

Research report

Recovery from methamphetamine induced long-term nigrostriatal dopaminergic deficits without substantia nigra cell loss

Dennis C. Harvey^a, Goran Lacan^a, Simon P. Tanious^a, William P. Melega^{a,b,*}^aDepartment of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095-1735, USA^bBrain Research Institute, UCLA School of Medicine, Los Angeles, CA 90095-1735, USA

Accepted 25 April 2000

Abstract

After administration of methamphetamine (METH) (2×2 mg/kg, 6 h apart) to vervet monkeys, long term but reversible dopaminergic deficits were observed in both in vivo and post-mortem studies. Longitudinal studies using positron emission tomography (PET) with the dopamine transporter (DAT)-binding ligand, [¹¹C]WIN 35,428 (WIN), were used to show decreases in striatal WIN binding of 80% at 1 week and only 10% at 1.5 years. A post-mortem characterization of other METH subjects at 1 month showed extensive decreases in immunoreactivity (IR) profiles of tyrosine hydroxylase (TH), DAT and vesicular monoamine transporter-2 (VMAT) in the striatum, medial forebrain bundle and the ventral midbrain dopamine (VMD) cell region. These IR deficits were not associated with a loss of VMD cell number when assessed at 1.5 years by stereological methods. Further, at 1.5 years, IR profiles of METH subjects throughout the nigrostriatal dopamine system appeared similar to controls although some regional deficits persisted. Collectively, the magnitude and extent of the dopaminergic deficits, and the subsequent recovery were not suggestive of extensive axonal degeneration followed by regeneration. Alternatively, this apparent reversibility of the METH-induced neuroadaptations may be related primarily to long-term decreases in expression of VMD-related proteins that recover over time. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system and aging

Topic: Drugs of abuse: amphetamines

Keywords: Methamphetamine; Neurotoxicity; Neurodegeneration; PET; Neuronal plasticity; Parkinson's disease

1. Introduction

The neurotoxic effects of methamphetamine (METH) on the striatal dopamine system are often characterized by long-term reductions in tyrosine hydroxylase (TH), the dopamine transporter (DAT), vesicular monoamine transporter-2 (VMAT) and dopamine (DA) levels [4,19,27,46,47,55,69]. These deficits persist from weeks in rodents to several years in humans [32] and non-human primates [71] and, accordingly, have been attributed to neurodegeneration. However, those results generally have

not provided direct evidence for the loss of nerve terminals and/or their corresponding substantia nigra cell bodies. Moreover, studies that have included determination of cell loss after neurotoxic METH exposure have not unequivocally established the correlate between substantia nigra cell loss and long-term METH-induced deficits. For example, decreases in substantia nigra cell number have been shown in the mouse [26,57] and not the rat [55] but neither study used unbiased stereological methods that are considered essential for obtaining accurate cell number estimates [41,68]. Nonetheless, long-term *striatal* dopaminergic deficits have generally been attributed to degeneration of striatal axonal terminals with an apparent sparing of cell bodies [47,53,59]. In this instance, the implicit supposition is that degeneration of *striatal* terminals occurred while indeterminate lengths of their remaining axons and corresponding cell bodies remained intact.

In our previous METH studies in the vervet monkey

*Corresponding author. Department of Molecular and Medical Pharmacology, 23-120 CHS, UCLA School of Medicine, Box 951735, Los Angeles, CA 90095-1735, USA. Tel.: +1-310-206-1797; fax: +1-310-825-2397.

E-mail address: wmelega@mednet.ucla.edu (W.P. Melega)

[34–37], we showed that acute METH administration protocols (2 doses of 2 mg/kg; 4 h apart) produced striatal dopamine deficits similar to those previously characterized as long-term METH neurotoxicity in both rodents and non-human primates [37,51,52,58,61,65]. However, further characterization of those long-term METH effects with 6-[18 F]fluoro-L-DOPA (FDOPA)-PET showed that an essentially complete recovery of DA synthesis capacity occurred over 8 months. Recently, an analogous recovery phenomenon for the striatal dopamine system has also been reported in rodents given neurotoxic doses of METH [6,18]. Generally, this recovery pattern has been attributed to degeneration/regeneration of striatal nerve terminals and/or compensatory collateral sprouting from residual terminals. By inference, the reversibility of the METH-induced changes was necessarily contingent on the preservation of residual *nigrostriatal* axons and ventral mid-brain dopamine (VMD) cell body integrity.

In the present study, we obtained further evidence for this phenomenon of reversible METH-induced dopaminergic deficits. In longitudinal PET studies on individual subjects, the magnitude of [11 C]WIN 35,428 (WIN) binding decreases was assessed in striatum at 1 week and the extent of subsequent recovery at 1.5 years. In other METH subjects, METH IR-profiles of TH, DAT and VMAT were characterized at 1 month post-METH in the VMD cell region, the medial forebrain bundle (MFB) projecting from the VMD, and in the striatum. Those studies were intended to establish if the striatal decreases in WIN binding extended throughout the nigrostriatal dopamine pathway and whether other indices of dopamine system integrity were also decreased. This regional analysis was again conducted at 1.5 years to determine the extent of recovery in those parameters in subjects whose PET studies at that time had shown significant striatal WIN-binding increases. Lastly, to establish whether VMD cell loss was associated with this profile of METH-induced neurotoxicity, cell numbers were determined in VMD regions.

2. Methods

2.1. Subjects

Subjects ($n=10$) were adult male vervet monkeys (*Cercopithecus aethiops sabaues*) (5 METH-treated, 5 controls); age 5–8 years, 6–9 kg. Prior to the study, the subjects were drug-naïve and group-housed except during the METH administration time period. In previous studies, we established that METH exposure for this age and weight range produced similar results for this METH dosage [35,37]. Animal care was in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health publication 865-23, Bethesda, MD).

2.2. METH administration

Doses were calculated as methamphetamine HCl (40 mg/ml) dissolved in 0.9% saline and filter-sterilized [37]. The animals were singly housed during the METH administration periods. Subjects ($n=5$) were administered METH i.m., 2×2 mg/kg, 6 h apart. After 2 days, the animals were returned to their outdoor enclosures for the duration of the study.

2.3. Sacrifice and perfusion

METH subjects were sacrificed at 1 month ($n=2$) and 1.5 years ($n=3$; range 1.3–1.6 years) post-METH for immunohistochemical analysis. The animals were initially anesthetized with ketamine (10 mg/kg, i.m.) and then deeply anesthetized with pentobarbital (50–75 mg/kg, i.v.). After Heparin Sodium (2000 units i.v.) was injected, the animals were transcardially perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.3, followed by a fixative solution (4% paraformaldehyde and 0.25% glutaraldehyde in PBS). Upon removal, the brain was oriented dorsal surface down in a polypropylene block with the supraorbital plane serving as the horizontal plane of reference. This plane was parallel to the anterior-posterior commissure line [16,23] so that the rostral tips of the temporal lobes defined two points in a coronal dissection plane that could be used for intersubject comparison. Perfused brain coronal blocks (0.5 cm thick) were placed in a post-fixative for 24 h, a cryoprotectant solution for 7–10 days and then stored at -10°C . Cryostat sections (30 μm) were obtained at -16°C (Reichert-Jung Frigidicut 2800) and stored in vials as free-floating libraries at -10°C in 30% (v/v) ethylene glycol and 30% (w/v) sucrose in 0.1 M PBS.

2.4. Imaging and image analysis

Microscopy and imaging were performed with an Olympus BX-60 fluorescent microscope equipped with an XYZ programmable motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY; Media Cybernetics, Silver Spring, MD) and a black-and-white digital camera connected to a computer equipped with supporting software (CRI-Inc., Boston, MA). ImagePro-Plus software version 3.01.00 (Media Cybernetics, Silver Spring, MD) was used for image analysis.

