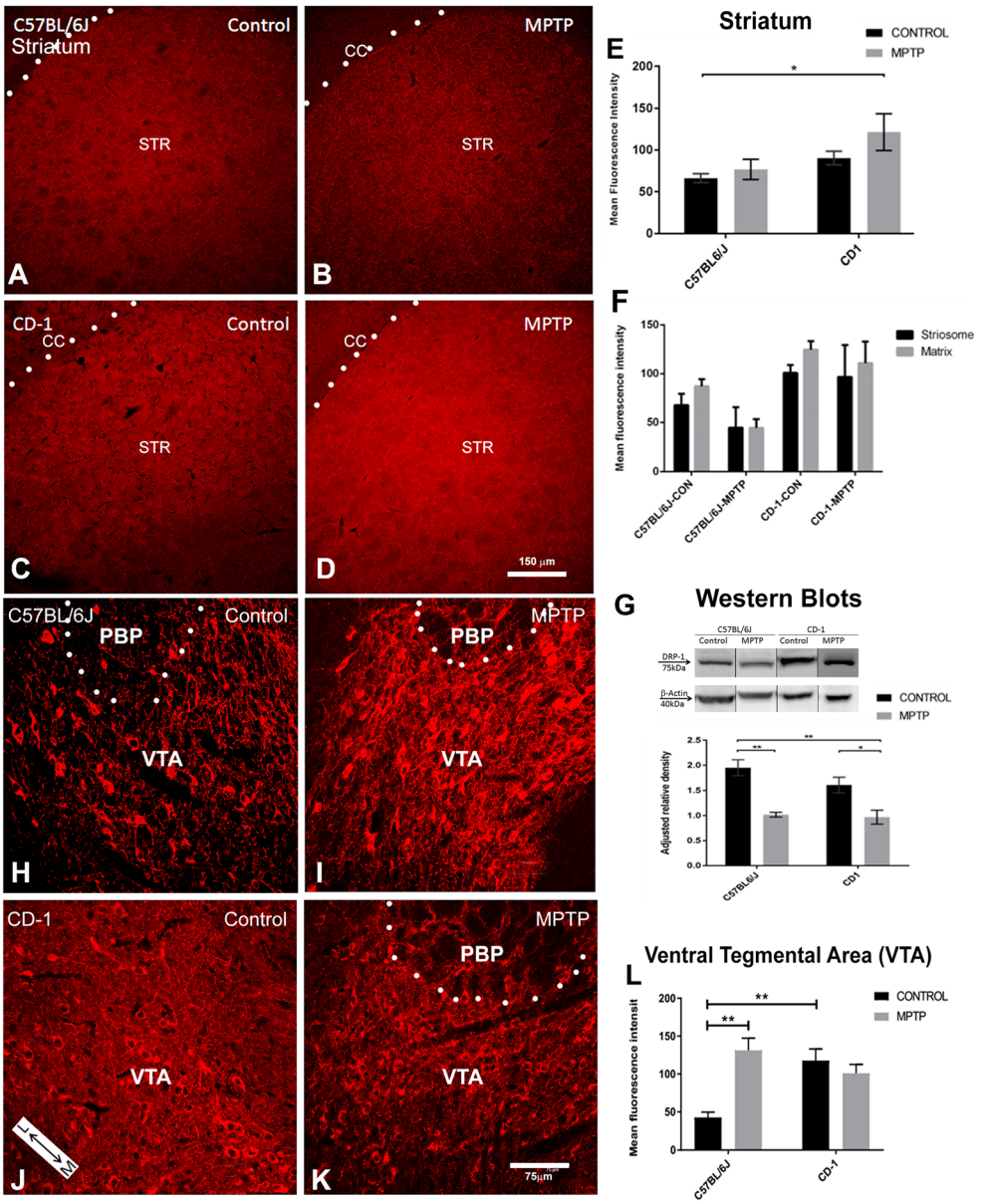

Fig. 2 Differences in basal expression pattern of Drp1 in the striatum and VTA. Representative laser scanning confocal photomicrographs showing immunostained sections containing corpus callosum (CC, dotted line in **a**–**d**) and dorsolateral striatum (scale bar = 150 μm for **a**–**d**) on the ventral aspect of CC. Note the insignificant differences in the baseline expression in the dorsolateral striatum of both the strains (compare **a** v/s **b** with compare **c** v/s **d**; histograms **e** and **f**). Note that Western blot-based quantitation showed significant reduction in the expression following MPTP administration in both the strains (histogram **g**; $^{**}p < 0.01$). Representative laser scanning confocal photomicrographs of ventral midbrain (**h**–**k**, scale bar = 75 μm) showing VTA and parabrachial pigmented nucleus (PBP, within dotted area) towards its lateral aspect (mediolateral orientation is shown by L↔M). Note the significantly lower basal expression in the VTA neurons of C57BL/6J than in CD-1 (compare **h** v/s **j**; $^{**}p < 0.01$; histogram **l**). Note the MPTP-induced upregulation in the expression in C57BL/6J neurons but not in CD-1 (compare **h** and **i** v/s **j** and **k**; $^{**}p < 0.01$; histogram **l**)

