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Brief mitochondrial inhibition causes lasting changes in motor behavior and corticostriatal synaptic physiology in the Fischer 344 rat

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Abstract

The striatum is particularly vulnerable to mitochondrial dysfunction and this problem is linked to pathology created by environmental neurotoxins, stimulants like amphetamine, and metabolic disease and ischemia. We studied the course of recovery following a single systemic injection of the mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP) and found 3-NP caused lasting changes in motor behavior that were associated with altered activity-dependent plasticity at corticostriatal synapses in Fischer 344 rats. The changes in synapse behavior varied with the time after exposure to the 3-NP injection. The earliest time point studied, 24 hours after 3-NP, revealed 3-NP-induced an exaggeration of D1 Dopamine (DA) receptor dependent long-term potentiation (LTP) that reversed to normal by 48 hours post-3NP exposure. Thereafter, the likelihood and degree of inducing D2 DA receptor dependent long-term depression (LTD) gradually increased, relative to saline controls, peaking at one month after the 3-NP exposure. NMDA receptor binding did not change over the same post 3-NP time points. These data indicate even brief exposure to 3-NP can have lasting behavioral effects mediated by changes in the way DA and glutamate synapses interact.

Keywords

dopamine; striatum; 3-NP; LTD; LTP

Excessive synaptic excitation is a mechanism implicated in neuron death under a variety of conditions including following exposure to stimulants, neurotoxins, stroke, and a number of age-related diseases (Wallace et al., 2007; Andre et al., 2010; Li et al., 2004; Lynch et al., 2002; Quinton et al., 2006; Sas et al., 2010; Sonkusare et al., 2005). Striatal lesions created by the neurotoxin examined in this study, 3-nitropropionic acid (3-NP), are minimized when

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animals are pretreated with NMDA receptor antagonists or by removal of corticostriatal afferents via decortications prior to 3-NP treatment (Beal et al., 1993; Karanian et al., 2006; Kim et al., 2000; Nasr et al., 2009). 3-NP has also been shown to cause a delayed, secondary, wave of cell death in neuronal cultures that requires NMDA receptor activation (Liot et al., 2009). Direct application of 3-NP to striatal brain slices increases synaptic activation of NMDA receptors, and our prior work showed that a low-dose systemic injection of 3-NP enhances NMDA receptor function for up to 24 hours after the injection (Akopian et al., 2008; Centonze et al., 2006).

Little is known about long-term behavioral and synaptic recovery following this early wave of toxin-induced pathology. This study adds to a growing body of evidence showing synaptic pathology without cell loss impacts behavior and that behavioral recovery is aligned with adaptive changes in synaptic function (Hasbani et al., 2000). Most studies examining 3-NP toxicity have monitored brain function after repeated, lesion-inducing injections of the neurotoxin. For example, repeated systemic 3-NP injections in Sprague-Dawley rats over 28 days (every other 4 days, 10 mg/kg) cause hypoactivity, bradykinesia and hind limb rigidity that is associated with substantial striatal cell loss (Koutouzis et al., 1994). Chronic low dose delivery of 3-NP (12 mg/kg via an Alzet pump) in Sprague-Dawley rats for one-month produces smaller selective lesions in the rostral-lateral striatum that is associated with an increase in DA turnover during the exposure (Beal et al., 1993). Lower dose chronic 3-NP in SD rats, obtained by adding 3-NP to drinking water for 1-month, also increased striatal DA turnover during the month-long period of exposure (Johnson et al., 2000). The present study shows a slow course of behavioral recovery is associated with changes occurring at excitatory corticostriatal synapses after a single low-dose systemic injection of 3-NP. The Fischer 344 rats used in this study are known for showing heightened, strain-dependent sensitivity to all forms of ischemia (Akopian et al., 2008; Aspey et al., 2000; 1998; Ouary et al., 2000).

EXPERIMENTAL PROCEDURES

Animals

The Fischer 344 strain used in this study is a long-time model-strain for aging studies, but more recently it has been used as a model of heightened vulnerability to hypoxia and ischemia (Aspey et al., 1998; 2000; Akopian et al., 2008; Ouary et al., 2000). Sprague-Dawley and Lewis rats show little effect from single injection of 3-NP (<20 mg/kg) and mice, which are much more resistant to 3-NP, recover fully by 5-days after being injected with repeated large doses of 3-NP (340 mg/kg over 7days) (Alexi et al., 1988; Akopian et al., 2008; Fernagut et al., 2002; Ouary et al., 2000).

Treatment with 3-NP

Beal et al. (1993) created large striatal lesions in Sprague Dawley rats with intraperitoneal (i.p.) injections of 3-NP (20 mg/kg each day for 5 days). Our intent was to impact synaptic function without creating lesions and thus we reduced the dose to a single i.p. injection of 16 mg/kg 3-NP (Akopian et al., 2008; Crawford et al., 2011). It was also important to reduce the dose of 3-NP to account for the heightened sensitivity of Fischer 344 rats to 3-NP and other forms of hypoxia (Akopian et al., 2008; Ouary et al., 2000). All rats were injected at one month of age and were allowed to survive from 24 hours to 3 months after the injection.

