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Methamphetamine Causes Degeneration of Dopamine Cell Bodies and Terminals of the Nigrostriatal Pathway Evidenced by Silver Staining

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Methamphetamine is a widely abused illicit drug. Recent epidemiological studies showed that methamphetamine increases the risk for developing Parkinson's disease (PD) in agreement with animal studies showing dopaminergic neurotoxicity. We examined the effect of repeated low and medium doses *vs* single high dose of methamphetamine on degeneration of dopaminergic terminals and cell bodies. Mice were given methamphetamine in one of the following paradigms: three injections of 5 or 10 mg/kg at 3 h intervals or a single 30 mg/kg injection. The integrity of dopaminergic fibers and cell bodies was assessed at different time points after methamphetamine by tyrosine hydroxylase immunohistochemistry and silver staining. The 3 × 10 protocol yielded the highest loss of striatal dopaminergic terminals, followed by the 3 × 5 and 1 × 30. Some degenerating axons could be followed from the striatum to the substantia nigra pars compacta (SNpc). All protocols induced similar significant degeneration of dopaminergic neurons in the SNpc, evidenced by amino-cupric-silver-stained dopaminergic neurons. These neurons died by necrosis and apoptosis. Methamphetamine also killed striatal neurons. By using D1-Tmt/D2-GFP BAC transgenic mice, we observed that degenerating striatal neurons were equally distributed between direct and indirect medium spiny neurons. Despite the reduced number of dopaminergic neurons in the SNpc at 30 days after treatment, there was a partial time-dependent recovery of dopamine terminals beginning 3 days after treatment. Locomotor activity and motor coordination were robustly decreased 1–3 days after treatment, but recovered at later times along with dopaminergic terminals. These data provide direct evidence that methamphetamine causes long-lasting loss/degeneration of dopaminergic cell bodies in the SNpc, along with destruction of dopaminergic terminals in the striatum.
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INTRODUCTION

Methamphetamine is an addictive illegal psychostimulant consumed by between 14.3 million and 53.1 million users worldwide, according to last estimations from the United Nations Office on Drugs and Crime (UNODC, 2013). Despite its high popularity, attributed to its wide availability, relative low cost, and long duration of psychoactive effects, methamphetamine is a neurotoxic drug that can produce long-lasting impairments in abusers (Krasnova and Cadet, 2009; McCann *et al*, 1998; Volkow *et al*, 2001a). Brain PET studies in human abusers showed dopamine transporter (DAT) reductions in the caudate nucleus and putamen that are associated with reduced motor skills (Volkow *et al*, 2001a,b; McCann *et al*, 1998). Furthermore, recent epidemiological studies provided evidence that the risk for developing Parkinson's disease (PD) is almost doubled in individuals with a history of methamphetamine use (Callaghan *et al*, 2012).

It is established that methamphetamine is a potent inducer of dopamine release and is toxic to dopamine neurons. Neurodegeneration of dopaminergic terminals in the striatum is evidenced by reductions in the immunoreactivity of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, and of DAT, accompanied by decreases in levels of dopamine and its metabolites (Ares-Santos *et al*, 2012, 2013a, b; Moratalla *et al*, 2014; Hotchkiss and Gibb, 1980; Granado *et al*, 2010, 2011a,b; Ricaurte *et al*, 1982,1984; Seiden *et al*, 1976; Wagner *et al*, 1980; Zhu *et al*, 2006a). Concomitant increases in reactive astrocytes and microglia in the striatum have also been described as indirect markers of this neurotoxicity.

However, a controversy exists regarding whether or not methamphetamine produces neurodegeneration of dopaminergic cell bodies in the substantia nigra pars compacta

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(SNpc); although most studies indicate that methamphetamine selectively injures dopamine terminals while dopaminergic cell bodies are spared (Krasnova and Cadet, 2009; Ricaurte *et al*, 1982), others report loss of dopaminergic neurons in the SNpc: TH-immunoreactive (TH-ir) revealed that methamphetamine causes a reduction in the number of TH neurons in the SNpc (Ares-Santos *et al*, 2012; Granado *et al*, 2011a, b, 2013; Hirata and Cadet, 1997; Sonsalla *et al*, 1996). Furthermore, recent reports from our laboratory have shown that methamphetamine not only reduces TH expression, which could be due to a reduction in its synthesis rather than neuronal loss, but also produces neuronal degeneration, evidenced by Nissl-stained apoptotic bodies, Fluorojade-stained neurons, and reduction in the total number of neurons in the SNpc (Ares-Santos *et al*, 2012; Granado *et al*, 2011a, b). However, to date, no direct proof of degeneration of nigral dopaminergic neurons has been reported.

The aim of this study was to test whether methamphetamine produces neurodegeneration of dopaminergic cell bodies in the SN by looking for direct evidence of degeneration of dopaminergic neurons. Moreover, we wanted to know if different regimens of methamphetamine delivery had different neurotoxic effects in the striatum and SNpc, and to test whether these effects were permanent or transient. We compared the effects of three different administration paradigms: a single high dose (bolus) (1×30 mg/kg), which models acute methamphetamine intoxication, *vs* repeated administration (binge) of lower doses (3×5 or 3×10 mg/kg), modeling methamphetamine use by humans (Bowyer *et al*, 2008). In addition, with the lowest dose protocol (3×5) we evaluated the time course of degeneration of dopamine terminals and cell bodies to determine the persistence of neurotoxic effects, and assessed locomotor activity and motor coordination at short (1 and 3 days) and long time points (7 and 30 days) after treatment to correlate neurotoxicity with functional behavioral changes.

We used the amino-cupric-silver (A-Cu-Ag) staining to identify damaged cell somas and processes. This technique, also known as de Olmos stain (de Olmos *et al*, 1994), specifically stains somatodendritic and terminal degeneration (Beltramino *et al*, 1993; de Olmos *et al*, 1981, 1994). We combined A-Cu-Ag staining with TH immunohistochemistry to determine if dopaminergic neurons in the SNpc degenerate after methamphetamine. In addition, we used it in D1R-tomato/D2R-eGFP BAC transgenic mice to identify specific neuronal populations sensitive to methamphetamine. We further evaluated the neurotoxic changes in the striatum by electron microscopy.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (3–4 months old, weighing 20–33 g) ($n = 10$–14 per group) from the Instituto de Investigación Médica Mercedes y Martín Ferreyra or from the Instituto Cajal (Harlan Iberica, Barcelona, Spain) were housed in groups of 4–6 per cage in conditions of constant temperature at $21 \pm 2\,^{\circ}$C, in a 12 h light/dark cycle, with free access to food and water. All experimental procedures conformed to European Community guidelines (2003/65/CE) and were approved by Cajal Institute's Bioethics Committee (following DC86/609/EU).

Treatment and Measurement of Rectal Temperature

(+)-Methamphetamine hydrochloride was obtained from Sigma-Aldrich (Madrid, Spain), dissolved in 0.9% (w/v) NaCl (saline) and injected in a volume of 10 ml/kg. Doses refer to the base. A single high dose of (+)-methamphetamine (30 mg/kg) or multiple lower doses (3 doses of 5 or 10 mg/kg, at 3 h intervals) were injected intraperitoneally in a room at constant temperature of $23 \pm 2\,^{\circ}$C. Control mice received saline. Mice were housed in groups of 4–6 per cage during treatment. Rectal temperature was measured every 30 min immediately before and after each methamphetamine injection and 1, 2, and 3 h after each drug administration as described before (Ares-Santos *et al*, 2012; Granado *et al*, 2010, 2011a, b). Animals were killed 1 or 3 days after treatment. To examine the time course of methamphetamine (3×5) effects, mice were killed 3 or 12 h, and 1, 3, 7, or 30 days after treatment.

Tissue Collection and Detection of Neuronal Degeneration by the A-Cu-Ag Stain

Animals were anesthetized with 6% chloral hydrate or sodium pentobarbital (50 mg/kg), and perfused transcardially with 4% paraformaldehyde in 0.2 M borate buffer (pH 7.4). Brains were left overnight in the skull and afterwards removed and placed in 30% sucrose. Brain sections of 50 μm were obtained in a freezing microtome or a vibratome and stored in 4% paraformaldehyde for A-Cu-Ag technique or immunohistochemistry. One hemisphere of each brain was cut in sagital and the other in coronal sections to study the anatomical neurodegeneration. Neuronal degeneration was analyzed by the A-Cu-Ag stain, which stains degenerating perikarya, dendrites, stem axons, and their terminal ramifications (synaptic endings) (de Olmos *et al*, 1994; Switzer, 2000). The A-Cu-Ag technique was set up in the lab after the training in the Laboratory of Experimental Neuroanatomy and Histology, Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET, Cordoba, Argentina).