following MPTP administration, in both the strains (Fig. 2g; $^{**}p < 0.01$).

The ventral midbrain sections showed the presence of VTA on the ventromedial aspect of the parabrachial pigmented nucleus (PBP) (Fig. 2h–k, within the dotted line). A significantly lower basal expression was noted in the VTA neurons of C57BL/6J than in CD-1 (Fig. 2, compare h and j; $^{**}p < 0.01$). MPTP induced an increase in expression in the C57BL/6J but not in CD-1 (Fig. 2, compare h with i and j with k; $^{**}p < 0.01$; histogram l). The mediolateral orientation (Fig. 2jM↔L) of the sections is uniform for all photomicrographs (Fig. 2a–d and h–k).

The medial subdivision of SNpc (SNpc M) was flanked by the PBP on the dorsal aspect and the substantia nigra pars

reticulata (SNpr) on the ventral aspect (Fig. 3a–d). Contrary to that seen in VTA, a relatively higher basal expression was noted in the medial nigral dopaminergic neurons of C57BL/6J than in CD-1 (Fig. 3; compare a with c; histogram e; $*p < 0.05$). MPTP did not alter the baseline expression significantly in the medial nigra of either strains (Fig. 3 compare a with b and c with d; histogram e); moreover, post-MPTP, the differences between the strains persisted (Fig. 3; histogram e).

An identical expression pattern of lower expression in CD-1 than in C57BL/6J was seen in the lateral nigra (SNpc L)