Rod walking behavioral analysis

Rod walking was assessed using a 60 cm long wooden dowel, 5 cm in diameter that was grooved and suspended between two platforms 60 cm above a padded surface. Rats were placed in the center of the rod facing one platform and latencies to reach the escape platform

were measured. Each rat was tested 3 times, with a maximum limit of 120 s. A rod walking score was calculated for each rat using the following scale: 0-fall, 1-clasp, 2-all paws on top, 3-takes steps, 4-reaches the platform (Friedmann and Gerhardt, 1992). The rat was then given a score that reflected the highest score of the three trials.

Two rod walking experiments were conducted. In the first experiment, baseline levels of rod walking were determined before rats were injected with 3-NP, and then they were examined again for rod walking performance 24 hr and 48 h post-injection. In the second experiment, rats were injected with 3-NP or saline and tested 2 weeks, 1 month, or 3 months after injection. Data from the rod walking experiment were analyzed with separate paired t-tests at the 24 hr and 48 hr time points. Data from the later time points (2 week, 1 month, and 3 months) were analyzed using a 2 x 3 (drug treatment time point) ANOVA.

Brain slice preparation

Experiments were performed on 2–5 month old Fischer 344 male rats (Harlan laboratories). All protocols were approved by the Animal Use and Care Committee at the University of Southern California and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy). Rats were anaesthetized with halothane and decapitated immediately. Their brains were removed and placed in cooled (1–4° C), modified-oxygenated artificial cerebrospinal fluid (aCSF). The modified aCSF replaced some of the sodium with sucrose to reduce tissue excitability during slice cutting (sucrose 124 mM, NaCl 62 mM). This solution maintained the osmotic balance found in normal aCSF. Normal aCSF was as follows: (concentrations in mM) NaCl 124, MgSO₄ 1.3, KCl 3.0, NaH₂PO₄ 1.25, NaHCO₃ 26, CaCl₂ 2.4, glucose 10.0, equilibrated with a 95% O₂ - 5% CO₂ mixture to obtain a pH value of 7.3 - 7.4).

Hemi-coronal striatal slices were cut at a thickness of 450 µm with a Vibratome (WPI). The slices were immediately placed in an oxygenated aCSF solution containing 30 µM bicuculline methiodide (BIC-aCSF) (Sigma) and they were slowly brought to room temperature (23° C). BIC was used to block gamma-amino butyric acid-A (GABA_A) receptor mediated inhibition as a means to isolate excitatory synaptic events. Slices remained in this solution for two hours prior to and throughout all recording sessions. Single slices were transferred to the recording chamber (Haas ramp style gas interface chamber), and bathed continuously with the oxygenated BIC-aCSF solution maintained at a temperature of 32° C.

Intracellular recording and extracellular stimulation

Bipolar insulated tungsten wire (50 µm diam.) stimulating electrodes were used for delivering paired and tetanizing extracellular stimuli to the border between the striatum and the overlying corpus callosum. Intracellular records were obtained at a 1mm distance from the extracellular electrodes. Test synaptic responses were delivered as pairs separated by an interstimulus interval (ISI) of 50 msec, with a constant current stimulus (10–500 µA) at a duration <0.2 msec. Responses to paired stimuli were sampled every 20 sec for a control period of 10 min prior to tetanization and a posttetanus sampling period of at least 20 minutes. Tetanic stimulation consisted of four trains of stimuli separated by 10 seconds. Each train lasted 1 sec and was delivered at a frequency of 100 hertz (Hz). The tetanus stimulation intensity was set to equal the threshold for orthodromic induction of action potentials. The intensity used to sample EPSPs was then set to half the intensity of the orthodromic threshold.

Intracellular records were obtained with glass microelectrodes pulled by a Flaming-Brown P-87 pipette puller. Electrodes filled with 2 M potassium acetate had resistance values

ranging from 100 to 160 Ω . Intracellular signals were amplified with an Axoclamp 2A (Axon Instruments) amplifier, digitized with a LABMASTER interface and stored on disk using pCLAMP software (Molecular Devices). Established electrophysiological criteria were used for including cells in this study (Akopian et al, 2008).

