Published in final edited form as:

Neurotoxicology. 2012 June ; 33(3): 321–331. doi:10.1016/j.neuro.2012.02.001.

Recovery of hypothalamic tuberoinfundibular dopamine neurons from acute toxicant exposure is dependent upon protein synthesis and associated with an increase in parkin and ubiquitin carboxy-terminal hydrolase-L1 expression

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Abstract

Hypothalamic tuberoinfundibular dopamine (TIDA) neurons remain unaffected in Parkinson disease (PD) while there is significant degeneration of midbrain nigrostriatal dopamine (NSDA) neurons. A similar pattern of susceptibility is observed in acute and chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse and rotenone rat models of degeneration. It is not known if the resistance of TIDA neurons is a constitutive or induced cell-autonomous phenotype for this unique subset of DA neurons. In the present study, treatment with a single injection of MPTP (20 mg/kg; s.c.) was employed to examine the response of TIDA *versus* NSDA neurons to acute injury. An acute single dose of MPTP caused an initial loss of DA from axon terminals of both TIDA and NSDA neurons, with recovery occurring solely in TIDA neurons by 16 h post-treatment. Initial loss of DA from axon terminals was dependent on a functional dopamine transporter (DAT) in NSDA neurons but DAT-independent in TIDA neurons. The active metabolite of MPTP, 1-methyl, 4-phenylpyridinium (MPP⁺), reached higher concentration and was eliminated slower in TIDA compared to NSDA neurons, which indicates that impaired toxicant bioactivation or distribution is an unlikely explanation for the observed resistance of TIDA neurons to MPTP exposure. Inhibition of protein synthesis prevented TIDA neuron recovery, suggesting that the ability to recover from injury was dependent on an induced, rather than a constitutive cellular mechanism. Further, there were no changes in total tyrosine hydroxylase (TH) expression following MPTP, indicating that up-regulation of the rate-limiting enzyme in DA synthesis does not account for TIDA neuronal recovery. Differential candidate gene expression analysis revealed a time-dependent increase in parkin and ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1) expression (mRNA and protein) in TIDA neurons during recovery from injury. Parkin expression was also found to increase with incremental doses of MPTP. The increase in parkin expression occurred specifically within TIDA neurons, suggesting that these neurons have an intrinsic ability to up-regulate parkin in response to MPTP-induced injury. These data suggest that TIDA neurons have a compensatory mechanism to deal with

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Conflict of interest statement

None.

toxicant exposure and increased oxidative stress, and this unique TIDA neuron phenotype provides a platform for dissecting the mechanisms involved in the natural resistance of central DA neurons following toxic insult.

Keywords

Parkin; Ubiquitin carboxy-terminal hydrolase-L1; Parkinson disease; MPTP; Tuberoinfundibular; Nigrostriatal; Recovery

1. Introduction

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by motor abnormalities, which are primarily due to extensive degeneration of nigrostriatal dopamine (NSDA) neurons, resulting in decreased quality of life and increased mortality (Schenkman et al., 2001; Wermuth et al., 1995). Synthesis and transmission of DA, one defining feature of NSDA neurons, has been intensively studied and proposed to contribute to the neurodegenerative process through the generation of reactive intermediates during the metabolism of cytoplasmic DA (Lotharius et al., 1999; Lotharius and O'Malley, 2000). However, separate DA neuronal populations with similar neurotransmitter synthetic and metabolic pathways, including hypothalamic tuberoinfundibular (TI) DA neurons, do not degenerate in the PD brain, suggesting other deleterious/protective factors may be involved in determining the fate of DA neurons in PD (Braak and Braak, 2000; Langston and Forno, 1978).

TIDA neurons originate in the arcuate nucleus (ARC) of the mediobasal hypothalamus (MBH) and project ventrally to the median eminence (ME) to terminate adjacent to hypophyseal portal vessels. Reuptake of DA in this system is mediated by high volume low affinity transporters and to a lesser extent, low volume high affinity DA transporter (DAT) (Lookingland and Moore, 2005). The molecular machinery for synthesis, vesicular storage, release, and metabolism of DA are otherwise virtually identical between TIDA and NSDA neurons. As such, pathological stresses that disrupt DA homeostasis should, theoretically, produce similar deleterious effects in both TIDA and NSDA neuronal systems.

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicant-based animal model produces a predictable injury and degeneration of NSDA nerve terminals and cell bodies. MPTP-based models efficiently recapitulate many of the molecular pathological features of PD, including oxidative stress, mitochondrial dysfunction, impairment of proteasome function, activation of programmed cell death, and neuroinflammation. Although there are noteworthy shortcomings for MPTP and other toxicant-based animal models (Chan et al., 1991a; Dauer and Przedborski, 2003; Litvan et al., 2007; Zang and Misra, 1993), these models offer an *in vivo* platform to investigate mechanisms of cellular dysfunction within post-mitotic, differentiated and PD relevant neurons. In addition, the observation that NSDA neurons are severely damaged, while TIDA neurons are resistant to acute doses of MPTP, administered systemically or centrally (Behrouz et al., 2007; Melamed et al., 1985; Mogi et al., 1988; Sundstrom et al., 1987; Willis and Donnan, 1987), make this a reasonable model for studying differences between these DA neuronal populations.