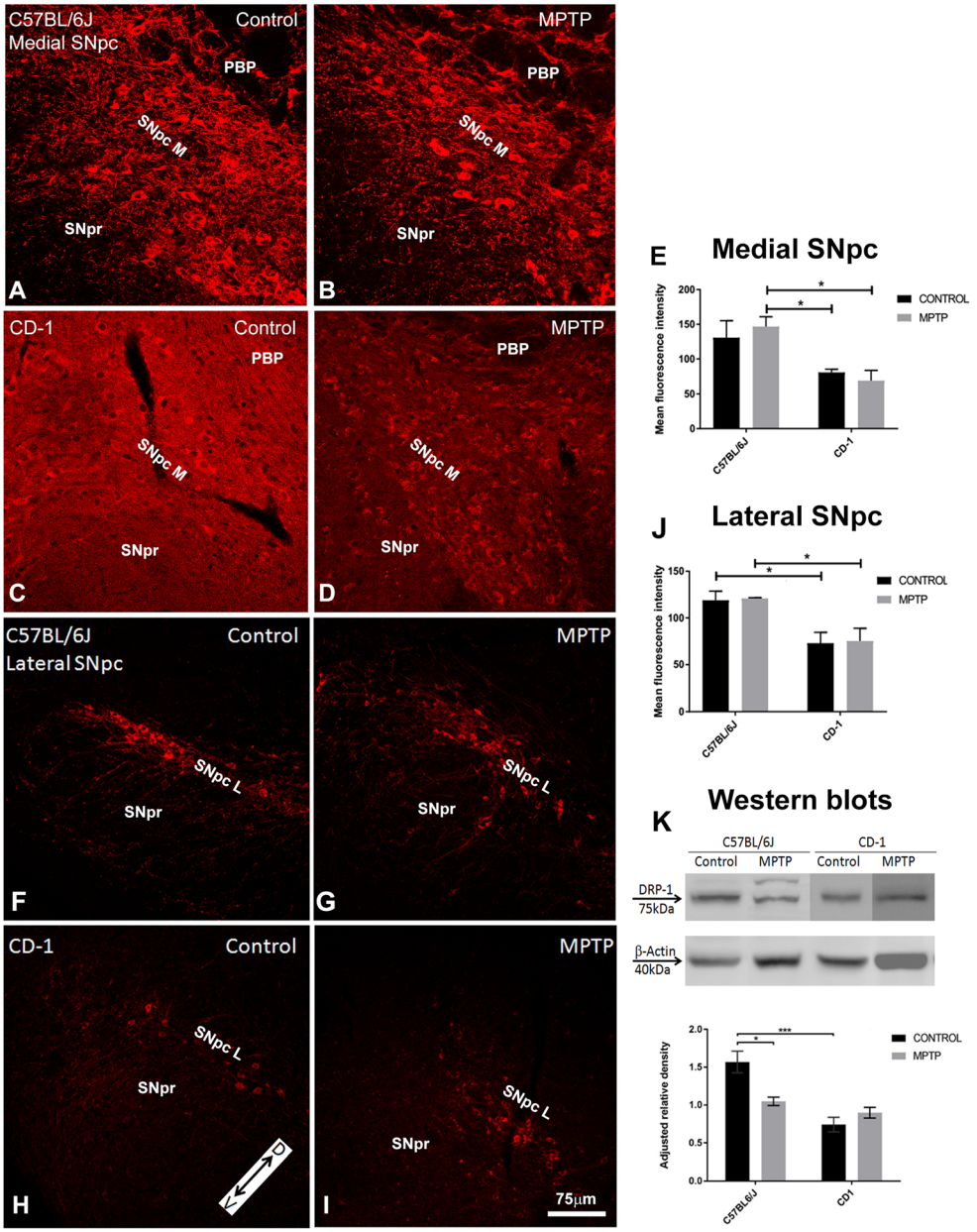

Fig. 3 Differences in basal expression of Drp1 in the nigra of C57BL/6J and CD-1 mice. Representative confocal photomicrographs showing localization of Drp1-labeled neurons in the medial nigra of C57BL/6J and CD-1 mice (**a**–**d**). Scale bar = 75 μm for all photomicrographs. The PBP is on the dorsal aspect and pars reticulata (SNpr) on the ventral aspect. The dorso-ventral orientation (**i**, D↔V) of the sections was uniform for all micrographs. Note the relatively higher basal expression in the medial nigral dopaminergic neurons of C57BL/6J than in CD-1 (compare **a** v/s **c**; histogram **e**; $*p < 0.05$). Note the absence of changes upon MPTP in both the strains (compare **a** v/s **b**; **c** v/s **d**; histogram **e**). **f**–**i** are the representative photomicrographs of the dopaminergic neurons within the lateral nigra (SNpc L compact band of neurons). Qualitative observations reveal fewer number of DRP1-expressing neurons in CD-1. Note the lower intensity of labeling in CD-1 than in C57BL/6J that remained unaltered following MPTP administration (compare **f** v/s **g** and **h** v/s **i**; histogram **j**; $*p < 0.05$). The representative Western blots show a band of 75 kDa of DRP-1 protein. The C57BL/6J showed higher Drp1 levels than CD-1 (histogram **k**; $***p < 0.001$). MPTP caused a significant reduction in C57BL/6J but not in CD-1 (histogram **k**; $*p < 0.05$)

(Fig. 3; f–i; compare f with h; histogram j; *$p < 0.05$), which remained unaltered following MPTP. The dorso-ventral orientation (Fig. 3iD↔V) of the sections is uniform for all photomicrographs (Fig. 3a–d, f–i).

The Western blots representing the differences in the overall nigra revealed a band of 75 kDa mol wt and were normalized against the β-actin bands (40 kDa) for quantification. Higher levels of Drp1 were noted in the SNpc of C57BL/6J than in CD-1 (Fig. 3; histogram k; ***$p < 0.001$). In response to MPTP, the C57BL/6J nigra showed a significant reduction (Fig. 3; histogram k; *$p < 0.05$) while the CD-1 nigra was spared of this loss (Fig. 3k).

