Published in final edited form as:

Eur J Neurosci. 2017 August ; 46(3): 1918–1932. doi:10.1111/ejn.13630.

Self-administration of methamphetamine alters gut biomarkers of toxicity

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Abstract

Methamphetamine (METH) is a highly abused psychostimulant that is associated with an increased risk for developing Parkinson's disease (PD). This enhanced vulnerability likely relates to the toxic effects of METH that overlap with PD pathology, for example, aberrant functioning of α -synuclein and parkin. In PD, peripheral factors are thought to contribute to central nervous system (CNS) degeneration. For example, α -synuclein levels in the enteric nervous system (ENS) are elevated, and this precedes the onset of motor symptoms. It remains unclear whether neurons of the ENS, particularly catecholaminergic neurons, exhibit signs of METH-induced toxicity as seen in the CNS. The aim of this study was to determine whether self-administered METH altered the levels of α -synuclein, parkin, tyrosine hydroxylase (TH), and dopamine- β -hydroxylase (D β H) in the myenteric plexus of the distal colon ENS. Young adult male Sprague-Dawley rats self-administered METH for 3 h per day for 14 days and controls were saline-yoked. Distal colon tissue was collected at 1, 14, or 56 days after the last operant session. Levels of α -synuclein were increased, while levels of parkin, TH, and D β H were decreased in the myenteric plexus in the METH-exposed rats at 1 day following the last operant session and returned to the control levels

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Conflict of interest

The authors declare no conflict of interest.

Competing interests

The authors declare no competing financial interests.

Authors contribution

AF carried out the immunofluorescence studies, conducted data acquisition and some analyses, helped with manuscript and figure preparation and critically revised the manuscript; ALP participated in the study concept and design, acquisition of colon tissue, provided technical support, and critically revised the manuscript; SMK contributed to the design of the study, performed the self-administration experiments, and provided critical review of the manuscript. TCN participated in the study concept and experimental design, and provided critical review of the manuscript; AM contributed to the conceptual and experimental design, supervised the project, conducted most analyses, constructed the figures and wrote the manuscript. All authors approved the final version of the manuscript.

Data accessibility

Data supporting the results can be available upon request.

after 14 or 56 days of forced abstinence. The changes were not confined to neurofilament-positive neurons. These results suggest that colon biomarkers may provide early indications of METH-induced neurotoxicity, particularly in young chronic METH users who may be more susceptible to progression to PD later in life.

Keywords

catecholamines; myenteric plexus; parkin; rat; α -synuclein

Introduction

Methamphetamine (METH) is a widely abused central nervous system (CNS) psychostimulant with a high potential for addiction. METH addiction can lead to dopaminergic (DAergic) deficits in the CNS and increase the risk for developing Parkinson's disease (PD) by as much as threefold as compared with individuals who do not use the drug (Garwood *et al.*, 2006; Callaghan *et al.*, 2012; Curtin *et al.*, 2015). This enhanced vulnerability may relate to the neurotoxic effects of METH that are similar to PD pathology. In laboratory rodents, METH-induced neurotoxicity is manifested by persistent decreases in DA, DA metabolites, DA transporters, and tyrosine hydroxylase (TH, the rate-limiting enzyme in DA synthesis) (Krasnova & Cadet, 2009; Yamamoto *et al.*, 2010; Kousik *et al.*, 2014). By evaluating several time points after the last operant session of self-administered METH in rats, Kousik and colleagues determined that reductions in TH immunoreactivity emerged in the striatum by 14 days of forced abstinence and that the number of TH-positive neurons in the substantia nigra *pars compacta* decreased by 28 days (Kousik *et al.*, 2014). These reductions tended to worsen over 56 days of forced abstinence. While the magnitude of TH deficit was considerable less than what is associated with a diagnosis of PD (Bernheimer *et al.*, 1973; Hornykiewicz & Kish, 1987), the neurodegenerative pattern was consistent with a preclinical 'trajectory' for the disease.

Other markers of PD neuropathology that have been observed in brains of experimental animals exposed to METH include changes to parkin and α -synuclein levels. Parkin is a ubiquitous ubiquitin-protein ligase with neuroprotective properties (Moore, 2006). α -Synuclein is a presynaptic protein, which has neurotoxic properties when oligomerized (Lo Bianco *et al.*, 2002; Roberts & Brown, 2015). In the hereditary form of PD, genes encoding for parkin and α -synuclein are mutated (Lesage & Brice, 2009), and in idiopathic PD, both proteins are aggregated in the brain (Baba *et al.*, 1998; Shimura *et al.*, 1999; Recasens & Dehay, 2014). High doses of METH non-contingently administered in binge (four injections of 8–10 mg/kg every 2 h) or chronic protocols (20–24 mg/kg/day for 5–14 days) increase striatal and/or nigral levels of α -synuclein (Yamamoto & Yang, 2012; Butler *et al.*, 2014; Jiang *et al.*, 2014; Gemechu *et al.*, 2015), as also seen with PD (Recasens & Dehay, 2014) and decrease parkin levels in the striatum (Moszczynska & Yamamoto, 2011). Overexpression of parkin attenuates damage to the nigrostriatal DA system in animal models of PD and in METH-induced neurotoxicity, indicating that the deficits in parkin may be involved in pathologies of these conditions (LoBianco *et al.*, 2004; Liu *et al.*, 2013). A pathological accumulation of α -synuclein can result in Lewy body-like intraneuronal

inclusions, and Lewy bodies within the substantia nigra *pars compacta* are a post mortem hallmark of PD. METH produces Lewy body-like intraneuronal inclusions in the substantia nigra *pars compacta* in experimental animals and humans (Fornai *et al.*, 2004; Quan *et al.*, 2005).

PD has historically thought to be a brain disease; however, peripheral factors may contribute to CNS pathology. It has been proposed that the pathology begins in the enteric nervous system (ENS) and progresses to the CNS via migration of inclusions rich in mis-folded α -synuclein (Braak *et al.*, 2006; Minguez-Castellanos *et al.*, 2007). α -Synuclein levels in the colonic ENS are increased in patients with PD (Shannon *et al.*, 2012a,b; Gold *et al.*, 2013) and in rats that self-administered METH, *prior* to TH deficits in the striatum (Napier *et al.*, 2013; Kousik *et al.*, 2014). Whether a similar pattern of pathology occurs with gut parkin and catecholaminergic systems remains unknown.