6-Hydroxydopamine lesions of the substantia nigra

The substantia nigra compacta and the medial forebrain bundle were injected unilaterally with 6-hydroxydopamine (6-OHDA) to lesion the dopamine-containing neurons of the substantia nigra compacta. Surgeries were performed under pentobarbital (50 mg/kg) anesthesia. Stereotaxic, unilateral 6-OHDA injections (2 mg/ml in 0.1% ascorbic acid dissolved in sterile saline) were made into both the rostral substantia nigra compacta (coordinates from bregma (mm): A -4.8, L 1.7, V -7.5) and the medial forebrain bundle (coordinates from bregma: A -4.3, L 1.2, V -7.5) (Paxinos and Watson, 1998). The compound was infused at a rate of 1 μ l/min with a total volume of infusion of 3 μ l (6 μ g of 6-OHDA/injection). The syringe was slowly withdrawn (1 mm/min) after it had been left in the place of infusion for 1 min. The animals were killed for *in vitro* analysis two weeks (24 hour post 3-NP group) or 6 weeks (one month post 3-NP group) after the 6-OHDA lesions. Corticostriatal long-term synaptic plasticity was compared between slices taken ipsilateral and contralateral to the unilateral 6-OHDA lesion to demonstrate the DA- dependence of the unique 3-NP-induced changes in synaptic plasticity seen at 24 hours and 1 month after the 3-NP injection. Each animal was tested three days prior to being injected with 3-NP for rotational behavior induced by apomorphine injection (0.5 mg/kg). Animals showing 10 rotations per minute or more were included in the study. Data on synaptic plasticity were obtained from striatal neurons located ipsilateral and contralateral to the lesion. The mesencephalic block containing both the lesioned and the intact substantia nigra was then fixed in 4% paraformaldehyde and processed for tyrosine hydroxylase (TH) immunocytochemistry. The TH immunocytochemistry was performed on 10–15 mesencephalic sections per animal, using the commercially available antibody from Chemicon (Temecula, CA). Immunopositive cells were counted in every other section through the rostral to caudal extent of the substantia nigra. With a section thickness of 60 μ m, we had approximately 15–20 sections per animal. 6-OHDA lesions produced an 85% or greater loss of TH positive neurons ipsilateral to the lesion as compared to the contralateral side (mean 24 hour post 3-NP: 90%, $n=3$; mean 1-month post 3-NP group: 91%, $n=3$) (Figs 3d, 4d).

DA receptor-based pharmacology

Brain slices taken from rats injected either 24 hours or 1 month after the 3-NP injection were evaluated for the sensitivity of the unique 3-NP-dependent changes in long-term plasticity to block of either D1 or D2 dopamine receptors. Prior work in many laboratories has established corticostriatal LTP is D1 DA receptor dependent and corticostriatal LTD is D2 DA receptor dependent (for review see Calabresi et al, 2007). The D1 DA receptor dependence of the 3-NP-induced enhancement of LTP seen 24 hours after the 3-NP injection was tested by bathing slices from these animals in the D1 receptor antagonist SCH 23390 (10 μ M)(Sigma) during the tetanus. The D2 DA receptor dependence of the 3-NP-induced enhancement of LTD seen 1 month after the 3-NP injection was tested by bathing slices from these animals in the D2 receptor antagonist l-sulpiride (10 μ M)(Sigma) during the tetanus.

Electrophysiological analysis

The peak amplitude of EPSPs was measured with respect to the potential measured just prior to the stimulus artifact off-line using CLAMPFIT analysis program (Molecular Devices). This procedure reduced, but did not eliminate the possibility of error created by summation

between the second response and the falling phase of the first response of the pair. Another way of minimizing this problem is to measure the ascending slope of the response, but previous work from our laboratory and others has shown identical outcomes for measurement of response amplitude and response ascending slope (Villar and Walsh, 1999; Kerr and Wickens, 2001).

Synaptic responses were sampled at 20 second intervals and the average of 3 samples (1 minute) was plotted for each minute of the experiment. Post-tetanic changes in response amplitude were calculated by expressing the amplitude of each one minute average as a percentage of the average response amplitude generated during the 10 min baseline sampling period. Post-tetanic plasticity was measured as the average plasticity occurring 0–3 min after the tetanus. Long-term, tetanus-induced plasticity was determined from the average change in EPSP amplitude measured from 16–20 minutes post-tetanus.

Groups were initially compared for differences in tetanus-induced plasticity by performing a repeated measures analysis of variance (ANOVA) across the entire post-tetanus sampling period. Post hoc comparisons were then performed between each group at 3–4 minutes posttetanus samples and at 16–20 minutes post-tetanus using two-tailed T-tests assuming unequal variance between samples ($p < 0.05$). Amplitude descriptive statistics (i.e. mean $\pm$ standard error of the mean (SEM)) were calculated for control, 3–4 minute post-tetanus and long-term post-tetanic (16–20 minutes post-tetanus) samples.

Some data from the early 24 and 48 hour post 3-NP time points was published previously in the Journal of Neuroscience and with their permission it is shown here to illustrate how long it takes for corticostriatal synapses to recovery from 3-NP treatment as well as to show the change in physiology seen throughout the recovery process (Akopian et al, 2008).

Homogenate [^3H]MK-801 binding assay

Rats were decapitated, their striata removed and frozen 24 h, 48 h, 2 week, 1 month, or 3 months after 3-NP or saline administration. On the day of assay, frozen striatal tissue was thawed on ice and homogenized in 15 volumes of 5mM Tris-HCl buffer, pH 7.4, 10 s using a Brinkmann Polytron. Homogenates were then centrifuged at 40,000 g for 20 min. The supernatant was discarded and the pellet washed twice under the conditions described above. The final pellet was suspended in ~ 10 volumes of buffer, pH 7.4. Protein concentrations for the final pellet were determined using the Bio-Rad Protein Assay with BSA as the standard.

Striatal homogenates were assayed in duplicate in test tubes containing 5 nM [^3H] (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801), 0.3 mM glycine, 2 mM glutamate, and 5 mM Tris- HCl buffer, pH 7.4. Nonspecific binding was determined in the presence of 100 μM unlabeled (+) MK-801. Incubation time was 90 min at 23°C. Incubation was terminated by vacuum filtration (Brandel, Gaithersburg, MD) over glass fiber filters (Brandel) previously soaked in 0.1% polyethylenimine. Filters were washed twice with ice-cold Tris-HCl buffer, and radioactivity was measured by liquid scintillation spectrometry.