C57BL/6J and CD-1 Contain Contrasting Basal Levels of HSD10 in the Striatum and SNpc

The basal expression of HSD10 in the dorsolateral striatum of C57BL/6J was significantly lesser than that of CD-1 (Fig. 4 compare a with c; histogram e; *$p < 0.05$). MPTP administration caused a mild reduction in expression but it was not statistically significant (Fig. 4 compare a with b and c with d; histogram e). The Western blot analysis of HSD10 showed a monomer at 27 kDa (Fig. 4f). There were no perceptible changes in either baseline or MPTP-induced changes in the expression patterns in both the strains.

The ventral midbrain sections stained for HDS10 showed the presence of VTA on the ventromedial aspect of the parabrachial pigmented nucleus (PBP; within the dotted line). We found comparable baseline expression of HSD10 within the dopaminergic neurons of VTA in C57BL/6J as well as CD-1 (Fig. 4, compare h and j, histogram l). MPTP administration resulted in a slight increase in its expression in the CD-1 neurons, although the increase was not statistically significant (Fig. 4, compare h with i and j with k; histogram l).

The medial subdivision of SNpc was on the dorsal aspect of the SNpr. In this sub-section of SNpc, the average intensity of HSD10 was higher in the control C57BL/6J mice when compared to CD-1 (Fig. 5 compare a with c). In response to MPTP, the CD-1 neurons showed an upregulation in expression (Fig. 5 compare b with d; histogram e; *$p < 0.05$). Differences in baseline observations were, similarly, noted in the lateral nigra albeit at a larger magnitude (Fig. 5 compare f with h; histogram j; **$p < 0.01$). In response to MPTP, the C57BL/6J nigra showed no changes in the lateral segment whereas the CD-1 showed a sizeable upregulation (Fig. 5 compare f and g and h and i; histogram j; ***$p < 0.001$). An increase in expression was also noted in the SNpr of CD-1. Thus, the magnitude of change was more pronounced in the lateral subdivision of SNpc followed by the medial subdivision and VTA. The changes seen in both the medial and lateral nigra were reflected in the overall nigra (Fig. 5k ***$p < 0.001$). The total HSD10 levels on immunoblots were comparable in the SNpc of both the strains, under control and MPTP administration conditions.

Discussion

The major observation of our study is that both HSD10 and DRP1 show susceptibility-specific expression patterns in the dorsolateral striatum, VTA, and SNpc of C57BL/6J and CD-1 mice. Among the brain regions, the SNpc is most susceptible to neuron loss in PD, which is replicated following MPTP administration. Between the two strains, the MPTP-sensitive C57BL/6J had higher baseline Drp1 levels in the lateral nigra which may suggest persistent occurrence of fission under normal conditions. On the contrary, in the CD-1 mice, MPTP administration caused an increase in HSD10 levels, suggesting that mitochondrial hyperfusion could be a compensatory attempt towards neuroprotection, following a toxic challenge.

Both Drp1 and HSD10 are proteins of pathological significance that control mitochondrial morphological transformations. We studied the expression of these proteins in response to a mitochondrial toxin, MPTP, in two mice strains that differently metabolize it thereby rendering each mice strain differentially susceptible. Mitochondrial networks reorganize themselves in accordance with the cellular metabolic states and are indicative of the internal energetic status of the cell. Signaling molecules that affect this process, in principle, link the metabolic reactions of the cell to the proteins that control mitochondrial morphology and distribution; for example, Drp1 reflects such a metabolic control.

Drp1 knock-out is lethal in mice and its activity is regulated by multiple factors including differential phosphorylation. In our study, we observed a higher basal expression of Drp1 in the SNpc of C57BL/6J in comparison to CD-1. This innate difference could enhance the baseline susceptibility of nigral neurons in C57BL/6J to degeneration, i.e., even without an exposure to MPTP. Higher basal Drp1 levels have the potential to create an environment of cellular stress with higher mitochondrial fission. Although the immunofluorescence intensity and hence the protein expression values remained stable in the neurons following MPTP administration, qualitative observations revealed an increase in number of neurons expressing the protein; thus, addition of MPTP may cause induction and progression of fission in several neurons.