2.5. Immunohistochemistry

All sections from METH and control tissues were immunostained in batches with identical antibody, substrate concentrations and incubation times (overnight for the primary antibody). For TH, sheep anti-TH polyclonal antibody (Pel-Freez, Rogers, AR) at 0.025 $\mu\text{g}/\text{ml}$ antibody

concentration (1:2,000) was used. In preliminary studies, various substrate controls were used to evaluate non-specific binding. With the primary antibody removed, the remainder of the immunostaining protocol did not generate specific immunoreactivity. Non-specific binding and serum blocking steps were not required. Sections were developed with a Vector *Elite* ABC Kit (ABC) (Vector Labs, Richmond, CA). The secondary antibody and ABC were diluted to 1:300, incubation time for both steps was 60–90 min. Sections were visualized using diaminobenzidine (DAB) (0.2 mg/ml) (Sigma, St. Louis, MO) with nickel (Ni) enhancement (6 mg/ml nickel ammonium sulfate tetrahydrochloride); development time, 15 min. For DAT, a serum blocking step was performed using 3% normal rat serum before the primary incubation with anti-DAT monoclonal antibody (Chemicon, Temecula, CA) (1:20,000); secondary antibody/ABC 1:400, 60–90 min; DAB-Ni, 20 min. For VMAT, a serum blocking step was also performed (3% normal goat serum) before the primary incubation with the rabbit anti-VMAT polyclonal antibody (Chemicon; Temecula, CA) (1:5000), the secondary antibody/ABC (1:400), 60–90 min; DAB-Ni, 15 min. For GFAP, rat anti-GFAP monoclonal antibody (Zymed Laboratories, So. San Francisco, CA) at 0.13 μ g/ml antibody concentration (1:7500); secondary antibody/ABC 1:400, 60–90 min; DAB-5 min. Sections were slide-mounted and allowed to dry overnight and then dehydrated in ethanol followed by Hemo-D xylene substitute and cover-slipped.

2.6. Cell counts

Following protocols described by West [67], VMD cell number estimates were obtained using unbiased stereological methods. Briefly, ventral midbrain regions containing the VMD cells were dissected out and serially sectioned through the extent of the structure. Every 10th section was immunostained for TH and then counterstained with Pyronin Y. The dual stain resulted in a distinctive red/brown color for identification of all TH-positive cell bodies. An area sampling grid (227 μ m spacing) was then applied to the coronal sections which included the entire extent of the VMD cell region. All grid points which fell within the boundaries of the VMD cells were sampled using the Optical Fractionator approach [22] using a thickness/height ratio of 1.5 (mean section thickness obtained by averaging all sections sampled). Summed ‘tops’ counts were obtained for each ventral midbrain subregion cell number estimate (N). To assess potential regional losses within the ventral midbrain, cell counts were obtained for four subregions: (1) the ventral tegmental area (VTA) (entire rostrocaudal extent), (2) the retrorubral area and pars lateralis region (RR/PL) were combined due to insufficient ‘tops’ counts for each region independently, (3) the substantia nigra pars compacta (SNc)-rostral dorsal tier, from its first appearance near the

caudal portions of the mamillary bodies past the region of the oculomotor (3rd) nerve, and (4) the SNc-caudal ventral tier, from its first appearance in the caudal regions of the oculomotor nerve to its disappearance in the caudal SN. The division of the SNc into rostral dorsal and caudal ventral tiers is consistent with that delimited for the human SNc [24] and characterized by the relative extent of calbindin-IR (VTA >>SNc) [33] (similar calbindin-IR profiles were also obtained in the vervet monkey, unpublished observations).

2.7. WIN 35,428 and WIN-PET studies

WIN-PET scans were conducted as previously described [35]. Briefly, 1 mCi/kg WIN was injected and a quantitative index of striatal WIN binding, the influx rate constant (K_i) value, was derived from multiple time graphic analysis of PET data acquired between 20 and 80 min [44,45].

3. Results

3.1. Immunohistochemistry

In the striatum at 1 month post-METH, a heterogeneous pattern of IR deficits was observed for dopaminergic phenotypic proteins as shown by sparing of IR in the ventromedial accumbens region relative to the decreases in the lateral and dorsal caudate and putamen (Fig. 1). Overall, IR loss was greater for DAT than for either TH or VMAT and without apparent heterogeneity of effect. All phenotypic protein IR recovered to near control levels by 1.5 years post-METH. The IR loss and subsequent recovery is shown in representative coronal sections that compare IR of striatal sections of controls and METH subjects at 1 month and 1.5 years (Fig. 1). These patterns of deficits were assessed in two different striatal sections: in an anterior section that contained the nucleus accumbens (shown in Fig. 1), and at the level of the lentiform nucleus and caudal striatum (data not shown).

In the VMD cell region, there was also an extensive loss and subsequent recovery of phenotypic protein IR after METH. As in the striatum, there was a gradient of effect for TH- and VMAT-IR losses: decreases in dorsomedial VMD cell region (which projects to the accumbens and ventromedial striatum) were relatively less than those in the ventrolateral region. DAT-IR loss (shown for a representative plane containing VMD cells in Fig. 2) was relatively greater than that for either TH- or VMAT-IR and without heterogeneity of effect. All three phenotypic protein IR profiles were similar to control values at 1.5 years, suggestive of extensive recovery.

In addition to the loss of phenotypic protein IR in the VMD neuropil, METH also effected an apparent loss of IR

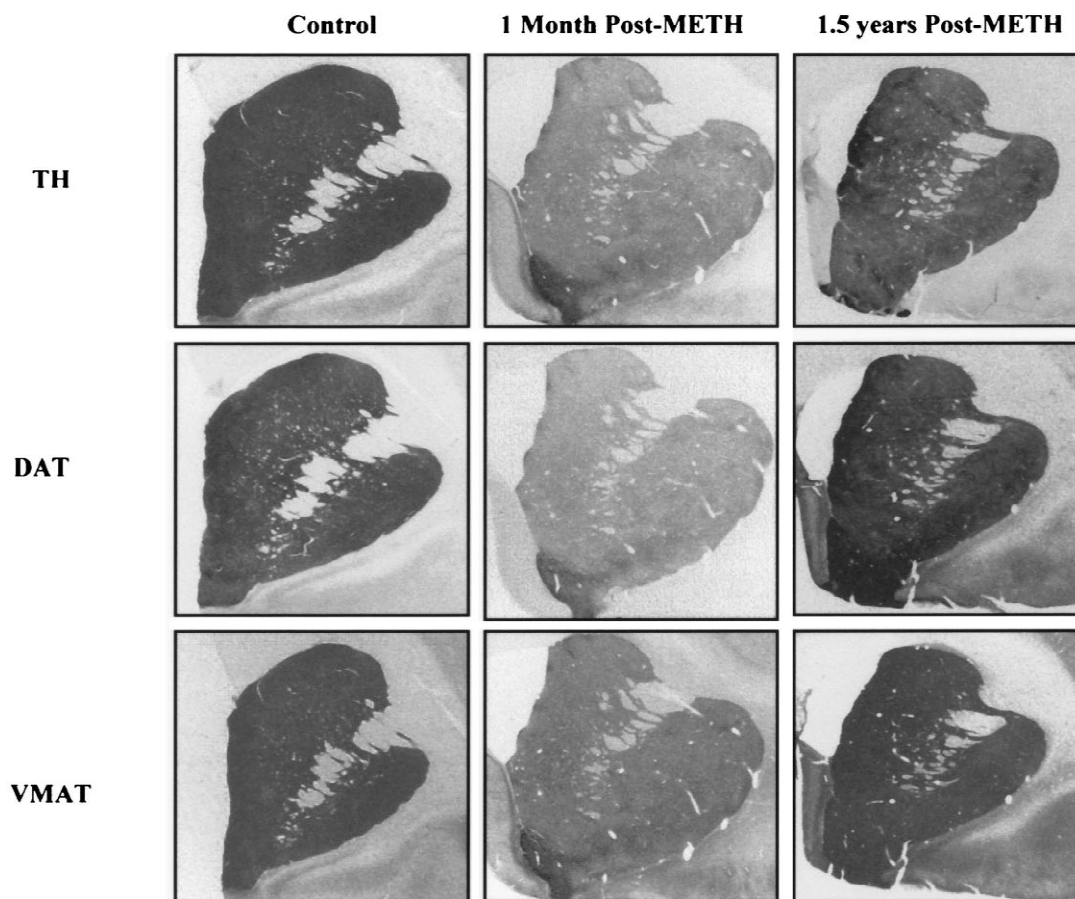


Fig. 1. Tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter-2 (VMAT) immunoreactivity (IR) in striatum for a control (left) and METH subjects after 1 month (middle) and 1.5 years (right).

within VMD cell bodies (Fig. 3A–C). However, visible changes in cell morphology (cell body profile or size changes, swelling or beading of dendritic processes, vacuole formation) or Nissl staining characteristics (assessed with cresyl violet) were not detected at either 1 month or 1.5 years post-drug (Fig. 3D–F).

