CaV1.3 L-Type Calcium Channels Increase the Vulnerability of Substantia Nigra Dopaminergic Neurons in MPTP Mouse Model of Parkinson’s Disease

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Mechanisms underlying the selective vulnerability of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) over those in the ventral tegmental area (VTA) to degeneration in Parkinson’s disease (PD) remain poorly understood. DA neurons of SNpc and VTA are autonomous pacemakers but pacemaking in SNpc but not in VTA is accompanied by calcium influx through L-type calcium channel, CaV1.3 contributing to increased intracellular calcium and hence to cell death. CaV1.3⁴²A, an alternatively spliced short variant of CaV1.3 has increased calcium influx. We, therefore studied the role of CaV1.3⁴² (full-length channel) and CaV1.3⁴²A in mouse SNpc in PD pathogenesis by quantifying mRNA levels of CaV1.3⁴² and CaV1.3⁴²A in SNpc and followed the change in their levels in MPTP induced parkinsonism mouse model. Using in situ hybridization and immunohistochemistry we observed the localization of mRNA of CaV1.3⁴² and CaV1.3⁴²A in tyrosine hydroxylase (TH) positive DA neurons. Further, mRNA levels of CaV1.3⁴²A were higher in SNpc as compared to the cortex. Upon MPTP treatment, mRNA levels of CaV1.3⁴² and CaV1.3⁴²A maintained their levels in SNpc in spite of the loss of ~50% of the DA neurons. This indicates that the expression of CaV1.3⁴² and CaV1.3⁴²A is maintained at a robust level during the degenerative process in the parkinsonism model.

Keywords: neurodegeneration, SNpc, MPTP, CaV1.3⁴²A, gene expression, alternative splicing

INTRODUCTION

Parkinson’s Disease (PD) is a debilitating movement disorder characterized by locomotor deficits including resting tremor, bradykinesia, rigidity and postural instability. Motor deficits result from the loss of dopaminergic (DA) neurons in the large midbrain nucleus, substantia nigra pars compacta (SNpc). SNpc DA neurons degenerate while neighboring DA neurons from the ventral tegmental area (VTA) remain relatively protected from neurodegeneration (Hirsch et al., 1988; Gibb and Lees, 1991; Dauer and Przedborski, 2003; Brichta and Greengard, 2014). A number of mechanisms have been implicated in PD neurodegeneration, namely oxidative stress possibly in relation with iron deposition (Sofic et al., 1988; Ayton and Lei, 2014), abnormal dopamine metabolism (Michel and Hefti, 1990; Pifl et al., 2014), mitochondrial dysfunction (Schapira et al., 1990; Park et al., 2009; Michel et al., 2016) and disruption of proteasomal or autophagic catabolism.
(Sherman and Goldberg, 2001; Betarbet et al., 2005; Carvalho et al., 2013; Michel et al., 2016). However, in PD none of these mechanisms provides in itself an explanation for the greater vulnerability of SNpc dopaminergic neurons over DA neurons in VTA.

There is also evidence to suggest that dysregulation in calcium homeostasis could potentially account for this selectivity (Surmeier et al., 2010, 2011, 2017; Schapira, 2013; Surmeier and Schumacker, 2013). In fact, it has been reported that DA neurons from VTA express higher levels of the calcium buffering protein, calbindin-D28K (Damier et al., 1999). Besides, DA neurons in SNpc but not in VTA are characterized by pacemaking activity that is accompanied with activity of a subset of L-type voltage-dependent calcium channels having a Cav1.3 pore that elevates intracellular Ca\(^{2+}\) (Nedergaard and Greenfield, 1992; Kang and Kitai, 1993; Mercuri et al., 1994; Chan et al., 2007; Surmeier et al., 2017). However, pacemaking in VTA DA neurons appears to depend primarily on HCN/voltage-gated sodium channels (Khaliq and Bean, 2010) and the cytosolic Ca\(^{2+}\) in VTA DA neurons during pacemaking is reported to be significantly less as compared to that in DA neurons in SNpc (Guzman et al., 2018). This has led to the hypothesis that Ca\(^{2+}\)-dependent pacemaking along with a poor calcium buffering capacity may cause calcium overload through L-type Cav1.3 channel activation in most vulnerable DA SNpc neurons making them preferentially at risk to degeneration (Surmeier et al., 2010, 2017; Liss and Striessnig, 2019).