MPTP did not affect the expression of Drp1 in the striatum of both the strains, as is supported by other studies which have demonstrated activity-dependent changes in localization (Filichia et al. 2016) possibly hinting at the occurrence of enhanced fission in the cells of nigral origin. This further explains the exclusivity of nigral neurons in terms of cell loss.

Mitochondrial hyperfusion is the lesser understood process of the two but holds greater implications towards neuroprotection. HSD10 is a multi-functional, mitochondrial enzyme

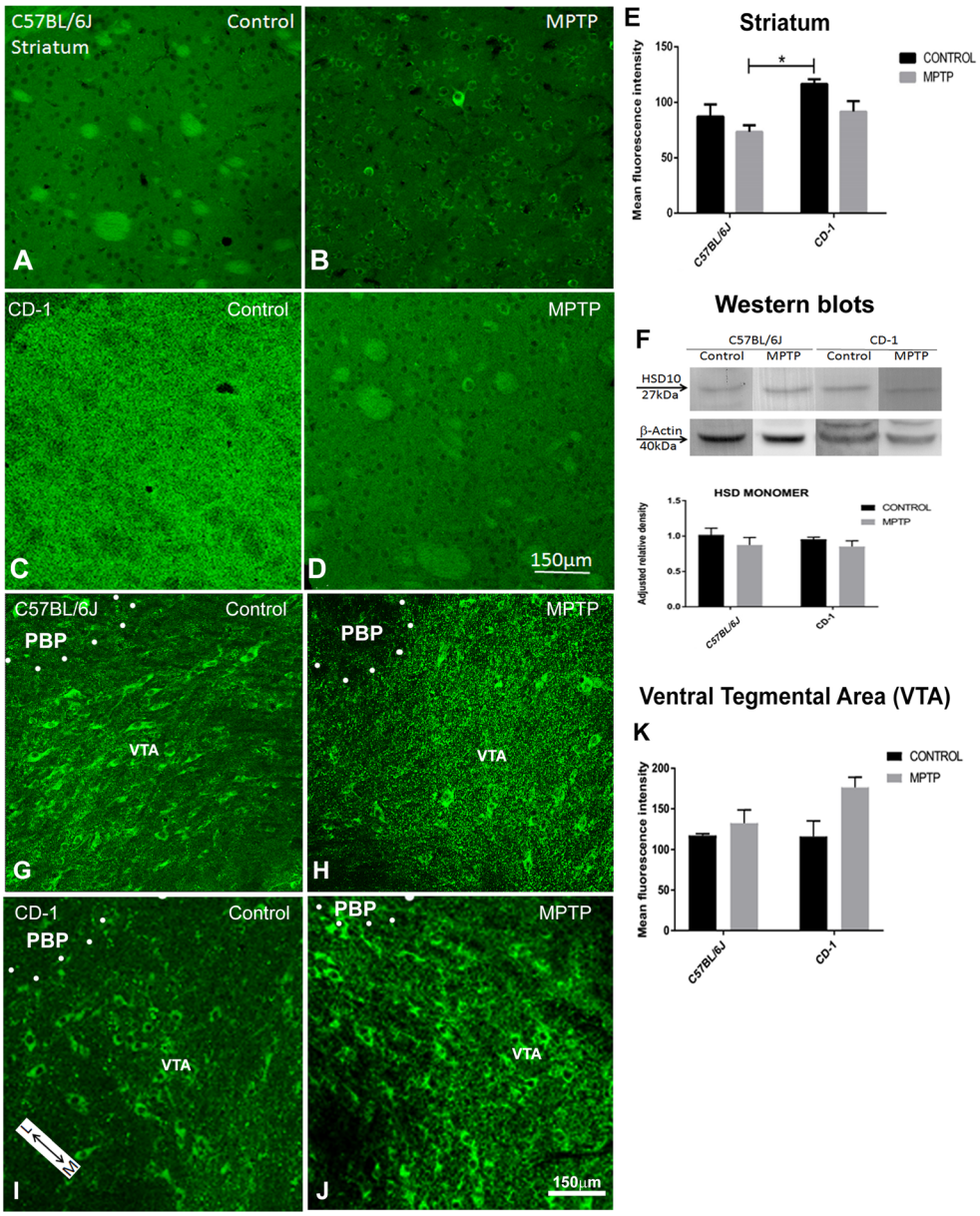

Fig. 4 HSD10 expression in the striatum and VTA of C57BL/6J and CD-1 mice. Representative laser scanning confocal photomicrographs showing immunostained sections of the dorsolateral striatum that was located on the ventromedial aspect of the corpus callosum (scale bar = 150 µm for **a**–**d**). The basal expression was significantly higher in CD-1 than C57BL/6J (compare **a** with **c**; histogram **e**; $*p < 0.05$). MPTP caused a mild reduction in expression (compare **a** with **b** and **c** with **d**; histogram **e**). Western blot analysis of HSD10 of monomer of 27 kDa (**f**) showed no perceptible differences in either baseline. MPTP-induced no alterations in the expression patterns in both the strains. Representative confocal photomicrographs