Binding densities were analyzed using 2 4 (drug treatment time point) ANOVAs. Separate saline controls were used at each age to control for possible age-dependent changes in binding densities. MK-801 binding provides an estimate of the surface expression of functionally active NMDA receptors (Cull-Candy et al, 2001). The activated (open) state of NMDA receptors, triggered in this study by tetanic activation of corticostriatal synapses, is the stage where MK-801 and dissociative anesthetics bind to the receptor (Young and Fagg, 1990). Wullner et al (1994) examined MK-801 binding 3 hours after a strong dose of 3-NP

and found changes in NMDA receptor binding in the Sprague-Dawley rat (Wullner et al, 1994).

RESULTS

Time course of behavioral recovery from a single, low-dose systemic injection of 3-NP

2-month old Fischer 344 rats received a single 16 mg/kg dose of 3-NP and were examined for 3-NP-induced behavioral and synaptic changes up to 3 months after the injection. The dose of 3-NP used in this study was determined parametrically to induce changes in synaptic function without creating associated striatal cell death (Akopian et al., 2008; Crawford et al., 2011). The Fischer 344 rat is by far the most sensitive rat strain to hypoxic challenges and consequently, the dose used in this study was far lower than that used in previous studies using Sprague-Dawley rats (Akopian et al., 2008; Beal et al., 1993; Borlongan et al., 1995; Brouillet et al., 1993; Ouay et al., 2000). We also selectively recorded from the dorsomedial striatum, which is distant from the ventral lateral striatum shown to be most vulnerable to 3-NP (Beal et al., 1993). Each brain slice used for in vitro physiological experimentation was screened for evidence of lesions in fresh brain slices, which appears as a white spot in the central-lateral striatum when using higher doses of 3-NP. Prior work by our laboratory did not reveal cell loss 24 hours after a single 3-NP injection using Nissl stain of cell bodies and no evidence of cell loss was found in slices used in this study (Akopian et al., 2008).

3-NP injected Fischer 344 rats were compared to saline control rats for weight gain over the 3 month course of our study. 3-NP injected rats lagged in weight gain at 48 hours post 3-NP ($p < 0.05$), but caught up to saline injected controls by 2 weeks post 3-NP injection and the 3-NP group maintained their weight, as compared to saline injected controls, out to 3 months post injection (Fig. 1b).

3-NP injected rats showed sustained motor dysfunction as determined by their performance on a balance beam (24 hours to 3 months post 3-NP injection). When compared to pre-injection performance, rod walking was impaired 24 hr ($n = 20$) and 48 hr ($n = 7$) after 3-NP administration [$t(19) = 7.26$, $P < 0.001$; $t(6) = 3.58$, $P < 0.01$, respectively] (see Fig. 1). Statistical analysis of data from the later time points (2 weeks, 1 month, and 3 months) showed that 3-NP treatment impaired the motor ability of rats when compared to saline-treated controls [drug treatment main effect, $F(1, 53) = 13.13$, $P < 0.001$] (see Fig. 1). A total of 56 3-NP injected rats were screened for performance on the balance beam (24 hours to 3 months post 3-NP) and of those 56 rats, 35 were used to examine 3-NP induced changes in corticostriatal synaptic plasticity. A total of 34 saline injected rats were screened for performance on the balance beam (24 hours to 3 months post saline injection) and of those 34 rats, 24 were used to examine corticostriatal synaptic plasticity.

Changes in corticostriatal synaptic plasticity associated with recovery from 3-NP

Corticostriatal synapse recovery from 3-NP exposure was examined 24 hours to 3 months after a single injection of 3-NP. A single systemic injection of 3-NP produced an abnormal increase in the expression of synaptic potentiation 24 hours after the injection at dorsomedial corticostriatal synapses ($p < 0.02$; d.f., 1,33; $F = 6.094$; repeated measures ANOVA) (saline = 16, 3-NP = 18). Post-hoc analysis of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) revealed a difference ($p < 0.05$, unpaired t-test), as did long-term plasticity (average 15–20 min post-tetanus) ($p < 0.025$, unpaired t-test) (Fig. 2). This abnormal expression of LTP disappeared by 48 hours post-3-NP injection, where no differences were observed between saline injected controls and the 48 hours-post-3NP group (Fig 2). The plasticity induced after a single exposure to 3-NP was not stable over time of recovery, however, with increased expression of LTD being seen at 2 weeks and one month after 3-NP

exposure. Rats injected two weeks earlier with a single dose of 3-NP showed greater synaptic depression over the entire sampling period ($p < 0.04$; d.f., 1,20; $F = 5.075$; repeated measures ANOVA) (saline = 16, 3-NP = 6). Post-hoc analysis of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) showed no difference ($p = 0.2343$, unpaired t -test), but increased LTD was seen in the 3-NP injected rats (average 15–20 min post-tetanus) ($p < 0.05$, unpaired t -test) (Fig. 2). Rats injected one month earlier with a single dose of 3-NP showed increased expression of synaptic depression across all post-tetanus sampling points at corticostriatal synapses ($p < 0.012$; d.f., 1,33; $F = 7.122$; repeated measures ANOVA) (saline = 20, 3-NP = 15). Post-hoc analysis of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) ($p < 0.02$, unpaired t -test) and long-term plasticity revealed differences (average 15–20 min post-tetanus) ($p < 0.05$, unpaired t -test) (Fig. 2). Differences in corticostriatal plasticity were not present at 3 months post 3-NP injection.

