The Journal of Neuroscience, December 1, 1998, *18*(23):10116–10127

Dopamine Modulates the Susceptibility of Striatal Neurons to 3-Nitropropionic Acid in the Rat Model of Huntington's Disease

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Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by chorea, psychiatric disturbances, and dementia. The striatum is the primary site of neuronal loss in HD; however, neither the mechanism of neurodegeneration nor the underlying cause of the selectivity for the striatum is understood. Chronic systemic injection of 3-nitropropionic acid (3-NP) into rats induces bilateral striatal lesions with many neuropathological features of HD and is widely used as a model of HD. In this study we examine the role striatal dopamine plays in 3-NP-induced striatal toxicity.

The effect of elevated striatal dopamine levels on 3-NP toxicity was examined by using acute administration of methamphetamine. After 7 d of 3-NP treatment, a single low dose of methamphetamine markedly increased the frequency of striatal lesion formation. This effect was mediated via dopamine receptors because it could be blocked by the administration of dopamine receptor antagonists. The effect of decreased striatal dopamine on 3-NP toxicity was examined by lesioning the nigrostriatal dopamine input to one striatum 7 d before 3-NP treatment was started. Removal of the dopamine input protected the denervated striatum from the neurotoxic effects of systemic 3-NP but did not prevent the formation of lesions in the intact striatum. Thus the formation of 3-NP lesions is critically dependent on an intact dopamine input.

Our data show that dopamine plays an important role in the formation of 3-NP lesions. We suggest that modulation of the dopaminergic system should be reevaluated as a potential drug target in the treatment for HD.

Key words: 3-nitropropionic acid; dopamine; 6-hydroxydopamine; rat; neurotoxicity; Huntington's disease; unilateral; striatum

Huntington's disease (HD) is a neurodegenerative disorder for which there is no treatment and that progresses relentlessly until death (Harper, 1996). The primary brain region affected in HD is the striatum; however, the mechanism underlying the selective loss of neurons from the striatum is not well understood.

Several animal models for HD exist (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976; Beal et al., 1986), the most recent being the chronic 3-nitropropionic acid (3-NP) model (Beal et al., 1993; Brouillet et al., 1993, 1995). 3-NP is a mitochondrial toxin that causes striatal neuropathology similar to that seen in HD (Beal et al., 1993; Bossi et al., 1993). A major advantage of this model over other models of HD is that the lesions that are produced are bilateral and striatal-specific, and they develop spontaneously after systemic administration of 3-NP. However, as in HD, the factors mediating the selective toxicity of 3-NP to striatal neurons are unknown.

One popular theory for the striatum selective neuronal loss after 3-NP treatment is that an energy deficiency makes striatal neurons more vulnerable to the neurotoxic effects of endogenous glutamate, thus allowing excitotoxicity to occur without an increase of glutamate to "excitotoxic" levels (Novelli et al., 1988; Simpson and Isacson, 1993). Indeed, a number of studies have shown that factors important in excitotoxicity, such as glutamate and nitric oxide (NO), play an important role in 3-NP toxicity (Beal et al., 1993; Fu et al., 1995a; Schulz et al., 1995). However, the actions of neither glutamate nor NO, alone or in combination, fully explain the sensitivity of striatal neurons to systemically administered 3-NP. Further, neither glutamate nor NO is found exclusively in the striatum, but both are also present in regions of the brain that are not vulnerable to 3-NP (Cotman et al., 1987; Bredt et al., 1990). This suggests that some additional factor mediates the selectivity of the neurotoxic action of 3-NP on striatal neurons.

Although there are a large number of neuroactive substances in the striatum that might be involved in 3-NP toxicity, one of the best candidates is dopamine. Dopamine is released in the striatum from nigrostriatal terminals (Björkland and Lindvall, 1984) and is neurotoxic after direct injection into the striatum (Filloux and Townsend, 1993; Ben-Shachar et al., 1995; Hastings et al., 1996). Further, it has been shown that the administration of amphetamine (which increases striatal dopamine levels) potentiates 3-NP toxicity after the administration of a large single dose of 3-NP (Bowyer et al., 1996). However, a possible role for dopamine in the neurotoxicity after chronic low-dose 3-NP treatment has not been examined.