of ventral midbrain (**g**–**j**) showing VTA and parabrachial pigmented nucleus (PBP, within the dotted area) towards its lateral aspect (L↔M: lateral medial orientation). Scale bar = 75 µm for all micrographs. Note analogous expression of HSD10 within the cytoplasm of the VTA neurons in C57BL/6J as well as CD-1 under control conditions (compare **h** and **j**, histogram **k**). The expression increased slightly upon MPTP administration in the CD-1 neurons (compare **i** and **j**; histogram **k**)

that controls several metabolic reactions. In our study, only faint cytoplasmic expression was observed in the cells within the dorsolateral striatum of C57BL/6J following MPTP treatment, which may suggest mild compensatory changes in the striatal cells. In the midbrain, the expression levels were relatively higher than in the striatum. The levels were unaffected in C57BL/6J following exposure to MPTP. Although the basal levels of HSD10 in CD-1 were lower than that in C57BL/6J, exposure to MPTP upscaled its expression. The fold increase was maximal in the lateral segment, followed by medial SNpc and VTA. The lateral segment of the nigra is specifically vulnerable to MPTP. Comparison of MPTP-induced cell loss in the SNpc of C57BL/6J and CD-1 showed a lateral nigra-specific neuronal loss of about 50–60% in C57BL/6J and ~