Dopamine's role in determining corticostriatal long-term plasticity in 3-NP injected rats

Corticostriatal LTP and LTD are both modulated by dopamine. Corticostriatal LTP increases with D1 receptor activation and LTD increases with D2 receptor activation (Akopian et al., 2008; Calabresi et al, 1997). The role played by DA modulation in 3-NP-induced extremes in long-term plasticity seen at 24 hours and one month after the 3-NP injection were investigated using 1) 6-OHDA lesions to reduce DA release by the nigrostriatal pathway and 2) by pharmacologically blocking either D1 and D2 dopamine receptors during the tetanus-induced induction protocol. Rats received unilateral 6-OHDA lesions 2 weeks prior to the 3-NP injection and were allowed to survive either 24 hours or one month after the 3-NP injection. Outcomes were compared between corticostriatal synapses studied ipsilateral and contralateral to the side of the 6-OHDA lesion. 6-OHDA treated rats were screened for apomorphine-induced rotational behavior and their midbrain was processed for tyrosine hydroxylase (TH) immunoreactivity of the substantia nigra compacta (see methods) (Figs 3d, 4d). TH staining in the substantia nigra ipsilateral to the 6-OHDA lesions was reduced by 85% or more in all rats studied (3 for 24 hours post 3-NP and 3 for 1 month post 3-NP) (Figs 3d, 4d).

Corticostriatal synapses examined on the side ipsilateral to the 6-OHDA lesion did not show the exaggerated synaptic potentiation seen in the contralateral side 24 hours after the 3-NP injection, demonstrating the LTP induced by 3-NP exposure 24 hours earlier required DA release during the tetanus (Fig. 3) ($p < 0.035$; d.f., 1, 12; $F = 5.95$ repeated measures ANOVA) (ipsilateral = 7, contralateral = 7). Post-hoc comparison of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) showed no difference, but long-term plasticity was different (average 15–20 min post-tetanus) ($p < 0.05$, unpaired t -test) (Fig. 3). Corticostriatal LTP in normal rodents has been shown to be D1 DA receptor dependent (for review see Calabresi et al, 2007) and we found the 24 hours post-3-NP induced increase in corticostriatal LTP was D1 receptor dependent as well, since it was blocked by bathing brain slices with the D1 receptor antagonist SCH 23390 (10 μ M) during the tetanus ($p < 0.05$; $df = 1, 18$; $F = 4.64$; repeated-measures ANOVA) (aCSF alone, $n = 16$; aCSF + SCH 23390, $n = 8$) (Fig. 3). *Post hoc* analysis of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) also revealed a difference ($p < 0.02$, unpaired t test), and a similar trend was seen for long-term plasticity (average 15–20 min post-tetanus; $p < 0.09$, unpaired t test) when comparing slices from rats injected 24 hours earlier with 3-NP that were bathed in saline or SCH 23390.

The DA-dependence of the increased expression of corticostriatal LTD seen 1 month after 3-NP exposure was also examined using the same strategy of showing 1) the DA requirement through 6-OHDA-mediated DA depletion and 2) the DA receptor based pharmacology for the induction of the enhanced LTD seen 1 month after the 3-NP injection. Corticostriatal synapses examined on the side ipsilateral to the 6-OHDA lesion did not show the exaggerated synaptic depression seen in the side contralateral to the 6-OHDA lesion 1

month after the 3-NP injection (Fig. 4) ($p < 0.03$ d.f., 1, 8; $F = 8.07$ repeated measures ANOVA) (ipsilateral = 5, contralateral = 5). Post-hoc comparison of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) showed no difference, but long-term plasticity approached was different (average 15–20 min post-tetanus) ($p < 0.05$, unpaired t -test) (Fig. 4). Corticostriatal LTD is D2 DA receptor dependent in normal rodents (for review see Calabresi et al, 2007), and we tested whether the 3-NP induced increase in corticostriatal LTD seen 1 month after 3-NP exposure was D2 receptor dependent as well by bathing brain slices from 3-NP injected rats in the selective D2 receptor antagonist 1-sulpiride (10 μ M). Comparison between neurons from slices taken from rats injected 1 month earlier with 3-NP and bathed in aCSF alone ($n = 16$) with those bathed in aCSF + 1-sulpiride ($n = 8$) during the tetanic induction revealed a significant difference across the entire post-tetanus sampling period ($p < 0.05$; $df = 1, 18$; $F = 4.64$; repeated-measures ANOVA) (Fig. 4). *Post hoc* analysis of post-tetanic plasticity (average 0–3 min post-tetanus plasticity) revealed a difference ($p < 0.02$, unpaired t test) as did long-term plasticity (average 15–20 min post-tetanus; $p < 0.04$, unpaired t test).

3-NP and [3 H] MK-801 binding in the dorsomedial striatum

[3 H]MK-801 binding measured in dorsomedial striatal homogenates did not reveal differences between 3-NP and saline injected rats at any of the post injection time points measured (24 hours to 3 months) (Fig. 5) (3-NP $n = 6$, saline $n = 6$). These data indicate 3-NP did not alter the number of NMDA receptors at any recovery time point.