These prior studies point to a progressive pathology that develops in both the gut and brain long after terminating chronic exposure to METH. To extend these observations, the aim of this study was to measure the levels of α -synuclein, parkin, and markers for catecholaminergic neurons (TH and dopamine- β hydroxylase (D β H), a noradrenergic marker) in the rat distal colon myenteric plexus harvested at different forced abstinence times following 2 weeks of METH self-administration. Our study found a temporal relationship among markers of ENS pathology to the duration of forced abstinence from METH self-administration. These findings are discussed in terms of a prior report from the Napier laboratory (Kousik *et al.*, 2014) showing progressive CNS neuropathology in the same rats used for this study. They may provide a better understanding of mechanisms that enhance the propensity of METH-addicted individuals to develop PD later in life.

Materials and methods

Animals

The total of 32 male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 225–250 g at arrival were housed in pairs and allowed access to food and water *ad libitum* for the duration of the study. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, Washington DC) with protocols approved by the Rush University Institutional Animal Care and Use Committee. The brains of these rats were used in two previous studies (Kousik *et al.*, 2014; Graves *et al.*, 2015), which detail the METH self-administration protocol, including jugular vein catheter implantation and operant procedures. Briefly overviewing the operant self-administration protocol, the rats were randomly assigned to the METH self-administering group, or to the saline-yoked controls. The operant chambers housed two levers, a left-side lever served as the ‘active’ lever, the pressing of which by a rat in the METH group resulted in a 0.1 mg/kg/0.1 mL METH infusion over 6 s, and the saline-yoked partner received a 0.1 mL saline infusion. A press of the right side, ‘inactive’, lever did not result in an infusion. Lever pressing by saline-yoked controls had no consequence. Minimal shaping was performed, and animals were never food restricted. The operant sessions were 3 h/day for 14 days. On days 1–7, rats self-administered METH on a fixed ratio-1 (FR-1; one lever press/infusion) schedule of reinforcement, and on days 8–14 the reinforcement schedule was altered to

FR-5. The average daily METH intake was 1.56 ± 0.22 mg/kg/3 h session (Kousik *et al.*, 2014; Graves *et al.*, 2015). Rats were euthanized by decapitation on the 1st ($n = 16$), 14th ($n = 8$), or 56th ($n = 8$) day following the last operant session. Distal colons, collected from the same rats we previously employed to study the progressive CNS effects of METH self-administration (Kousik *et al.*, 2014; Graves *et al.*, 2015), were excised and flushed before being snap frozen and stored at -80°C prior to usage. The tissue was post-fixed in 4% paraformaldehyde (PFA) for 24 h, washed with 1x PBS, and then kept in 20% and 30% glycerol in PBS for 24 h at 4°C .

Immunohistochemistry

Colon sections were cut at $20\text{ }\mu\text{m}$ and mounted on gel-coated microscope slides. The sections were washed three times for 5 min at room temperature (RT) with phosphate-buffered saline (PBS), permeabilized for 5 min at RT with 0.1% Triton X-100 (Fisher, Waltham, MA, USA) diluted in PBS-Triton-x 100 (PBST), blocked with 5% bovine serum albumin (BSA) (Santa Cruz Biotechnology, Dallas, TX, USA) in PBST (1 h, RT), washed again in PBS and treated with 1x citrate buffer (Fisher) for 40 min at $70\text{--}80^{\circ}\text{C}$. Each section was double-stained (for 1 h at RT) with an antibody against neurofilament (mouse, 1: 100, Cell Signaling, Beverly, MA, USA) and antibody against either TH (rabbit, 1: 400, EMD Millipore, Temecula, CA, USA), D β H (rabbit, 1: 100, Abcam, Cambridge, MA, USA), α -synuclein (mouse, 1: 400, BD Biosciences, San Jose, CA, USA), or parkin (mouse, 1: 400, Cell Signaling), all diluted in 5% BSA/PBST. After washing, the sections were incubated (1 h, RT, in the dark) with an appropriate secondary antibody conjugated to either Alexa 488 (1: 2000, Life Technologies, Grand Island, NY, USA), Alexa 594 (1: 2000, Life Technologies), or Cy3 (1: 1000, GE Healthcare, Piscataway, NJ, USA) diluted in 5% BSA/PBST. Sequential staining was used in cases where the antibodies for both antigens were raised in the same species. Negative controls (no primary or secondary antibody) were obtained to ensure the specificity of the staining. Subsequently, the slices were washed, incubated (5 min, RT) with DRAQ5 (Life Technologies) to label nuclei, and washed again.

Outlining of the plexus areas and data analyses were performed in duplicates, with blinding to treatment employed. Colon slices were double-labeled for neurofilament and α -synuclein, parkin, TH, or D β H. Immunoreactivities were measured within each of the delineated myenteric plexus areas on each slice (z-stacks of $15\text{--}17\text{ }\mu\text{m}$ in depth), using Leica TCS SPE-II confocal microscope with spectral detector and Application Suite Advanced Fluorescence software. All data were averaged first per slice and then per rat, and normalized to saline-yoked controls after each experiment. This approach normalized differences in the immunoreactivity across the experimental days and allowed for standardization across the treatment groups.

Measurement of myenteric plexus area in the distal colon

The myenteric plexus (Auerbach's plexus) is embedded in the connective tissue lying between the longitudinal and circular layers of the external gut musculature (Fig. 1). Functionally, enteric neurons can be grouped into intrinsic primary afferent (sensory) neurons, interneurons, and effector (motor) neurons. Neurofilament immunoreactivity marks subpopulations of myenteric neurons, one of them being Dogiel type II neurons, which are

primary afferent neurons (Brehmer, 2007). Neurofilament immunoreactivity has been used to delineate the myenteric plexus and measure its size within the colon (Bjorklund *et al.*, 1984; Roman *et al.*, 2001) as well as to assess colonic abnormalities (Kluck *et al.*, 1986). To visualize myenteric plexus within the distal colon tissue, every sixth 20- μm -thick colon slice was collected, mounted onto glass slides, labeled for neurofilament protein as described above, and visualized (z-stack depth: 15–17 μm) using Leica TCS SPE-II confocal microscope with spectral detector (Leica Microsystems Inc., Buffalo Grove, IL). Five to 10 myenteric plexus sites per slice (over four slices per rat) within $512 \times 512 \mu\text{m}$ areas were manually delineated (Fig. 2) until the plexus sizes were equal in all experimental groups. This approach prevented the possibility of potential changes in toxicity indices to be due to differences in myenteric plexus sizes. The μm^2 of delineated areas were measured using the Leica Application Suite Advanced Fluorescence software. The area sizes were first averaged per slice and, subsequently, per rat.