Immunohistochemistry

Immunostaining was carried out on free-floating sections with standard avidin–biotin immunocytochemical protocols (Granado *et al*, 2008a; Ortiz *et al*, 2010). Endogenous peroxidase activity was removed by incubation in 3% H_2O_2 for 10 min. This step was avoided if immunostaining was performed in silver-stained tissue. The specific primary antibodies (Ab-I) used were as follows: rabbit anti-TH (1 : 1000; Chemicon International, Temecula, CA); rat anti-GFP (1 : 1000; Nacalai Tesque, Kyoto, Japan); and rabbit anti-Tmt (DsRed) (1 : 1000; Clontech). For immunofluorescence experiments, we used Alexa Fluor 488- and 594-conjugated secondary antibodies (1 : 400–500; Invitrogen, Eugene, OR).

Quantitative Assessment of Degeneration of Dopaminergic Terminals in the Striatum

Quantification of TH expression and silver staining in the striatum was carried out using pictures of the striatal sections taken with a $\times 4$ lens in an optic microscope equipped with a Leica DFC 290 HD videocamera. An image analysis system (Analytical Imaging Station; Imaging Research, Linton, UK) was used to convert color intensities into a gray scale and to quantify the area of staining in the striatum as a proportion of pixels in the striatum that show staining (stained area) in relation to total pixels in the striatum (scanned area). We refer to this as proportional stained area (Darmopil *et al*, 2008, 2009). The threshold was chosen in saline animals and applied to all animals.

Electron Microscopy

Degeneration of striatal fibers was confirmed at the ultrastructural level by EM following the protocol described previously (Rivera *et al*, 2002). Cryoprotected tissue was frozen with liquid nitrogen and thawed in cold 0.1 M PB. Sections (40 μm thick) were cut with a Vibratome (Leica Microsystems GmbH, Wetzlar, Germany) and immunostained for 3-nitrotyrosine (primary antibody from Dr Martinez-Murillo; diluted 1 : 2000) to mark protein nitration (Castro-Blanco *et al*, 2003), or for TH (1 : 1000). After DAB reaction, sections were washed with PBS, postfixed in 1% osmium tetroxide in 0.1 M PB, dehydrated in graded ethanols (1% uranyl acetate was included at the 70% ethanol), mounted on Durcupan ACM resin (Fluka) slides under a plastic coverslip, and cured for 3 days at 56 °C. Selected areas of the caudate–putamen were dissected out, re-embedded in Durcupan, and cut in ultrathin sections (1–0.5 μm thick) with Ultramicrotome (Leica Microsystems), mounted on Formvar-coated grids, stained with lead citrate, and examined in a Jeol 1200 EX electron microscope.

Stereological Quantification of Degenerating Neurons in the Striatum and in SNpc

The number of striatal A-Cu-Ag-stained neurons was counted unilaterally in every 4th sections of the striatum of saline- and methamphetamine-treated animals ($n = 4$–6) stained for A-Cu-Ag. The degeneration of neurons in the SNpc was assessed by counting TH-ir neurons, A-Cu-stained neurons, and TH/A-Cu-Ag doubled-stained neurons unilaterally in every 4th section of the SNpc of all experimental groups ($n = 4$–6) in which TH immunostaining was performed following the A-Cu-Ag technique (de Olmos *et al*, 2009). The optical fractionator, Stereoinvestigator program (Microbrightfield Bioscience, Colchester, VT), was used as described (Ares-Santos *et al*, 2012; Espadas *et al*, 2012) by an experienced observer unaware of treatment conditions. The outline of the striatum and SNpc (including SN pars lateralis) were drawn at low power ($\times 2$) using defined anatomic landmarks (Ares-Santos *et al*, 2012; Baquet *et al*, 2009; Granado *et al*, 2008c), and the number of cells was counted at higher power ($\times 20$ for the striatum and $\times 100$ for the SNpc). At these magnifications, A-Cu-Ag-stained cell bodies were easily identified among the terminal degeneration in the striatum and among TH-ir, TH/A-Cu-Ag-stained or A-Cu-Ag non-dopaminergic neurons in the SNpc. To avoid double counting, neurons were counted when their nuclei were optimally visualized, which occurred only in one focal plane. Only A-Cu-Ag-stained particles with the size and morphology of degenerating cells or apoptotic bodies were counted. Neurons that stained for both TH and A-Cu-Ag were only counted if there was a silver-stained cell body surrounded by TH staining. Although this criterion may have excluded some dopaminergic degenerating neurons, it should have reliably excluded all non-dopaminergic degenerating cells. Some neurons with extremely faint signs of TH expression were not counted as TH-expressing neurons (see Figure 4f). Results are expressed as bilateral estimations.

Identification of Striatal Degenerating Neurons

For these studies, we used D1R-tomato/D2R-eGFP BAC transgenic mice obtained by crossing D1R-tomato with D2R-eGFP BAC transgenic mice obtained from MMRRC, SC, and from Jackson Labs, ME. These mice were backcrossed for six generations to C57BL/6N mice using a speed congenic protocol, D1R-tomato/D2R-eGFP mice (Suárez *et al*, 2013) were administered a single high dose of methamphetamine (1×30 mg/kg), killed 1 day later, and processed for silver staining in combination with immunohistochemistry for GFP or tomato red (Tmt) proteins. The number of total striatal A-Cu-Ag-stained neurons positive for GFP or Tmt was quantified by stereology as described for striatal degenerating neurons ($n = 5$).

Histopathological Studies in SNpc

Coronal midbrain sections (30 μm) containing SNpc of animals treated with saline or methamphetamine (3×5 mg/kg) were mounted on slides and dried overnight, rehydrated, and stained for hematoxylin and eosin (H&E) or for Fluoro-Jade C as described before (Granado *et al*, 2008b, 2011a, b). Nissl staining was performed on SNpc sections after TH immunohistochemistry.

Locomotor Activity and Motor Coordination

Horizontal and vertical movements were recorded in the same animals in naive conditions and after 1, 3, 7, and 30 days of treatment with saline or methamphetamine (3×5) ($n = 10$) in 60 min sessions as described previously (Granado *et al*, 2008c). Motor coordination was measured at the same time points in the same animals, right after the locomotor activity evaluation, using an accelerating rotarod apparatus (Hugo Basile, Rome, Italy) as described previously (Rodrigues *et al*, 2007).

Statistics

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using Student's *t*-test and two-way ANOVA. Relevant differences were analyzed pair-wise by *post hoc* comparisons with Student–Newman–Keuls and Tukey's test, to determine specific group differences. All statistical analyses and graphical representations were performed using Sigma Plot 11.0 program and the threshold for statistical significance was set at $p < 0.05$.

RESULTS

Methamphetamine-Induced Hyperthermia Peaks at 30 min after Each Administration

Animals treated with methamphetamine in all three different administration protocols showed significant increases in rectal temperature compared with saline-treated animals (Figure 1a). The multiple dose regimens produced hyperthermia, peaking 30 min after each administration, compared with saline-treated animals ($p<0.001$). The 3×10 regimen induced greater hyperthermia than the 3×5, which induced a slight hypothermia 2 h after the first injection, in comparison with saline-treated animals ($p<0.05$). A single high dose (30 mg/kg) of methamphetamine induced a single peak of hyperthermia in the first 30–60 min after the injection that was similar to those

