Published in final edited form as:

Neurotoxicology. 2018 July ; 67: 121–128. doi:10.1016/j.neuro.2018.05.003.

Prolonged increase in ser31 tyrosine hydroxylase phosphorylation in substantia nigra following cessation of chronic methamphetamine

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Abstract

Methamphetamine (MA) exposure may increase the risk of motor or cognitive impairments similar to Parkinson's disease (PD) by middle age. Although damage to nigrostriatal or mesoaccumbens dopamine (DA) neurons may occur during or early after MA exposure, overt PD-like symptoms at a younger age may not manifest due to compensatory mechanisms to maintain DA neurotransmission. One possible compensatory mechanism is increased tyrosine hydroxylase (TH) phosphorylation. In the rodent PD 6-OHDA model, nigrostriatal lesion decreases TH protein in both striatum and substantia nigra (SN). However, DA loss in the SN is significantly less than that in the striatum. An increase in ser31 TH phosphorylation in the SN may increase TH activity in response to TH loss. To determine if similar compensatory mechanisms may be engaged in young mice after MA exposure, TH expression, phosphorylation, and DA tissue content were evaluated, along with dopamine transporter expression, 21 days after cessation of MA (24 mg/kg, daily, 14 days). DA tissue content was unaffected by the MA regimen in striatum, nucleus accumbens, SN, or ventral tegmental area (VTA), despite decreased TH protein in SN and VTA. In the SN, but not striatum, ser31 phosphorylation increased over 2-fold. This suggests that increased ser31 TH phosphorylation may be an inherent compensatory mechanism to attenuate DA loss against TH loss, similar to that in an established PD model. These results also indicate the somatodendritic compartments of DA neurons are more vulnerable to TH protein loss than terminal fields following MA exposure.

Keywords

Tyrosine hydroxylase; Parkinson's disease; Aging; Phosphorylation; Methamphetamine; Dopamine transporter

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1. Introduction

The 2013 Substance Abuse and Mental Health Services Administration reported methamphetamine (MA) use can begin in adolescence (SAMHSA, 2014; DAWN, 2014) with some studies finding the onset age ranging from 14 to 18-years old (Uhlmann et al., 2014). With increasing MA use in this cohort, the serious motor and cognitive consequences in the developing brains of our youth cannot be overstated as this drug has a half-life of 12-hours during which time normal circulating levels of dopamine increase up to 600 times their normal levels (Tompkins-Dobbs and Schiefelbein, 2011; Buck & Siegel, 2015; Luikinga et al., 2017). Previous exposure to MA can lead to long-lasting abnormalities in brain structure, chemistry, and function (Chang et al., 2007; Sekine et al., 2003; Sekine et al., 2006; Volkow et al., 2001), persisting even after prolonged periods of drug abstinence (Sonsalla et al., 1996; Wilson et al., 1996a, b; Simon et al., 2002; Segal et al., 2003; Sekine et al., 2003; McCann et al., 2008; Kuczenski et al., 2009; Keller et al., 2011). This is true even with doses that are comparatively lower than those used in acute regimens (Keller et al., 2011; Segal et al., 2003), and after abuse during adolescence (Spear, 2000). Furthermore, MA use has been shown to particularly jeopardize neuronal integrity in the substantia nigra (SN), a brain area associated with Parkinson's related motor decline (Thrash et al., 2009; Todd et al., 2013; Mursaleen and Stamford, 2016). Taken together, the literature suggests that in addition to the risks for cognitive and motor impairments, MA abuse during adolescence or young adulthood may predispose risk for developing Parkinson's disease (PD) symptoms in middle age.