Co-localization analysis

The co-localization between neurofilament and α -synuclein, parkin, TH, or D β H within in each of the outlined myenteric plexus area was estimated using Pearson's correlation analysis. The co-localizations, expressed as Pearson's correlation coefficients, between the toxicity markers were estimated in the same manner. Pearson's correlation coefficient measures the overlap of two fluorescent intensities and characterizes their linear dependency, thus suggesting a potential molecular interaction between two proteins. Scattergrams show the overall relationship between signal intensities, including a degree of overlap. Pearson's coefficient is largely insensitive to differences in mean intensity of a fluorescent signal; therefore, we used Leica software to measure mean signal intensities of each fluorophore within areas where the two signals overlapped. For example, mean parkin intensity within an overlap of neurofilament and parkin provides information whether there is more or less parkin present in neurofilament-positive Dogiel type II neurons.

Statistical analysis

The changes over time in outcome measures associated with METH self-administration when compared with saline-yoked controls were evaluated using a mixed-model analysis of variance (ANOVA) (treatment—the between-subject variable and time—the within-subject variable), which does not require the assumption of sphericity. A mixed linear model ANOVA was selected to analyze the repeated measures effect because the present data have uneven sample sizes for differing time points within groups. Mixed linear modeling allows for the estimation of missing data in a repeated measures design (Field, 2013). This model used a diagonal repeated covariance type that assumes no correlation between time points and maximum likelihood (ML) estimation. Repeated measures correlations as well as heterogeneity of variance were accounted for by a diagonal matrix, and pairwise comparisons of main effects were corrected with Sidak's procedure. Co-localization was measured using Pearson's correlation analysis. Pearson's correlation coefficients were further analyzed by mixed-model ANOVA followed by Sidak *post hoc* test. Mean intensity data in co-localization areas collected at 1-day time point were analyzed by Student's unpaired *t*-tests. For all analyses, statistical significance was set at $P < 0.05$. Data were analyzed using SPSS program (IBM, Armonk, NY, USA). Power of analysis was calculated

using G*Power software (v.3.1.9.2). A priori power estimates based on prior literatures suggested a large effect size ($d = 1.2$), which gave an estimated sample size of $n = 8$. *Post hoc* power analysis was performed to determine power for the analyses reported herein, and the powers were as follows: 1.0 for parkin, 0.98 for α -synuclein, 1.0 for TH, and 1.0 for D β H (immunofluorescence data) and 0.98 for parkin, 0.53 for α -synuclein, 0.99 for TH, and 1.0 for D β H (co-localization data).

Results

Neurofilament immunoreactivity in the myenteric plexus of rat distal colon

Neurofilament immunoreactivity has been used to identify the myenteric plexus and estimate its size in experimental animals (Bjorklund *et al.*, 1984; Roman *et al.*, 2001). In the rat distal colon, the neurofilament antibody strongly labeled myenteric plexus ganglia (clusters of neuronal cell bodies) (Fig. 2A–F) and slightly labeled glial cells (data not shown). There was no significant difference in the size of myenteric plexus between the METH group and saline-yoked control group. The average size of the myenteric plexus was $698 \pm 51 \mu\text{m}^2$ in the saline-yoked controls and $696 \pm 55 \mu\text{m}^2$ in rats that self-administered METH (Student's *t*-test, $P = 0.66$, $n = 32$). There was also no difference in total intensity of neurofilament immunoreactivity within the myenteric plexus between these two treatment groups at any of the assessed time points (Figs 3E, 4, 5, 6E).

METH self-administration increases the levels of α -synuclein in the myenteric plexus of rat distal colon

The mixed-model ANOVA revealed a significant main effect of time ($F_{2,22,14} = 3.76$, $P = 0.04$) but no effect of treatment ($F_{1,30,94} = 2.18$, $P = 0.15$, $n = 32$) on α -synuclein immunoreactivity in the myenteric plexus. However, there was a significant time x treatment interaction ($F_{2,22,14} = 3.72$, $P = 0.04$). Pairwise comparisons revealed that there was an increase in total α -synuclein immunoreactivity within the myenteric plexus in rats that self-administered METH, as compared to saline-yoked controls, at 1 day after the last operant session (mean: +49%, $P = 0.02$, $n = 8$ rats/group, Sidak *post hoc* test) but not at 14 or 56 days. α -Synuclein immunoreactivity was not similarly increased in all METH-exposed rats (median: +21%) (Fig. 3D). α -Synuclein immunoreactivity returned to levels identified for the saline-yoked controls by 14 days of forced abstinence. The total immunoreactivity of neurofilament stayed at the control values for 56 days since the forced abstinence from METH (Fig. 3E). Next, it was determined whether METH self-administration had an effect on α -synuclein immunoreactivity within the neurofilament-positive neurons. There was no significant main effect of time or treatment, and no significant interaction between treatment and time for colocalization between α -synuclein and neurofilament immunoreactivity (mixed-model ANOVA, $P = 0.54$, $n = 32$, data expressed as Pearson's correlation coefficients) (Fig. 3G). Pearson's coefficient is largely independent of signal intensity; therefore, mean intensity of α -synuclein immunofluorescence within the α -synuclein-neurofilament co-localization area was recorded at 1 day after the last operant session and found not to be significantly different between the rats that self-administered METH and the saline-yoked controls. However, non-significant tendency for an increase in α -synuclein immunofluorescence in neurofilament-positive cells was noted (Fig. 3F).