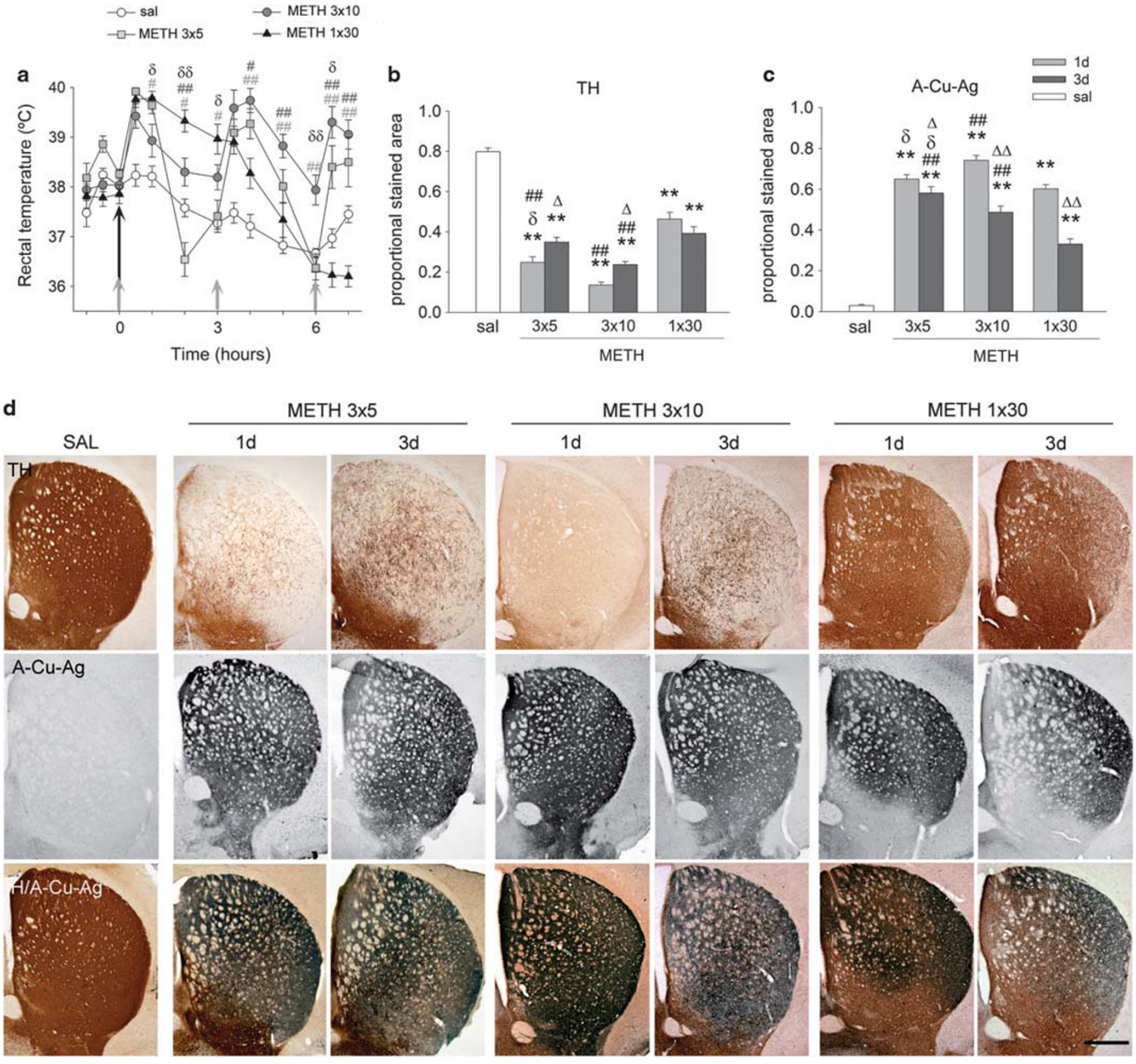


Figure 1 Single administration of methamphetamine (1×30) induced less dopaminergic terminal degeneration in the striatum than multiple administration treatments (3×5 or 3×10). (a) Methamphetamine produced hyperthermia after each injection, with three peaks in the multiple administration regimens, and a single and more sustained peak of hyperthermia after single administration of (1×30 mg/kg). Data represent mean ± SEM, $n=10$–14 per group. Arrows indicate drug injection. (b and c) Histograms show the proportional stained area of tyrosine hydroxylase (TH) and amino-cupric-silver staining in the striatum. Data represent mean ± SEM, $n=4$–6 per group. (d) Photomicrographs of striatal sections from mice 1 and 3 days after treatment with saline (sal) or methamphetamine (3×5), (3×10), or (1×30), stained for TH (top), A-Cu-Ag (middle), or TH/A-Cu-Ag (bottom). $^{*}p<0.05$, $^{**}p<0.001$ *vs* sal, $^{\#}p<0.05$, $^{\#\#}p<0.001$ *vs* (1×30), $^{\delta}p<0.05$, $^{\delta\delta}p<0.001$ *vs* (3×10), $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.001$ *vs* 1 day. Statistical analysis was performed by two-way analysis of variance and *post hoc* Newman–Keuls analysis. Bar indicates 500 μm (d).

produced by multiple lower dose regimens. However, in the single high-dose protocol, hyperthermia was sustained, and significantly higher than following multiple lower doses for 3 h ($p<0.05$), and gradually decreased until reaching control levels at 5 h after administration ($p<0.05$ *vs* saline). Body temperature in these animals continued decreasing and resulted in slight hypothermia (Figure 1a) 7 h after the injection ($p<0.05$).

A Single High Dose of Methamphetamine Induced Less Degeneration of TH-ir Terminals than Multiple Lower Doses

One day after methamphetamine administration, a marked overall decrease in the density of TH-ir terminals in the striatum was evident compared with the intense TH staining in saline-treated animals (Figure 1b and d). At this time point, quantitative image analysis revealed significant decreases in TH-ir in all treatment paradigms compared with saline ($p<0.001$): 70% in the 3×5 group, 83% in the 3×10 group, and 42% in the 1×30 group. As expected, multiple dose regimens induced a dose-dependent TH terminal loss in the striatum ($p<0.001$). The multiple lower dose protocols produced greater reductions in TH-ir in the striatum than the single high dose ($p<0.001$), with only some scattered TH fibers visible throughout the whole striatum. In the single high-dose regimen, striosomes of the dorsolateral part of the caudoputamen were the most affected areas, with severe TH-ir loss compared with the rest of the striatum. Three days after treatment, the levels of striatal TH-ir were still reduced in methamphetamine-treated animals compared with saline animals ($p<0.001$). However, a slight increase in the density of TH terminals was seen in the multiple dose regimens ($p<0.05$). This tendency to recover TH-ir levels was not observed in the single high-dose group (Figure 1b and d).

In parallel with changes observed in striatal TH-ir, a marked overall increase in the density of A-Cu-Ag-stained terminals in the striatum (Figure 1c and d), with a widespread punctuate silver deposition (see Figure 2a), was evident in methamphetamine-treated animals one day after the treatment compared with saline-treated animals ($p<0.001$), indicating that methamphetamine produces strong terminal degeneration in the striatum (Figure 1). TH-ir was performed on A-Cu-Ag-stained striatal sections to see if the signals of the two techniques were inversely associated. The increase in A-Cu-Ag-stained degenerating terminals in the striatum was complementary to the loss of TH-ir, strongly suggesting that dopaminergic fibers are the ones that degenerate after methamphetamine.

The multiple dose regimens induced a dose-dependent effect on A-Cu-Ag staining levels in the striatum, as the 3×10 protocol had greater effect than the 3×5 protocol ($p<0.05$). The single high-dose administration (1×30) had less effect on the integrity of dopaminergic terminals than multiple lower dose protocols ($p<0.001$). This high dose produced a stronger silver deposition in the striosomes and in the dorsolateral part of the caudoputamen than in the rest of the striatal areas (Figure 1c and d). Silver deposits in striatal dopaminergic fibers were still detected 3 days after the treatment with methamphetamine in comparison with saline animals ($p<0.001$). However, the intensity of A-Cu-Ag signal in the striatum was reduced compared with 1 day after treatment (Figure 1c and d). This degeneration was accompanied by microgliosis (assessed by Iba-1-ir) and astrogliosis (GFAP-ir), peaking 1 and 3 days after treatment, respectively (data not shown), in agreement with previous reports (Ares-Santos *et al*, 2012; O'Callaghan and Miller, 1994; LaVoie *et al*, 2004).