Although damage to nigrostriatal dopamine (DA) neurons may occur with MA use in adolescence or young adult, the onset of Parkinson's type symptoms may not manifest until middle age (Guilarte, 2001; Garwood et al., 2006; Callaghan et al., 2010, 2012; Curtin et al., 2015; Kish et al., 2017). In those with MA use disorders, loss of DA, TH, and dopamine transporter (DAT) in striatum is evident (Wilson et al., 1996a; McCann et al., 2008; Moszczynska et al., 2004). However, because the loss of TH or DAT following MA exposure (Wilson et al., 1996b) is not at the threshold of PD symptoms of > 70%, as established in human (Bernheimer et al., 1973; Hornykiewicz and Kish, 1986) and animal models (Bezard et al., 2001) alike, earlier work concluded that MA use may not produce the pathology associated with locomotor symptoms of PD (Moszczynska et al., 2004). However, it is important to note that these studies were conducted in post-mortem tissue or imaging studies obtained from young adults (Wilson et al., 1996a; McCann et al., 2008). A study of those who formerly abused MA in their 30 s revealed an increased risk of PD prior to age 50 (Callaghan et al., 2012). Therefore, any MA-related loss of DA-regulating proteins that already existed in early adulthood could conceivably set the stage for the development of PD symptoms with advancing age, the top risk factor for PD (Collier et al., 2011). Given the loss of DA-regulating proteins from MA use but the lack of PD-like symptoms, mechanisms of neural resiliency, despite MA-insult, could mask PD-like symptoms.

Common pathologies occurring in both MA and PD models include oxidative stress, impaired mitochondrial function, apoptosis, and excitotoxicity (Mark et al., 2004; Cadet et al., 2007; Chotibut et al., 2014, 2017). The regulation of TH phosphorylation during loss of DA neurons may reveal the status of TH stability or activity (Johnson et al., 2018). In the 6-

OHDA PD model, TH protein loss in the SN exceeds DA loss and is associated with an increase in ser31 TH phosphorylation therein (Salvatore, 2014). This suggests that DA biosynthesis capacity is increased in the SN in response to TH protein loss. While the loss of DA in striatum has been a focus of motor impairment in PD, there is evidence that loss of TH protein in the SN is associated with locomotor deficits in animal models of PD or aging (Emborg et al., 1998; Bezard et al., 2001; Salvatore et al., 2009a) and increased TH and DA content in the SN of aged rats is associated with increased locomotor activity (Pruett and Salvatore, 2013; Salvatore et al., 2017). Here, we evaluated the impact of previous MA exposure on TH protein expression and phosphorylation in conjunction with DA tissue content to evaluate the possibility of a similar response in TH regulation in the nigrostriatal dopamine pathway, as observed in an established PD toxin model.

2. Methods

2.1. Methamphetamine administration

2.1.1. Animals—C57BL/6 J male mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) at 4–5 weeks of age, being within the adolescent age range for rodents (postnatal days 28–42) (Spear, 2000). Mice were housed in an AALAC accredited animal care facility at Meharry Medical College, under constant temperature and a 12-h light/dark cycle (lights on: 7 a.m.–7 p.m.), with food and water provided ad libitum. Experimental procedures complied with the NIH Guide for the Care and Use of Laboratory Animals, ARRIVE guidelines, and conducted with the approval of Meharry Medical College Institutional Animal Care and Use Committee. All efforts were made to minimize stress and discomfort and to decrease the number of animals utilized in this study. The animals were gently handled every day for five days prior to experimentation, and throughout the study.

2.1.2. Drug treatment rationale—Methamphetamine (MA) addiction is clinically defined as escalating drug use, an inability to reduce the amount of drug use, an increased amount of time recovering from the effects of drug use, and persistent drug use that causes physical and/or mental problems (among 11 criteria) that regularly occurs over a 12-month period (American Psychiatric Association, 2013). Depending on the chronicity of drug use over time, withdrawal may last anywhere from 2- to 3-weeks, months, and sometimes years (McGregor et al., 2005; Zorick et al., 2010). Therefore, given these characteristics of MA use, MA intake is likely to become neurotoxic over time, which is the translational framework of preclinical drug use correlates as measured in this study. In the United States, arrest reports of drivers show MA blood levels can be above 300 mg/l (Logan, 1996). To examine MA-induced neurotoxicity in animal models, various non-contingent high doses of MA have been used, including 60 mg/kg/day (Fumagalli et al., 1998), 40 mg/kg/day (Wallace et al., 1999), 32 mg/kg/day (Armstrong and Noguchi, 2004), and 30 mg/kg/day (Cadet et al., 2011; O’Callaghan and Miller, 1994; Sonsalla et al., 1996). We selected a daily dose of 24 mg/kg/day MA. We used a comparatively lower dose because MA was administered for 14 days longer than the previous reports (Cadet et al., 2011; Fumagalli et al., 1998; O’Callaghan and Miller, 1994; Sonsalla et al., 1996). The mice (N = 10; 5 MA, 5 saline) received 14 days of intraperitoneal (i.p.) injection of 24 mg/kg/day MA, followed by 21 days of drug abstinence. The animals were randomly assigned to MA or saline groups.