The initial responses of TIDA and NSDA neurons to a single systemic injection of MPTP are similar, with both neuronal populations losing axon terminal DA stores by 4 h post treatment (Behrouz et al., 2007). Differences between these neuronal populations become evident by 8 h post-MPTP, when TIDA neurons begin recovering from DA loss, eventually reaching full convalescence by 16 h. In contrast, NSDA neuronal DA stores remain depleted

at 16 h and show evidence of apoptosis by 72 h post-MPTP (Behrouz et al., 2007; Perry et al., 1985; Pileblad et al., 1984, 1985). A similar differential resistance/sensitivity to toxicant induced injury is observed in TIDA and NSDA neurons in primary culture (Behrouz et al., 2007). The narrow window of time during which resistant TIDA neurons recover and susceptible NSDA neurons remain damaged allows for examination of intrinsic, cell autonomous mechanisms underlying their differential responses. The studies presented herein test the hypothesis that up-regulation of protective proteins following MPTP facilitate recovery of TIDA neurons from disruptive effects of acute toxicant-induced neuronal injury.

2. Materials and methods

2.1. Animals

All experiments were conducted in 8–10 week old male C57Bl/6J mice purchased from Jackson Laboratories (Bar Harbor, MA). Animals were housed two to four per cage, maintained in a light-controlled (12 h light/dark cycle; lights on 0600 h) and temperature-controlled (22 ± 1 °C) room, and provided with food and tap water *ad libitum*. The Michigan State University Institutional Animal Care & Use Committee approved all experiments using live animals (AUF 08/08-123-00).

2.2. Drugs

All drugs were purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in 0.9% sterile saline on the day of the experiment. Doses were calculated as the free base of the respective drug.

Single injection MPTP—Mice received a single injection of either vehicle (10 ml/kg; s.c.) or MPTP (20 mg/kg; s.c.) and the experiment was terminated at 4, 8, 12, 16, 24 or 32 h after the MPTP injection.

Cycloheximide—Mice were treated with 2 injections of vehicle (10 ml/kg; i.p.) or the protein synthesis inhibitor cycloheximide (120 mg/kg; i.p.) 2 and 4 h after a single injection of MPTP.

GBR-12909—Mice were treated with an injection of either vehicle (10 ml/kg; i.p.) or the DAT blocker GBR-12909 (10 mg/kg; i.p.) 30 min prior to a single injection of MPTP. The experiment was terminated 4 h after MPTP treatment.

2.3. Tissue preparation

At the appropriate time following drug administration, mice were killed by decapitation and brains were rapidly removed and placed on an ice-cooled glass stage. Under a dissecting microscope, the ME was collected and the remaining brain was quickly frozen on dry ice. Consecutive frozen coronal sections (500 μ m) were prepared throughout the rostrocaudal extent of the regions containing subpopulations of interest using a cryostat set at -10 °C (CTD-Model Harris, International Equipment Co., Needham, MA) and the regions of interest were microdissected using a modification of the method described previously (Palkovits, 1973). These tissue samples were used for neurochemical, Western blotting and RNA analyses, and were processed according to the appropriate protocols described below.

2.4. Neurochemical analyses

Microdissected brain tissue samples were placed into cold tissue buffer (0.1 M phosphate–citrate buffer pH 2.5) and sonicated with three consecutive 1 sec bursts (Heat Systems Ultrasonics, Plainview, NY). Protein was pelleted by centrifugation at $12,000 \times g$ (Beckman

Coulter Microfuge, Palo Alto, CA) for 1 min. The content of DA in supernatants was determined with high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED) using a Waters 515 HPLC pump (Waters Corporation, Milford, MA) and an ESA Coulochem 5100A electrochemical detector with an oxidation potential of +0.4 V. DA content was quantified by comparing the peak heights of each sample to the peak heights of standards. MPP⁺ content in supernatants was determined using HPLC coupled with mass spectrometry (MS) as described previously (Lehner et al., 2011).

Tissue pellets were re-suspended in 1 N NaOH and assayed for protein (Lowry et al., 1951). To correct for differences in sample size the DA and MPP⁺ content was normalized to the amount of protein in each sample and expressed as a concentration in ng DA or pg MPP⁺ per mg protein.

2.5. Western blot analyses

Microdissected brain samples were sonicated in cold homogenization buffer (TBS containing 1% SDS, 0.1 mM PMSF, 1 mM DTT with Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany) pH 7.4, and centrifuged (10,000 × *g* 10 min)). The supernatants containing total cytoplasmic protein were removed and placed into fresh microcentrifuge tubes. The supernatants were assayed for protein content using the Bradford protein method (Thermo Fisher, IL, USA). Protein (15–20 µg) from each sample was run on polyacrylamide gels and transferred to 0.45 µm FL-PVDF membrane (Millipore, Pittsburgh, MA, USA) by electrophoresis and reacted with 1:1000 rabbit anti-parkin (Cell Signaling), 1:800 rabbit anti-UCHL1 (Cell Signaling), 1:1000 mouse anti-β-actin (Cell Signaling) and 1:20,000 mouse anti GAPDH (Sigma) primary antibodies overnight at 4 °C and incubated with IR Dye 800-conjugated goat anti-rabbit (Li-Cor Biosciences) and 680-conjugated goat anti-mouse (Li-Cor Biosciences) secondary antibodies (1:15,000 dilution in blocking buffer) for 1 h at room temperature. Membranes were washed and bound antibodies were visualized with the Odyssey infrared imager (Li-Cor Biosciences). The density of each band was quantified by measuring the infrared absorbance using the Odyssey infrared imager and Odyssey software (Version 3.0, Li-Cor Biosciences). Relative density was obtained by normalizing the band density of parkin to that of the control protein used to account for variations in loading of samples onto the gel. GAPDH or β-actin were used as the control proteins and their detection and visualization was linear. Expression levels of GAPDH or β-actin were similar among the compared brain regions regardless of treatment. Each FL-PVDF membrane contained representative samples from all experimental conditions.