Throughout the striatum, the METH exposure resulted in large increases in GFAP-IR. At 1 month post-drug, cells with typical astrocyte morphology intensely immunostained for GFAP throughout the rostro-caudal extent of the striatum (accumbens, caudate, and putamen) (Fig. 4). By 1.5 years post-METH, these increases were not observed although GFAP-IR was still visibly greater than that in controls.

Dopamine phenotypic protein immunostaining of the MFB (Fig. 5) was observed in coronal sections of the rostral VMD cell region (seen as an 'eyebrow') from below the thalamus past the mamillary bodies to the level of the caudal hypothalamus where it ascended dorsally toward the pallidum and striatum. TH-, DAT- and VMAT-IR in the MFB was greatly reduced at 1 month post-METH through this rostro-caudal extent and had distinctly re-

covered by 1.5 years in terms of both area and intensity of IR response (Fig. 5; data shown for TH-IR only).

3.2. Cell counts

The majority of TH-positive cells in the VMD cell region (Table 1) were contained within the dorsal and ventral tiers of the SNc (64%) with the remainder in the VTA (20%) and RR/PL (16%). Cell counts were similar for control and 1.5 year post-METH subjects. The apparent absence of TH-positive cell body loss after METH was observed in all subregions. In the caudal ventrolateral SNc, cell number estimates were slightly lower than those in controls but this difference was not significant ($P > 0.1$). In preliminary studies of VMD cell integrity at 1 month post-METH, a loss of VMD cell profile counts was not detected even though protein IR was markedly decreased (data not shown). However, at that time point, quantitation of VMD cells was not conducted because decreases in their TH-IR precluded the unambiguous identification needed for accurate cell counts [42]. Further, determination of similar cell counts in METH and control subjects at that

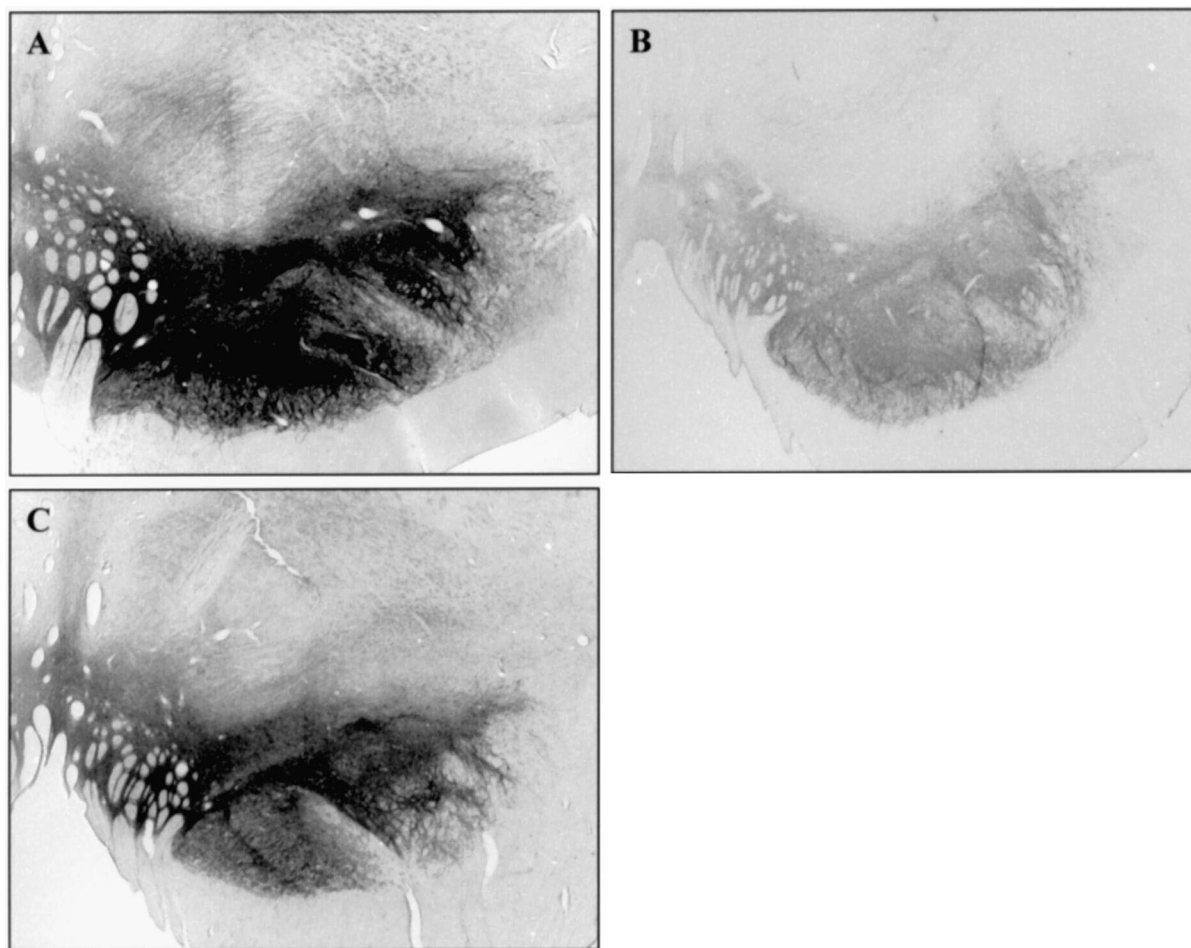


Fig. 2. DAT-IR in the ventral midbrain dopamine (VMD) cell region where the ventral tegmental area, and both dorsal and ventral tiers of the substantia nigra-pars compacta are present for a control (A), and a METH subject after 1 month (B), and 1.5 years (C). METH effected a loss of DAT-IR throughout the VMD cell region at 1 month that recovered to near control levels at 1.5 years.

time point would not have provided conclusive evidence for the absence of METH-induced cell degeneration insofar as VMD cell death following neurotoxic lesions may be a protracted process over several weeks or months [3]. Therefore, unbiased stereological counts were conducted at 1.5 years post-METH, when any cell loss subsequent to the acute METH exposure would have been completed.

3.3. WIN-PET results

At 1 week post-METH, 2 of the 3 METH subjects for the long-term recovery study received WIN-PET scans to establish that the magnitude of WIN binding decreases was comparable to that observed previously with a similar METH dose protocol [35]. Multiple time graphic analysis was used to obtain influx rate constant (Ki) values to provide an index of WIN binding to the DAT. The 1 week post-METH Ki values for these subjects were 4.8×10^{-3} and 3.1×10^{-3} which corresponded to ~75–85% decreases relative to previous referent values (2.0×10^{-2} , S.D. $4.0 \times$

10^{-3} ; $n=6$) [35]. These decreases were similar to results from previous studies (4.0×10^{-3} , S.D. 5.5×10^{-4} ; $n=3$). All 3 METH subjects received WIN-PET scans at 1.5 years post-drug. (range 1.3–1.6 years). Their mean Ki value of 1.8×10^{-2} (S.D. 1.0×10^{-3}) was similar to the referent values above and was suggestive of DAT recovery (Fig. 6).