Several alternatively spliced variants of the full-length channel Cav1.3\(_{42}\) have been reported, among them is Cav1.3\(_{42A}\), a short splice variant that incorporates in a mutually exclusive manner, exon 42A instead of exon 42 (Bock et al., 2011; Huang et al., 2013). Exon 42A contains a stop codon and translation of the protein terminates before encoding the C-terminal modulatory domain, which results in a splice variant product with different electrophysiological properties compared to the full-length channel. The calcium current density through Cav1.3\(_{42A}\) is about 2.5 times greater than in Cav1.3\(_{42}\) (Singh et al., 2008). Further, it has been reported that the activation range of Cav1.3\(_{42A}\) is shifted to a more negative potential by \(-\sim 10\) mV (Singh et al., 2008). Moreover, these variants show greater insensitivity to inhibition by dihydropyridines (DHPs) as compared to other C-terminus variants and the full-length channel (Huang et al., 2013). Thus, the activation potential of Cav1.3\(_{42A}\) is closer to the resting membrane potential and there is greater calcium influx through this variant as compared to other C-terminus variants and full-length channel (Tan et al., 2011; Huang et al., 2013). Further, the presence of these channels has been demonstrated in the mouse and human brain (Singh et al., 2008; Bock et al., 2011).

Owing to the atypical electrophysiological properties of Cav1.3\(_{42A}\), we hypothesized that the expression of this splice variant in SNpc could potentially play a role in DA cell death by causing perturbations in calcium homeostasis. To this aim we studied the expression of mRNA encoding Cav1.3\(_{42A}\) and Cav1.3\(_{42}\) (full-length) in midbrain DA neurons in naïve mice and evaluated if the transcription of these channels was affected during neurodegeneration mediated by MPTP in mouse model of PD.

**MATERIALS AND METHODS**

**Animals and MPTP Dosing**

Animal experiments were carried out on C57BL/6J male mice (3–4 months; 25–30 g) procured from Central Animal Facility of Indian Institute of Science, Bangalore, India. All experiments were carried out according to institutional guidelines for the use and care of animals. Animal experiments were approved by the institutional animal ethical review board, named “Institutional Animal and Ethics Committee” of the Indian Institute of Science (Protocol# CAF/Ethics/267/2012). Handling of animals was done according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All experiments were performed in adherence to ARRIVE guidelines. All efforts were made to minimize animal suffering, reduce the number of animals used and to use alternatives to *in vivo* techniques, if available. Animals were housed in groups and had access to pelleted diet and water, *ad libitum*. The sample size for experiments with untreated animals was 6–11. For the MPTP mouse model of dopaminergic loss (Jackson-Lewis and Przedborski, 2007), 30 mg/kg body weight 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma–Aldrich Cat# M0896) dissolved in normal saline was given subcutaneously to the mice as a single dose or daily for 14 days. MPTP injections were carried out in an isolated clean air room in the Central Animal Facility, Indian Institute of Science, Bangalore, India. The controls were injected with an equivalent volume of normal saline. Animals were allocated to treated or control groups in a random manner. The sample sizes for MPTP experiments were 4–11 for each group. Animals were sacrificed 24 h after the last MPTP dose.

Adequate safety precautions were followed in the proper handling of MPTP during preparation and injection, and in the disposal of materials and samples contaminated with MPTP and its metabolites. Protective gear was worn during the preparation and injection of MPTP. Syringes and needles that were used for injection were incinerated after a single-use. Mice undergoing MPTP treatment were housed in separate cages and the contaminated bedding material and feed were incinerated upon disposal.

**Mouse Brain Dissection**

Animals were decapitated following cervical dislocation. Cortex, ventral midbrain and striatum were dissected out under cold and sterile conditions. For dissection of SNpc, the whole mouse brain was placed on mouse brain matrix (Ted Pella, Inc., Cat# 15050) and 1 mm thick slices of the brain were obtained. Then, SNpc was dissected out from these slices under a dissecting stereomicroscope using anatomical markers.

**RNA Isolation and cDNA Synthesis**

All the reagents and glassware used for RNA isolation, cDNA synthesis, *in situ* hybridization and immunohistochemistry are available.
were made RNase free. RNA from mouse brain tissue was isolated using TRIzol reagent (Invitrogen Cat# 15596018) and bromochlorophenol (BCP; Molecular Research Centre, Inc., Cat# BP151; Chomczynski and Sacchi, 2006). Total RNA (500 ng) was used for first-strand cDNA synthesis using random hexamers, dNTPs and reverse transcriptase from the High capacity cDNA reverse transcription kit (Applied Biosystems Cat# 4368814).