DISCUSSION

A single, relatively low systemic dose of the mitochondrial respiration inhibitor 3-NP produced early as well as long-lasting changes in motor behavior that were associated with changes in corticostriatal synaptic function in Fischer 344 rats. The dorsolateral striatum is the most vulnerable region for hypoxia-induced damage created by either systemic NP exposure or by occlusion of the middle cerebral artery (Beal et al., 1993; Hamilton and Gould, 1987). We recorded distant from the vulnerable dorsolateral striatum in the dorsomedial striatum to reduce the risk of recording from degenerating neurons. Our interest was to gain insight into the neurotransmitter and receptor based mechanisms of damage created by 3-NP at corticostriatal synapses as well the role transmitters and receptors play in recovery. This study supports the concepts that ischemic damage to synapses, without neuronal loss, occurs with mild infarcts or heart attacks and that recovery of damaged synapses contributes to restoration of neurological function (Hasbani et al., 2000).

We used tetanus induced long-term plasticity as a tool to probe the striatum for changes in the behavior of corticostriatal synapses that might be associated with the initial pathology caused by acute exposure to 3-NP and then the same methods were used to track the course of synaptic recovery. Many studies have found the read-out from tetanus-induced corticostriatal plasticity to be an excellent tool for gaining insight into how altered synaptic function may contribute to the pathology and/or the behavioral problems seen in basal ganglia disease (Akopian et al, 2008; Calabresi et al, 1997; 2001; Centonze et al, 2006; Martella et al, 2009; Sivilly et al, 2011 for review see Calabresi et al, 2007). We found DA-dependent corticostriatal LTP increases for up to 24 hours after a single injection of 3-NP, and then the tetanus-induced plasticity returning to normal by 48 hours post-3-NP injection. At 2 weeks after 3-NP exposure, we found increased DA-dependent LTD, which was even more pronounced at one month post injection. The tetanus-induced plasticity returned to that expected for dorsomedial corticostriatal neurons by 3 months post-3-NP (Akopian and Walsh, 2006).

Calabresi et al (2001) found NMDA receptor function is increased within seconds of bath application of high dose 3-NP in brain slices and our work shows brief systemic exposure to 3-NP increases NMDA receptor function for up to 24 hours (Akopian et al., 2008). Dalbem et al. (2005) also found a more intense, multiple-day, multiple-3-NP injection regime increased corticostriatal LTP expression for up to 48 hours after the last injection in Wistar rats (Dalbem et al., 2005). Together, these studies provide synaptic evidence supporting a role for excess NMDA receptor activity in 3-NP's excitotoxic damage to the striatum (Jenkins et al., 1996; Nasr et al., 2009; Pang and Geddes, 1997). Wullner et al (1994) reported a single 30 mg/kg systemic injection of 3-NP in 10 month old Sprague-Dawley rats caused a dramatic increase in MK-801 binding to striatal NMDA receptors 3 hours after the injection. We found in the 2 month old Fischer rat that by 24 hours after a smaller dose of 3-NP (16 mg/kg) that MK-801 binding had not changed, indicating the enhancement of NMDA receptor function observed at 24 hours post 3-NP occurs through a mechanism other than an increase in the number of NMDA receptors.

3-NP's primary pharmacological effect is block of succinic acid dehydrogenase (complex II) (Huang et al., 2006; Nishimura et al., 2008). This direct pharmacological action leads to increased reactive oxygen species (ROS) production, reduced ATP production and the formation of mitochondrial permeability transition pores (Huang et al., 2006; Nishimura et al., 2008). One consequence of a drop in ATP production is reduced activity of Ca^{2+} pumps and the Na^+/K^+ ATPase, which causes neurons to depolarize. This depolarization creates a secondary risk of Ca^{2+} overload by enabling Ca^{2+} entry through NMDA receptors and voltage-dependent Ca^{2+} channels (for review see Brouillet et al., 2005). These multiple actions of 3-NP combine to trigger calpain, caspase and JNK pathways of necrosis and apoptosis (Bizat et al., 2003; Garcia et al., 2002; Puerta et al., 2010). Evidence also exists for a delayed, secondary, NMDA receptor-mediated wave of apoptosis with 3-NP exposure in cultured striatal neurons (Brouillet et al., 2005; Liot et al., 2009; Pang and Geddes, 1997).