METH self-administration decreases the levels of parkin in the myenteric plexus of rat distal colon

There was a significant main effect of time ($F_{2,20.12} = 18.53$, $P = 0.000027$) and treatment ($F_{2,26.64} = 11.80$, $P = 0.002$) on parkin immunoreactivity as well as a significant treatment $\times$ time interaction ($P = 0.000027$, mixed-model ANOVA, $n = 32$). Compared to the saline-yoked controls, rats that self-administered METH displayed significant reduction in total parkin immunoreactivity in the myenteric plexus at 1 day after the last METH self-administration session (-58% , $P = 0.000015$, Sidak *post hoc* test, $n = 8$ rats/group) (Fig. 4D). Parkin immunoreactivity recovered by the 14th day of forced abstinence from METH and remained at levels similar to that reported for saline-yoked controls at 56 days of forced abstinence (Fig. 4D). The total neurofilament immunoreactivity stayed at the control values for 56 days since the forced abstinence from METH (Fig. 4E). The pattern of changes in co-localization between parkin and neurofilament immunoreactivity (Pearson's correlation coefficients) followed the pattern observed for total parkin immunoreactivity; however, the main effect of treatment was not statistically significant ($P = 0.14$, mixed-model ANOVA, $n = 32$). A significant main effect of time ($F_{2,22} = 4.87$, $P = 0.02$) and a significant time $\times$ treatment interaction was observed ($F_{2,22} = 4.96$, $P = 0.02$) (Fig. 4G). The variance was due to parkin deficit at 1 day of forced abstinence (-17% , $P = 0.004$, Sidak *post hoc* test, $n = 8$ rats/group). Mean intensity of parkin immunofluorescence within the parkin-neurofilament co-localization area was significantly reduced in METH self-administering rats at 1 day after the last operant session compared with their saline-yoked counterparts (-61% , $t_{14} = 3.29$, $P = 0.005$, two-tailed Student's *t*-test, $n = 8$ rats/group) (Fig. 4F).

At 1 day after the last operant session, there was no statistically significant difference in parkin co-localization with α -synuclein between rats that self-administered METH and the saline-yoked controls (Pearson's coefficients ~ 0.35 , $t_6 = 0.91$, $P = 0.39$, two-tailed unpaired Student's *t*-test, $n = 8$ rats/group) (Fig. 5A–C). The two proteins did not always co-localize within the myenteric plexus, which was particularly visible in colons from the METH-exposed rats. METH self-administration significantly decreased parkin immunofluorescence within the α -synuclein immunofluorescence and significantly increased α -synuclein immunofluorescence within the parkin immunofluorescence (-74% , $t_6 = 5.77$, $P = 0.001$ and 3.3-fold, $t_6 = 6.17$, $P = 0.0008$, respectively, Student's *t*-test, $n = 8$ rats/group) (Fig. 5C), suggesting that cells that lose parkin also experience an increase in α -synuclein. Of note, there was not statistically significant correlation between the two proteins either in the controls or in the METH-exposed rats at 24 h after the last operant session.

Methamphetamine self-administration reduces the levels of catecholaminergic markers in the myenteric plexus of rat distal colon

There was a significant main effect of time ($F_{2,16.12} = 5.21$, $P = 0.02$) and treatment ($F_{1,19.79} = 7.80$, $P = 0.01$) on TH immunoreactivity in the myenteric plexus; there was also a significant time $\times$ treatment interaction ($P = 0.02$, mixed-model ANOVA, $n = 32$). The follow-up *post hoc* analysis determined that the immunoreactivity of TH was significantly decreased in rats that self-administered METH at 1 day of forced abstinence from METH compared with saline-yoked controls (-50% , $P = 0.000006$, Sidak *post hoc* test, $n = 8$ rats/group) and returned to the control values by the 14th day of forced abstinence (Fig. 6D). The

total neurofilament immunoreactivity stayed at the control values for 56 days since the forced abstinence from METH (Fig. 6E). Statistically significant main effect of treatment ($F_{1,23.3} = 5.30$, $P = 0.03$) but not of time was found after the mixed-design ANOVA of co-localization between TH and neurofilament immunoreactivity ($n = 32$ rats); the effect was due to TH deficit at 1 day after forced abstinence (-20% , $P = 0.002$, Sidak *post hoc* test, data expressed as Pearson's coefficients) (Fig. 6G). The mean intensity of TH immunofluorescence within the neurofilament-positive neurons significantly decreased in METH self-administering rats compared to saline-yoked controls at 1 day after the last operant session (-39% , $t_{14} = 3.96$, $P = 0.001$, two-tailed Student's *t*-test, $n = 8$ rats/group) (Fig. 6F). Kousik and colleagues previously determined that reductions in TH immunoreactivity ($\sim 17\%$) emerged in the striatum by 14 days of forced abstinence and reached -50% by the 56th day of forced abstinence (Kousik *et al.*, 2014), suggesting that TH decreases faster and recovers faster in the colon than in the brain.

At 1 day after the last operant session, there was minimal co-localization between TH and α -synuclein immunoreactivities in the control rats as well as in rats that self-administered METH (Fig. 7A). This was expected because TH innervation of the myenteric plexus is not extensive and because α -synuclein resides mostly in cell bodies while TH resides in axons and their terminals. There was no statistical correlation between the TH and α -synuclein immunofluorescence (Fig. 7C, D). In contrast, parkin immunofluorescence visibly co-localized with the TH immunofluorescence (Fig. 7B). There was a statistically significant correlation between parkin and TH immunofluorescence in saline-yoked controls (Pearson's correlation coefficient $r = 0.85$, $r^2 = 0.71$, $P = 0.008$, $n = 8$ rats/group) (Fig. 7E) but not in METH-exposed rats (Fig. 7F).