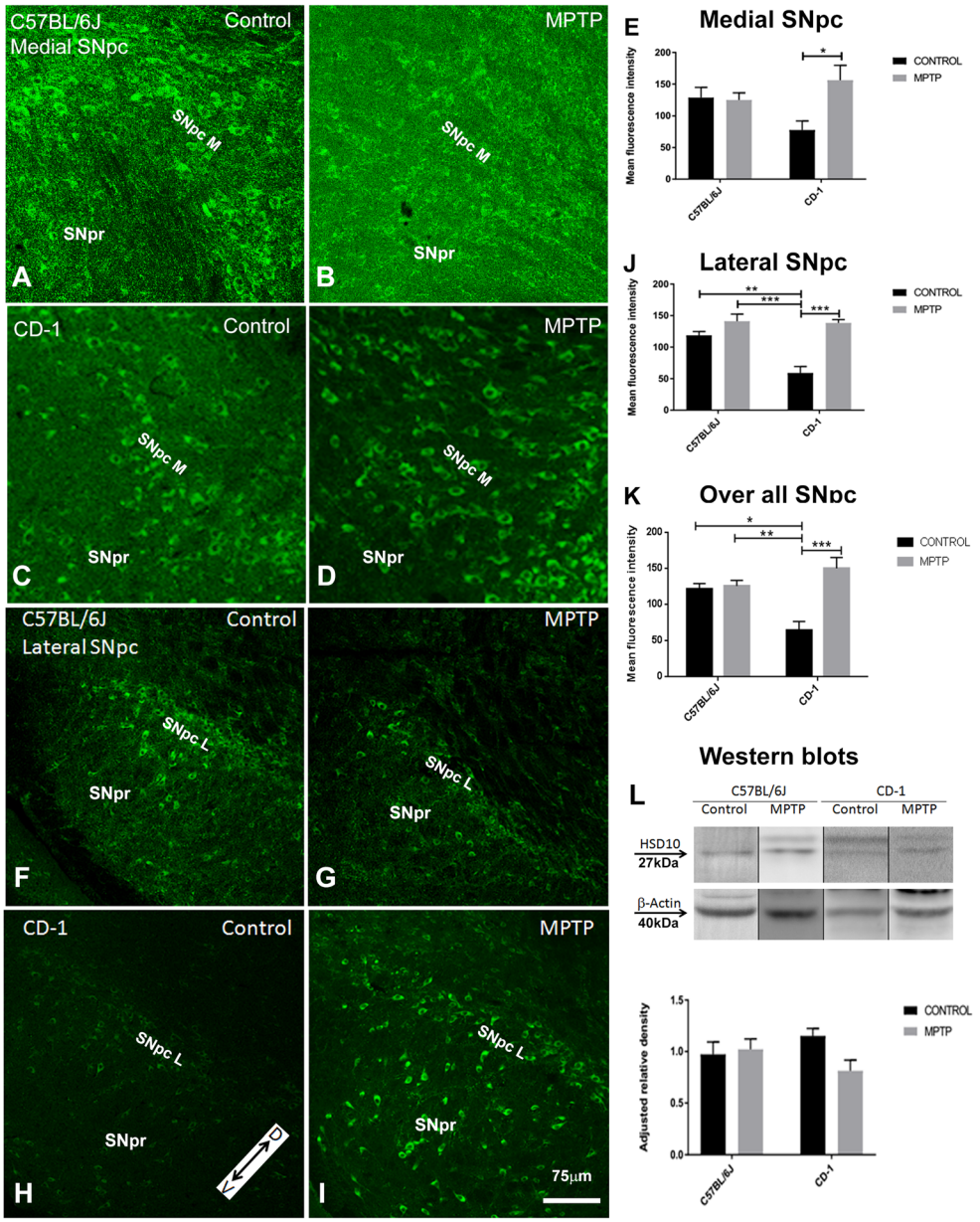

Fig. 5 HSD10 expression in the medial and lateral nigra. Representative confocal photomicrographs, showing cytoplasmic localization of HSD10 in the neurons of the medial (**a**–**d**) and lateral nigra of C57BL/6J and CD-1 mice (**f**–**i**). Scale bar = 75 μm for all micrographs. In the medial nigra, note the relatively higher basal expression in C57BL/6J compared to CD-1 (**a** v/s **c**). MPTP induced an upregulation in the CD-1 neurons (**c** v/s **d**; histogram **e**; $*p < 0.05$) but not in C57BL/6J. The pars reticulata (SNpr) appears on the ventral aspect of the medial nigra (SNpcM). Qualitative observations showed increased expression in the SNpr in the CD-1 following MPTP administration. Note the differences in baseline expression in the lateral nigra (**f** v/s **h**; histogram **j**; $**p < 0.01$). MPTP-induced a significant upregulation in CD-1 (**f** v/s **g** and **h** v/s **i**; histogram **j**; $***p < 0.001$). An increase in expression was also noted in the SNpr of CD-1 (**h** v/s **i**). The 27-kDa monomer on immunoblots represents HSD10 protein on Western blots (**l**). Note comparable HSD10 levels in the SNpc of both the strains, in controls, and upon MPTP administration (histogram **l**)

15% in CD-1 (Vidyadhara et al. 2017). In another study, the calbindin expression in the lateral SNpc was found to be more in CD-1, which was considered to be neuroprotective (Vidyadhara et al. 2016). Thus, such an increase in the HSD10 expression in the lateral segment may be relevant and appears to be neuroprotective even in this instance. Interestingly, the VTA of CD-1 which is more resistant to MPTP exhibits the highest intensity of HSD10 expression following MPTP. Thus, this upregulation of HSD10 could be a component of the stress-induced mitochondrial hyperfusion (Wai and Langer 2016) and explains the compensatory neuroprotective mechanism of the midbrain VTA/DA

neurons of the CD-1 against degeneration. A strong possibility arises that hyperfusion is helpful, by balancing and counteracting the MPTP-mediated mitochondrial fission. Absence of such responses in C57BL/6J might impede any compensatory response towards MPTP. It may also have a compounding influence on the intrinsically higher Drp1 levels.