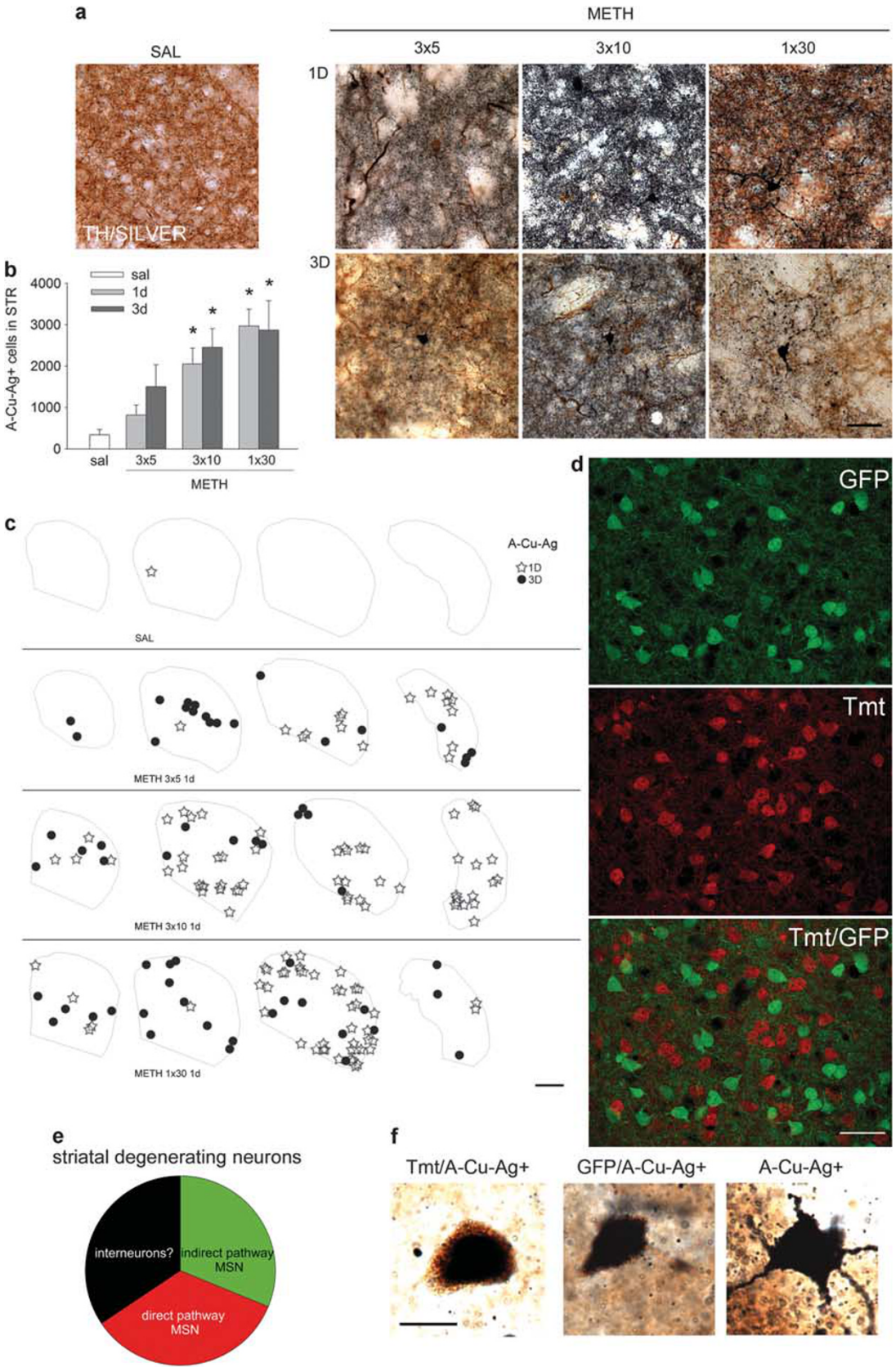
Methamphetamine Induces Striatal Cell Death

A-Cu-Ag-stained neurons were observed in the striatum of methamphetamine-treated animals (Figure 2). The lowest dose (3×5) did not cause a significant increase in striatal A-Cu-Ag-stained neurons compared with saline, but higher doses (3×10 and 1×30) did. The 3×10 dose increased stained neurons in the striatum by five- to sevenfold at 1 and 3 days after treatment and the 1×30 dose increased stained neurons by $\sim$9-fold at 1 and 3 days. There was no significant difference between numbers of stained neurons at 1 and 3 days for either dosing paradigm (Figure 2b). Degenerating striatal cells were generally more abundant in the ventromedial part of the striatum, although at 1 day after treatment with the 1×30 protocol, they were also observed frequently in dorsolateral parts of the striatum (Figure 2c).

Degenerating Neurons were Equally Distributed between Direct Pathway MSNs, Indirect Pathway MSNs and Other Striatal Neurons

No colocalization of Tmt and GFP proteins was found in the striatal neurons of D1R-Tmt/D2R-eGFP BAC transgenic mice (Figure 2d). In these mice, Tmt fluorescence identifies

Figure 2 Methamphetamine produced degeneration of striatal neurons. (a) Photomicrographs of tyrosine hydroxylase/amino-cupric-silver (TH/A-Cu-Ag)-stained sections of the striatum of mice treated with saline (sal) or methamphetamine (3×5), (3×10), or (1×30) 1 day or 3 days after the treatment, at high magnification. (b) Histogram showing the number of A-Cu-Ag-positive cells in the striatum counted by stereology in sections of mice treated with saline or methamphetamine (3×5), (3×10), or (1×30) 1 day or 3 days after the treatment (mean ± SEM, $n=4$–6 per group). Treatment with methamphetamine (3×10) or (1×30) induced the appearance of A-Cu-Ag-stained neurons in the striatum. Data represent mean ± SEM, $n=4$–6 per group, $^{*}p<0.05$ *vs* sal. (c) Stereoinvestigator drawings of the striatum of mice treated with saline or methamphetamine (3×5), (3×10), or (1×30), showing the distribution of A-Cu-Ag-stained neurons at different rostrocaudal levels at 1 day (stars) and 3 days (dots) after treatment. (d) Striatal neurons in D1-Tmt/D2-GFP BAC transgenic mice did not coexpress GFP and Tmt proteins. (e) Among the degenerating striatal cells, about one-third were direct pathway medium spiny neurons (MSNs), another third indirect pathway MSN, and the remaining third were other types of striatal neurons ($n=5$). (f) Photomicrographs of Tmt/A-Cu-Ag-, GFP/A-Cu-Ag-, or A-Cu-Ag-positive neurons in the striatum of D1-Tmt/D2-GFP BAC transgenic mice 1 day after treatment with methamphetamine (1×30). Statistical analysis was performed by two-way analysis of variance and *post hoc* Newman–Keuls analysis. Bar indicates 30 μm (a), 25 μm (d), 500 μm (c), or 10 μm (f). BAC, bacterial artificial chromosome; GFP, green fluorescent protein; Tmt, tomato red.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direct pathway MSN
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GFP/A-Cu-Ag+
A-Cu-Ag+

medium spiny neurons (MSNs) of the direct pathway and GFP fluorescence identifies MSNs of the indirect pathway. A third (34%) of the A-Cu-Ag-stained striatal cells observed 1 day after the treatment with the single high dose (1×30) colocalized with Tmt-positive neurons (D1R, direct pathway MSN), and another third (31%) colocalized with GFP-positive neurons (D2R, indirect pathway MSN). The remaining third (35%) of degenerating neurons did not colocalize with either red or green fluorescence, suggesting that other populations of striatal neurons are affected as well (Figure 2e and f).

Degenerating Axons were Observed in the Nigrostriatal Pathway

In sagital brain sections from animals treated with methamphetamine, but not in saline animals, some A-Cu-Ag-stained axons were observed in the nigrostriatal pathway leading from the SNpc, via medial forebrain bundle (MFB), to the striatum (Figure 3). However, A-Cu-Ag staining was not as abundant as in the striatum. No appreciable loss of TH axons in the MFB was revealed by visualization of sagital sections of methamphetamine-treated animals at low magnification ($\times 2.5$), despite the significant loss of dopaminergic terminals in the striatum.

Methamphetamine Administration Induced Degeneration of Dopaminergic Neurons in the SNpc

As reported previously (Ares-Santos *et al*, 2012; Granado *et al*, 2011a, b), methamphetamine administration resulted in significant loss of TH-expressing neurons in the SNpc. One day after methamphetamine administration, the number of TH-ir cell bodies in the SNpc was reduced by 22% in the 3×5 paradigm, 34% in the 3×10, and 21% in the 1×30 group. The number of TH-expressing neurons in this area remained stable 3 days after treatment with each of the different regimens of the drug (Figure 4a and b).

This reduction in the number of TH neurons was at least partially due to degeneration of dopamine neurons, as TH soma colocalized with A-Cu-Ag deposits (TH/A-Cu-Ag) in the SNpc (Figure 4c and g). The percentage of double-stained neurons at 1 day after treatment with methamphetamine represented 7.1% of the total number of dopaminergic neurons in the 3×5 group, 14.8% in the 3×10 group, and 13% in the 1×30 group. The percentage of double-stained neurons remained similar 3 days after treatment in all the protocols (Figure 4c). The estimated percentage of double-stained neurons was lower than the percentage of TH-ir neuron loss, which could indicate that some neurons do lose TH expression before degenerating or without degeneration (see Figure 4f).