2.6. RNA isolation and quantitative real time PCR

Total RNA from microdissected tissue was extracted using the MELT total nucleic acid isolation with DNase digestion with on-bead TURBO-DNA digestion (Ambion, Austin, TX). RNA concentration was determined using a Nanodrop and quality was assessed using a picochip bioanalyzer (AGILENT Technologies). RNA was incubated with oligo(dT) primers and dNTP mix at 65 °C for 5 min to promote primer annealing. The mixture was incubated with 0.1 M DTT, a reverse transcriptase (Superscript II, Invitrogen) and RNase inhibitor (RNaseIn, Ambion) at 42 °C for 1 h to allow for reverse transcription. The mixture was then heated to 70 °C for 15 min in order to destroy the reverse transcriptase. And resulting cDNA was stored in –80 °C until use with real time PCR. Primers were identified using Primer3 software and synthesized by the macromolecular and structural facility (Michigan State University, East Lansing, MI) and sequences are available upon request. All primer sets were tested to ensure a single product as assessed by 2% agarose gel electrophoresis and melt-curve analysis. GAPDH was used as the reference gene, was similar across brain regions and did not change with treatment. No template reactions were

used as negative controls. Fold change between regions was determined using the $\Delta\Delta C_t$ method, which compares the C_t values of the samples of interest with a control or calibrator, in this case substantia nigra (SN) from untreated mice. PCR efficiencies of all primer sets were therefore tested to ensure similarity to the GAPDH primer (85–100%).

2.7. Laser capture microdissection

Parkin mRNA expression in TIDA neurons was determined using laser capture microdissection (LCM) of TH-immunoreactive cells in the ARC (PixCell II Laser Microdissection System, Arcturus Engineering Incorporated; Mountain View, CA, USA). LCM utilizes a near-infrared power laser microbeam to melt a thermoplastic ethyl vinyl acetate membrane which overlays the tissue of interest. The melted membrane adheres to the selected cells, which can be lifted and secured in a microfuge tube containing the RNA extraction solution. The surrounding tissue remains intact. This technique, coupled with rapid immunofluorescent TH staining allows for sampling of DA neurons without major contamination from other neuronal types and glia.

Mice were killed 8 h after MPTP or saline treatment and TH immunoreactive cells were harvested using laser capture. Prior to cell dissection, coronal 10 μm sections through the hypothalamus were obtained in a cryostat at -10°C , mounted on slides and kept frozen on dry ice. Sections were stained with immunofluorescence according to previously described methods (Greene et al., 2005). Briefly, sections were fixed in ice-cold acetone for 1 min, rinsed and incubated with primary mouse anti-TH antibody (1:40; Zymed, CA) for 2 min, rinsed again and incubated with Alexa Fluor 488 goat anti mouse secondary antibody (Invitrogen, CA) for 2 min, rinsed, dipped in cresyl violet and dehydrated in an ethanol gradient followed by xylene.

LCM was performed using a PixCell II Laser Microdissection System (Arcturus Engineering Incorporated; Mountain View, CA, USA). The following parameters were used for dissection of TH-immunofluorescent cells: spot size = 7 μm ; power = 45–85 mW; and duration = 750–1100 μs . After rapid staining, slides containing the sections were cleaned using a prep strip and placed onto the microscope. Capsure HS caps (Arcturus Engineering Inc., CA) were loaded onto the LCM and after focusing on the appropriate sections an HS cap was positioned on top of the tissue so that the cells of interest were in the center of the cap. The same HS cap was used to collect 300–500 cells from each brain region per animal. The cap was placed into the alignment tray and 10 μl of extraction buffer was placed into the fill port of the ExtractSure™. The tube was placed into the port, covered with a heating block (preheated to 42°C) and incubated for 30 min at 42°C to extract the cells. The cells were collected at the bottom of the tube by centrifugation for 2 min at $800 \times g$ and were stored frozen at -80°C . Cells were extracted from the cap and mRNA was isolated using an Extractsure adapter and PicoPure isolation kit (Arcturus) with DNase digestion (Qiagen). RNA quality was assessed for a subset of samples using a Bioanalyzer Picochip (Agilent Technologies, CA) and was optimal. Parkin and GAPDH mRNA levels were determined by real time quantitative PCR as described above.