**Quantitative Real-Time PCR**
Quantitative real-time PCR (qRT-PCR) was performed using SYBR green chemistry with primer pairs designed to distinguish the full-length Ca\textsubscript{V}1.3 and splice variant. The nucleotide sequences for primers used for mouse gene expression analysis and the PCR conditions are provided in Supplementary Tables S1, S2, respectively. Further, the specificity of the primers as assessed by the presence of a single band at the desired size measured through gel electrophoresis has been represented in Supplementary Figure S1. Three endogenous controls, namely 18S rRNA, β-actin and GAPDH were used for normalization when cDNA from untreated mouse tissue was analyzed. β-actin and/or GAPDH normalization was performed in subsequent experiments as reported. Further, cell-type-specific normalizations were performed with tyrosine hydroxylase (TH), DAT, GAD1, and VGlut2. The samples were analyzed in duplicates or triplicates. Data from all samples have been reported and no exclusion of outliers has been performed.

**Fluorescent in situ Hybridization (FISH) and Immunohistochemistry**
Male C57BL/6j mice brains were isolated and fixed in 4% paraformaldehyde (w/v) for 12 h following decapitation after cervical dislocation. Fixed brains were then allowed to sink in 30% sucrose before embedding in tissue freezing system (Leica Microsystems Nussloch GmbH Cat# 0201 08926). Coronal sections measuring 14 \( \mu \)m in thickness were cut through midbrain under RNase free conditions using a Cryostat (Leica Microsystems). The sections were hydrated, acetylated, and treated with 25 \( \mu \)g of proteinase K (Roche Cat# 03115852001) for 7 min at 37\(^{\circ}\)C. The sections were then rinsed with phosphate buffer and dehydrated using ethanol gradient. Digoxigenin-labeled sense (control) and antisense RNA probes were synthesized using SP6 and T7 polymerases (Roche Cat# 11175025910), respectively from Ca\textsubscript{V}1.3\textsubscript{42A} and Ca\textsubscript{V}1.3\textsubscript{42A} cDNA sequences that were cloned into dual promoter pCRII vector (Invitrogen Cat# K206001). The sequences of the primers used for Ca\textsubscript{V}1.3\textsubscript{42A} and Ca\textsubscript{V}1.3\textsubscript{42A} amplification are as follows: mouse Ca\textsubscript{V}1.3\textsubscript{42}, full-length (NM_028981.2; Forward, GGGAAGTACCCTGGCAGAACC; Reverse, GGAATCTGGGGCAATGTCACTGCG) and Ca\textsubscript{V}1.3\textsubscript{42A} splice variant (Forward, CAGATGCTTGAAAAGATGGTC; Reverse, CTTCCCTGCCGAGGAGTGC). The sections were hybridized with sense and antisense probes (100 ng/\( \mu \)l) overnight in a humid chamber at 45\(^{\circ}\)C following wash, incubation with 0.5% blocking agent (from Invitrogen TSA Kit #21 Cat# T20931). Signal was developed using a peroxidase-labeled anti-DIG antibody (Roche Cat# 11207739310) at a concentration of 1 in 250 followed by tyramide signal amplification (Invitrogen TSA Kit #21 Cat# T20931) and finally incubation with fluorescein-conjugated streptavidin (Vector Laboratories Cat# SA-5001) at a concentration of 1 in 500. Absence of fluorescence signal on the sections hybridized with the sense probes has been represented in Supplementary Figure S2.

Immunohistochemistry (IHC) was performed for investigating the co-localization of the expression of calcium channel isoforms with marker of DA neurons, TH. IHC was performed on the same sections on which FISH was performed. The sections were first rinsed in phosphate buffer followed by blocking and overnight incubation in anti-TH rabbit antibody (Millipore Cat# AB152, RRID:AB_390204). Sections were then washed and incubated in Goat Anti-Rabbit IgG H+L (Alexa Fluor\textregistered 594; Thermo Fisher Scientific, Cat# A-21207, RRID:AB_141637) followed by washing. The sections were then mounted in Vectashield\textregistered© mounting medium (Vector Laboratories Cat# H-1000) and imaged as z-stacks using a Zeiss LSM 780 confocal microscope using LD LCI plan-apochromat 25\( \times \)/0.8 Oil objective using 488 nm and 594 nm lasers for low magnification images and plan-apochromat 100\( \times \)/1.4 Oil objective using 488 nm and 594 nm lasers for high magnification images. The maximum intensity projection that is used for representation was derived using Zeiss ZEN black software and orthogonal reconstruction was performed using Zeiss ZEN blue software.