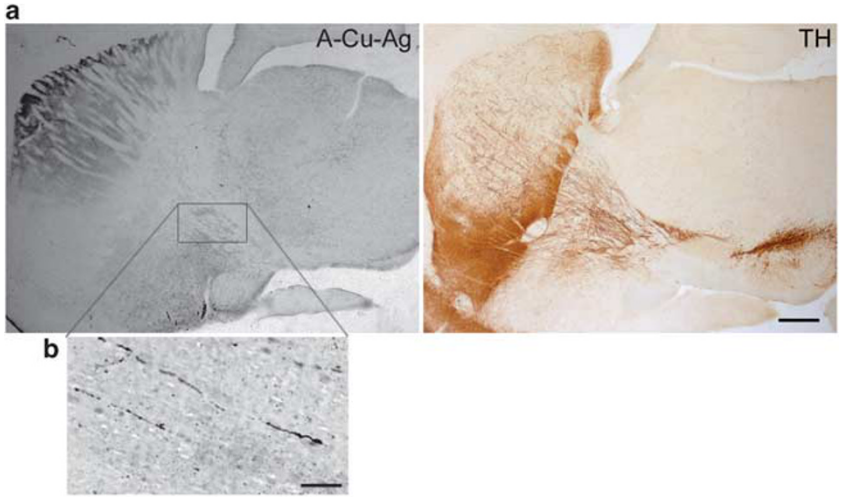
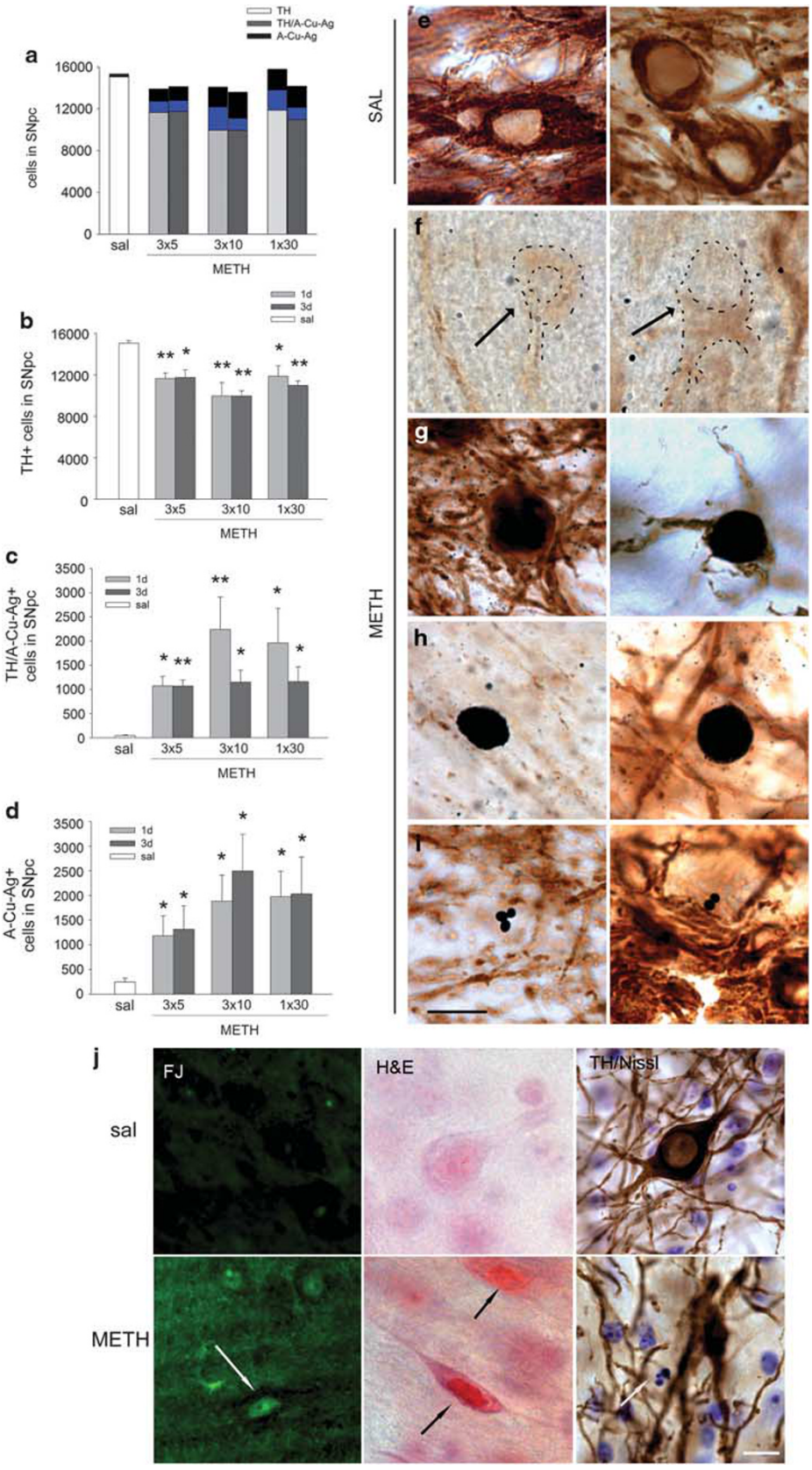


Figure 3 Some degenerating nigrostriatal axons are observed in sagital sections of methamphetamine-treated animals. (a) Representative photomicrographs of sagital sections of the brain of a mouse that received methamphetamine (3×5) stained for amino-cupric-silver (A-Cu-Ag) or tyrosine hydroxylase (TH) 1 day after drug delivery. (b) Note that some degenerating A-Cu-Ag axons can be observed in the nigrostriatal pathway among many intact TH-stained axons. Bar indicates 500 μm (a) and 30 μm (b).