Our previous report (North et al., 2013) suggests that behavioral and electrochemical responses to MA exposure maximize after 21 days of drug abstinence. Therefore, at the end of the drug abstinence period, the animals were anesthetized with isoflurane and decapitated for dissection of striatum, SN, nucleus accumbens (NAc), and ventral tegmental area (VTA). The tissues were dissected and immediately frozen on dry ice prior to processing.

2.2. Tissue analysis for dopamine, tyrosine hydroxylase, and dopamine transporter

Frozen tissues were sonicated immediately after removal from dry ice in perchloric acid/EDTA solution. The solubilized sample was spun to precipitate inherent protein and the supernatant was processed for analysis of DA and the DA metabolite dihydroxyphenylacetic acid (DOPAC). The protein pellet was sonicated in 1% SDS solution (Salvatore et al., 2012a) and processed for analysis of total protein and then further processed for western blot analyses of TH (Millipore, rabbit, cat#AB152, Temecula, 1:1000 dilution) and site-specific TH phosphorylation at ser19 (Phosphosolutions, Aurora, CO), (given its relationship to TH loss (Salvatore, 2014) and excitotoxicity (Chotibut et al., 2014, 2017) ser31 (characterization described in Salvatore et al., 2009a), ser40 (Phosphosolutions) (Salvatore and Pruett, 2012). Limited tissue content in some regions prevented complete analysis of some of these phosphorylation sites. Increased ser31 phosphorylation alone can increase L-DOPA biosynthesis (Salvatore et al., 2001), and there is abundant evidence for its role in DA biosynthesis in vivo (Salvatore et al., 2009a,b; Salvatore and Pruett, 2012, Salvatore, 2014; Dadalko et al., 2015; Salvatore et al., 2016; Senthilkumaran et al., 2016), the priority was to analyze this site first. Dopamine transporter was analyzed using a 2 µg/ml dilution of anti-goat dopamine transporter primary (Santa Cruz sc-1433).

2.3. Statistics

A two-tailed unpaired Student's t-test was used, comparing the MA recipients versus the saline-injected control group. In both experiments, outliers were detected with alpha set to 0.05 as dictated by the sample size using the Grubb's test. Statistical significance was set at $p < 0.05$. Statistical outcomes with $p < 0.10$ were noted as trends.

3. Results

3.1. MA increases locomotion and body temperature

Previously, we have shown 14 days of intraperitoneal (i.p.) injection of 24 mg/kg/day MA increases locomotor activity (North et al., 2013). Consistent with previous reports, MA exposure also increases body temperature (Supplemental Fig. 1).

3.2. Tyrosine hydroxylase protein

Previous exposure to MA affected TH protein expression only in the somatodendritic compartments of both the nigrostriatal and mesoaccumbens DA pathways. There was no TH loss in striatum (Fig. 1A) or NAc (Fig. 1C), the terminal field compartments of the nigrostriatal and mesoaccumbens pathways. TH protein expression in the SN (Fig. 1B) or VTA (Fig. 1D) was ~50% of the vehicle control group, indicating that the normal expression levels of TH protein in the somatodendritic compartments of the nigrostriatal and mesoaccumbens pathways were reduced by previous MA exposure.