**Statistical Analyses**
Analysis of relative gene expression from the qRT-PCR data was done using \( \Delta \Delta \text{Ct} \) method. The thresholds were set manually. Data were analyzed using Graphpad Prism (Graphpad Prism Inc., San Diego, CA, USA). Shapiro–Wilk test was performed on all datasets to test for normality and statistical tests were carried out accordingly. Statistical significance was determined using one-way ANOVA followed by student’s Newman–Keuls post-test for multiple comparisons and student’s t-test for comparison between two groups for data that passed normality test. Two-tailed Mann–Whitney U test was used for comparison between two groups when the data did not pass the test for normality.

**RESULTS**
**Ca\textsubscript{V}1.3\textsubscript{42A} and Ca\textsubscript{V}1.3\textsubscript{42} Expression in Ventral Midbrain, Cortex, and Striatum in Mice**
 mRNA levels of Ca\textsubscript{V}1.3\textsubscript{42A} and Ca\textsubscript{V}1.3\textsubscript{42} were assayed in the ventral midbrain of C57BL/6\textsubscript{L} naïve mice using qRT-PCR. mRNA levels for Ca\textsubscript{V}1.3\textsubscript{42A}, the short splice variant of Ca\textsubscript{V}1.3 containing exon 42A, were four times greater in the ventral midbrain as compared to the cortex and striatum (Figure 1A). Conversely, mRNA levels of full-length Ca\textsubscript{V}1.3 (Ca\textsubscript{V}1.3\textsubscript{42}) were greater in the cortex and striatum than in the ventral midbrain (Figure 1B). When comparing relative mRNA levels for Ca\textsubscript{V}1.3\textsubscript{42A} and Ca\textsubscript{V}1.3\textsubscript{42}, we found that Ca\textsubscript{V}1.3\textsubscript{42A}
mRNA levels represented about 0.5 times that of Cav1.342 in the ventral midbrain (Figure 1C). However, in the cortex and striatum mRNAs encoding Cav1.342A were much less abundant (approximately 0.06 times) than mRNAs for Cav1.342 (Figures 1D,E, respectively).

Co-localization of Cav1.342A and Cav1.342 With TH Positive Dopaminergic Neurons in SNpc

The available antibodies against Cav1.3 are highly non-specific. Therefore, in situ hybridization was used to identify and localize the expression of Cav1.342A and Cav1.342 in the midbrain. Combined fluorescent in situ hybridization for Cav1.342A and immunohistochemistry for TH allowed us to demonstrate that mRNAs encoding the short splice variant were present in SNpc DA neurons in control mice as indicated by orthogonal reconstruction (Figure 2A). Likewise, Cav1.342 mRNA was also co-localized with TH positive neurons in SNpc as seen from orthogonal reconstruction (Figure 2A). When using qRT-PCR, we found that mRNA levels of Cav1.342A were approximately four-fold higher in the SNpc as compared to the cortex (Figure 2B). In contrast, mRNA levels for the full-length channel Cav1.342 were lower in the SNpc as compared to the cortex (Figure 2C). These observations are also consistent with mRNA expression data reported for Cav1.342 and Cav1.342A in the midbrain and cortex in Figure 1.

mRNA Levels of Cav1.342A and Cav1.342 in SNpc After Acute Exposure to MPTP

MPTP, which is known to cause a selective loss of DA neurons in SNpc when administered to mice serves as a model of neurodegeneration akin to that seen in PD. In the present study, male C57BL/J6 mice received a single dose of MPTP (30 mg/kg body weight dose) administered subcutaneously. The animals were sacrificed 1 day after the dose. A single dose of MPTP did not result in the loss of TH mRNA levels in the SNpc (Figure 3A).

Subsequently, mRNA levels of Cav1.342A and Cav1.342 were assessed in SNpc. However, normalization of Cav1.342A and Cav1.342 mRNA to TH mRNA revealed that the expression of mRNA encoding Cav1.342A was significantly increased in the SNpc (Figure 3B) while Cav1.342 presented no significant change in mRNA expression (Figure 3C).