4. Discussion

Nigrostriatal DA system alterations after METH exposure have been characterized in various animal species and in humans by both in vivo and post-mortem studies. Generally, in rodents, administration of METH doses between 0.1 and 2 mg/kg ('low dose') [52,66] have been associated with behavioral effects but not neurotoxicity. In contrast, multiple METH doses between 4–10 mg/kg ('high dose'), have resulted in a reduction of DA con-

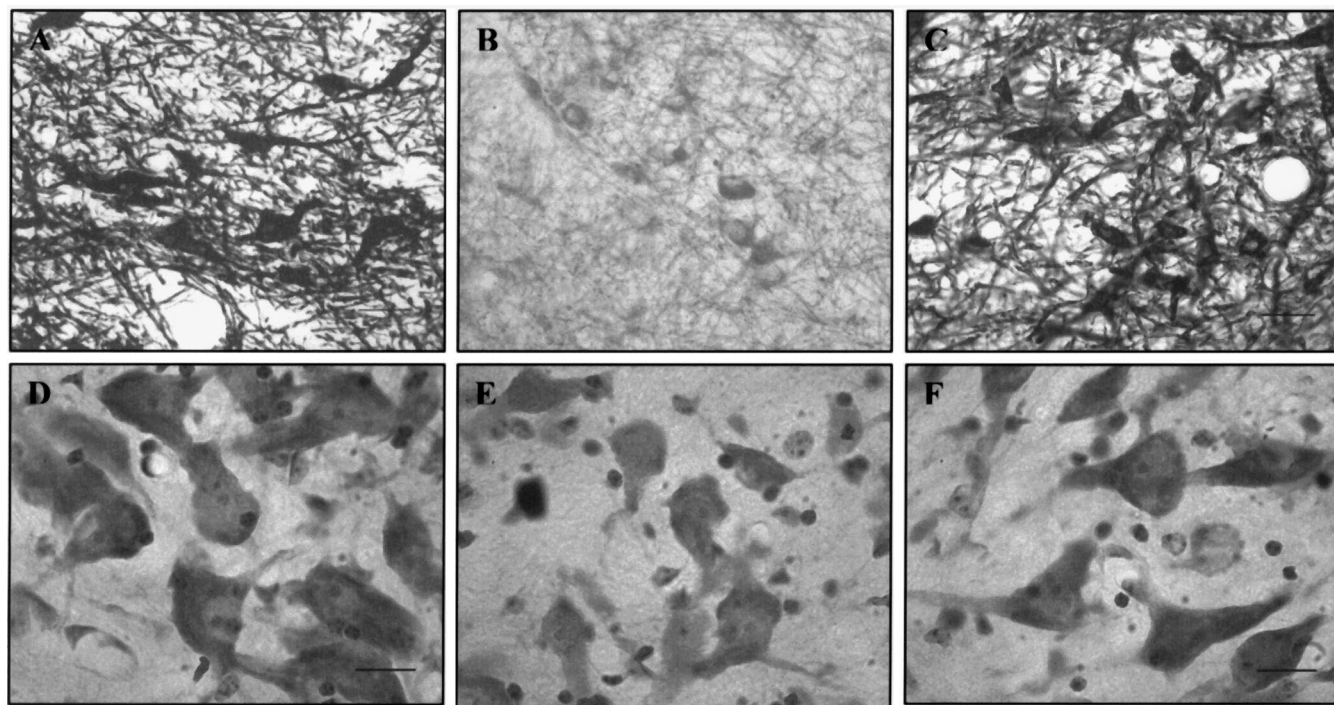


Fig. 3. TH-IR in the ventral midbrain dopamine (VMD) cell region for a control (A), and a METH subject after 1 month (B) and 1.5 years (C). Note the loss and subsequent recovery of immunostaining within VMD cell bodies and associated dendritic arbor. Cell morphology of VMD cell bodies, as shown by Cresyl violet–Nissl staining, of the METH subjects after 1 month (E) and 1.5 years (F) was similar to that for a control subject (D) (scale bar=20 μ m).

centrations and DA system-related proteins [49,61]. In addition, 'high dose' METH and other substituted amphetamines [1,47] have resulted in fiber swelling, decreases of fine fiber density, and an increased number and size of varicosities (beading) of both dopaminergic and serotonergic projection fibers [40,56]. An apparent recovery of serotonergic fibers has been observed after doses of neurotoxic methylenedioxymethamphetamine [12], however, for most of these studies, the irreversibility of the neuronal deficits was not established.

In non-human primate studies, 'high dose' METH dosage protocols have resulted in neurotoxicity as characterized by extensive striatal DA system deficits [49,50,54]. Interestingly, longitudinal studies after chronic METH [71] or amphetamine [36] dosing regimens of 10–14 days showed that those decreases either partially or fully recovered over time. Recently, it has been demonstrated that even relatively lower METH dosages (0.5–2 mg/kg), approximating overdose levels in naïve human users, resulted in decreases of WIN 35,428 and tetra-benazine binding, and dopamine concentrations in striatum [35,48,63]. Further, the striatal deficits induced by these lower METH dosage protocols were shown in PET studies to be reversible [34,35]. Thus, both 'high' and 'low' dose' METH protocols in non-human primate studies have resulted in a range of dopaminergic deficits that were reversible.

The results of our present studies have shown that a METH administration protocol of 2 $\times$ 2 mg/kg METH (6 h

apart) resulted in nigrostriatal dopamine system deficits. Specifically, the results of the PET study at 1 week suggested apparent decreases in striatal DAT availability while the immunohistochemical study at 1 month post-METH showed decreases in DA phenotypic markers. Additionally, at that time point, increases in GFAP immunoreactivity and morphological changes were observed in astrocytes. Generally, these glial alterations are prominent among a range of reactive gliosis responses to CNS traumatic brain injury [39] that are typically associated with a breach of the blood–brain barrier or drug-induced neurotoxic insults, e.g., MPTP, 6-hydroxydopamine (6-OH DA), and METH [15,21]. The precise function of this GFAP response remains unclear but it may reflect increased metabolic demands placed on subpopulations of activated astrocytes, or a response to the presence of cellular breakdown products consequent to impaired neuronal function. This type of gliosis can persist for weeks to months or can be permanent as astrocytes are incorporated into a glial scar which fulfills some structural support role function [8]. In our studies, the GFAP-IR was prominent at 1 month but greatly diminished at 1.5 years although at that time it was still above control levels.

Collectively, the observed deficits in TH-, DAT-, and VMAT-IR in the striatum, MFB and substantia nigra at 1 month can be considered as supportive evidence for *nigrostriatal degeneration*. But such an interpretation also requires additional consideration of the PET and post-mortem results that showed recovery of those parameters