In this study we examined the effects of altering striatal dopamine levels on the neurotoxicity induced by chronic 3-NP treatment. We found that increased striatal dopamine release potentiated the formation of striatal 3-NP lesions and that decreased dopamine levels prevented lesion formation in the striatum. Our data strongly support the suggestion of a central role for dopamine in the formation of striatal lesions in 3-NP-intoxicated animals.

Received July 1, 1998; revised Aug. 25, 1998; accepted Sept. 15, 1998.

This work was supported by grants from the Wellcome Trust and the Hereditary Disease Foundation. We thank Mr. Roger Hart for excellent photography and Mrs. Wendy Leavens for expert technical assistance.

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MATERIALS AND METHODS

Animals. Female Sprague Dawley rats (200–250 gm) were used for all experiments. All rats were housed under standard conditions (12 hr light/dark cycle) with free access to food and water.

Drugs. 6-Hydroxydopamine (6-OHDA) was dissolved in 0.9% saline and 0.1% ascorbic acid and diluted to a concentration of 2 mg/ml. 3-NP for injection was dissolved in PBS (100 mM), and the pH was adjusted to 7.4 with 5N sodium hydroxide. Methamphetamine, SCH23390, and pargyline were dissolved in PBS, and sulpiride was dissolved in 1% aqueous lactic acid (50 mg/ml) and then diluted in PBS. All drugs were supplied by Sigma (Poole, UK).

Unilateral 6-OHDA lesions of the medial forebrain bundle. At 60 min before lesioning, rats ($n = 11$) received 35 mg/kg pargyline by intraperitoneal injection. Rats were then anesthetized with Avertin (10 ml/kg, i.p.) and placed in a stereotaxic frame (Kopf, Tujunga, CA) with the incisor bar 5 mm above the interaural line. 6-OHDA (8 μg) was injected into the left medial forebrain bundle at a constant rate over 8 min. The coordinates used were posterior 2.2 mm and lateral 1.5 mm from the bregma and ventral 7.9 mm from the dura mater, according to the atlas of Pellegrino and Cushman (1967). After the injection the needle was left *in situ* for 2 min before being slowly withdrawn, and the incision was closed with interrupted silk sutures.

Chronic 3-NP treatment. Two different protocols were used for the administration of 3-NP. For the 3-NP/methamphetamine experiments, rats received daily subcutaneous injections of either 3-NP [12 mg/kg per day ($n = 42$)] or PBS ($n = 4$) for 7 d. On the seventh day, 4 hr after their last 3-NP injection, 34 of the rats were given an intraperitoneal injection of either methamphetamine (5 mg/kg; $n = 15$) or saline ($n = 19$). The remaining eight rats were given an intraperitoneal injection of methamphetamine (5 mg/kg) 24 hr after the last 3-NP injection. All saline-treated rats received methamphetamine 4 hr after the last saline injection. One of the methamphetamine-treated rats displayed no drug-induced behavioral changes of any kind and was excluded from the study. All of the other animals were allowed to recover for a further 7 d before death.

3-NP treatment of 6-OHDA-lesioned rats ($n = 11$) began 7 d after lesioning. A stepwise protocol was used, with the dose of 3-NP, from an initial dose of 12 mg/kg, increasing by 3 mg/kg every 4 d until the behavioral symptoms consistent with lesion formation were observed. The development of ataxia, piloerection, and hind limb recumbency was used as an index of striatal lesion formation (Gould and Gustine, 1982; Hamilton and Gould, 1987). On the day these behaviors appeared, 3-NP treatment was stopped. Rats were killed 7 d later.

A control group of unlesioned rats ($n = 22$) was treated in parallel with 3-NP, and a second group of unlesioned rats ($n = 8$) was used as a vehicle control group. Vehicle-treated rats received treatment until all 3-NP-treated rats had developed the behavioral symptoms of lesion formation.