2.8. Statistical analyses

Power analyses were conducted to determine optimal sample size required for each experiment. One-way analysis of variance (ANOVA) tests were used to detect statistical significance between two or more groups on a single independent variable. Two-way ANOVAs were used to detect statistical significance between two or more groups when there were two independent variables in the study. Repeated measures ANOVA was used when comparing brain regions within the same animal. A p value of less than or equal to

0.05 was considered statistically significant. If the ANOVA revealed an interaction of statistical significance Tukey's test was used for multiple comparisons among groups.

3. Results

3.1. Time course of the neurochemical responses of TIDA and NSDA neurons to a single injection of MPTP

Fig. 1 depicts the time course response of TIDA and NSDA neurons to a single systemic injection of MPTP. ME and striatum (ST) DA levels (reflecting DA stores in the terminal regions of TIDA and NSDA neurons, respectively), were significantly decreased 4 h after MPTP treatment. ME DA levels recovered and were similar to controls by 24 h post-MPTP. In contrast, DA levels in the ST decreased further past the 4 h time point and remained low (~25% of controls) for up to 24 h post-MPTP.

3.2. Effects of blockade of DAT on MPTP-induced DA depletion in TIDA and NSDA neurons

Systemic MPTP readily crosses the blood brain barrier and is converted to 1-methyl-4-phenylpyridinium (MPP⁺), which is taken up into NSDA neurons *via* DAT (Bezard et al., 1999; Gainetdinov et al., 1997; Kitayama et al., 1992). To determine if MPTP disrupts DA stores in TIDA neurons *via* the same DAT-dependent mechanism as NSDA neurons, mice were pre-treated with the DAT blocker GBR-12909 prior to MPTP administration. The experiment was terminated 4 h following MPTP, a time point when a significant loss of DA was observed in both the ME and the ST. ME DA levels decreased 4 h following MPTP treatment (Fig. 2, Left Panel), regardless of pre-treatment with GBR-12909 or saline. In contrast, pretreatment with GBR-12909 blocked the MPTP-induced decrease in ST DA (Fig. 2, Right Panel). These results confirm previous reports of DAT dependency for MPTP-induced toxicity of NSDA neurons, and indicate that a DAT independent mechanism is responsible for MPTP-induced DA depletion in TIDA neurons.

3.3. Time course of MPP⁺ accumulation in brain regions containing axon terminals of TIDA and NSDA neurons following a single injection of MPTP

TIDA recovery from a single injection of MPTP could be due to differential distribution and elimination of its active metabolite in the ME and ST, thus MPP⁺ concentrations were determined in the terminal field regions of NSDA and TIDA neurons following MPTP treatment. Compared to saline-treated animals, there is a significant accumulation of MPP⁺ in the ME by 4 h post-MPTP administration, which is slowly eliminated by 24 h post-injection (Fig. 3, Left Panel) with an approximate half life of 5.9 h. The accumulation and elimination of MPP⁺ in the terminal regions of TIDA neurons (ME) mirrored the changes in DA concentrations within this region, *i.e.*, ME DA concentrations reaches its lowest point at 4 h post-MPTP and recovers to pretreatment levels while MPP⁺ is concurrently eliminated. MPP⁺ concentrations in the ST (Fig. 3, Right Panel) reached maximal accumulation at 4 h followed by a relatively rapid elimination of the toxicant over the next 20 h with an approximate half life of 1.8 h. In contrast to ME DA, ST DA remains depleted for up to 24 h post MPTP without any appreciable recovery. The absolute maximal concentrations of MPP⁺ were four times higher in the ME compared to the ST, with MPP⁺ concentrations of 365 ± 37 pg/ μ g in the ME and 78 ± 9 pg/ μ g in the ST. Elimination constants were calculated for MPP⁺ in the ME and ST. MPP⁺ has a significantly higher elimination constant (Table 1) in the ST (0.38 ± 0.02) than in the ME (0.11 ± 0.01).

3.4. Effects of blockade of protein synthesis on MPTP-induced DA depletion in TIDA and NSDA neurons

In order to determine if TIDA neuronal recovery is mediated *via de novo* synthesis of protective proteins, terminal DA concentrations were measured in the presence of the

protein synthesis inhibitor cycloheximide. Fig. 4 (Left Panel) demonstrates the effects of cycloheximide on DA concentrations in the ME of MPTP-treated mice. The experiment was terminated 16 h following MPTP, a time point when TIDA neurons show full recovery from MPTP-induced DA depletion (Fig. 1) (Behrouz et al., 2007). Consistent with our initial observation, DA levels in the ME were similar to vehicle-injected controls 16 h after treatment with MPTP. Cycloheximide had no effect on ME DA concentrations in vehicle-injected control mice, but prevented full recovery of DA concentrations in ME of MPTP-treated mice. In contrast, the MPTP-induced loss of ST DA was similar in vehicle- and cycloheximide-treated mice (Fig. 4; Right Panel).

3.5. Time course of tyrosine hydroxylase (TH) protein expression following a single injection of MPTP

Given that the ability of TIDA neurons to recover from MPTP is dependent on new protein synthesis, it is possible that there is a compensatory increase in TIDA expression of TH, the rate limiting enzyme in DA synthesis. In order to determine if increased DA synthesis, *via* an up-regulation of TH, is responsible for the recovery of ME DA following MPTP, total TH protein was measured by Western blot over a 24 h period. Following acute MPTP treatment total TH protein is unchanged in both the ME and the ST for 24 h post toxicant administration (Fig. 5).