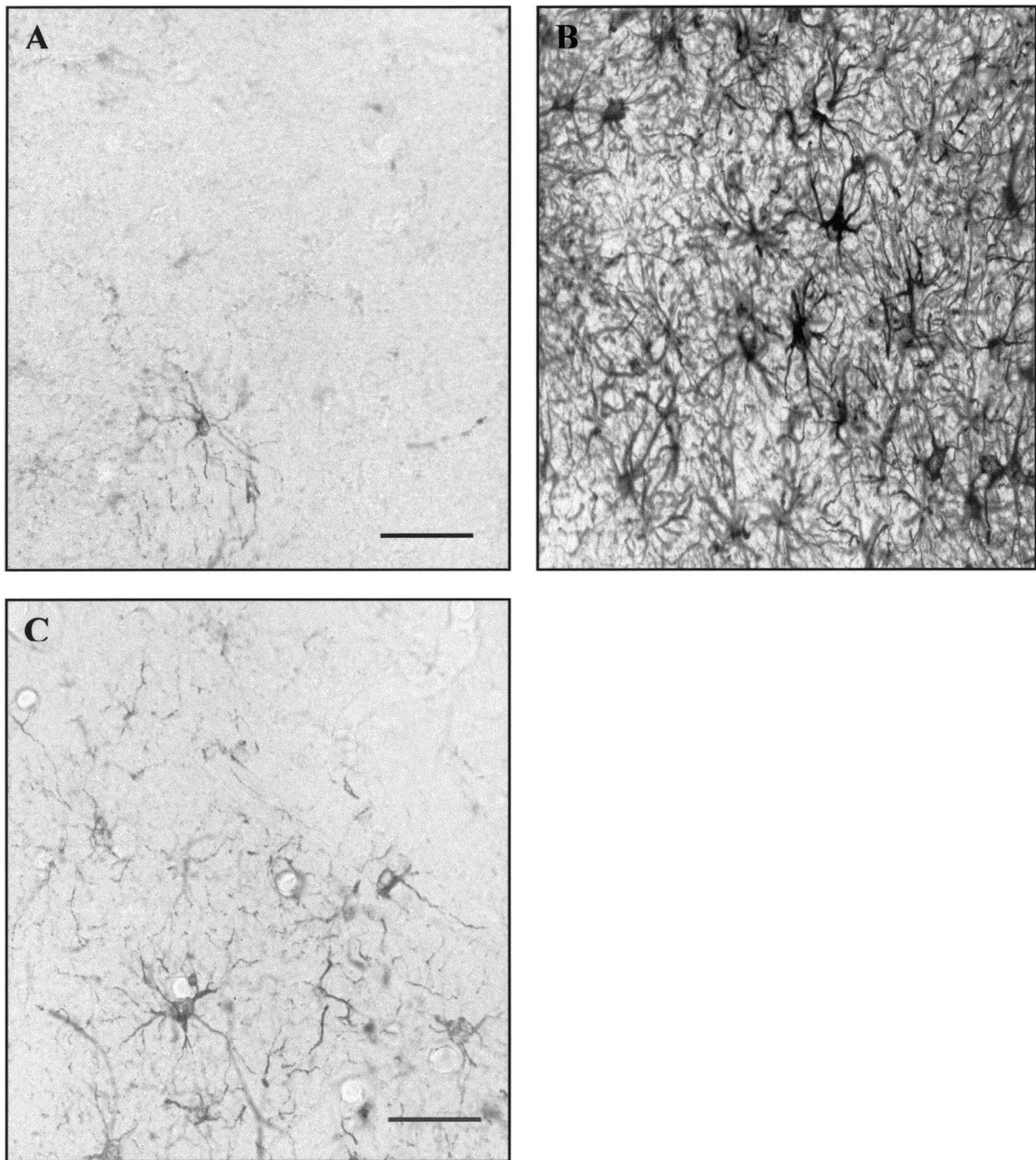


Fig. 4. GFAP-IR of astrocytes in striatum for a control (A), and a METH subject after 1 month (B) and 1.5 years (C). The extensive increase in GFAP-IR observed at 1 month was markedly reduced at 1.5 years although still greater than control levels (scale bar=40 μ m).

at 1.5 years. That is, if the decreases observed at 1 month are to be associated with nigrostriatal degeneration, then the subsequent PET and IR-increases become attributable to *nigrostriatal regeneration*, insofar as the IR decreases were observed throughout the nigrostriatal pathway. However, the tenability of a degeneration hypothesis to account exclusively for the observed results is weakened by the necessary supposition of axons that not only regenerate but

also migrate appropriately into the striatum from the VMD cell region.

Further, if nigrostriatal degeneration had occurred, it may have resulted in at least a partial loss of VMD cells. To address the possibility of VMD cell death being associated with the striatal dopaminergic alterations, we obtained stereologically-based cell counts on VMD nuclei from long-term recovered METH and control subjects. The

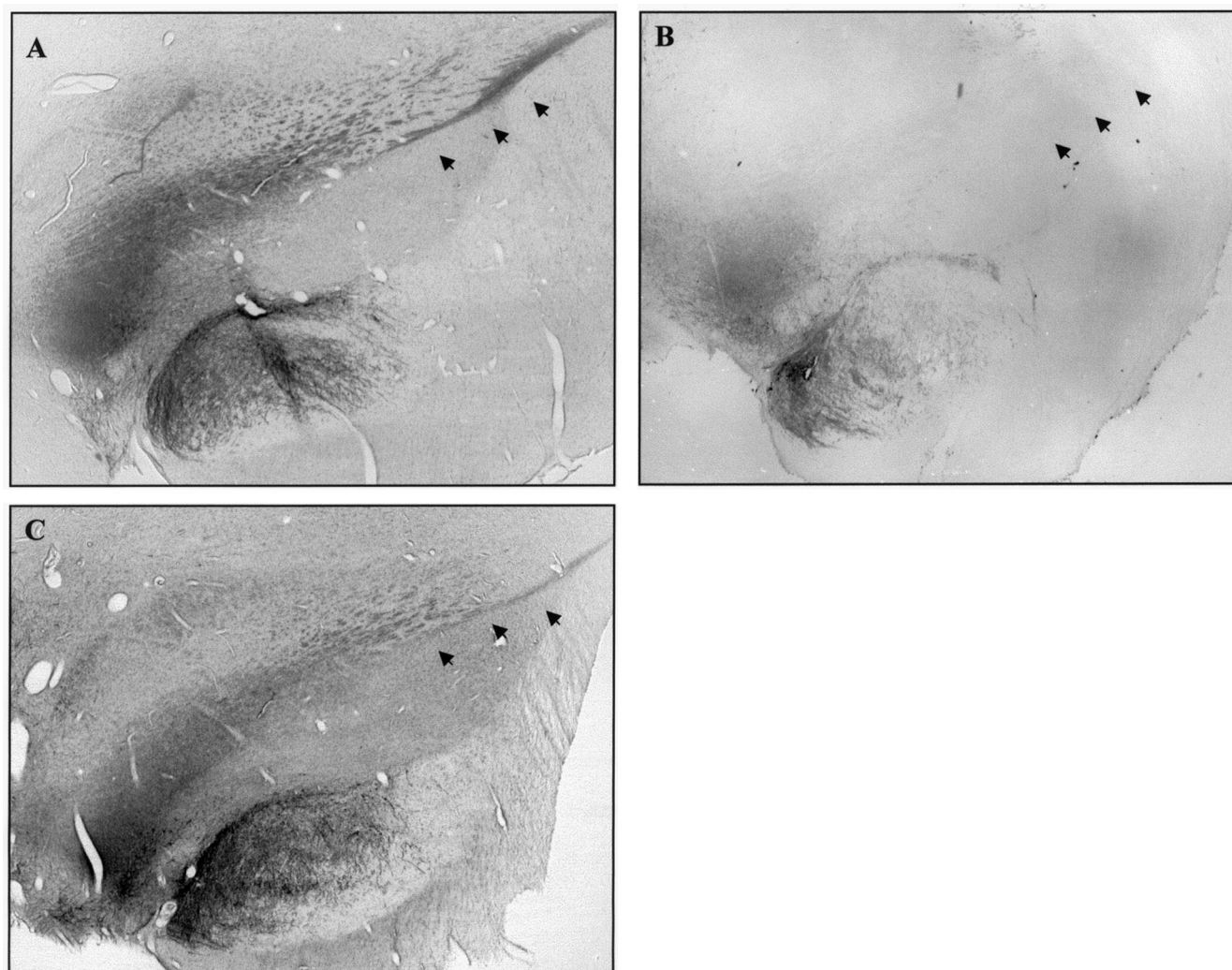


Fig. 5. TH-IR in a coronal section through the rostral ventral midbrain dopamine cell region and medial forebrain bundle in a control (A) and a METH subject after 1 month (B) and 1.5 years (C). The immunoreactivity loss in the lateral portions of the MFB (arrows) at 1 month showed significant recovery at 1.5 years.

Table 1

Estimates of subregional ventral midbrain dopamine cell numbers, as obtained by unbiased stereological methods, were similar for control and METH subjects after 1.5 years

Substantia nigra TH-positive cell number estimates — by region

	Control				METH			% Control
	Mean	S.D.	% CV		Mean	S.D.	% CV	
VTA ^a	29,785	5599	18	VTA	33,731	1158	3	113
RR/PL ^b	24,989	3401	13	RR/PL	24,653	2077	8	99
DMPC ^c	45,181	1789	4	DMPC	46,419	1054	2	103
VLPC ^d	53,381	2397	4	VLPC	50,854	2688	5	95
Total	153,336	10627	7	Total	155,657	6372	4	102

^a VTA=ventral tegmental area.

^b RR/PL=retrobulbar field/pars lateralis combined estimate.

^c DMPC=dorsomedial pars compacta.

^d VLPC=ventrolateral pars compacta.