These results were further validated by using dopamine transporter (DAT) as another marker for dopaminergic neurons. MPTP treatment did not result in significant loss of DAT mRNA transcript levels in SNpc (Figure 3D). Upon normalization of Cav1.342A and Cav1.342 to DAT mRNA, there was a statistically significant increase in mRNA expression of Cav1.342A (Figure 3E) but there was no significant difference in expression of Cav1.342 mRNA (Figure 3F). There was also no significant change when Cav1.342A and Cav1.342 mRNA levels were normalized to mRNA levels for glutamate decarboxylase 1 (GAD1) and vesicular glutamate transporter 2 (Vglut2), markers of GABAergic (Figures 3G,H) and glutamatergic (Figures 3I,J).
neurons, respectively. Our results indicate the differential expression of CaV1.342A but not of CaV1.342 following acute exposure to MPTP.

**mRNA Levels of CaV1.342A and CaV1.342 in SNpc After Sub-chronic Exposure to MPTP**

Further, to assess the effect of sub-chronic exposure to MPTP on the mRNA expression of CaV1.342A and CaV1.342, male C57BL/J6 mice received MPTP that was administered subcutaneously once a day for 14 days. This treatment regimen resulted in a 45% loss of TH mRNA levels in the SNpc (Figure 4A). mRNA levels of CaV1.342A and CaV1.342 were then assessed in SNpc. Normalization of CaV1.342A and CaV1.342 mRNA to TH mRNA resulted in significantly increased expression of mRNA encoding CaV1.342A and CaV1.342, respectively, in the SNpc (Figures 4B,C). Further, MPTP treatment resulted in a 47% loss of DAT mRNA transcript levels in SNpc (Figure 4D). Upon normalization of CaV1.342A and CaV1.342 to DAT mRNA, there was a statistically significant increase in mRNA expression (Figures 4E,F). Moreover, the increased expression of CaV1.342A or CaV1.342 mRNA was not observed in the VTA from animals after sub-chronic MPTP exposure (Supplementary Figure S3). MPTP treatment did not change the expression of GAD1 (Figure 4G) and VGlut2 (Figure 4J). No significant difference in mRNA expression was observed when CaV1.342A and CaV1.342 mRNA levels were normalized to mRNA levels for GAD1 (Figures 4H,I) and Vglut2 (Figures 4K,L) and β-actin (Figures 4M,N), respectively.
DISCUSSION

It has been postulated that Cav1.3 L-type calcium channels, which contribute to the pacemaking activity of SNpc DA neurons may also play a role in their vulnerability to degeneration. Indeed, autonomous pacemaking leads to increase in basal mitochondrial oxidative stress in SNpc DA neurons, presumably as a direct consequence of the Ca\(^{2+}\) load (Foehring et al., 2009; Guzman et al., 2010; Surmeier et al., 2017; Liss and Striessnig, 2019). In support of this hypothesis, it has been shown that blocking Cav1.3 with a calcium channel antagonist, isradipine, afforded protection against neurodegeneration in the MPTP, 6-OHDA and rotenone rodent model of PD (Chan et al., 2007, 2010; Ilijic et al., 2011). Further, Guzman et al. have demonstrated that chronic, systemic isradipine treatment led to reduced cytosolic Ca\(^{2+}\) in SNpc...
FIGURE 4 | Modulation of Ca\textsubscript{$\alpha$}1.3\textsubscript{42A} and Ca\textsubscript{$\alpha$}1.3\textsubscript{42} mRNA levels in the SNpc of MPTP-treated mice after 14 days of treatment. (A) TH mRNA expression in the SNpc of mice treated subcutaneously with MPTP (30 mg/kg body weight) for 14 days. MPTP treatment led to a 45% reduction of TH mRNA levels in the SNpc (p = 0.0052, t = 3.152, df = 19). qRT-PCR data were normalized to mRNA levels of β-actin. (B) Relative mRNA levels for Ca\textsubscript{$\alpha$}1.3\textsubscript{42A} (p = 0.0048, t = 3.19, df = 19) and (C) Ca\textsubscript{$\alpha$}1.3\textsubscript{42} (Mann–Whitney U = 17, n\textsubscript{1} = 10, n\textsubscript{2} = 11, p = 0.0062, two-tailed) increased in the SNpc in response to MPTP treatment when the mRNA signal was normalized to TH. (D) mRNA levels of DAT were reduced by 47% upon MPTP treatment when normalized to mRNA levels of β-actin (Mann–Whitney-U = 19, n\textsubscript{1} = 9, n\textsubscript{2} = 11, p = 0.0200, two-tailed). (E) Relative mRNA levels for Ca\textsubscript{$\alpha$}1.3\textsubscript{42A} (p = 0.0227, t = 2.522, df = 16) and (F) Ca\textsubscript{$\alpha$}1.3\textsubscript{42} (p = 0.0114, t = 2.859, df = 16) (Continued)
DA neurons and lowered mitochondrial oxidant stress. It has been shown that knockdown of Cav1.3 resulted in reduced dendritic Ca$^{2+}$ oscillations in SNpc DA neurons eliciting their importance in the process (Guzman et al., 2018). This prompted us to study the expression of mRNAs encoding the full-length channel Cav1.3$_{42A}$ and its C-terminally truncated splice variant Cav1.3$_{42A}$ in SNpc. Besides, we evaluated the transcripts of the two-channel isoforms in the MPTP mouse model of PD.