3.6. Changes in gene and protein expression in TIDA and NSDA neurons following a single injection of MPTP

Considering changes in TH expression do not appear to be involved in the differential susceptibility of TIDA and NSDA neurons to MPTP, changes in other candidate gene and protein expression following toxicant administration were investigated. Fig. 6 depicts expression levels of genes involved in PD pathogenesis in tissue obtained from the ARC and SN (cell body regions of TIDA and NSDA neurons, respectively) in mice treated with a single injection of MPTP or its saline vehicle. Mice were killed by decapitation 8 h after injection, a time point when TIDA neurons have begun recovering from MPTP-induced DA loss while NSDA neurons continue to lose DA (Behrouz et al., 2007). Parkin mRNA expression increased following MPTP treatment in the ARC, but not in the SN (Fig. 6). A significant increase was also detected in ubiquitin carboxy terminal hydrolase-L1 (UCH-L1) mRNA expression in the ARC (but not in SN) following MPTP. In contrast, levels of PINK1, DJ-1, α -synuclein and LRRK2 mRNA were not altered in either the ARC or the SN of MPTP-treated mice as compared with vehicle-treated controls.

Further examination of parkin and UCH-L1 expression demonstrates an increase in parkin and UCH-L1 protein in brain regions containing TIDA (but not in NSDA) neurons following MPTP administration. The MPTP-induced increase in parkin protein in the area of the MBH containing the ARC was time-dependent, with progressively increasing protein levels at 6, 12, 24 and 36 h following MPTP (Fig. 7). In contrast, parkin protein expression remains static in the SN for up to 12 h following toxicant administration, followed by a small decrease at 24 and 36 h. Similarly, UCH-L1 was significantly up-regulated in the ARC at 16 h post-MPTP (though to a lesser extent than parkin) and was decreased in the SN by the 8 h time point and remained low for the remainder of the 24 h time course (Fig. 8). The MPTP-induced up-regulation of parkin protein expression in the ARC was found to be dose-dependent (Fig. 9, Left Panel), while there was a decrease in parkin protein expression in the SN at higher doses (Fig. 9, Right Panel).

To verify that changes in gene expression were up-regulated within TIDA neurons, parkin mRNA expression was assessed 8 h after saline or MPTP treatment in phenotypically identified TH-immunoreactive (IR) TIDA neurons located in the ARC and SN using LCM.

TH-positive cells were collected from the ARC and SN as exemplified in Fig. 10 (Panels A–C). A marked increase in parkin mRNA was observed in TIDA neurons 8 h following acute MPTP treatment, at the time when TIDA neurons are beginning to recover DA in the ME (Fig. 10). Consistent with the above protein and mRNA expression data, no change in parkin mRNA expression was observed in TH-IR cells of the SN at this time point.

4. Discussion

The ability of DA neurons to recover following toxicant exposure may be important in treatment of PD progression (especially in early stages) since there is likely a population of neurons that are injured but not yet doomed to undergo cell death, similar to DA neurons following an acute dose of MPTP. The experiments presented herein examine how TIDA neurons (which survive in PD) differ from the highly susceptible NSDA neurons following an acute injury with the dopaminergic toxicant MPTP. The current study utilized a single injection of MPTP in order to identify early events that occur following toxicant administration. The small dose and short time course following MPTP causes dysfunction within the DA terminal, most likely due to displacement of vesicular DA and ATP depletion with subsequent reactive oxygen species formation, which is thought to be independent of any overt cell death (Chan et al., 1991b; Jackson-Lewis et al., 1995; Lotharius and O'Malley, 2000). TIDA neurons recover from acute MPTP-induced DA loss *via* a mechanism that requires synthesis of new proteins, but is independent of an increased expression of the DA synthetic enzyme TH. Considering that *de novo* protein synthesis is required for TIDA recovery and total TH protein concentrations remain unchanged, it is unlikely that an increase in DA synthesis, mediated by a change in TH activity, could account for the differential susceptibility of TIDA *versus* NSDA neurons following acute MPTP exposure. Thus, increased expression of compensatory factors other than TH is a plausible mechanism to explain the rapid recovery of TIDA neurons from acute MPTP-induced injury.

A time- and dose-dependent increase in parkin protein occurs following MPTP in TIDA neurons. Additionally, MPTP also induced increases in another protein related to the ubiquitin-proteasome system, UCHL-1, suggesting that a coordinated compensatory response may function to allow the unique recovery of TIDA neurons from acute toxicant exposure. In contrast, NSDA neurons do not recover from acute MPTP exposure and do not appear able to rapidly increase expression of these proteins, rather, there is a gradual decrease in parkin and UCHL-1 expression in the SN. Taken together, these data suggest that TIDA neurons have a compensatory ability to recover from an acute toxicant exposure that may be dependent, in part, on the ability of these neurons to increase expression of neuroprotective proteins like parkin and UCHL-1.