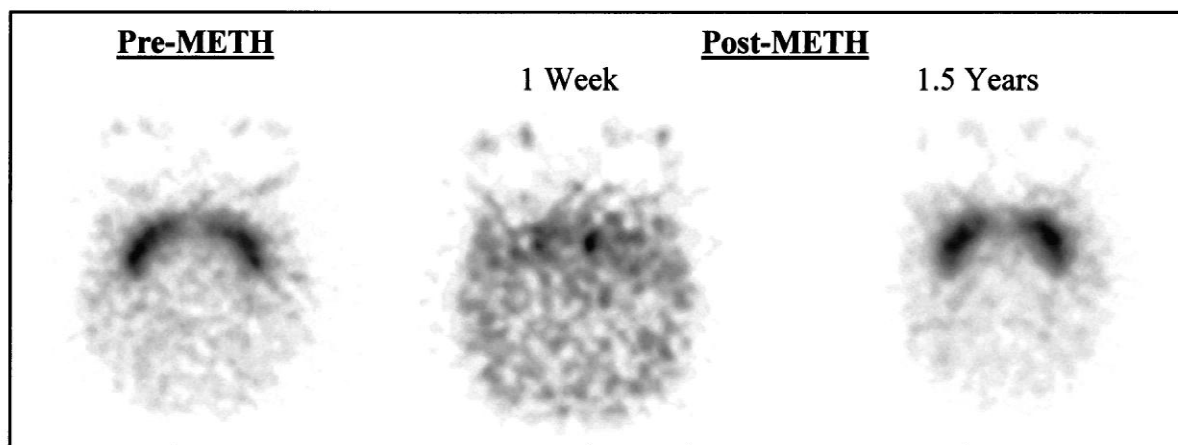


Fig. 6. PET images (summed activity between 60 and 90 min) are represented for the horizontal plane of striatum with the highest [^{11}C]35,428 WIN binding of a referent monkey (left) and a METH subject at two time points. WIN binding for the METH subject, as indexed by changes in the influx rate constant (K_i), was decreased by 80% at 1 week (middle) and by <10% at 1.5 years (right) relative to referent values.

similar results that were obtained for the two groups did not provide support for even a limited VMD cell loss component. Thus, it was apparent that the striatal deficits characterized at 1 month did not progress to VMD cell loss, as has been observed for the protracted time course of nigrostriatal degeneration induced by 6-OH-DA in rodents [2,11,20,60] and by MPTP in monkeys [43]. Rather, it is likely that in our study, the preservation of VMD cell integrity was a critical factor that allowed for the apparent recovery of the dopamine system.

Nonetheless, it could still be argued that degeneration of axonal terminals in striatum occurred as has been characterized for serotonergic terminals after parachloroamphetamine exposure [70]. However, from our results a similar conclusion of exclusive 'terminal' loss could not be inferred since we showed IR deficits that extended from the striatum to the VMD cells. Therefore, based on our data, we propose an alternative hypothesis for the reversibility of the dopaminergic deficits. Namely, METH exposure resulted primarily in long-term decreases of DA phenotypic protein expression rather than a frank loss of nigrostriatal terminals. These effects may have been due to a corresponding down-regulation of mRNA levels. Evidence in support of METH affecting neurotransmitter protein expression has been previously shown in rodents by decreases in TH and VMAT mRNA levels after METH [72]. Likewise, analogous regulatory responses have been demonstrated after trauma to the brain in which the time course of increases and decreases in GFAP expression were paralleled by corresponding changes in GFAP mRNA levels [39]. It may also be that vulnerability of transcription pathways to reactive oxygen species (ROS) resulted in a long term alteration in protein expression. Previously, short-term, reversible changes in DAT-ligand binding and uptake rates, and the activities of some monoamine synthesis enzymes [13,14] have been attributed to ROS

after METH-induced increases in extracellular DA concentrations [5,7,25,28,31].

Presently, our results suggest that multiple mechanisms of neurotoxicity are initiated by METH's pharmacodynamic actions. For example, the greater effect by METH on DAT (a neuronal membrane protein) versus that on TH and VMAT (cytosolic and vesicle-associated proteins respectively) and the regional differences in responsivity to METH may be related to relative differences in the levels of neurotoxic factors or to abundances of METH-sensitive moieties on proteins in the axonal projections. It should be noted that in some regions of caudate and putamen at 1.5 years, DA phenotypic protein IR did not appear to return fully to pre-drug control levels in terms of either observed staining intensity or fiber density. Also, in the VMD cell region, IR decreases, although relatively minor, were consistently observed, indicating that some long-term effects persisted within its dendritic arbor. This type of recovery suggests that compensatory mechanisms aimed at restoring function were activated, although the nigrostriatal system may be now more vulnerable to further deficits promoted by oxidative stress or other neurotoxicants [5,30]. Lastly, our results do not exclude *some* degree of METH-induced striatal terminal degeneration since specific assays for determination of neuronal degeneration were not conducted (e.g., anterograde labeling of DA nigrostriatal tracts [9,10], or silver staining [29]).

The use of these results and prior studies of animal models of METH toxicity to interpret the consequences of human METH abuse has remained tenuous for many years, primarily due to the absence of human data for comparison. But recently, a post-mortem study of human METH abusers characterized various striatal deficits that were similar to those previously shown in animal studies, e.g., decreases in striatal WIN-binding (20–50%) and dopamine concentrations (40–50%) [69]. However, the

decreases in the human study were not attributed to nerve terminal loss because VMAT levels were not correspondingly reduced. At present, VMAT integrity is considered an index of neuronal degeneration since its levels are apparently less susceptible to regulation or alterations by pharmacological manipulation [17,38,62]. Accordingly, without VMAT loss, those data have been interpreted as evidence for the preservation of neuronal vesicles and the terminals that contained them and, therefore, it was concluded that nigrostriatal degeneration had not occurred. It should be noted that those results [69] represent one study of METH abusers who died within 24 h from the effects of METH or from other causes. However, these post-mortem results have been corroborated, in part, by PET imaging studies of chronic METH abusers in which 20–30% decreases in striatal WIN and [^{11}C]methylphenidate binding were measured [32,64]. Thus, extant results of both post-mortem and in vivo studies provide evidence that METH-induced neuroadaptations occur in humans. Further PET studies can be used to determine whether these deficits are reversible upon abstinence from METH as has been observed in the non-human primate studies.

In conclusion, these results confirm and extend our previous reports on the apparent reversibility of dopaminergic deficits in the vervet monkey after acute exposure to neurotoxic METH. Elucidation of mechanisms underlying these neuroadaptations, whether regulatory or degenerative/regenerative, may provide new targets for pharmacological manipulation of dopamine system function.

Acknowledgements

The authors thank Debbie Pollack and Grenvill Morton of the Sepulveda VAMC Research Service–Nonhuman Primate Research Laboratory for supervision of monkey care; Judy Edwards, Waldemar Ladno and Brian Stauffer for expert technical assistance with the PET studies. This research was supported, in part, by a contract from the U.S. Department of Energy (DOE) DE-FC03-87ER60615 and a grant from PHS/NIDA-DA11237.