The total protein levels as assessed by immunoblots did not show any differences in HSD10 expression in both the striatum and midbrain. There are a couple of reasons proposed for this lack of correlation between fluorescence-based quantification and protein blotting. Immunohistochemistry-based quantification essentially focused on the fluorescence intensity within TH-positive DA neurons, and demarcations of different subsections of the nigra were possible. The total protein levels by immunoblotting summate the effects on all the sub-regions as well as all the major cell types within the regions, including the glia. This observation further underpins earlier studies that highlight the differential response of individual cell types, i.e., neurons and glia, to MPTP and its influence on the overall vulnerability of a region to neurodegeneration. Both astrocytes and microglia proliferate following MPTP treatment in the SNpc of C57BL/6J (Kohutnicka et al. 1998), and it may be reasonable to believe that glial mitochondria entail these alterations.

Drp1 receptor mitochondrial fission factor (MFF) modulates the mitochondrial size without affecting their properties like trafficking efficiency or ATP generation (Lewis et al. 2018). MFF downregulation results in elongation of presynaptic mitochondria which augments their capacity for Ca^{2+} uptake during neurotransmission, leading to reduced presynaptic Ca^{2+} accumulation. In developing axons, this controls neurotransmitter release causing branching through fission-dependent regulation of presynaptic mitochondrial size. In our study, in C57BL/6J, the baseline expression of the DRP1 protein was higher, which may be an indirect indicator of upregulated MFF and persistent fission. On the contrary, in CD-1, MPTP administration caused a decrease in DRP1, which was complemented by an increase in HSD10, suggesting an increase in mitochondrial length. Since elongated mitochondria have higher capacity of Ca^{2+} uptake, it is likely that the CD-1 mitochondria combat Ca^{+2}-induced excitotoxic stresses by morphological rearrangements maintaining the mitochondrial morphology and hence its homeostatic balance.

CD-1 is also endowed with neuroanatomical and biochemical advantages, including higher neuronal reserve in SN, striatal volume, etc., in addition to higher cellular calbindin levels, when compared to C57BL/6J (Vidyadhara et al. 2016). The neurons of CD-1 were smaller than those of C57BL/6J. Between C57BL/6J and CD-1, differences in volume of the regions, size of the neurons, and ratio of neuron to glia are capable of influencing the basal levels.

Human studies have reported differences in prevalence of PD as a function of race or ethnicity. Among the prominent differences between Caucasian and Asian-Indian midbrains was the absence of nigral neuronal loss with age (Muthane et al. 1994; Alladi et al. 2009). In addition, their nigral neurons retained the expression of GDNF receptors (Alladi et al. 2010a) and also showed a compensatory increase in endoplasmic reticular chaperone protein GRP-78 (Alladi et al. 2010b). We hypothesize that the curtailed age-related loss of nigral neurons in the less vulnerable Asian-Indians may be an upshot of the balanced ratio of fission-fusion inducing proteins that result in/from persistent GDNF responsiveness. The mitochondrial homeostasis may strike a balance between cellular energy generation and utilization which may help the neurons thrive longer despite the age-related increase in endotoxins. Thus, differences may exist in the mitochondrial morphology in different populations that are differentially susceptible to the disease. It is probable that these molecular phenomena are interlinked. Thus, we provide indirect evidence that an imbalance in mitochondrial fission-fusion dynamics may potentiate the risk of individuals to PD.

Mitochondria have been consistently associated with neurodegeneration, as one of its most reliable mark. The variations between populations of different ethnicities appear relevant and may be associated with mitochondrial dynamics. To distill it further, one may look at the expression of proteins at the interface of mitochondria and metabolism that are subjected to multiple post-translational modifications and focus on potential protective factors in this pool. Since mitochondria are vital to development, knock-outs of most of its proteins are lethal; therefore, inhibiting these proteins may not be a viable therapeutic option. The concluding emphasis would be on identifying biomolecules that can counteract and render neuroprotection, via these quintessential processes.

Acknowledgements The authors are grateful to Dr. G.H. Mohan, Head Veterinarian at the National Centre for Biological Sciences, Bengaluru, for providing breeding colonies of the CD-1 mice strain. We thank Dr. Vidyadhara D.J. and Dr. Yarreiphang H. for their help in initiating the experiments.

Funding This work was supported by the Science and Engineering Research Board, Department of Science and Technology, Government of India, to PAA (No. SR/SO/HS-0121/2012). SA is a NIMHANS M.Phil. fellow.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interest.

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