The qRT-PCR analysis revealed that Cav1.3$_{42A}$ transcripts were expressed more abundantly in the ventral midbrain including SNpc when compared to the cortex, whereas the opposite was true for Cav1.3$_{42}$ transcripts. Interestingly, mRNAs encoding the full-length channel Cav1.3$_{42}$ as well as its truncated splice variant Cav1.3$_{42A}$ were found to be selectively increased in the SNpc of MPTP-treated mice.

While the presence of the full-length isoform of Cav1.3 channels Cav1.3$_{42}$ and that of its short splice variant Cav1.3$_{42A}$ has been reported in the mouse (Bock et al., 2011; Tan et al., 2011) and human brain (Singh et al., 2008), there is no study comparing the regional expression of the two mRNA isoforms in the brain. By coupling in situ hybridization to immunohistochemistry, we demonstrated that both Cav1.3$_{42}$ and Cav1.3$_{42A}$ mRNA expressed in TH positive neurons in the SNpc. Furthermore, we showed that mRNA levels for the splice variant Cav1.3$_{42A}$ were higher in mouse ventral midbrain as compared to cortex or striatum whereas the opposite was true for Cav1.3$_{42}$ transcripts. Cav1.3$_{42A}$ mRNA expression was also higher in the SNpc in comparison to the cortex in concurrence with the above. This indicates that the L-type calcium channel isoform Cav1.3$_{42A}$ substantially contributes to calcium influx in midbrain DA neurons in the SNpc. Since calcium current density through Cav1.3$_{42A}$ is about 2.5 times greater than the full-length channel isoform (Singh et al., 2008), one may assume that calcium influx through this channel is more pronounced in midbrain DA neurons than cortical neurons. It may be noted that the pacemaking activity inherent to DA neurons is accompanied by Cav1.3 L-type calcium channels in the SNpc but not in the VTA where it relies on Nav1/HCN channels (Guzman et al., 2010; K Halliday and Bean, 2010). This may render nigral DA neurons more vulnerable to calcium overload through activation of Cav1.3$_{42A}$ channels. The differential vulnerability between SNpc and VTA DA neurons may be further exacerbated by the fact that DA neurons from the VTA also contain high levels of the calcium buffering protein Calbindin-D28K while most vulnerable DA neurons in the SNpc are lacking this protein (Damiar et al., 1999).

In the present study, we also examined mRNA expression changes, if any, of the L-type calcium channel and its variant in the SNpc in response to both acute and sub-chronic treatment regimens with the DA neurotoxin MPTP. In fact, the expression of Cav1.3$_{42A}$ was significantly higher in SNpc after 24 h of MPTP exposure although the levels of TH and DAT mRNA were not significantly affected (Figure 3). However, after 14 days of MPTP treatment, when the TH and DAT mRNA levels were reduced by approximately 50%, the mRNA levels of Cav1.3$_{42}$ and Cav1.3$_{42A}$ were maintained and were similar to vehicle controls. Since Cav1.3$_{42}$ and Cav1.3$_{42A}$ are predominantly expressed in SNpc by DA neurons, a ~50% reduction of the number of these neurons induced by sub-chronic MPTP treatment should be reflected by a significant decrease in transcripts encoding the two-channel isoforms. This was not the case, however, upon normalization to β-actin indicating that there is a compensatory increase in the expression of the channel transcripts in the surviving SNpc DA neurons. Further, when mRNA expression for Cav1.3$_{42}$ and Cav1.3$_{42A}$ was normalized to TH and DAT mRNA, we observed a significant increase in transcripts encoding Cav1.3$_{42A}$ after 1 day of single dose of MPTP and both channel isoforms after 14 days of exposure to MPTP. This suggested that DA neurons may produce more transcripts to possibly preserve their activity in surviving neurons in response to neurodegeneration triggers.