3.3. Dopamine transporter

DAT expression was reduced by the MA regimen in both the nigrostriatal and mesoaccumbens pathways and in both terminal field and somatodendritic compartments, with significant DAT loss in striatum (Fig. 2A), NAc (Fig. 2C), and VTA (Fig. 2D). Greater variability in DAT expression in both treatment groups in the SN likely affected detecting significant difference, despite a 40% overall decrease in DAT in the MA group (Fig. 2B).

3.4. Dopamine tissue content

Dopamine tissue levels were unaffected by the MA regimen in the somatodendritic or terminal field compartments of nigrostriatal or mesoaccumbens pathways, striatum (Fig. 3A), SN (Fig. 3B), NAc (Fig. 3C), or the VTA (Fig. 3D). Given the role for TH in DA biosynthesis, this outcome suggests less vulnerability of TH loss in the terminal fields, but not the somatodendritic compartments.

Our approach to determine DA and TH expression in the same tissue source permitted evaluation of DA against TH protein across the test subjects. This determination in DA regions is correlative with ser31 TH phosphorylation (Salvatore et al., 2009a,b; Salvatore and Pruett, 2012). There was an increase in DA tissue content against the remaining TH protein lost following MA exposure in the SN (Fig. 4B), with a trend toward significance in the VTA ($p = 0.051$, Fig. 4D), but not in the striatum or nucleus accumbens.

3.5. TH phosphorylation

In the MA exposed mice, there was a significant increase in ser31 TH phosphorylation in the SN of mice previously exposed to MA (Fig. 5A). No difference in ser31 TH phosphorylation was observed in the striatum. Ser19 phosphorylation increases following nigrostriatal lesion (Salvatore, 2014), and trends in ser19 phosphorylation in opposing directions were observed in striatum and SN, with a 46% increase in ser19 TH phosphorylation in the SN (Fig. 5C). Ser40 TH phosphorylation was unaffected by MA in either compartment of the nigrostriatal pathway (Fig. 5D).

4. Discussion

The MA regimen of this study revealed that DA biosynthesis capacity within the somatodendritic compartments of both DA pathways is more vulnerable than that in the terminal fields following cessation of MA exposure. Whereas DAT loss occurred in the striatum and nucleus accumbens, TH protein loss was specific for the SN and VTA. As the evidence for increased PD risk or PD-like motor impairment in former MA users of an average age of 40 is increasing in prevalence (Callaghan et al., 2012; Curtin et al., 2015), we note that TH protein loss in our study may signify the possibility that TH loss or pathology in the SN of former MA users (Todd et al., 2013) may also be a contributing factor to MA-induced motor impairment. Quantitative studies of nigral TH or other DA markers have yet to be conducted in MA users (Kish et al., 2017), but the data from other preclinical MA impact studies warrant such an evaluation given the reports of nigral TH loss (Keller et al., 2011; Ares-Santos et al., 2014; Kousik et al., 2014) along with these results from our work.

The human data of MA users has revealed that DA-regulating proteins of the nigrostriatal pathway are vulnerable to loss following MA exposure (Wilson et al., 1996a; McCann et al., 1998; Moszczynska et al., 2004). Earlier studies of DA protein loss in striatal regions revealed significant DA loss (~50%), but not at the Parkinson level of > 70% in the putamen (Moszczynska et al., 2004). However, the average age of the 20 chronic MA users evaluated in this study was 31 years old. Therefore, aging may be one factor in MA addiction that determines the threshold and amount of DA loss whereby PD-like motor symptoms could manifest.

The ventral tegmental area (VTA) lies adjacent to the SNc and also contains a dense population of DA neurons; however, these VTA DA neurons remain relatively spared in PD (Damier et al., 1999), but recent evidence also suggests some vulnerability in PD (Alberico et al., 2015). Multiple neuronal properties such as the size of soma, the rate and magnitude of intracellular Ca²⁺ buffering, basal oxidative stress levels, and the frequency of spontaneous firing activity of DA neurons have been shown to be different between SNc and VTA (Guzman et al., 2010; Krashia et al., 2017). However, in the case of MA exposure, TH expression in the VTA DA neurons may be equally vulnerable (Keller et al., 2011). Given the particular vulnerability of TH loss in the SN (and noted similar vulnerability in the VTA) compared with the terminal field compartments following chronic MA exposure, despite cessation, it can be concluded from our study that a history of MA exposure at a young age could accelerate loss of nigral TH that occurs during aging.