Figure 4 Methamphetamine induces degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc). (a–d) Left, histograms show the number of tyrosine hydroxylase (TH)- (b), TH/amino-cupric-silver (A-Cu-Ag)- (c), A-Cu-Ag-positive cells (d), and the accumulated mean of each of them (a) in the SNpc, counted by stereology in sections of mice treated with saline (sal) or methamphetamine (3×5), (3×10), or (1×30) 1 day or 3 days after the treatment (mean ± SEM, $n = 4$–6 per group). *,**$p < 0.05$, 0.001 *vs* sal. Statistical analysis was performed by Student's *t*-test. Right, high magnification photomicrographs of nigral sections stained for TH/A-Cu-Ag illustrating TH-stained neurons in saline animals (e), and neurons seen in animals treated with methamphetamine 3×5, 3×10, or 1×30 (f–i). (f) Neurons that have only faint remaining TH staining. (g) Degenerating dopaminergic neurons stained with TH and A-Cu-Ag. (h) Degenerating neurons that do not express TH. (i) A-Cu-Ag-stained apoptotic bodies. Bars indicate 10 μm. (j) Degeneration of nigrostriatal neurons may be caused by different death pathways. Photomicrographs of neurons in the SNpc of mice show normal neurons in a saline control (upper row), and degenerating neurons 1 day after treatment with methamphetamine (lower row). Positive FluoroJade staining, eosinophilic necrotic red neurons stained with hematoxylin and eosin (H&E), and Nissl-stained apoptotic bodies were observed in mice after methamphetamine (3×5) treatment but not in saline animals. Bar indicates 10 μm.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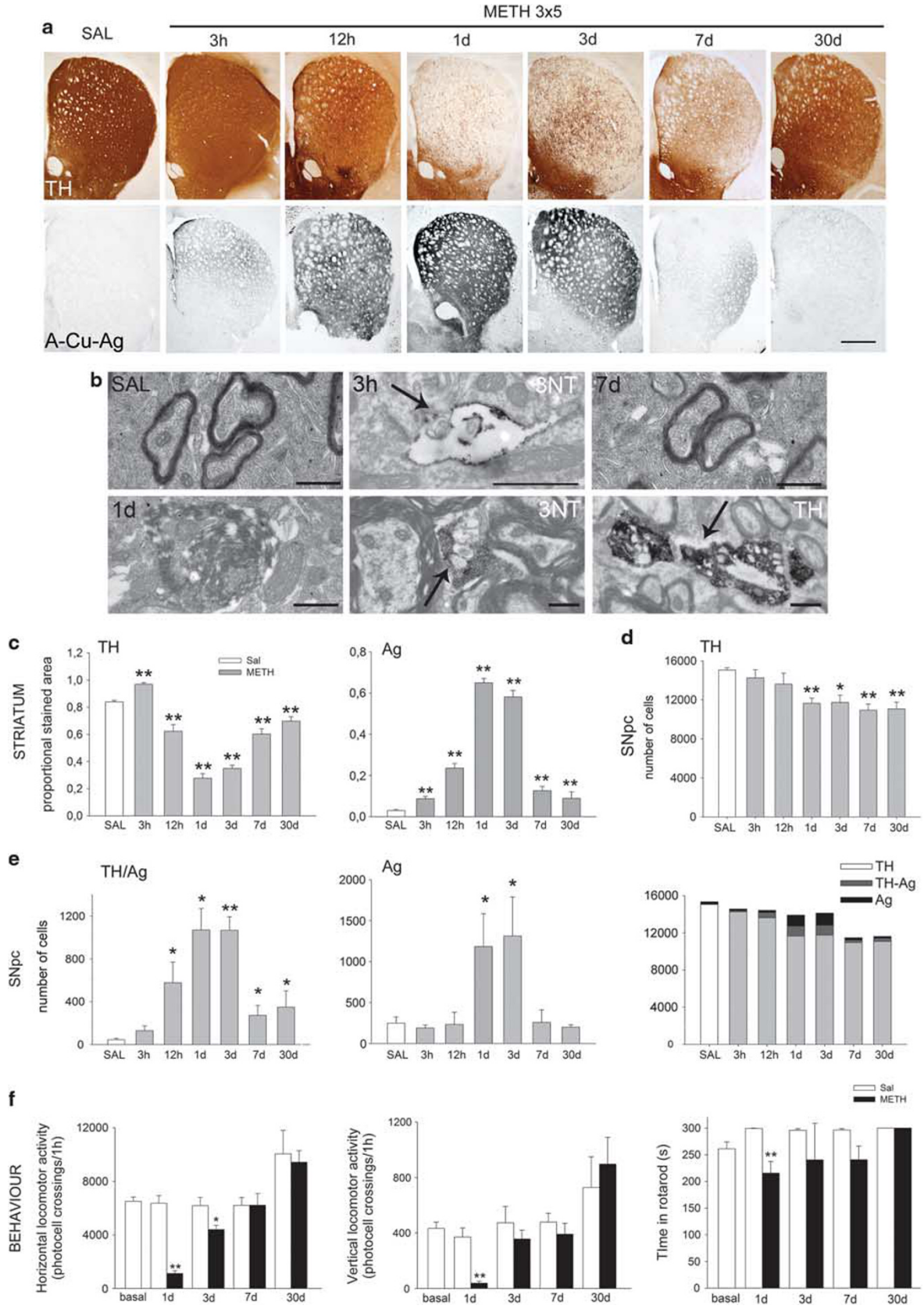
1500
1000
500
d
A-Cu-Ag+ cells in SNpc
SAL
METH
e
f
g
h
i
j
FJ
H&E
TH/Nissl
sal
METH


a
SAL
METH 3x5
3h
12h
1d
3d
7d
30d
TH
A-Cu-Ag
b
SAL
3h
3NT
7d
1d
3NT
TH
c
STRIATUM
proportional stained area
TH
Sal
METH
Ag
SAL 3h 12h 1d 3d 7d 30d
d
SNpc
number of cells
TH
e
SNpc
number of cells
TH/Ag
Ag
TH
TH-Ag
Ag
f
BEHAVIOUR
Horizontal locomotor activity (photocell crossings/1h)
Vertical locomotor activity (photocell crossings/1h)
Time in rotarod (s)
basal 1d 3d 7d 30d

Some degenerating or apoptotic cell bodies stained for A-Cu-Ag but not for TH were seen in this area (Figure 4d, h, and i). These could be dopaminergic neurons that degenerate after the loss of TH expression or non-dopaminergic degenerating neurons. The number of these TH-negative but A-Cu-Ag-positive soma was significantly greater 1 and 3 days after treatment with any of methamphetamine regimens ($p<0.05$) than saline. No significant changes in the estimate of non-dopaminergic-A-Cu-Ag-positive neurons (A-Cu-Ag) were observed at 3 days *vs* 1 day after reatment (Figure 4d).

Dopaminergic Neurons in the SNpc Degenerate by Necrosis or Apoptosis

Degeneration of neurons in the SNpc 1 day after treatment with methamphetamine (3×5) was also detected by FluoroJade staining (Figure 5). As evidenced by H&E, some degenerating neurons in this area after methamphetamine (3×5) were necrotic (eosinophilic necrotic neurons) (Fujikawa *et al*, 2010), whereas there were no eosinophilic neurons in the SNpc following saline treatment (Figure 4j). In addition, as described previously (Ares-Santos *et al*, 2012), Nissl-stained apoptotic bodies were observed in mice with methamphetamine (3×5) but not in saline animals, indicating that apoptosis mediates degeneration of some of these neurons (Figure 4j).

Time Course of Methamphetamine-Induced Loss of TH-ir Terminals in the Striatum

Three hours after methamphetamine (3×5) administration, TH-ir in the striatum was more intense ($p<0.001$) and the signal was more diffuse, appearing to extend into cortical areas, compared with the TH staining in saline-treated animals (Figure 5a and c). Silver staining was already slightly increased ($p<0.01$). At 12 h after treatment, TH-ir was decreased (by 25%, $p<0.001$), whereas silver staining was further increased compared with saline ($p<0.001$). As indicated by TH-ir, dopamine terminal loss was greatest at 1 day after treatment, with a 70% reduction compared with saline levels ($p<0.001$). Afterwards, there was a progressive recovery of dopamine terminals at 3, 7, and 30 days after treatment: striatal TH-ir levels were 58%, 28%, and 16% below saline controls, respectively ($p<0.001$). The persistence of reduced TH-ir at 30 days indicates that recovery was not complete, at this time point. Striatal A-Cu-Ag staining also peaked at 1 day after treatment and decreased over time (7 and 30 days), but was still slightly increased over saline control at 30 days ($p<0.001$) (Figure 5a and c).

EM Confirmed Terminal Degeneration

To confirm that increases in A-Cu-Ag staining in the striatum after methamphetamine were due to fiber degeneration, evidence for neurodegeneration was obtained by EM. Striatal sections from saline- and methamphetamine (3×5)-treated animals were processed for EM (Figure 5b). Normal looking neuropil with typical dendritic and axonal striatal profiles were observed in saline-treated animals (Figure 5b, SAL). Striatal terminals looked normal 3 h after methamphetamine (3×5), although nitration of intrinsic striatal dendrites was observed (Figures 5b, 3h). At 1 day after treatment, several fibers had a characteristic degenerating morphology, similar to that reported after MPTP treatment (Cochiolo *et al*, 2000). These fibers exhibited an abnormal collection of altered membranous structures (Figure 5b, bottom row, left). Some nitrated terminals were observed at this time point (Figure 5b, bottom row, middle). In addition, vacuolated TH-ir fibers with characteristic degenerating morphology were observed 1 day after the treatment. Seven days after drug injection, the morphology of most striatal axons and synaptic terminals was similar to that seen in saline-treated animals. At this time, terminals containing densely packed small synaptic vesicles and establishing typical synaptic contacts were observed (Figure 5b).