Administration of selective D_1 and D_2 dopaminergic antagonists. The role of dopamine receptors was examined by pretreating the rats with a selective D_1 (SCH23390) (Bischoff et al., 1986; Hollis and Strange, 1992) or D_2 (sulpiride) antagonist before methamphetamine administration. Rats for the dopamine antagonist study were treated for 1 week with 3-NP at a dose of 12 mg/kg ($n = 30$). On the final day of 3-NP treatment, rats were divided into three groups of 10 animals and given single injections of the D_1 antagonist SCH23390 (100 μg/kg, i.p.), the D_2 antagonist sulpiride (200 mg/kg, i.p.), or both antagonists together.

All rats were given their final 3-NP injection 4 hr before methamphetamine injection. Sulpiride has a slow onset of action (Fujiwara, 1992) and hence was given 5 hr before methamphetamine injection. SCH23390 was administered 1 hr before methamphetamine injection. All rats then received 5 mg/kg methamphetamine, and behavioral changes were observed. All rats were killed 1 week later.

Drug-induced behavioral changes. The behavioral changes induced by the administration of methamphetamine, SCH23390, or sulpiride were observed and recorded until drug-induced behaviors had subsided; this generally took 3–4 hr. Drug-induced behaviors were classified into several categories: hyperactivity, stereotypical sniffing and head bobbing, and piloerection. Gait alterations were classified into one of two categories: wobbly gait (uncoordinated use of the back legs and sideways falling) and hind limb recumbency (legs splayed out to either side).

A measure of catalepsy induced by either sulpiride or SCH23390 was obtained by placing the hind legs of the rat on a platform 5 cm above the bench on which the front legs were resting. If the rat did not significantly alter its body posture within 30 sec, it was considered to be cataleptic.

Tissue preparation. Rats were anesthetized with Avertin (10 ml/kg, i.p.) and perfused transcardially with 200 ml of ice-cold heparinized (500 U) PBS, followed by 300 ml of ice-cold paraformaldehyde (2% in PBS). The flow rate of perfusion was 25 ml/min. The brains were dissected out of the skull and post-fixed overnight in 2% paraformaldehyde and then cryoprotected in 30% sucrose solution for 2 d. Subsequently, the brains were frozen in powdered dry ice and stored at −80°C until they were processed for histochemical and immunocytochemical studies. Coronal sections (30 μm thick) were cut with a cryostat (Leica, Milton-Keynes, UK).

Histochemical staining. Sections for cresyl violet staining were mounted onto gelatin-coated slides and stained using a 1% solution of cresyl violet acetate (Sigma). NADPH diaphorase staining was performed on free-floating cryostat sections by incubation for 60 min in the dark at 37°C in a reaction mixture containing 50 mM PBS, 5 mM magnesium chloride, 2 mg/ml NADPH (reduced form; Sigma), and 1 mg/ml nitroblue tetrazolium (Sigma).

Immunocytochemistry. Nonspecific binding was blocked by incubating free-floating coronal sections in blocking solution (3% normal deer serum in PBS containing 0.2% Triton X-100) with 0.02% sodium azide at 4°C overnight. Sections were then incubated in either polyclonal rabbit antisera or monoclonal mouse antibodies. The antisera/antibodies used were polyclonal rabbit antisera raised against either purified tyrosine hydroxylase (TH; Affiniti, Exeter, UK) or purified glial fibrillary acidic protein (GFAP; Sigma) and a monoclonal mouse antibody raised against the microglial cell marker cluster of differentiation 11b (CD11b; Serotec, Oxford, UK). The sections were incubated for 7 d at 4°C in blocking solution, with 0.02% sodium azide and primary antisera/antibodies diluted to a concentration of 1:1000 for CD11b, 1:2000 for TH, and 1:5000 for GFAP. Then the sections were washed five times, for 5 min each, in wash solution (PBS containing 0.02% Triton X-100) and incubated at 4°C overnight in a horseradish peroxidase-labeled secondary antibody (1:1000; Vector Laboratories, Peterborough, UK) raised against the host animal of the primary antibody in blocking solution without azide. Then the sections were washed five times, for 5 min each, with wash solution and developed with 3,3′-diaminobenzidine (0.5 mg/ml; Sigma) in 50 mM Tris buffer, pH 7.6, containing 0.009% hydrogen peroxide.