References

- [1] K.J. Axt, L.A. Mamounas, M.E. Molliver, Structural features of amphetamine neurotoxicity in the brain, in: A.K. Cho, D.S. Segal (Eds.), *Amphetamine and Its Analogs: Psychopharmacology, Toxicology, and Abuse*, Academic Press, Inc, San Diego, CA, 1994, pp. 315–358.
- [2] A. Bjorklund, C. Rosenblad, C. Winkler, D. Kirik, Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease, *Neurobiol. Disease* 4 (1997) 186–200.
- [3] K. Bowenkamp, D. David, P. Lapchak, M. Henry, A. Granholm, B. Hoffer, T. Mahalik, 6-hydroxydopamine induces the loss of the dopaminergic phenotype in substantia nigra neurons of the rat: a possible mechanism for restoration of the nigrostriatal circuit mediated by glial cell line-derived neurotrophic factor, *Exp. Brain Res.* 111 (1996) 1–7.
- [4] J.F. Bowyer, R.R. Holson, Methamphetamine and amphetamine neurotoxicity, in: L.W. Chang, R.S. Dyer (Eds.), *Handbook of Neurotoxicology*, Marcel Dekker, Inc, New York, 1995, pp. 845–870.
- [5] J. Cadet, C. Brannock, Free radicals and the pathobiology of brain dopamine systems, *Neurochem. Int.* 32 (1998) 117–131.
- [6] W. Cass, M. Manning, Recovery of presynaptic dopaminergic functioning in rats treated with neurotoxic doses of methamphetamine, *J. Neurosci.* 19 (1999) 7653–7660.
- [7] J. Cubells, S. Rayport, G. Rajendran, D. Sulzer, Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress, *J. Neurosci.* 14 (1994) 2260–2271.
- [8] M. Eddleston, L. Mucke, Molecular profile of reactive astrocytes implications for their role in neurologic disease, *Neuroscience* 54 (1993) 15–36.
- [9] A. Eisch, J. Marshall, Methamphetamine neurotoxicity: dissociation of striatal dopamine terminal damage from parietal cortical cell body injury, *Synapse* 30 (1998) 433–445.
- [10] A. Eisch, L. Schmued, J. Marshall, Characterizing cortical neuron injury with Fluoro-Jade labeling after a neurotoxic regimen of methamphetamine, *Synapse* 30 (1998) 329–333.
- [11] A. Emmi, H. Rajabi, J. Stewart, Behavioral and neurochemical recovery from partial 6-hydroxydopamine lesions of the substantia nigra is blocked by daily treatment with glutamate receptor antagonists MK-801 and CPP, *J. Neurosci.* 16 (1996) 5216–5224.
- [12] C. Fischer, G. Hatzidimitriou, J. Wlos, J. Katz, G. Ricaurte, Reorganization of ascending 5-HT axon projections in animals previously exposed to the recreational drug (+/–)3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'), *J. Neurosci.* 15 (1995) 5476–5485.
- [13] A. Fleckenstein, R. Metzger, D. Wilkins, J. Gibb, G. Hanson, Rapid and reversible effects of methamphetamine on dopamine transporters, *J. Pharm. Exp. Therapeutics* 262 (1997) 834–838.
- [14] A. Fleckenstein, D. Wilkins, J. Gibb, G. Hanson, Interaction between hyperthermia and oxygen radical formation in the 5-hydroxytryptaminergic response to a single methamphetamine administration, *J. Pharm. Exp. Therapeutics* 283 (1997) 281–285.
- [15] J. Francis, J. Von Visger, G. Markelonis, T. Oh, Neuroglial responses to the dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mouse striatum, *Neurotoxicol. Teratol.* 17 (1995) 7–12.
- [16] C. Francois, G. Percheron, J. Yelnik, S. Heyner, A histological atlas of the macaque (*Macaca mulatta*) substantia nigra in ventricular coordinates, *Brain Res. Bull.* 14 (1985) 349–367.
- [17] K. Frey, M. Kilbourn, T. Robinson, Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine, *Eur. J. Pharmacol.* 334 (1997) 273–279.
- [18] S. Friedman, E. Castañeda, G. Hodge, Long-term monoamine depletion, differential recovery, and subtle behavioral impairment following methamphetamine-induced neurotoxicity, *Pharmacol. Biochem. Behav.* 61 (1998) 35–44.
- [19] F. Fumagalli, R.R. Gainetdinov, K.J. Valenzano, M.G. Caron, Role of dopamine transporter in methamphetamine-induced neurotoxicity: evidence from mice lacking the transporter, *J. Neurosci.* 18 (1998) 4861–4869.
- [20] D. Gash, C.M. Kearns, GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo, *Brain Res.* 672 (1995) 104–111.
- [21] M. Gordon, W. Schreier, X. Ou, L. Holcomb, D. Morgan, Exaggerated astrocyte reactivity after nigrostriatal deafferentation in the aged rat, *J. Comp. Neurol.* 388 (1997) 106–119.