Aging itself is associated with greater loss of TH in SN versus that in the striatum in rodents (Salvatore et al., 2009a; Salvatore and Pruett, 2012), non-human primates (Emborg et al., 1998), and humans (Haycock et al., 2003; Ross et al., 2004). Accordingly, augmenting TH expression in the SN may increase locomotor activity in aged rats (Pruett and Salvatore, 2013). Therefore, the increase in reports of PD like motor impairment in humans with a history of MA or amphetamine use (Callaghan et al., 2010, 2012; Christine et al., 2010; Guilarte, 2001) suggests that the acceleration of aging-related TH loss in the SN could be a contributing factor in these individuals. A longitudinal study of locomotor function in previously exposed MA rats would reveal if the expected acceleration of aging-related motor decline does occur due to TH or DA loss in the SN.

Moreover, the increase in ser31 phosphorylation in the SN in conjunction with TH protein loss may represent presynaptic plasticity to loss of nigral DA. This increase may be associated with increased DA synthesis reported after MA exposure (Larsen et al., 2002) and also is observed in the SN following 6-OHDA lesion of the nigrostriatal pathway (Salvatore, 2014). This increase may be one possible mechanism by which locomotor impairment does not manifest until loss of TH protein reaches a critical threshold (Agid et al., 1973; Hornykiewicz, 1993; Bezard et al., 2001; Zigmond et al., 2002). *in situ* and *in vivo* evidence supports that ser31 TH phosphorylation increases TH activity (Salvatore et al., 2001; Salvatore and Pruett, 2012; Shi et al., 2012; Salvatore et al., 2016). One possible mechanism for this increase may be a MA-related increase in GDNF expression in the SN (Valian et al., 2017). Reduced GDNF expression exacerbates aging-related locomotor impairment following MA exposure compared to mice with intact GDNF signaling (Boger et al., 2007). GDNF increases ser31 TH phosphorylation in the SN and ERK phosphorylation (Salvatore

et al., 2004, 2009b) and ERK is a protein kinase established to phosphorylate ser31 (Haycock et al., 1992; Salvatore et al., 2001), and is also activated by MA exposure (El Ayadi and Zigmond, 2011). We speculate that since aging decreases ser31 TH phosphorylation (Salvatore et al., 2009a,b), this MA-related increase in ser31 TH phosphorylation could be reduced with increasing age and thereby, with TH protein loss already at-hand, precipitate an aging-related Parkinsonism at earlier age.

Another possible explanation for loss of TH protein in the SN may be related to glutamate excitotoxicity. MA exposure appears to increase glutamate release (Mark et al., 2004), and TH loss can be mitigated by increasing glutamate uptake early (Salvatore et al., 2012b; Chotibut et al., 2014), after nigrostriatal lesion in conjunction with reduced ser19 TH phosphorylation (Salvatore et al., 2012b; Chotibut et al., 2014). Although the increase in ser19 TH phosphorylation observed in the SN in this study did not reach statistical significance, the increase is similar in magnitude following 6-OHDA lesion. Ser19 phosphorylation may play a role in TH loss by promoting TH proteolysis (Nakashima et al., 2011). Therefore, one possible mechanism of MA-related loss of TH expression in the SN may be related to glutamate-related excitotoxicity.