To assess potential alterations in noradrenergic (NEergic) innervation, the immunoreactivity of D β H was examined in the myenteric plexus. The D β H signal was observed in the axons and appeared to be also present in a few perikarya (Fig. 8A–C). There was a significant main effect of time ($F_{2,20.88} = 6.01$, $P = 0.01$) and treatment ($F_{1,17.25} = 54.67$, $P = 0.0000009$) as well as a significant time $\times$ treatment interaction ($P = 0.01$, mixed-model ANOVA, $n = 32$). When compared with saline-yoked controls, the immunoreactivity of D β H showed a significant decrease on the 1st and 14th day after the last day of self-administration (1 day: -55% , $P = 0.00000002$, $n = 8$ rats/group; 14 days: -45% , $P = 0.002$, $n = 4$ rats/group, Sidak *post hoc* test) (Fig. 8D). The D β H immunoreactivity returned to 80% of the control levels by 56 days of forced abstinence (Fig. 8D). The total neurofilament immunoreactivity stayed at the control values for 56 days after the operant task (Fig. 6E). Mixed-model ANOVA of D β H immunoreactivity within neurofilament immunoreactivity (analysis of Pearson's correlation coefficients) revealed a main treatment effect ($F_{1,15.8} = 12.14$, $P = 0.003$). The follow-up with Sidak *post hoc* test revealed a significant decrease in co-localization of D β H and neurofilament immunoreactivity at 1 day (-21% , $P = 0.00012$, $n = 8$ rats/group) and 14 days (-20% , $P = 0.04$, $n = 4$ rats/group) of forced abstinence (Fig. 8F). Moreover, mean intensity of D β H immunofluorescence within the D β H-neurofilament co-localization area recorded at 1 day after the last operant session was significantly decreased in rats that self-administered METH compared with saline-yoked controls (-38% , $t_{14} = 3.33$, $P = 0.0007$, two-tailed Student's *t*-test, $n = 8$ rats/group).

Daily METH intake appeared to influence the most immunoreactivity of parkin (Pearson's correlation analysis, $r = -0.61$, $r^2 = 0.38$), but not immunoreactivities of other toxicity markers (not shown) at 1 day of forced abstinence. However, the correlation missed statistical significance ($P = 0.07$, Pearson's one-tailed correlation analysis) (Fig. 9).

Discussion

The present study determined that METH self-administration produced marked deficits in the levels of parkin, TH, and D β H in the colonic myenteric plexus of the rat at the time when α -synuclein was upregulated. The changes were not confined to neurofilament-positive neurons. Our study also found a temporal relationship between the markers of ENS pathology and the duration of forced abstinence from METH self-administration.

METH abuse is associated with an increased risk for developing PD later in life, (Callaghan *et al.*, 2012; Curtin *et al.*, 2015), even in individuals who have remained abstinent for decades (Garwood *et al.*, 2006). In rats, METH self-administration produces an abstinent period-dependent, progressive decrease in TH-positive neuronal elements in the nigrostriatal DA system (Kousik *et al.*, 2014) and upregulates α -synuclein in colonic myenteric plexus at 1 day of abstinence (Napier *et al.*, 2013). In the present study, we also found an elevation in distal colon α -synuclein; however, most rats displayed moderate increases in α -synuclein protein. Co-localization analysis of neurofilament and α -synuclein staining revealed that α -synuclein was expressed mostly in the neurofilament-positive neurons of the myenteric plexus, which are Dogiel type II neurons and that α -synuclein upregulation occurred inside and outside the neurofilament-positive neurons. Fourteen days after METH, there was less co-localization of neurofilament and α -synuclein, which may reflect a migration of α -synuclein toward sympathetic neuronal cell bodies (Pan-Montojo *et al.*, 2012) and/or uptake by glial cells (Lee *et al.*, 2010). α -Synuclein co-localized with parkin in many, but not in all, myenteric plexus cells, which was not unexpected as these two proteins are not always present in the same cell types (Taguchi *et al.*, 2014). α -Synuclein may be a substrate for parkin in the colon as it is in the brain (Choi *et al.*, 2001; Shimura *et al.*, 2001); therefore, the observed decrease in parkin may have been partially responsible for the observed increase in α -synuclein at 1 day of forced abstinence. Apparent individual variability in α -synuclein levels in rats that self-administered METH at 1 day of forced abstinence may be explained by individual differences in α -synuclein migration and/or degradation by parkin in response to METH.

Increased levels of gut α -synuclein are reported for several neuropathologies. PD is one example wherein increased α -synuclein in the myenteric plexus of the distal colon is thought to be a preclinical indicator of developing PD (Olanow, 2012; Shannon *et al.*, 2012a,b; Visanji *et al.*, 2014). A similar temporal profile may also be relevant to the ability of METH to enhance vulnerability for developing PD (Callaghan *et al.*, 2012; Curtin *et al.*, 2015).

High doses (4×7.5 or 10 mg/kg, every 2 h) of non-contingently-administered binge METH lead to parkin deficits of 28–34% in the striatum at 1 and 24 h after the last injection of the drug in rats (Moszczynska & Yamamoto, 2011; Killinger *et al.*, 2014). In the rat myenteric

plexus, we observed a 58% decrease in total parkin immunoreactivity, a 61% decrease in parkin-neurofilament colocalization, and a 17% decrease in intensity of parkin signal within the co-localization area at 1 day after the cessation of METH self-administration. These results suggest that parkin levels decreased mainly within the neurofilament-positive neurons, such as Dogiel type II neurons, which are primary afferent neurons in the myenteric plexus (Brehmer, 2007). Given low dose of self-administered METH, compared to the doses in the binge protocols (Moszczynska & Yamamoto, 2011; Killinger *et al.*, 2014), it may be that gut parkin is more sensitive to the METH-induced oxidative damage in the ENS than it is in the CNS. In the CNS, the deficit in parkin is caused by oxidative damage to the protein induced by DA-dependent oxidative stress (LaVoie *et al.*, 2005, 2007; Moszczynska & Yamamoto, 2011). While not the major catecholaminergic system in the ENS, DAergic neurons are found in the myenteric plexus (Li *et al.*, 2004); therefore, DA-dependent oxidative stress could have taken place in the colon in METH self-administering rats and decrease parkin levels. In support of this notion, daily intake of METH tended to negatively correlate with parkin levels.

In *Drosophila*, loss of parkin function is associated with decreased TH levels without DAergic neuronal cell loss (Greene *et al.*, 2003; Cha *et al.*, 2005). In view of this finding, loss of the correlation between TH and parkin immunoreactivity at 1 day after exposure to METH suggests that METH may transiently disrupt a potential regulation of TH levels by parkin in the myenteric plexus.

METH-induced DA-mediated oxidative stress is also a contributing factor to DAergic deficits in the CNS of rodents and humans (Yamamoto *et al.*, 2010), including reduction in TH levels and activity. In the ENS, we found a decrease in TH immunoreactivity, with cytoplasmic and axonal reductions being evident. In contrast to our finding, Natale and colleagues (Natale *et al.*, 2008) reported no changes in TH immunoreactivity in the ENS of mouse small intestine after mild doses of non-contingent binge METH (4×5 mg/ kg). There were several differences in study design that may have influenced the results reported by Natale *et al.* (2008) including different areas assessed (distal colon vs. small intestine), dose and protocol of METH administration, and time of kill and tissue analyses. Regarding the latter point, in the study by Natale and colleagues, mice were killed at 7 days after the last day of METH administration at which point, alterations in TH immunoreactivity may have returned to normal. Of interest, two other DAergic toxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, are reported to decrease TH immunoreactivity in the myenteric plexus in rodents (Anderson *et al.*, 2007; Natale *et al.*, 2008; Drolet *et al.*, 2009).