- [22] H.J.G. Gundersen, P. Bagger, T.F. Bendtsen, S.M. Evans, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J.R. Nyengaard, B. Pakkenberg, F.B. Sorensen, A. Vesterby, M.J. West, The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis, *APMIS* 96 (1988) 857–881.
- [23] G.M. Halliday, I. Tork, Comparative anatomy of the ventromedial mesencephalic tegmentum in the rat, cat, monkey and human, *J. Comp. Neurol.* 252 (1986) 423–445.
- [24] R. Hassler, Zur Pathologie der Paralysis Agitans und des postenzephalitischen Parkinsonismus, *J. Psychol. Neurol.* 48 (1938) 387–476.
- [25] T. Hastings, D. Lewis, M. Zigmond, Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections, *Proc. Natl. Acad. Sci. USA* 93 (1996) 1956–1961.
- [26] H. Hirata, J.L. Cadet, p53-knockout mice are protected against the long-term effects of methamphetamine on dopaminergic terminals and cell bodies, *J. Neurochem.* 69 (1997) 780–790.
- [27] M. Laruelle, R.J. Iyer, M.S. Al-Tikriti, Y. Zea-Ponce, R. Malison, S.S. Zoghbi, R.M. Baldwin, H.F. Kung, D.S. Charney, P.B. Hoffer, R.B. Innis, C.W. Bradberry, Microdialysis and SPECT measurements of amphetamine-induced dopamine release in nonhuman primates, *Synapse* 25 (1997) 1–14.
- [28] M. LaVoie, T. Hastings, Dopamine quinone formation and protein modification associated with the striatal neurotoxicity of methamphetamine: evidence against a role for extracellular dopamine, *J. Neurosci.* 19 (1999) 1484–1491.
- [29] J.C. Linder, S.J. Young, P.M. Groves, Electron microscopic evidence for neurotoxicity in the basal ganglia, *Neurochem. Int.* 26 (1995) 195–202.
- [30] Y. Liu, R. Edwards, The role of vesicular transport proteins in synaptic transmission and neural degeneration, *Ann. Rev. Neurosci.* 20 (1997) 125–156.
- [31] G. Marek, G. Vosmer, L. Seiden, The effects of monoamine uptake inhibitors and methamphetamine on neostriatal 6-hydroxydopamine (6-OHDA) formation, short-term monoamine depletions and locomotor activity in the rat, *Brain Res.* 516 (1990) 1–7.
- [32] U.D. McCann, D.F. Wong, F. Yokoi, V. Villemagne, R.F. Dannals, G.A. Ricaurte, Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: Evidence from positron emission tomography studies with [^{11}C]WIN-35,428, *J. Neurosci.* 18 (1998) 8417–8422.
- [33] D.A. McRitchie, C.D. Hardman, G.M. Halliday, Cytoarchitectural distribution of calcium binding proteins in midbrain dopaminergic regions of rats and humans, *J. Comp. Neurol.* 364 (1996) 121–150.
- [34] W. Melega, G. Lacan, A. DeSalles, M. Phelps, Long term methamphetamine-induced decreases of [^{11}C]WIN 35,428 binding in striatum are reduced by GDNF: PET studies in the vervet monkey, *Synapse* 35 (2000) 243–249.
- [35] W. Melega, G. Lacan, D. Harvey, S. Huang, M. Phelps, Dizocilpine and reduced body temperature do not prevent methamphetamine-induced neurotoxicity in the vervet monkey: [^{11}C]WIN-35,428-positron emission tomography studies, *Neurosci. Lett.* 258 (1998) 17–20.
- [36] W.P. Melega, J. Quintana, M.J. Raleigh, D.B. Stout, D.C. Yu, K.P. Lin, S.C. Huang, M.E. Phelps, 6- ^{18}F Fluoro-L-DOPA-PET studies show partial reversibility of long-term effects of chronic amphetamine in monkeys, *Synapse* 22 (1996) 63–69.
- [37] W.P. Melega, M.J. Raleigh, D.B. Stout, G. Lacan, S.C. Huang, M.E. Phelps, Recovery of striatal dopamine function after acute amphetamine- and methamphetamine-induced neurotoxicity in the vervet monkey, *Brain Res.* 766 (1997) 113–120.
- [38] L. Naudon, R. Raisman-Vozari, R. Edwards, I. Leroux-Nicollet, D. Peter, Y. Liu, J. Costentin, Reserpine affects differentially the density of the vesicular monoamine transporter and dihydrotetrabenazine binding sites, *Eur. J. Neurosci.* 8 (1996) 842–846.
- [39] W.T. Norton, D.A. Aquino, I. Hozumi, F.C. Chiu, C.F. Brosnan, Quantitative aspects of reactive gliosis: a review, *Neurochem. Res.* 17 (1992) 877–885.
- [40] E. O'Hearn, G. Battaglia, E. De Souza, M. Kuhar, M. Molliver, Methylenedioxymphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: immunocytochemical evidence for neurotoxicity, *J. Neurosci.* 8 (1988) 2788–2803.
- [41] B. Pakkenberg, H.J.G. Gundersen, Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator, *J. Microscopy* 150 (1988) 1–20.
- [42] H. Pakkenberg, B. Andersen, R. Burns, B. Pakkenberg, A stereological study of substantia nigra in young and old rhesus monkeys, *Brain Res.* 693 (1995) 201–206.
- [43] A. Parent, B. Lavoie, The heterogeneity of the mesostriatal dopaminergic system as revealed in normal and parkinsonian monkeys, *Adv. Neurobiol.* 60 (1993) 25–33.
- [44] C. Patlak, R. Blasberg, Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data. Generalizations, *J. Cereb. Blood Flow Metab.* 5 (1985) 584–590.
- [45] C. Patlak, R. Blasberg, J. Fenstermacher, Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data, *J. Cereb. Blood Flow Metab.* 3 (1983) 1–7.
- [46] C. Pu, C. Vorhees, Developmental dissociation of methamphetamine-induced depletion of dopaminergic terminal and astrocyte reaction in rat striatum, *Dev. Brain Res.* 72 (1993) 325–328.
- [47] G.A. Ricaurte, R.W. Guillery, L.S. Seiden, C.R. Schuster, R.Y. Moore, Dopamine nerve terminal degeneration produced by high doses of methylamphetamine in the rat brain, *Brain Res.* 235 (1982) 93–103.
- [48] G.A. Ricaurte, U.D. McCann, Neurotoxic amphetamine analogues: effects in monkeys and implications for humans, *Ann. NY Acad. Sci.* 648 (1992) 371–382.
- [49] G.A. Ricaurte, C.R. Schuster, L.S. Seiden, Long-term effects of repeated methylamphetamine administration on dopamine and serotonin neurons in the rat brain: a regional study, *Brain Res.* 193 (1980) 153–163.
- [50] T. Robinson, J. Becker, Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis, *Brain Res. Rev.* 11 (1986) 157–198.
- [51] T. Robinson, Z. Mocsary, D. Camp, I. Whishaw, Time course of recovery of extracellular dopamine following partial damage to the nigrostriatal dopamine system, *J. Neurosci.* 14 (1994) 2687–2696.
- [52] T. Robinson, J. Yew, P. Paulson, D. Camp, The long-term effects of neurotoxic doses of methamphetamine on the extracellular concentration of dopamine measured with microdialysis in striatum, *Neurosci. Lett.* 110 (1990) 193–198.
- [53] L.J. Ryan, J.C. Linder, M.E. Martone, P.M. Groves, Histological and ultrastructural evidence that d-amphetamine causes degeneration in neostriatum and frontal cortex of rats, *Brain Res.* 518 (1990) 67–77.
- [54] L.S. Seiden, M.W. Fischman, C.R. Schuster, Long-term methamphetamine induced changes in brain catecholamines in tolerant rhesus monkeys, *Drug Alcohol Depend.* 1 (1976) 215–219.
- [55] L.S. Seiden, G.A. Ricaurte, Neurotoxicity of methamphetamine and related drugs, in: H.Y. Metzler (Ed.), *Psychopharmacology: the Third Generation of Progress*, Raven Press, New York, 1987, pp. 359–366.
- [56] W. Slikker Jr., S.F. Ali, A.C. Scallet, C.H. Frith, G.D. Newport, J.R. Bailey, Neurochemical and neurohistological alterations in the rat and monkey produced by orally administered methylenedioxymethamphetamine (MDMA), *Toxicol. Appl. Pharmacol.* 94 (1988) 448–457.
- [57] P.K. Sonsalla, N.D. Jochnowitz, G.D. Zeevalk, J.A. Oostveen, E.D. Hall, Treatment of mice with methamphetamine produces cell loss in the substantia nigra, *Brain Res.* 738 (1996) 172–175.
- [58] L. Steranka, E. Sanders-Bush, Long-term effects of continuous exposure to amphetamine on brain dopamine concentration and

- synaptosomal uptake in mice, *Eur. J. Pharmacol.* 65 (1980) 439–442.
- [59] R. Switzer, Strategies for assessing neurotoxicity, *Neurosci. Biobehav. Rev.* 15 (1991) 89–93.
- [60] D.M. Togasaki, Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine, *Neuroscience* 67 (1995) 631–647.
- [61] M. Trulsson, M. Cannon, T. Faegg, J. Raese, Effects of chronic methamphetamine on the nigral-striatal dopamine system in rat brain: tyrosine hydroxylase immunohistochemistry and quantitative light microscopic studies, *Brain Res. Bull.* 15 (1985) 569–577.
- [62] T. Vander Borgh, M. Kilbourn, T. Desmond, D. Kuhl, K. Frey, The vesicular monoamine transporter is not regulated by dopaminergic drug treatments, *Eur. J. Pharmacol.* 294 (1995) 577–583.
- [63] V. Villemagne, J. Yuan, D. Wong, R. Dannals, G. Hatzidimitriou, W. Mathews, H. Ravert, J. Musachio, U. McCann, G. Ricaurte, Brain dopamine neurotoxicity in baboons treated with doses of methamphetamine comparable to those recreationally abused by humans: evidence from [¹¹C]WIN-35,428 positron emission tomography studies and direct in vitro determinations, *J. Neurosci.* 18 (1998) 419–427.
- [64] N. Volkow, L. Chang, G. Wang, J. Fowler, D. Franceschi, S. Gatley, C. Wong, R. Hitzemann, N. Pappas, In vivo evidence that methamphetamine abuse produces long lasting changes in dopamine transporters in human brain, *J. Nucl. Med.* 40 (1999) 110 P.
- [65] G. Wagner, G. Ricaurte, C. Johanson, C. Schuster, L. Seiden, Amphetamine induces depletion of dopamine and loss of dopamine uptake sites in caudate, *Neurology* 30 (1980) 547–550.
- [66] G.C. Wagner, G.A. Ricaurte, L.S. Seiden, C.R. Schuster, R.J. Miller, J. Westley, Long-lasting depletions of striatal dopamine and loss of dopamine uptake sites following repeated administration of methamphetamine, *Brain Res.* 181 (1980) 151–160.
- [67] M. West, New stereological methods for counting neurons, *Neurobiol. Aging* 14 (1993) 275–285.
- [68] M.J. West, L. Slomianka, H.J.G. Gundersen, Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator, *Anat. Rec.* 231 (1991) 482–497.
- [69] J. Wilson, K. Kalasinsky, A. Levey, C. Bergeron, G. Reiber, R. Anthony, G. Schmunk, K. Shannak, J. Haycock, S. Kish, Striatal dopamine nerve terminal markers in human, chronic methamphetamine users, *Nature Med.* 2 (1996) 699–703.
- [70] M. Wilson, M. Molliver, Microglial response to degeneration of serotonergic axon terminals, *Glia* 11 (1994) 18–34.
- [71] W.L. Woolverton, G.A. Ricaurte, L.S. Forno, L.S. Seiden, Long-term effects of chronic methamphetamine administration in rhesus monkeys, *Brain Res.* 486 (1989) 73–78.
- [72] Y. Zhang, J.A. Angulo, Contrasting effects of repeated treatment vs. withdrawal of methamphetamine on tyrosine hydroxylase messenger RNA levels in the ventral tegmental area and substantia nigra zona compacta of the rat brain, *Synapse* 24 (1996) 218–223.