Our previously published data indicated that an intrinsic neuronal mechanism (rather than extrinsic factors such as toxicant distribution or elimination) most likely accounts for the differential susceptibility of TIDA *versus* NSDA neurons to toxicant-induced degeneration (Behrouz et al., 2007). TIDA neurons have been thought to be resistant to the toxicant MPP⁺ due to lower expression of the high affinity DAT (Annunziato et al., 1980; Demarest and Moore, 1979; Revay et al., 1996). However, our previous findings and the data presented herein indicate that this is not likely to be the case. Pharmacological blockade of DAT prior to administration of MPTP does not prevent initial loss of DA stores in TIDA neurons as it does in NSDA neurons, suggesting the mode of MPP⁺ entry into TIDA neurons is DAT-independent. Previous reports characterizing a low affinity high volume transporter in TIDA neurons provide further evidence that these neurons utilize different mechanisms for uptake of DA-like substances (for review see Lookingland and Moore, 2005). Neurons in the ARC express high levels of the organic cation transporter-3 (OCT3) and this transporter shows

high affinity for MPP⁺ (Cui et al., 2009; Gasser et al., 2009), suggesting that OCT3 may be the low affinity uptake transporter that allows MPP⁺ accumulation in TIDA neurons.

Peak concentrations of MPP⁺ are higher in regions containing axon terminals of TIDA neurons as compared to NSDA neurons, and the half-life of MPP⁺ is shorter in the ST than in the ME. These data are consistent with previous reports demonstrating that MPP⁺ does effectively enter as well as accumulate within hypothalamic DA neurons and is eliminated slower than in NSDA neurons (Del Zompo et al., 1993; Speciale et al., 1998). Regardless of the mode of MPP⁺ entry into the cell, both neuronal populations respond to toxicant treatment in a similar fashion, *i.e.*, loss of DA stores in axon terminal regions is similar by 4 h after systemic administration, at a time when MPP⁺ concentrations peak. As such, a difference in the mode of toxicant entry into the cell is an unlikely explanation for the unique ability of TIDA neurons to recover from an acute injury.

Critical events likely occur between 4 and 16 h following MPTP administration, when TIDA neurons are able to completely recover from DA loss while NSDA neurons demonstrate continued and sustained DA loss. TIDA axons terminate in the ME, a circumventricular region where the blood brain barrier is relatively porous, potentially creating a micro-environment of increased toxicant exposure. In this context, it is possible that TIDA neurons are specially suited to rapidly up-regulate proteins, like parkin and UCHL-1, which allow for survival in the face of exposure to hazardous molecules that produce acute injury. In line with this hypothesis, blockade of protein synthesis between 4 and 10 h following MPTP significantly impairs the ability of TIDA neurons to recover from DA loss by 16 h after toxicant treatment. On the other hand, blockade of protein synthesis during this same epoch following MPTP treatment does not affect DA loss in NSDA neurons. Although the protein synthesis inhibitor, cyclohexamide, used in the current study has been shown to have off target effects, cyclohexamide had no effect on terminal DA concentrations in vehicle treated animals and did not affect MPTP-induced DA loss in NSDA neurons. As such, cyclohexamide appears to be a reasonable tool to investigate the contribution of protein synthesis in the unique ability of TIDA neurons to recover following MPTP (Matthaei et al., 1988).

Although increases in parkin and UCH-L1 were observed in the ARC following MPTP exposure, this data does not necessarily mean these increases were occurring specifically within TIDA neurons. It is possible that other cell types, both neuronal and non-neuronal, within this brain region could be contributing to the observed up-regulation of protein synthesis. That said, increases in parkin expression were observed specifically within TIDA neurons isolated utilizing LCM, identifying one specific cell type in which these compensatory changes can occur following MPTP treatment. It should also be noted that the acute MPTP-induced increase in parkin mRNA in TH-IR cells of the MBH does not exclude the possibility that other cell types may be contributing to TIDA recovery or NSDA susceptibility, through the differential regulation of neuroprotective proteins (such as parkin), neuronal or glial-derived trophic support, inflammation or reactive gliosis (Hung and Lee, 1996; Hurley et al., 2003; Schwartz et al., 1993; Teismann and Ferger, 2001). The compensatory up-regulation of parkin mRNA within TIDA neurons in response to an acute neuronal disruption is quite likely a component of a broad, yet region specific response to injury.

It is plausible that the differential susceptibility of NSDA and TIDA neurons is a reflection of the intrinsic, neuron specific ability of TIDA neurons to selectively up-regulate protective protein expression. The observations that the same proteins that are up-regulated in toxicant and PD resistant TIDA neurons are also decreased in susceptible NSDA neurons are consistent with this hypothesis. Of course, the data presented in this manuscript are largely

correlative regarding the individual proteins involved (*e.g.*, parkin and UCHL-1) and requires further investigation to validate a causal link between differential expression of specific proteins and recovery from toxicant-induced neuronal injury.