Lesion volume measurement and cell counting. Cresyl violet-stained sections were analyzed for total neuronal counts. Cells within lesions on both sides of the brain were measured. Six fields (300 μm^2 each, two from each of three sections) from all five animals in each group were counted to compare the survival of neurons within striatal lesions induced by 3-NP and methamphetamine with the survival of those induced by 3-NP and saline. Lesions were mapped by using camera lucida drawings so that equivalent striatal areas could be counted from saline-treated control animals. The whole of the lesion area or its equivalent area was used for NADPH diaphorase-positive neuron counts.

The volume of the 3-NP-induced striatal lesions was measured from NADPH diaphorase-stained sections by image analysis software (Q500, Leica). The striatal lesion was taken as the area of loss of NADPH diaphorase staining. (The area of loss of NADPH diaphorase staining corresponded to the area of neuronal loss, as defined by cresyl violet staining, but had a better defined edge for analysis purposes.) At least four serial sections were measured for each lesion. The lesion volume was estimated by the following formula: Volume $= d(a_1 + a_2 + a_3 + \dots)$, where d = distance (in mm) between serial sections and $a_1, a_2, a_3 \dots$ = areas (mm^2) of the lesion for individual serial sections.

In 6-OHDA-lesioned animals the volume of the 3-NP-induced striatal lesion on the 6-OHDA-lesioned (left) side of the brain was expressed as a percentage of the volume of the striatal lesion on the intact (right) side.

Counts of dopamine neurons were generated from six sections at the level of the substantia nigra, which had been stained for TH immunoreactivity. The number of TH-positive neurons surviving in the substantia nigra pars compacta (SNpc) or ventral tegmental area (VTA) on the 6-OHDA-lesioned side was expressed as a percentage of the number of SNpc or VTA neurons on the intact side.

Statistics. The significance of numerical data was determined by a one-way ANOVA, followed by Newman–Keuls *post hoc* test to examine differences between treatment groups. Population data were analyzed either with Fisher's exact test, for 2×2 contingency tables, or with the χ^2 test for larger contingency tables. A paired Student's t test was used to examine the volume relationship between the left and right striatal lesions of 3-NP-treated rats. Linear regression analysis was used to examine the correlation between dopaminergic neuronal survival and striatal lesion volume. All statistical calculations were performed with the statistical program Prism (GraphPad Software, San Diego, CA).

Table 1. Methamphetamine-induced changes after 3-NP intoxication

Chronic/acute treatment (time after last chronic injection)	Behavioral changes (Number of rats displaying behavior/Total number of rats)			Number of rats with lesions
	Hyperactivity/stereotypy	Wobbly gait	Splayed hind legs	
Saline/Meth (4 hr)	4/4	0/4	0/4	0/4
3-NP/Saline (4 hr)	0/19	0/19	0/19	2/19
3-NP/Meth (4 hr)	14/14	14/14	10/14	14/14[a]
3-NP/Meth (24 hr)	8/8	5/8	2/8	3/8[b]

[a] $p < 0.0001$ compared with 3-NP/Saline (4 hr); [b] $p = 0.136$ compared with 3-NP/Saline (4 hr).

RESULTS

Behavioral effects of low-dose chronic 3-NP

None of the rats treated with 12 mg/kg 3-NP for 7 d showed the pronounced behavioral signs of 3-NP toxicity associated with lesion formation (see below). This is consistent with previous findings that lesion formation in rats treated with low doses of 3-NP is infrequent and very variable, with only 1 of 10 rats likely to have a lesion after 7 d (Beal et al., 1993; Wüllner et al., 1994; D. Reynolds and A. J. Morton, unpublished observations).

Behavioral effects of acute methamphetamine

A few minutes after methamphetamine was administered to saline-treated control rats, all displayed a marked increase in locomotor and exploratory behavior (Table 1). This period of hyperactivity lasted ~30 min before being replaced gradually by stereotypy. Stereotypic sniffing and head bobbing predominated for the next 2–3 hr, although locomotion was not abolished completely. Stereotypy began to decline ~3 hr after the methamphetamine injection, and movement returned to preinjection levels after 4–5 hr. No gait or postural changes were observed in these animals, nor was piloerection observed.