The myenteric plexus receives NEergic innervation from the sympathetic ganglia; however, intrinsic NE-producing neurons are absent from the ENS. Similarly, D β H-positive neurons are absent from the ENS in most, but not all, species. Thus, D β H-positive cell bodies are present in the myenteric plexus in the ferret (Sann *et al.*, 1998). As in the ferret, we detected in rats D β H immunoreactivity in the cytoplasm of some pericarya of the colonic myenteric plexus. Compared to saline-yoked controls, the levels of D β H were significantly decreased in rats that self-administered METH for at least 14 days of forced abstinence from the drug. The D β H deficit persisted longer than TH deficit, suggesting the existence of separate TH-

containing and D β H-containing neuronal cell bodies in rat colonic myenteric plexus. In support of existence of separate populations of TH and D β H neurons in the myenteric plexus, previous studies in the ENS determined that TH and D β H are produced by neurons of different phenotypes (Anlauf *et al.*, 2003; Li *et al.*, 2004). The reductions in TH and D β H may reflect inhibition of synthesis of these catecholaminergic markers subsequent to METH-induced release of DA and NE, or the increase in their degradation. Given that the deficits in the measured biomarkers dissipated within 56 days of forced abstinence from METH, they may not reflect degeneration of catecholaminergic neurons of the myenteric plexus, but rather the effects of METH on the turnover of these markers. Alternatively, if some catecholaminergic neurons did degenerate, the surviving neurons may have compensated so that the biomarkers eventually normalize. This has been observed with DAergic markers in the CNS of METH abusing humans (Volkow *et al.*, 2001).

The temporal profile of the gut changes precipitated by METH is of interest. Within the brain of rats used here for gut evaluations, nigrostriatal TH is not altered 1 day after self-administration, but striatal TH decreases at 14 days post-METH and progressively worsens through at least 56 days of abstinence; nigral TH-positive neuronal loss begins by at 28 days of forced abstinence (Kousik *et al.*, 2014). Here, we reveal that parkin, TH, and D β H are reduced 1 day after terminating METH, and we previously observed an increase in α -synuclein, which was mirrored in the current study. All of these outcomes indicate an early dysregulation in catecholaminergic systems that normalize overtime while pathology is potentially initiated and progresses in the CNS via migration of α -synuclein. Such a gut/brain temporal relationship is consistent with the theory proposed by Braak *et al.* (2006) suggesting that (at least α -synuclein) dysregulation is initiated in the gut to subsequently promote pathology in the brain in PD. It may be that a similar progression is initiated by METH abuse, which, in susceptible individuals, may increase vulnerability to subsequent development of PD. Apparently normal levels of α -synuclein, parkin, TH, and D β H in METH-exposed myenteric plexus cells at 56 days after the last operant session may be due to compensatory alterations of their synthesis or degradation (Tong *et al.*, 2000; Bianco *et al.*, 2008; George *et al.*, 2010), and/or due to axonal sprouting as seen in brains of PD patients (Arkadir *et al.*, 2014). PD is characterized by degeneration of both DAergic neurons in the substantia nigra *pars compacta* and NEergic neurons in the locus coeruleus (Bernheimer *et al.*, 1973; Hornykiewicz & Kish, 1987; Zarow *et al.*, 2003). DAergic projections are the most affected while NEergic axons and terminals are largely spared in METH neurotoxicity (Yamamoto *et al.*, 2010), with the possible exception of the NEergic innervation in the frontal cortex (in some species) (Seiden *et al.*, 1976). Our finding of marked and persistent deficit in neuronal D β H suggests that NEergic neurons of the ENS might be more sensitive to METH than those of the CNS.

Neither total neurofilament immunoreactivity nor the plexus area significantly differed between the METH self-administering and saline-yoked rats. These outcomes suggest that METH self-administration did not decrease neurofilament levels or cause neurodegeneration of the neurofilament-positive elements in the myenteric plexus of the rat distal colon (Sanchez *et al.*, 2003), which does not exclude a potential METH-induced damage to neurons other than those synthesizing neurofilament protein.

In summary, the present investigation demonstrates that self-administration of physiologically relevant doses of METH promotes deficits in parkin, TH, and D β H in rat myenteric plexus of the distal colon prior to when deficits in TH immunoreactivity were reported in the striatum (Kousik *et al.*, 2014). Although α -synuclein and all three biological markers in METH self-administering rats returned to levels that were not significantly different from their saline-yoked counterparts at 56 days following forced abstinence from METH, long-term chronic abuse of the drug in humans may lead to persistent changes in these markers. These deficits, together with the parallel increase in α -synuclein, may serve as early indicators of METH-induced neurotoxicity to the CNS (e.g., persistent deficits in catecholaminergic neurotransmission) and potential PD progression in human METH users before the onset of clinically identified motor impairments. The distal colon may provide an easily accessible tissue for early screening of biochemical markers that can be used to evaluate potential neurodegeneration/neurotoxicity. Our study also demonstrated a link between α -synuclein and parkin in the ENS, a finding that may aid the understanding of a potential progression of METH-induced neurotoxicity and PD from the ENS to the CNS.

Acknowledgments

We thank Sean Callan for help with statistical analysis. This work was supported by National Institutes of Health grants DA034783 and DA024923, and the Center for Compulsive Behavior and Addiction at Rush University Medical Center, Chicago, IL.

Abbreviations

CNS	central nervous system
DAergic	dopaminergic
DβH	dopamine- β -hydroxylase
ENS	enteric nervous system
FR	fixed ratio
METH	methamphetamine
PD	Parkinson's disease
TH	tyrosine hydroxylase

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Fig. 1.