Given that the recovery of TIDA neuronal DA is observed as early as 8 h after MPTP administration, it is likely that mechanisms that allow for a prompt recovery of function must be readily available *via* differential basal expression or induced expression in response to cellular stress. Basal mRNA expression levels of several candidate genes including parkin, UCH-L1, pink1, DJ1, α -synuclein and Lrrk2 were similar in ARC and SN of control animals. While these observations are drawn from a small subset of candidate genes involved in PD, they are consistent with the hypothesis that induced expression (and not basal expression) is the key process underlying the differential response of TIDA and NSDA neurons to acute MPTP exposure. Both parkin and UCH-L1 levels increase in ARC (but not in the SN) 8 h following MPTP, suggesting that rapid and differential up-regulation of protective gene expression may represent an important component for the restoration of TIDA neuronal integrity following an initial injury. Further examination of the time course of parkin and UCH-L1 expression following MPTP demonstrates an increase in protein levels in the ARC that temporally parallels the recovery from DA loss in TIDA axon terminals. In contrast, parkin and UCH-L1 protein expression is initially unaltered in NSDA neurons followed by a decrease, a pattern which correlates with further DA loss and notable absence of recovery of DA integrity in axon terminals. The molecular mechanisms regulating the differential neuronal expression of these proteins following acute injury in TIDA *versus* NSDA neurons is not known and is a compelling focus for future investigation. However, prior to this it will be necessary to determine if parkin and UCH-L1 are necessary and sufficient for TIDA recovery, in addition to investigating whether these proteins are acting independently or in concert with other neuroprotective or anti-apoptotic proteins.

5. Conclusions

The unique ability of TIDA neurons to recover from a toxicant-induced injury is dependent on induced synthesis of new proteins other than TH and is independent of toxicant entry or elimination. Further, an endogenous increase in parkin and UCH-L1 expression correlates with TIDA neuronal recovery from injury. The observations reported herein provide an opportunity to characterize novel up- and down-stream mechanisms underlying the ability of TIDA neurons to increase protein expression in the face of toxicant exposure and increased oxidative stress. The molecular steps that allow rapid and robust induction of protein expression in TIDA neurons following injury may reveal a novel and attractive candidates for putative disease modifying therapies for PD.

Acknowledgments

This work was supported by the Michigan State University Foundation and National Institute of Health Grants NS04905701 and NS065338-01A2. The authors would like to thank Dr. Jim Greene for kindly training Dr. Behrouz in Laser Capture Microscopy and RNA isolation techniques.

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Fig. 1.

The time course of effects of a single injection of MPTP on DA concentrations in ME and ST. Male C57Bl/6J mice ($n = 8/\text{group}$) were treated with MPTP (20 mg/kg; s.c.) and killed by decapitation either 4 or 24 h later. Saline (10 ml/kg; s.c.) treated animals were killed 8 h post-injection and were used as zero time controls. DA levels are presented as percent of these saline-treated controls. Actual zero time control values (mean ng DA per μg protein) were 97 ± 7 and 102 ± 8 . Columns represent means of groups and vertical lines represent ± 1 standard error of the mean. (*) Represent values for MPTP-treated mice that were significantly different ($p < 0.05$) from those of saline-treated zero time controls.

Fig. 2.

The effect of GBR-12909 pre-treatment on MPTP-induced DA loss in the ME and ST. Male C57Bl/6J mice (8/group) were treated with GBR-12909 (10 mg/kg; i.p.) or vehicle 30 min prior to injection with MPTP (20 mg/kg; s.c.; black columns) or its saline vehicle (10 ml/kg; s.c.; white columns). Animals were killed by decapitation 4 h after MPTP. DA concentrations are expressed as ng per mg protein for each region. Bars represent means of groups and vertical lines represent + 1 standard error of the mean. (*) Represents values for MPTP-treated mice that were significantly different ($p < 0.05$) from those for saline-treated controls.

Fig. 3.

Time course of the effects of a single injection of MPTP on DA and MPP+ concentrations in the ME (Left Panel) and ST (Right Panel). Mice were injected with MPTP (20 mg/kg; s.c.) and decapitated 4, 8, 16 or 24 h later. Zero time control mice were injected with saline (10 ml/kg; s.c.) and killed 4 h later. DA (solid lines) concentrations were calculated as a percent of control animals and are represented on the left Y-axis. Actual zero time control values (mean ng DA per μ g protein) were 104 ± 5 for the ST and 220 ± 16 for the ME. MPP+ (dashed line) concentrations were calculated as pg/ μ g protein and are represented on the right Y-axis. Symbols represent means of groups ($n = 8$ /group) and vertical bars indicate ± 1.0 SEM. Filled symbols represent values from MPTP-treated groups that are significantly different from vehicle-treated controls ($p < 0.05$).

Fig. 4.

The effects of cycloheximide on MPTP-induced DA loss in the ME and ST. Male C57Bl/6J mice ($n = 8/\text{group}$) were treated with 2 injections of cycloheximide (120 mg/kg) or saline (10 ml/kg; s.c.), 2 and 4 h after a single injection of MPTP (20 mg/kg; s.c.; black columns) or its saline vehicle (10 ml/kg; s.c.; white columns). All animals were decapitated 16 h after MPTP administration. DA concentrations are presented as ng per mg protein. Columns represent means of groups and vertical lines represent ± 1 standard error of the mean. (*) Indicates values for MPTP-treated mice that were significantly different ($p < 0.05$) from those for saline-treated controls.

Fig. 5. The time course of effects of a single injection of MPTP on total TH protein concentrations in the ME and ST. Male mice ($n = 8/\text{group}$) were treated with MPTP (20 mg/kg; s.c.) and killed by decapitation either 4, 8, 16 or 24 h later. Saline (10 ml/kg; s.c.) treated animals were killed 4 h post-injection and were used as zero time controls. The ME and ST were microdissected and protein was isolated from each region. Total TH concentrations were determined by Western blotting and normalized to β -actin. Total TH protein values are represented as mean fold change from saline $\pm$ 1 standard error of the mean. Representative blots from all groups are shown above line graph.