3-NP induced pathology at excitatory synapses is accompanied by pathology in nigrostriatal DA synapses as well (Crawford et al., 2011; Pandey et al., 2009; Villaran et al., 2008). High levels of DA are released in response to 3-NP's acute block of cellular respiration. Rapid dumping of DA interacts with 3-NP's direct action on complex II to further reduce cellular respiration and to increase the production of reactive oxygen species (Pandey et al., 2009; Villaran et al., 2008). High DA by itself increases H_2O_2 and hydroxyl radical formation and DA is readily oxidized to form highly reactive DA-quinones, which act on mitochondria to decrease respiration (Hastings et al., 1996; Jana et al., 2007; Pandey et al., 2009). This study and our previous work indicates that the delayed recovery of DA synapses, which lasts up to 24 hours after 3-NP exposure, may be indirectly involved in the excessive activation of NMDA receptors as well. A period of low DA release occurs for 24 hours after the acute 3-NP-induced overflow of DA and this condition tips the balance toward D1 over D2 receptor activation (Crawford et al., 2011; Akopian et al., 2008). Prior depletion of DA by a 6-OHDA lesion eliminated the enhancement of NMDA receptors seen 24 hours after 3-NP, indicating DA was necessary for this pathology to occur. A critical feature of the pathological enhancement of NMDA receptor function seen at 24 hours post-3-NP is that it is produced when DA release is still recovering. The lower DA release present at 24 hours post 3-NP conditions appears to be optimal for enhancing NMDA receptor function via a D1 dopamine receptor mechanism (Fig. 3)(Akopian et al, 2008). Adding DA directly to brain slices taken from animals injected 24 hours earlier with 3-NP eliminates the enhanced NMDA receptor function by correcting for the 3-NP-induced lowering of DA release in brain slices. NMDA receptor numbers do not change during the first 24 hours after 3-NP, but their modulation by dopamine does (Figs. 3, 5). NMDA receptor activity is similarly increased in the cortex after stroke, where a critical period of NMDA receptor-mediated vulnerability exists with recovery from mild to severe middle cerebral artery occlusion and,

much like we see with 3-NP, NMDA receptor-dependent LTP is enhanced during the initial recovery from cortical infarcts as well (Hagemann et al., 1998; Humm et al., 1999; for review see Hasbani et al., 2000).

We found previously that DA content and release recovered 48 hours after 3-NP exposure and the increase in DA release in these brain slices was associated with a return of D2 receptor-dependent LTD (Akopian et al., 2008; Crawford et al., 2011). The return of DA, the activation of D2 receptors and its affect on corticostriatal synapse function has been shown in the MPTP model of DA depletion to be critical for recovery of basal ganglia related behaviors (Vanleeuwen et al., 2010). We found the long-term course of recovery from 3-NP was associated with an increase in corticostriatal LTD out to one month after 3-NP exposure (as compared to saline injected age-matched controls). This outcome is consistent with increased D2 receptor modulation of corticostriatal synapses one month after 3-NP (fig. 4). The LTD seen at one month post 3-NP is DA-dependent, since it's expression is eliminated if the substantia nigra is lesioned by 6-OHDA before the rat is injected with 3-NP. The increased LTD is also eliminated by block of D2 DA receptors, which is consistent with the role played by D2 receptors in modulating corticostriatal LTD (Fig. 4) (Kreitzer and Malenka, 2005; Wang et al, 2006; for review see Calabresi et al, 2007). The increased LTD expression seen in Fischer 344 rats 1 month post 3-NP could also be an over-reaction of glutamate synapse plasticity, as has been shown to occur early in cortical neurons recovery from stroke (Ward, 2005). One mechanism to explain this kind of mechanisms would be an increase in GluA2 AMPA receptor subunit expression as we have seen in our laboratory at corticostriatal synapses during recovery in the MPTP treated mouse (VanLeeuwen et al, 2010). Our findings indicate synapse injury without cell loss can contribute to pathological changes in motor behavior and that the recovery process for restoring synaptic function is slow and possibly incomplete, even at 3 months post 3-NP (Hasbani et al., 2000). It is possible more complete recovery from 3-NP-induced brain damage can be achieved by rigorous, activity based intervention. For example, a critical period of heightened activity based neuroplasticity exists within 30 days after the brain injury (Jones and Schallert, 1992; Murphy and Corbett, 2009; Vanleeuwen et al., 2010).

It is important to emphasize that this study used the Fischer 344 rat and that the Fischer 344 rat differs significantly from other rat strains in a number of physiological measures. The National Institute on Aging chose the inbred Fischer 344 rat to be the preferred strain for studying aging for its genetic homogeneity and because it does not become obese with advancing age (Mosoro, 1990). However, questions about Fischer 344 rat vulnerability to age-related kidney disease and certain cancers emerged and caution was advised in generalizing findings from this genetically restricted strain (Lipman et al., 1996; Hazzard et al., 1992; Weindruch and Masoro, 1991). We use the Fischer 344 rat in our laboratory and have done so for many years do look at the effect of aging and diseases of aging on corticostriatal plasticity (Akopian and Walsh, 2006; Dunia et al., 1996; Ou and Walsh, 1997; Ou et al., 1997; Siviý et al., 2011). In this study, we found as have others that Fischer rats were far more sensitive to the chemical hypoxia caused by 3-NP and reports also exists for Fischer rats showing heightened sensitivity to middle cerebral artery occlusion (Akopian et al., 2008; Aspey et al., 1998; 2000; Ouary et al., 2000). Fischer 344 rats also show developmental differences in social behavior that are associated with lower DA release and reduced corticostriatal LTD expression when compared to SD rats, which should be considered when evaluating our findings on DA modulation of corticostriatal synapses (Akopian et al., 2008; Siviý et al., 2011).