Time Course of Methamphetamine-Induced Loss of Dopamine Neurons in the SNpc

The number of TH-ir cell bodies in the SNpc showed a tendency to decrease at 3 and 12 h (4% and 8%, respectively) after methamphetamine (3×5). A significant reduction (22%, $p<0.001$) was observed 1 day after treatment and persisted at 3, 7, and 30 days (Figure 5d and e). Degenerating dopamine neurons were first detected by TH/Ag staining at 12 h after methamphetamine ($p<0.05$), comprising about 4% of total dopamine neurons in the SNpc (Figure 5e). The number of TH/Ag double-stained neurons peaked at 1 day and 3 days after methamphetamine, representing 7% of the total number of dopaminergic neurons. The percentage of TH/Ag neurons decreased significantly at 7 and 30 days (2%) after treatment but remained elevated

Figure 5 Time course of methamphetamine (3×5) effects: neurotoxicity in the striatum, substantia nigra pars compacta (SNpc), and motor behavior. (a) Photomicrographs of tyrosine hydroxylase- (TH) or amino-cupric-silver- (A-Cu-Ag) stained sections of the striatum of mice treated with saline (sal) or methamphetamine (3×5) 3 h, 12 h, 1 day, 3 days, 7 days, and 30 days after the treatment. Bar indicates 500 μm. (b) Ultrastrutural evidence of nitration of a striatal dendrite at 3 h after methamphetamine (3 h, upper middle) and, at 1 day after methamphetamine, nitration (lower middle), a degenerating terminal (lower left), and degenerating TH-immunoreactive (TH-ir) axon (lower right) in the striatum. Bar indicates 0.5 μm. (c) Histograms show the proportional stained area of TH and amino-cupric-silver staining in the striatum. Data represent mean ± SEM, $n=4$–6 per group. (d and e) Histograms show the number of TH- (d), TH/A-Cu-Ag- (e, left), A-Cu-Ag-positive cells (e, middle), and the accumulated mean of each (e, right) in the SNpc, counted by stereology in sections of mice treated with saline or methamphetamine (3×5), 3 h, 12 h, 1 day, 3 days, 7 days and 1 month after the treatment (mean ± SEM, $n=4$–6 per group). (f) Methamphetamine induced a decrease in horizontal locomotor activity 1 and 3 days after treatment with methamphetamine (3×5), with return to control levels at 7 days after treatment (f, left). Methamphetamine (3×5) also induced a decrease in the vertical motor activity of the animals at 1 day after treatment, returning to control levels at 3 days, and remaining stable 7 days after the treatment (f, middle). Methamphetamine (3×5) impaired motor coordination, as animals showed reduced latency time on an accelerating rotarod 1 day after treatment with the drug. Motor coordination returned to normal at 3 and 7 days after treatment with methamphetamine (f, right). $^{*}p<0.05$, $^{**}p<0.001$ *vs* sal. Statistical analysis was performed by Student's *t*-test.

compared with control levels ($p<0.05$) (Figure 5e). These results indicate that the degeneration wave peaks 1–3 days after methamphetamine, although a small number of dopaminergic neurons degenerate at later times (Figure 5e). By contrast, the number of A-Cu-Ag-positive, TH-ir-negative neurons in the SNpc was significantly increased 1 and 3 days after methamphetamine 3×5 ($p<0.05$), but no significant differences *vs* control animals were observed before or after these time points (see Figure 5e).

Methamphetamine Temporarily Decreases Motor Activity and Motor Coordination

Mice treated with methamphetamine (3×5) showed drastic reductions in horizontal and vertical locomotor activity 1 day after treatment, scoring an average of 1115 and 38 photocell crossings in a 60-min period, respectively, compared with saline animals (an average of 6364 horizontal and 370 vertical crossings, $p<0.001$; Figure 5f). At 3 days after treatment, vertical locomotor activity returned to normal levels (365 crossings) but horizontal locomotor activity remained reduced (4393 crossings, $p<0.05$) despite partial TH-terminal recovery. At 7 days after treatment with methamphetamine, both horizontal (6219 crossings) and vertical (391 crossings) locomotor activity had returned to normal levels. In the rotarod test of motor coordination, naive mice quickly adapted to the accelerating rod, reaching the cutoff time (300 s) in the acquisition day (Figure 5f). However, 1 day after methamphetamine mice were unable to reach the cutoff time, and achieved an average latency time of just 215 s ($p<0.001$ *vs* saline animals). At 3 and 7 days after methamphetamine, the latency time increased to 240 s, not significantly different from saline animals. These results indicate that motor behavior is drastically impaired at 1 and 3 days after methamphetamine and then recovers, consistent with the time course of TH loss in the striatum.

DISCUSSION

We found that multiple low doses of methamphetamine produced a greater, but still dose-dependent, loss of dopaminergic terminals than a single higher dose. There was also degeneration of striatal neurons after treatment with methamphetamine, with degenerating neurons equally divided between direct pathway MSNs, indirect pathway MSNs, and other striatal neurons. We show for the first time that methamphetamine kills dopaminergic neurons in the SNpc of mice, which can be detected 1 day after the treatment. Despite the significant differences between the effects of the three regimens on the striatum, similar toxicity was observed in the SNpc following all three delivery paradigms. With the multiple low dose (3×5) paradigm, loss of dopamine terminals started as soon as 3–12 h after the last injection, peaked at 1 day and was followed by partial reinervation of the striatum, although dopamine terminal deficits persisted for more than 30 days after the last dose. EM confirmed fiber degeneration, first showing degenerating dopamine fibers and nitrated terminals 1 day after the treatment with the drug. In parallel, the loss of dopamine neurons in the SNpc started by 12 h after the last injection, peaking by 1 day. The number of TH-ir neurons did not change at 3, 7, or 30 days after drug administration, indicating long-lasting loss of TH-ir neurons. These neurotoxic changes had functional consequences: animals showed deficits in locomotor activity and motor coordination with a time course consistent with the observed dopaminergic terminal degeneration, peaking at 1–3 days after methamphetamine and returning to normal levels by 7 days after the treatment.

The A-Cu-Ag technique is highly sensitive detecting degenerating neurons soma, dendrites, axons, and synaptic terminals (de Olmos *et al*, 2009; de Olmos *et al*, 1994; Switzer 2000). Degeneration is seen as black stained objects against a pale unstained background (of normal unaffected components). Staining is thought to result from the precipitation of ionic silver around chemical reducing groups present in damaged subcellular structures, like proteins dismantled by proteolitic mechanism (Beltramino *et al*, 1993; Switzer, 2000). This technique has the advantage of selectively identifying degenerating nerve cell components while producing a high contrast image that is relatively easy to observe and can be combined with immunohistochemistry, facilitating identification of the phenotype of degenerating neurons (de Olmos *et al*, 2009). Results from this study agree with previous reports with other suppressive silver methods that indicated that methamphetamine produces destruction and loss of dopaminergic terminals in the striatum (Ricaurte *et al*, 1982, 1984). The concomitant reductions of TH-ir fibers along with the presence of A-Cu-Ag-stained terminals (Figures 1–3) reveal the selective degeneration of dopaminergic terminals in the striatum 1 day after methamphetamine. This is also evidenced by the presence of degenerating striatal terminals detected by EM.

Multiple doses of methamphetamine (3×5 or 3×10) were clearly more toxic to the dopaminergic terminals in the striatum than single administration (1×30) (Figure 1b–d). This difference could be due to the different effects of these regimens on blockade of DAT or VMAT2, resulting in cytosolic accumulation of dopamine, which have been shown to be greater and longer lasting for multiple administrations of methamphetamine than for a single injection (Fleckenstein *et al*, 1999; Metzger *et al*, 2000). In addition, the hyperthermic response was greater and had more peaks for multiple administration regimens than for single administration (Figure 1a), which may potentiate the neurotoxic effects of methamphetamine although hyperthermia is not solely responsible for methamphetamine-induced neuropathology (Albers and Sonsalla, 1995; Ares-Santos *et al*, 2012, 2013b; Granado *et al*, 2011a; Urrutia *et al*, 2013).

Consistent with previous reports, methamphetamine induced degeneration of striatal cells 1 day after treatment (Zhu *et al*, 2006a, b) and degenerating neurons were still visible 3 days later (Figure 2a and b). Surprisingly, no differences were observed in the number of degenerating striatal neurons between the (3×10) and (1×30) protocols despite the significant difference they present in neurotoxic effects on dopaminergic terminals in the striatum (Figure 1). Previous reports showed that striatal apoptosis is independent of the hyperthermic response and that multiple administration of methamphetamine (4×10 mg/kg)

was less effective in inducing apoptotic cell death assessed by TUNEL staining 24 h after treatment than single bolus administration of 1×30 mg/kg (Zhu *et al*, 2006a), in line with our results.

Notably, this is the first study to report that the degenerating neurons that appear in the striatum following methamphetamine treatment are equally distributed between direct and indirect projection pathways neurons. A third of the degenerating Ag-stained striatal neurons did not colocalize with either Tmt-D1R or GFP-D2R (Figure 2e and f). This could mean that some striatal projection neurons have lost the expression of Tmt/GFP proteins before degenerating, or that they are interneurons. As interneurons comprise a much smaller cell population in the striatum (5–10%) relative to projection neurons (90–95%) (Kawaguchi *et al*, 1995), the equal absolute numbers of degenerating cell populations (direct and indirect pathway projection neurons and putative interneurons) suggest that interneurons are selectively more vulnerable to methamphetamine toxicity. In line with this, previous work showed that projection neurons are less vulnerable to methamphetamine neurotoxicity than parvalbumin interneurons or cholinergic interneurons, although somatostatin interneurons are refractory to methamphetamine neurotoxicity (Zhu *et al*, 2006b). Alternatively, it is possible that the loss of striatal projection neurons is due to systemic rather than specific toxicity as opposed to that of the interneurons.