The myenteric plexus in the distal colon of the rat. A representative immunofluorescence labeling (red) of neurofilament-positive neurons in rat distal colon. The enteric nervous system (ENS) is composed of two ganglionated neuronal networks: myenteric (Auerbach's) and submucosal (Meissner's) plexuses. The myenteric plexus is embedded in the connective tissue lying between the longitudinal and the circular layer of the external gut musculature. Bar: 100 μ m. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 2.

Neurofilament immunoreactivity in the distal colon myenteric plexus. Rats that self-administered methamphetamine (METH) for 14 days and saline-yoked controls were sacrificed at 1 ($n = 16$), 14 ($n = 8$), and 56 ($n = 8$) days following the last self-administration session. Distal colon tissue slices were immunolabeled with neurofilament antibody to identify intrinsic neurons of the enteric nervous system (ENS). Five to 10 areas containing myenteric plexus neurons were manually outlined on each slice taken from the colons of (A–C) saline-yoked rats and (D–F) rats that self-administered METH. The average size of the outlined myenteric plexus was $698 \pm 51 \mu\text{m}^2$ in the saline-yoked controls and $676 \pm 55 \mu\text{m}^2$ in rats that self-administered METH ($n = 32$). Bar: $10 \mu\text{m}$. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 3.

The effect of self-administered METH on α -synuclein immunofluorescence in rat myenteric plexus of the distal colon. In figures A–C, examples of fluorescence imaging of neurofilament (green) and α -synuclein (red) staining are provided for rats sacrificed at 1 day (A), 14 days (B), or 56 days (C) following the last operant session. Saline-yoked rats are shown in the top images, and METH self-administering rats are shown in the bottom images. Scatter plots show co-localization of neurofilament (green, x-axis) and α -synuclein (red, y-axis) immunofluorescence. The enlarged images of α -synuclein red immunofluorescence correspond to areas outlined by white rectangles. (D) There was a significant main effect of time on α -synuclein immunoreactivity ($F_{2,22.14} = 3.76$, $P = 0.04$) as well as a significant time $\times$ treatment interaction ($F_{2,22.14} = 3.72$, $P = 0.04$) (mixed-model ANOVA, $n = 32$). Compared to saline-yoked controls, there was an overall 49% increase (median +21%) in total α -synuclein immunoreactivity in the myenteric plexus of rats that self-administered METH at 1 day after the last operant session ($P = 0.02$, Sidak *post hoc* test, $n = 8$ rats/group). α -Synuclein immunoreactivity returned to those identified for the saline-yoked controls by the 14th day of forced abstinence. (E) The total immunoreactivity of neurofilament stayed at the control values for 56 days since the forced abstinence from METH. (F) Mean intensity of α -synuclein immunofluorescence within the co-localization recorded at 1 day after the last operant session was not significantly increased in rats that self-administered METH compared to the saline-yoked controls. (G) No significant main effect of time, main effect of treatment, or treatment $\times$ time interaction was found by mixed-

model ANOVA for co-localization of α -synuclein and neurofilament immunofluorescence (measured by Pearson's correlation coefficient) in METH self-administration group compared to saline-yoked group (mixed-model ANOVA, $n = 32$). The data are expressed as mean $\pm$ SEM. d, day/ days; METH, methamphetamine; NF, neurofilament; SAL, saline. Bars: 10 μ m. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 4.

The effect of self-administered METH on parkin immunofluorescence in rat myenteric plexus of the distal colon. In figures A–C, examples of fluorescence imaging of neurofilament (green) and parkin (red) staining are provided for rats sacrificed at 1 day (A), 14 days (B), or 56 days (C) following the last operant session. Saline-yoked rats are shown in the top images, and METH self-administering rats are shown in the bottom images. Scatter plots show co-localization of neurofilament (green, x-axis) and parkin (red, y-axis) immunofluorescence. The enlarged images of parkin immunofluorescence correspond to areas outlined by white rectangles. (D) There was a significant main effect of time ($F_{2,22,12} = 18.53$, $P = 0.000027$) and treatment ($F_{2,26,64} = 11.80$, $P = 0.002$) on parkin immunoreactivity as well as a significant interaction between treatment and time ($P = 0.000027$, mixed-model ANOVA, $n = 32$). Compared to the saline-yoked controls, rats that self-administered METH displayed significant reduction in total parkin immunoreactivity in the myenteric plexus at 1 day after the last METH self-administration session (-58% , $P = 0.000015$ Sidak *post hoc* test, $n = 8$ rats/group). Parkin immunoreactivity recovered by 14th day of forced abstinence from METH and remained at levels similar to that reported for saline-yoked controls at 56 days of forced abstinence. (E) The total neurofilament immunoreactivity stayed at the control values for 56 days since the forced abstinence from METH. (F) Mean intensity of parkin immunofluorescence within the parkin-neurofilament co-localization area was significantly reduced in METH self-administering rats sacrificed at 1 day after the last operant session compared to their saline-yoked counterparts. (-61% , t_{14}

= 3.29, $P = 0.005$, two-tailed Student's t -test, $n = 8$ rats/group). (G) The pattern of changes in co-localization between parkin and neurofilament immunoreactivity (Pearson's correlation coefficients) followed the pattern observed for total Parkin immunoreactivity; there was a significant main effect of time on parkin immunoreactivity ($F_{2,22} = 4.87$, $P = 0.02$) and significant time x treatment interaction ($F_{2,22} = 4.96$, $P = 0.02$) were detected (mixed-model ANOVA, $n = 32$). The effect was due to parkin deficit at 1 day of forced abstinence (-17% , $P = 0.004$, Sidak *post hoc* test, $n = 8$ rats/group). The data are expressed as mean $\pm$ SEM. d, day/days; METH, methamphetamine; NF, neurofilament; SAL, saline. Bars: 10 μm . ** $P < 0.01$, *** $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 5.

The effect of self-administered METH on co-localization of parkin and α -synuclein immunofluorescence. At 1 day after the last operant session, there was detectable co-localization between parkin and α -synuclein immunoreactivities in the saline-yoked rats as well as in rats that self-administered METH (B) (yellow arrowheads). Parkin and α -synuclein immunoreactivity do not always co-localized (white arrows). (C) At 1 day after the last operant session, there was no statistically significant difference in parkin co-localization with α -synuclein between rats that self-administered METH and the saline-yoked controls (data expressed as Pearson's coefficients, $n = 8$ rats/group). METH self-administration significantly decreased parkin immunofluorescence within the α -synuclein immunofluorescence and significantly increased α -synuclein immunofluorescence within the parkin immunofluorescence (-74% , $t_6 = 5.77$, $P = 0.001$ and 3.3 - fold, $t_6 = 6.17$, $P = 0.0008$, respectively, Student's t -test, $n = 8$ rats/group). The data are expressed as mean $\pm$ SEM. d, day/days; METH, methamphetamine; SAL, saline; +, positive. ** $P < 0.01$, *** $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 6.