CONCLUSION

Our studies provide insight into synaptic mechanisms contributing to striatal vulnerability to mitochondria toxicity as well as to how striatal circuitry changes with recovery over an extended time after 3-NP exposure. We found as have others that brief hypoxic events can have lasting behavioral and synaptic effects (Crawford et al., 2011; Hankey et al., 2000; Heim et al., 1999). 3-NP is the active compound responsible for a number of outbreaks in China of basal ganglia pathology stemming from ingestion of *Arthritium* fungus infected sugar cane (He et al., 1995). The first symptoms of *Arthritium* ingestion were gastrointestinal followed, in the most severe cases, by coma. Emergence from the coma revealed development of dystonia associated with putaminal hypodensity, as detected by CT examination (He et al., 1995). The striatum is particularly vulnerable to metabolic compromise, with a number of clinical conditions linked by the common thread of mitochondrial dysfunction leading to putaminal necrosis, dystonia and or chorea (Johnston and Hoon, 2000). Reduction in blood flow by stroke, cardiac arrest and head injury preferentially damages the striatum as do a number of rare organic acidurias (Brouillet et al., 2005; Hawker and Lang, 1990; Kumar et al., 2011; Mlynash et al., 2010; Wajner and Goodman, 2011). Our data and others indicate striatal vulnerability may lie in a combination of factors unique to striatal circuitry including DA toxicity, increased D1-receptor linked over-activation of NMDA receptors and possibly striatal-specific differences in mitochondria vulnerability (Brustovetsky et al., 2003; Mirandola et al., 2010).

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Brief 3-NP exposure has lasting effects on corticostriatal synapses

3-NP-induced alteration of corticostriatal synapses involves changes in DA modulation

Altered D1 and D2 receptor activation determines acute versus lasting changes

Fig. 1. 3-NP associated changes in rod walking performance and weight gain. Rats in the 24 Hr, 48 Hr and 2 week group were weighed and tested on the rod walking test before (control) and after being injected with 3-NP (16.5 mg/kg, i.p.). The remaining groups were treated with 3-NP (16 mg/kg, i.p.) or saline and tested 2 weeks, 1 month, or 3 months post injection. A: Time course of 3-NP induced difference in rod walking performance. B: Comparison of weight gain in 3-NP and saline injected rats over the course of the 3 month post injection study. *Significantly different from control or saline-treated rats.

Fig. 2.

Time-course of change in tetanus-induced plasticity at corticostriatal synapses following injection with 3-NP. Short-term and long-term tetanus induced plasticity were calculated over a 3 month each post-injection recovery period (3-NP and saline). A: Increased post-tetanic potentiation (calculated 0–3 minutes post-tetanus) was seen 24 hours after the 3-NP injection. This trend reversed to increased post-tetanic depression by 1 month recovery from the 3-NP injection. B: Increased long-term potentiation (calculated at 15–20 minutes post-tetanus) was seen 24 hours after the 3-NP injection. This trend reversed to increased long-term depression at 2 weeks and 1 month recovery from the 3-NP injection.

*Significantly different from control or saline-treated rats.

Fig. 3.

Abnormal short- and long-term potentiation produced 24 hours after 3-NP is D1 DA receptor dependent. A: Plot of tetanus-induced change in EPSP amplitude in rats injected 24 hours earlier with either 3-NP or saline. B: Plot of tetanus-induced change in EPSP amplitude from rats injected with 3-NP 24 hours earlier after receiving a unilateral 6-OHDA lesion 2 weeks prior to the 3-NP injection. Recordings were made from the dorsomedial striatum ipsilateral and contralateral to the side of the 6-OHDA dopaminergic lesion. C: The D1 receptor antagonist SCH 23390 blocks the abnormal increase in short- and long-term potentiation seen in rats injected 24 hours earlier with 3-NP. D: Image of TH staining of coronal section taken through the midbrain of unilateral 6-OHDA lesioned rat used to study long-term plasticity 24 hours after 3-NP (B). The 6-OHDA lesion occurred 2 weeks prior to the 3-NP injection (calibration = 500 μ m).

Fig. 4. Abnormal short- and long-term depression produced at corticostriatal synapses 1 month after 3-NP exposure is D2 DA receptor dependent. **A:** Plots of tetanus-induced change in EPSP amplitude 1 month after an injection of saline or 3-NP. **B:** Plots of tetanus-induced change in EPSP amplitude from rats injected with 3-NP 1 month earlier after receiving a unilateral 6-OHDA lesion 2 weeks prior to the 3-NP injection. Recordings were made from the dorsomedial striatum ipsilateral and contralateral to the side of the 6-OHDA dopaminergic lesion. **C:** The D2 receptor antagonist sulpiride blocks the abnormal increase in short- and long-term depression seen in rats injected 24 hours earlier with 3-NP. **D:** Image of TH staining of coronal section taken through the midbrain of unilateral 6-OHDA lesioned rat used to study long-term plasticity 1 month after 3-NP (**B**). The 6-OHDA lesion occurred 2 weeks prior to the 3-NP injection (calibration = 500 μ m).

Fig. 5.

NMDA receptor binding does not change following recovery from 3-NP exposure. [^3H]MK-801 binding was measured in dorsomedial striatal homogenates 24 hours, 48 hours, 2 weeks, 1 month and 3 months after a single i.p. injection of 3-NP. 3-NP animals were compared to age and post-injection matched saline controls. No differences in [^3H]MK-801 binding were found at any of the post injection time points (24 hours to 3 months).