Fig. 6. Normalized mRNA expression levels of parkin, UCH-L1, LRRK2, α -synuclein, DJ1, and Pink1 in the ARC and SN of mice injected with either saline or MPTP. Male C57Bl/6J mice ($n = 8$ /group) were treated with a single injection of MPTP (20 mg/kg; s.c.; black columns) or its saline vehicle (10 ml/kg; s.c.; white columns) and killed 8 h later. ARC and SN were microdissected and mRNA was isolated from each region. Real time PCR analysis was performed and expression levels of Parkin, UCH-L1, Lrrk2, α -synuclein, DJ1 and Pink1 were normalized to GAPDH expression levels in each region. Columns represent means of groups and vertical lines represent ± 1 standard error of the mean. (*) Values for MPTP-treated mice that were significantly different from those in vehicle-treated control mice.

Fig. 7.

The time course of effects of a single injection of MPTP on parkin protein expression in the ARC and SN. Male mice ($n = 8/\text{group}$) were treated with MPTP (20 mg/kg; s.c.) and killed by decapitation either 6, 12, 24 or 36 h later. Saline (10 ml/kg; s.c.) treated animals were killed 12 h post-injection and were used as zero time controls. The area of the mediobasal hypothalamus or ventral mesencephalon containing the ARC or SN, respectively, was microdissected and protein was isolated from each region. Parkin levels were determined by Western blotting and normalized to GAPDH. Parkin protein values are represented as mean fold change from saline $\pm$ 1 standard error of the mean. Representative blots from all groups are shown above line graph. (*) Represent values for MPTP-treated mice that were significantly different ($p < 0.05$) from those of saline-treated zero time controls.

Fig. 8.

The time course of effects of a single injection of MPTP on UCH-L1 protein expression in the ARC and SN. Male mice ($n = 8/\text{group}$) were treated with MPTP (20 mg/kg; s.c.) and killed by decapitation either 4, 8, 16 or 24 h later. Saline (10 ml/kg; s.c.) treated animals were killed 4 h post-injection and were used as zero time controls. The area of the mediobasal hypothalamus or ventral mesencephalon containing the ARC or SN, respectively, was microdissected and protein was isolated from each region. UCH-L1 levels were determined by Western blotting and normalized to β -actin. UCH-L1 protein values are represented as mean fold change from saline $\pm$ 1 standard error of the mean. Representative blots from all groups are shown above line graph. (*) Represent values for MPTP-treated mice that were significantly different ($p < 0.05$) from those of saline-treated zero time controls.

Fig. 9.

Dose-response changes in parkin expression in the ARC and SN. Male C57Bl/6J mice were injected with either saline or MPTP (10, 20 or 30 mg/kg; s.c.) and killed 12 h later. The area of the mediobasal hypothalamus or ventral mesencephalon containing the ARC (Left Panel) or SN (Right Panel) were microdissected and protein was isolated from each region. Parkin protein concentrations were determined by Western blotting and normalized to GAPDH. Columns represent means of groups and vertical bars represent + 1 standard error of the mean ($n = 8/\text{group}$). Representative blots from all groups are shown above histograms. (*) Indicates values significantly different from saline control, (**) indicates values significantly different from saline and 10 mg/kg MPTP-treated group. Corresponding striatal DA concentrations were 205 ± 11 , 159 ± 14 , 137 ± 17 and 72 ± 11 ng/mg protein following saline or 10, 20 or 30 mg/kg MPTP, respectively.

Fig. 10.

Parkin mRNA from laser captured TH-immunoreactive cells in the ARC and SN. Male C57Bl/6J mice were treated with either saline (1 ml/kg; s.c.) or MPTP (20 mg/kg; s.c.) and sacrificed 8 h later. 10 μ m frozen sections were rapidly fixed and stained for TH. Panel A shows TH-IR cells before laser capture. Panel B shows the same section after cells have been captured while Panel C shows Cap with captured cells. Panel D, mRNA extracted from ~500 cells/animal was amplified and then analyzed by Q-PCR. Parkin mRNA is calculated as a fold change over vehicle-treated levels. All mRNA levels were normalized to GAPDH mRNA levels. Bars and line represent means and standard error, respectively ($n = 4$ /group). (*) Values for MPTP-treated mice that were significantly different from those in vehicle-treated control mice.

Table 1

Half lives and elimination constants of MPP⁺ in the terminal regions of TIDA and NSDA neurons following a single injection of MPTP. Mice were injected with MPTP (20 mg/kg; s.c.) and decapitated 4, 8, 16 or 24 h later. Zero time control mice were injected with saline (10 ml/kg; s.c.) and killed 4 h later. Elimination constants were calculated by plotting the linear regression of the natural log of MPP⁺ concentrations from all time points and obtaining the slope. Half lives were then calculated by dividing this value into the natural log of 2.

	Half life (h)	Elimination constant (3SE)
ME	5.9	0.11 ± 0.01
ST	1.8	0.38 ± 0.02 *

* Represents MPP⁺ elimination constants within the striatum that were significantly different ($p < 0.05$) than those of the median eminence.