This is also the first study to provide direct evidence of degeneration of dopaminergic neurons in the SN (pars compacta and pars lateralis) after exposure to methamphetamine (Figure 4), confirming previous studies that reported a decrease of TH-ir neurons, (Ares-Santos *et al*, 2012; Granado *et al*, 2011a, b; Sonsalla *et al*, 1996). Although it has been proposed that axonal degeneration, rather than neuronal loss, has the dominant causative role in the clinical manifestations of PD (Burke and O'Malley, 2012), the permanent loss of a small fraction of the dopaminergic cells in the SNpc significantly diminishes the capacity for protective compensation following future insults. Ricaurte *et al* (1982) previously used a silver technique and found no evidence of TH-soma loss after methamphetamine. The discrepancy between our studies is likely the result of timing: they looked at 6 weeks after methamphetamine and our time-course experiment showed the biggest cell body degeneration at 1–3 days after methamphetamine. This is consistent with silver-suppressive studies with other neurotoxic agents (Switzer, 2000) that indicate that degenerating cell bodies can only be detected shortly after the injury. Several nigrostriatal degenerating axons stained for A-Cu-Ag could be followed from SNpc to the striatum via the MFB (Figure 3). However, degenerating axons and dopaminergic neurons in the SNpc after methamphetamine exposure represent a small percentage compared with the massive terminal damage observed in the striatum.

The mechanisms mediating methamphetamine-induced degeneration of dopaminergic neurons in the SNpc seem to include more than one known death pathway. We have reported previously the appearance of Nissl-stained apoptotic cell bodies in the SNpc 1 day after treatment with methamphetamine (Ares-Santos *et al*, 2012). On the other hand, we have also observed necrotic eosinophilic neurons following methamphetamine. Necrosis and apoptosis can occur either as distinct conditions, in combination, or as sequential events (Davidson *et al*, 2001). Results from *in vitro* research with dopaminergic cell culture models point to activation of the apoptotic cascade involving caspase-3 and DNA fragmentation in neurodegeneration of these cells and suggest that other factors may contribute to cell death, including ER stress, ubiquitin dysfunction, and autophagic impairment (Kanthasamy *et al*, 2011; Larsen *et al*, 2002).

Our time-course experiment with the lowest multiple-dose regimen of methamphetamine (3×5) showed that dopamine terminal and cell body loss had already started at 3–12 h and peaked at one day for the terminals and between 1 and 3 days after treatment for the cell bodies (Figure 5a–e). From this time onwards, the number of silver-stained cell bodies decreased, but remained significant 7 and 30 days after treatment compared with saline-treated animals. As the A-Cu-Ag staining only labels cells actively undergoing degeneration, these later appearing silver-stained cells may be damaged neurons that initially survive methamphetamine, but eventually degenerate and die (Figure 5e). In line with this, there was no recovery of TH-ir neuron numbers in the SNpc even 30 days after the treatment, confirming the idea that, unlike the partial recovery of terminals in the striatum, the loss of striatal neurons is permanent. It is likely that the major source of TH terminal regrowth is the spared dopaminergic neurons in the SNpc. Neurodegeneration in the striatum and SNpc were accompanied by strong glial activation, in agreement with previous results (Ares-Santos *et al*, 2012; O'Callaghan and Miller, 1994; LaVoie *et al*, 2004).

Although non-selective systemic methamphetamine toxicity might contribute to the selective loss of dopamine (Halpin and Yamamoto, 2012), the motor impairment we observed is due to the loss of dopamine terminals in the striatum as has been shown in drug abusers (Volkow *et al*, 2001a,b; McCann *et al*, 1998). Thus, the loss of dopamine markers after methamphetamine neurotoxicity may be the cause of the motor impairment we observed: a drastic, but transient, decrease in horizontal and vertical movement and motor coordination. The timing of these changes paralleled the degeneration and partial recovery of dopamine terminals, and complete recovery of these motor indicators suggested they were not related to the permanent loss of nigral neurons. Our results with methamphetamine further confirm the requirement for certain dopamine levels in the striatum for motor activity and motor coordination (Rodrigues *et al*, 2007). Once dopamine terminals have recovered to at least 78%, they produce sufficient dopamine for the neurotoxicity to be subsymptomatic. The finding that the persistent 25% loss of nigral neurons produces no motor symptoms mirrors previous findings in animal models of PD, in which motor impairment appears only after an 80% loss of striatal dopamine and 30–60% loss of nigral dopamine neurons (Burke and O'Malley, 2012; Fearnley and Lees, 1991), indicating that methamphetamine could destroy many dopamine neurons with no PD symptomatology (Davidson *et al*, 2001).

The dying-back axonopathy hypothesis in PD suggest that degeneration begins in the distal axon and proceeds toward the cell body (Burke and O'Malley, 2012). In contrast, our

finding that degenerative processes in the terminal and neuronal body occurred simultaneously suggest a direct effect of methamphetamine administration on SNpc neurons and terminals, as opposed to retrograde degeneration. A large body of evidence indicates that the molecular mechanisms of terminal degeneration are separate and distinct from those of neuron somatic degeneration (Burke and O'Malley, 2012; Coleman, 2005; Raff *et al*, 2002; Ries *et al*, 2008). This idea is further supported by the fact that genetic inactivation of Nrf2 potentiates dopamine terminal loss without affecting survival of dopamine neurons in the SNpc (Granado *et al*, 2011b). In line with this, we observe no significant differences between the effects of single high dose and multiple lower doses on methamphetamine-induced dopaminergic neuronal loss, despite the clear dose dependence of dopamine terminal loss in the striatum. Further investigation is required to characterize the process leading to neurodegeneration and to define the distinct mechanisms that may mediate terminal and somatic neurodegeneration.

Our results are consistent with the recent report that methamphetamine abusers have higher risk for future development of PD (Callaghan *et al*, 2012) and with other reports of neurotoxic effects of methamphetamine in human abusers that indicate poor motor performance associated with DAT loss in the caudate nucleus and putamen (Volkow *et al*, 2001a,b; McCann *et al*, 1998). Similar partial recovery of dopaminergic markers in the striatum has been reported in human methamphetamine abusers after periods of abstinence (Volkow *et al*, 2001a). Persistent dopamine terminal loss has been also documented after 11 months (Volkow *et al*, 2001a) or 3 years of abstinence (McCann *et al*, 1998) in line with the loss we see at 30 days. However, to date, there are no published reports of anatomical evidence of dopamine neuronal destruction in SNpc of human methamphetamine abusers. Some evidence of neurodegenerative changes exists: for example, a specific decrease in pigmented neurons in SN of human abusers, similar to that seen in PD patients (Büttner and Weis, 2006; Büttner, 2011). Moreover, the morphology of the SN (as measured by transcranial sonography) in individuals with a history of stimulant abuse, including methamphetamine, is abnormal, and is associated with reduced dopamine uptake in the striatum and increased risk for development of PD (Todd *et al*, 2013). Our results indicating very low, but significant, rates, around 0.17%, considering 1.72×10^6 total neuronal population (Rosen and Williams, 2001), of striatal cell death after methamphetamine are also consistent with the small decrease (5%) in the neuronal marker *N*-acetylaspartate described in the striatum of abstinent methamphetamine abusers (Ernst *et al*, 2000).

In summary, the data presented here provide anatomical evidence of dopamine cell body degeneration and a persistent loss of dopaminergic cell soma after exposure to methamphetamine in mice, making it clear that some neurotoxic effects of this drug are long-lasting despite partial regeneration of dopaminergic terminals in the striatum. The lower neuronal content and/or the priming and damage of the spared neurons represent a likely vulnerability factor for further neurotoxic insults (like other neurotoxins, age, or genetic risk factors for PD). As it is true for other susceptibility factors for PD, the methamphetamine-increased susceptibility does not mean that all methamphetamine users will develop PD. The low disease concordance in relatives and the low penetrance of some genetic mutations described as risk factors for PD suggests that a combination of insults or interaction between multiple predisposing factors, 'multiple hits', is required for developing PD (Sulzer, 2007). Our data indicate that methamphetamine abuse is highly likely to represent one such hit, predisposing abusers to PD.

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The authors declare no conflict of interest.

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