In rodents, while about 70% of SNpc neurons are DA neurons, about 29% of SNpc neurons are GABAergic and about 1–2% are glutamatergic (Nair-Roberts et al., 2008). It was therefore, essential to demonstrate that the increased expression of Cav1.3$_{42}$ and Cav1.3$_{42A}$ mRNA resulted from the loss of DA neurons after MPTP treatment and not from changes affecting other neuronal populations. Consistent with this view, the expression of Cav1.3$_{42}$ and Cav1.3$_{42A}$ transcripts remained unchanged in the SNpc when mRNA signals were normalized to either mRNA for GAD1, a GABAergic neuron marker or for Vglut2, a glutamatergic neuron marker.

The upregulation observed in the expression of Cav1.3$_{42A}$ and Cav1.3$_{42}$ mRNA upon normalization to TH and DAT mRNA levels could, therefore, have the following plausible explanations: (i) While the observed change in the expression of Cav1.3 channels in MPTP treated mouse SNpc could be explained as a retention of phenotype, it is important to note that about 50% loss in the number of TH positive neurons along with loss of Nissl positive neurons has been observed in the SNpc of MPTP treated mice (Saeed et al., 2009). Since Cav1.3 channels are expressed in neurons, a resultant reduction in their mRNA expression in SNpc is, therefore, expected.
concomitant to the reduction in the number of TH positive neurons in SNpc similar to that seen for the mRNA expression of TH and DAT. (ii) The Cav1.3A2A and Cav1.3A2 mRNA upregulation could be a result of their upregulation in non-TH expressing (non-dopaminergic) neurons. To address this issue, we showed that the expression of mRNA for the markers of GABA-ergic neurons, i.e., GAD1 and glutamatergic neurons, i.e., VGluT2 did not change. Further, the expression of Cav1.3A2A and Cav1.3A2 when normalized to GAD1 and VGluT2 expression did not differ in MPTP treated SNpc as compared to controls. (iii) The upregulation of Cav1.3A2A and Cav1.3A2 mRNA results from their increased expression in the surviving TH neurons. In the light of the present results demonstrating a decrease in TH and DAT expression and an increase in Cav1.3A2A and Cav1.3A2 mRNA levels, it is likely that the surviving DA neurons in SNpc are expressing greater levels of Cav1.3A2A and Cav1.3A2 mRNA.

Further, an increase in the expression of Cav1.3A2A upon normalization with TH and DAT 24 h after a single exposure to MPTP, in which case, there is no downregulation of TH and DAT also suggests that there is potentially an increase in the expression of Cav1.3 channels in vulnerable DA neurons in SNpc on exposure to MPTP. This set of results supports the view that mouse DA neurons in SNpc may be at risk to degenerate presumably because they have to cope with a larger influx of calcium through Cav1.3 L-type calcium channels during neurodegeneration (Surmeier and Schumacker, 2013). Possibly related to present observations, Lieberman et al. (2017) demonstrated that there is a build-up of cytosolic calcium in cultured DA neurons from SN as opposed to VTA following treatment with the active metabolite of MPTP, MPP+. To conclude, the sustained high levels of expression of Cav1.3A2 and its variant Cav1.3A2A indicates the possible contribution of these two channel isoforms to degeneration of dopaminergic neurons in the sub-chronic MPTP mouse model of PD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal and Ethics Committee of Indian Institute of Science, Bangalore, India (Protocol# CAF/Ethics/267/2012).

AUTHOR CONTRIBUTIONS

The research project was conceptualized by VR, organized by AV and VR and executed by AV. Statistical analysis was designed and executed by AV and reviewed and critiqued upon by VR. First draft of the manuscript was written by AV and VR.

FUNDING

We acknowledge the grant support from Tata Trusts India. AV also thanks the Council of Scientific and Industrial Research (CSIR) for research fellowship.

ACKNOWLEDGMENTS

We thank Prof. Etienne C. Hirsch and Dr. Patrick P. Michel, ICM, Paris for the discussions and help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00382/full#supplementary-material.

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

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