The MA regimen we used did not show loss of TH protein at the time of evaluation post-MA, although there was a significant decrease in DAT expression in the striatum and nucleus accumbens. This degree of DAT loss does not affect TH expression or ser31TH phosphorylation in striatum, although complete DAT loss does affect both measures (Salvatore et al., 2016). Partial or complete loss of DAT in the SN, on the other hand, has no effect on TH expression or ser31 TH phosphorylation. DAT expression in the somatodendritic regions is comparatively much lower than the cognate terminal field regions (Keller et al., 2011). Therefore the changes in TH in the SN are likely independent of the expression levels of DAT per se. Evidence supports that DA tissue content is more dependent upon TH function in the somatodendritic regions compared to the terminal fields (Salvatore and Pruett, 2012). Therefore, as chronic exposure of the DAT to MA would conceivably predispose the nigrostriatal somatodendritic compartment to continual DA efflux (Khoshbouei et al., 2003; Goodwin et al., 2009), the burden to replenish lost DA stores in the SN may activate signaling mechanisms by which ser31 is increased.

In summary, TH expression is especially vulnerable in the somatodendritic compartments of nigrostriatal and mesoaccumbens neurons following MA exposure. Increased ser31 TH phosphorylation may be a compensatory mechanism to offset DA loss that would otherwise occur following TH protein loss (Keller et al., 2011). We did not observe the compartmental vulnerability to MA exposure with regard to DAT expression, wherein loss was observed in striatum and nucleus accumbens. We speculate that the particular vulnerability of TH protein loss in the SN may be unrelated to DAT expression and instead related to nigra-specific changes in signaling, as evidenced by increased TH phosphorylation. Given that aging decreases nigral ser31 and TH protein expression (Cruz-Muros et al., 2007; Salvatore et al., 2009b; Salvatore and Pruett, 2012), it stands to reason that this compensatory mechanism would be adversely affected by aging, and therefore compromising DA biosynthesis. As such, this and other MA studies that have revealed a particular vulnerability of nigral TH

loss should be investigated in human studies to examine whether nigral TH loss could be related to motor impairments similar to PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was funded in part by the National Institutes of Health grant awards DA026947 and NS071122 to HK and AG040261 to MFS.

None.

We wish to thank Victoria Fields, Charles Dempsey, Ashley North, and Jarod Swant for excellent technical support.

Abbreviations

DA	dopamine
TH	tyrosine hydroxylase
PD	Parkinson's disease
MA	methamphetamine
DAT	dopamine transporter
SN	substantia nigra
6-OHDA	6-hydroxydopamine
GDNF	glial cell line-derived neurotrophic factor

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.neuro.2018.05.003>.

Fig. 1. Tyrosine hydroxylase (TH) loss following chronic MA exposure in nigrostriatal and mesoaccumbens pathways

A. Striatum (Str). TH protein expression in striatum was unaffected 21 days after cessation of chronic MA ($t = 0.27$, ns, $df = 8$). **B. Substantia nigra (SN).** TH protein expression in SN decreased to 53% of the vehicle (Veh) control group 21 days after cessation of chronic MA ($t = 3.75$, $**p < 0.01$, $df = 6$). **C. Str, representative western blot.** Total TH standard curve range, 0.5, 1.0. and 2.0 ng TH protein and 12 µg of striatal protein loaded, with associated Ponceau stain for each sample producing the TH signal. **D. SN, representative western blot.** Total TH standard curve range, 0.5, 1.0. and 2.0 ng TH protein and 10 µg of SN protein loaded, with associated Ponceau stain for each sample producing the TH signal. **E. Nucleus Accumbens (NAc).** TH protein expression in nucleus accumbens was unaffected 21 days after cessation of chronic MA ($t = 0.24$, ns, $df = 7$). **F. Ventral tegmental area (VTA).** TH protein expression in VTA decreased to 47% of the vehicle (Veh) control group 21 days after cessation of chronic MA ($t = 3.90$, $**p < 0.01$, $df = 7$). **G. NAc, representative western blot.** Total TH standard curve range, 0.5, 1.0. and 2.0 ng TH protein and 8 µg of NAc sample protein loaded, with associated Ponceau stain for each sample producing the TH signal. **H. VTA, representative western blot.** Total TH standard curve range, 0.5, 1.0. and 2.0 ng TH

protein and 12 µg of VTA sample protein loaded, with associated Ponceau stain for each sample producing the TH signal.