The effect of self-administered METH on tyrosine hydroxylase (TH) immunofluorescence in rat myenteric plexus of the distal colon. In figures A-C, examples of fluorescence imaging of neurofilament (green) and TH (red) staining are provided for rats sacrificed at 1 day (A), 14 days (B), or 56 days (C) following the last operant session. Saline-yoked rats are shown in the top images, and METH self-administering rats are shown in the bottom images. Scatter plots show co-localization of neurofilament (green, *x*-axis) and TH (red, *y*-axis) immunofluorescence. The enlarged images of TH immunofluorescence correspond to areas outlined by white rectangles. (D) There was a significant main effect of time ($F_{2, 16.12} = 5.21$, $P = 0.02$) and treatment ($F_{1, 19.79} = 7.80$, $P = 0.01$) on TH immunoreactivity in the myenteric plexus; there was also a significant time $\times$ treatment interaction ($P = 0.02$, mixed-model ANOVA, $n = 32$). The immunoreactivity of TH was significantly decreased in rats that self-administered METH at 1 day of forced abstinence from METH compared with saline-yoked controls (-50% , $P = 0.000006$, Sidak *post hoc* test, $n = 8$ rats/group). (E) The total neurofilament immunoreactivity stayed at the control values for 56 days since the forced abstinence from METH. (F) The mean intensity of TH immunofluorescence within neurofilament-positive neurons significantly decreased in METH self-administering rats compared to saline-yoked controls at 1 day after the last operant session (-39% , $t_{14} = 3.96$, $P = 0.001$, Student's two-tailed *t*-test, $n = 8$ rats/group). (G) There was a significant main effect of treatment ($F_{1, 23.3} = 5.30$, $P = 0.03$) on TH immunoreactivity; the effect was due to

TH deficit at 1 day after forced abstinence (-20% , $P = 0.001$, mixed-model ANOVA followed by Sidak *post hoc* test, data expressed as Pearson's coefficients) The data are expressed as mean $\pm$ SEM. d, day/days; METH, methamphetamine; NF, neurofilament; SAL, saline; TH, tyrosine hydroxylase. Bars: 10 μm . ** $P < 0.01$, *** $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 7.

The effect of self-administered METH on co-localization of tyrosine hydroxylase (TH) immunofluorescence with α -synuclein (α -syn) or parkin immunofluorescence. There was no detectable co-localization between TH and α -synuclein immunoreactivities in the control rats as well as in rats that self-administered METH (A). In contrast, parkin immunofluorescence visibly co-localized with the TH immunofluorescence in both groups (B). There was no statistically significant correlation between the TH and α -synuclein immunofluorescence in the saline-yoked controls (C) or METH-exposed rats (D). There was a statistically significant correlation between parkin and TH immunofluorescence in the saline-yoked controls (E) but not in METH-exposed rats at 1 day of forced abstinence (F). d, day/days; METH, methamphetamine; NF, neurofilament; SAL, saline. Bars: 10 μ m. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 8.

The effect of self-administered METH on dopamine-β-hydroxylase (DβH) immunofluorescence in rat myenteric plexus of the distal colon. In figures A–C, examples of fluorescence imaging of neurofilament (green) and DβH (red) staining are provided for rats killed at 1 day (A), 14 days (B), or 56 days (C) following the last operant session. Saline-yoked rats are shown in the top images, METH self-administering rats are shown in the bottom images. Scatter plots show co-localization of neurofilament (green, *x*-axis) and DβH (red, *y*-axis) immunofluorescence. The enlarged images of DβH immunofluorescence correspond to areas outlined by white rectangles. (D) The DβH signal was observed in the axons as well as in some perikarya. There was a significant main effect of time ($F_{2,20.88} = 6.01$, $P = 0.01$) and treatment ($F_{1,17.25} = 54.67$, $P = 0.0000009$) as well as a significant time $\times$ treatment interaction ($P = 0.01$, mixed-model ANOVA, $n = 32$). When compared with saline-yoked controls, the immunoreactivity of DβH showed a significant decrease on the 1st and 14th day after the last day of self-administration (-55% , $P = 0.00000002$, $n = 8$ rats/group and -45% , $P = 0.002$, $n = 4$ rats/group, respectively, Sidak *post hoc* test). The DβH immunoreactivity returned to near-control levels by 56 days of forced abstinence. (E) The total neurofilament immunoreactivity stayed at the control values for 56 days since the forced abstinence from METH. (F) Mean intensity of DβH immunofluorescence within the DβH-neurofilament co-localization area recorded at 1 day after the last operant session was significantly decreased in rats that self-administered METH compared with saline-yoked controls (-38% , $t_{14} = 3.33$, $P = 0.0007$, two-tailed *t*-test, $n = 8$ rats/group). (G) Mixed-model

ANOVA of co-localization of D β H immunoreactivity with neurofilament immunoreactivity revealed the main treatment effect ($F_{1,15.8} = 12.14$, $P = 0.003$) on D β H signal. The follow-up with Sidak *post hoc* test revealed a significant decrease in colocalization of D β H and neurofilament immunoreactivity at 1 day (-21% , $P = 0.00012$, $n = 8$ rats/group) and 14 days (-20% , $P = 0.04$, $n = 4$ rats/group). The data are expressed as mean $\pm$ SEM. d, day/days; D β H, dopamine- β -hydroxylase; METH, methamphetamine; NF, neurofilament; SAL, saline. Bars: 10 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 9.

The effect of daily METH intake on parkin immunofluorescence in rat myenteric plexus of the distal colon. Daily METH intake appeared to negatively correlate with parkin immunoreactivity measured at 1 day of forced abstinence (Pearson's one-tailed correlation analysis). METH, methamphetamine; RFUs, relative fluorescence units; SA, self-administration. [Colour figure can be viewed at wileyonlinelibrary.com].