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Fig. 2. DAT protein expression following chronic MA exposure in nigrostriatal and mesoaccumbens pathways

A. DAT protein expression in striatum decreased to 62% of the vehicle (Veh) control group 21 days after cessation of chronic MA ($t = 3.01$, $*p < 0.05$, $df = 7$). **B.** DAT protein expression in SN decreased to 60% of the vehicle (Veh) control group 21 days after cessation of chronic MA, though this difference was not statistically significant ($t = 1.55$, ns, $df = 6$). **C.** DAT protein expression in nucleus accumbens decreased to 54% of the vehicle (Veh) control group 21 days after cessation of chronic MA ($t = 4.44$, $**p < 0.01$, $df = 6$). **D.** DAT protein expression in VTA decreased to 41% of the vehicle (Veh) control group 21 days after cessation of chronic MA ($t = 4.84$, $**p < 0.01$, $df = 6$). **Representative blot of DAT loss in the MA group in striatum (E) and nucleus accumbens (F).** 10 µg total protein was loaded for striatum and 20 µg for nucleus accumbens. Ponceaustain of respective DAT-immunoreactive samples shown below DAT image.

Fig. 3. Dopamine (DA) tissue content following chronic MA exposure in nigrostriatal and mesoaccumbens pathways

DA tissue content was not affected by MA exposure in either compartment of the nigrostriatal or mesoaccumbens pathways. DA values are presented as mean $\pm$ SEM ng DA per mg protein. **A. Striatum.** Veh 137 ± 3 , MA 123 ± 7 . ($t = 1.56$, ns, $df = 7$). **B. SN.** Veh 4.3 ± 0.6 , MA 3.8 ± 0.2 . ($t = 0.83$, ns, $df = 7$). **C. Nucleus accumbens** Veh 95 ± 9 , MA 80 ± 7 ($t = 1.38$, ns, $df = 7$). **D. VTA** Veh 5.2 ± 0.7 , MA 5.3 ± 1.2 . ($t = 0.95$, ns, $df = 8$).

Fig. 4. Dopamine (DA) tissue content normalized to total TH protein following chronic MA exposure in nigrostriatal and mesoaccumbens pathways

Results are expressed as mean $\pm$ SEM ng DA per ng TH. **A. Striatum.** Veh 1333 ± 232 , MA 1421 ± 228 . ($t = 0.26$, ns, $df = 7$). **B. SN.** Veh 43 ± 3 , MA 65 ± 8 . ($t = 2.45$, $*p < 0.05$, $df = 8$). **C. Nucleus accumbens** Veh 662 ± 67 , MA 655 ± 146 ($t = 0.04$, ns, $df = 8$). **D. VTA** Veh 40 ± 6 , MA 54 ± 5 . ($t = 2.29$, $p = 0.051$, $df = 8$).

Fig. 5. TH phosphorylation stoichiometry following chronic MA exposure in nigrostriatal pathway

A. ser31 TH phosphorylation. In striatum (str), no significant difference in ser31 TH phosphorylation was observed between the control vs. MA group ($t = 1.46$, ns, $df = 7$). In the SN, ser31 TH phosphorylation was increased over 2-fold in the MA group ($t = 3.72$, $**p < 0.01$, $df = 6$). **B.** Representative western blot image of ser31 TH phosphorylation to represent MA effect. Total TH loaded for the assessment was 5 ng. **C. ser19 TH phosphorylation Str (left), SN (right).** In striatum, a trend toward decreased ser19 TH phosphorylation was observed in the MA group ($t = 1.92$, $p = 0.10$, $df = 6$), whereas in the SN, a trend toward increased ser19 TH phosphorylation was observed in the MA group ($t = 1.89$, $p = 0.10$, $df = 7$). **D. ser40 TH phosphorylation Str (left), SN (right).** In striatum, no significant difference in ser40 TH phosphorylation was observed between the control vs. MA group ($t = 0.13$, ns, $df = 8$). In the SN, ser40 TH phosphorylation was not affected in the MA group ($t = 1.63$, ns, $df = 4$).