Pretreatment of rats with 3-NP for 1 week had no effect on initial behavioral responses to methamphetamine administered 4 hr after the final 3-NP injection, and within a few minutes all rats displayed an increase in locomotor and exploratory activity that was very similar to that of the saline-treated control rats. However, marked differences in the behavior of the two groups rapidly became apparent, with all of the 3-NP-treated rats developing a wobbly, uncoordinated gait within 20 min of the methamphetamine injection. In 4 of the 14 animals the gait remained wobbly for several hours and then gradually returned to normal. However, hind limb movement of the remaining 10 animals became progressively more uncoordinated, resulting first in sideways falling during walking and finally in hind limb recumbency with the back legs splayed out from the body. Despite severe coordination problems, these animals were able to move, but they were propelled forward mainly by the front legs with little contribution from the hind legs, which performed paddle-like movements. Hind limb recumbency persisted for ~1 hr before a gradual recovery of hind limb function was observed. The majority of rats displaying hind recumbency also developed piloerection that lasted for the duration of the observation period.

The eight rats to whom methamphetamine (5 mg/kg) was administered 24 hr after the last 3-NP injection displayed a similar, but less severe, set of behavioral changes as compared with those given methamphetamine 4 hr after the final 3-NP injection. All rats displayed hyperactivity that gradually gave way to stereotypy after ~30 min. However, only five of eight developed a wobbly, uncoordinated gait, and of those only two animals progressed to hind limb recumbency.

The day after acute methamphetamine treatment, all rats appeared behaviorally normal. A slight decrease in body weight was recorded, but this weight was regained by the next day.

Methamphetamine increases frequency of 3-NP lesions

Histological examination of the brains of rats treated with methamphetamine 4 hr after the last 3-NP injection showed that all 14 had bilateral striatal lesions (Table 1; Fig. 1). By comparison, only 2 of the 19 brains from rats treated with 3-NP and saline displayed striatal lesions, and none of the animals that received methamphetamine alone displayed lesions. Thus, chronic pretreatment of rats with a low dose of 3-NP dramatically increased the striatal toxicity of a small, normally nontoxic, dose of methamphetamine ($p < 0.0001$). The increased toxicity of methamphetamine critically depended on the time interval between the final 3-NP injection and the methamphetamine injection, because delaying methamphetamine treatment until 24 hr after the last 3-NP injection reduced the number of rats displaying striatal lesions to a level not statistically different from the frequency of lesions induced by 3-NP and saline (Table 1; $p = 0.136$).

Extrastriatal brain regions are not damaged after 3-NP/methamphetamine treatment

Lesions induced by chronic 3-NP are primarily striatal. However, extrastriatal damage to the thalamus and hippocampus has been reported in some animals after prolonged treatment with 3-NP (Hamilton and Gould, 1987; Beal et al., 1993; Allen et al., 1994; Miller and Zaborszky, 1997). Here, neuronal damage caused by the acute administration of methamphetamine to 3-NP-treated rats was restricted to the striatum, and no damage was seen in the extrastriatal regions of any rat after 3-NP/methamphetamine treatment.

Characterization of neuronal loss induced by 3-NP/methamphetamine treatment

The histopathology of the lesions induced by 3-NP/methamphetamine was similar, but not identical, to that of lesions induced by 3-NP treatment alone. In both cases, bilateral lesions were found in the dorsolateral striatum and became more ventrolateral at the caudal extremes of the lesion (Fig. 1*a,b*). However, the lesions induced by 3-NP/methamphetamine were generally smaller [4.16 ± 0.42 mm^3 for the 4 hr group ($n = 14$) and 3.82 ± 0.64 mm^3 for the 24 hr group ($n = 3$)] than those induced by 3-NP alone [7.64 ± 0.63 mm^3 ($n = 2$)], and there was a different pattern of neuronal loss within each type of lesion.

The loss of NADPH diaphorase neuropil staining was used to delineate the borders of the lesions as well as to determine the survival of NADPH diaphorase neurons. In unlesioned brain, NADPH diaphorase staining showed strong neuropil staining throughout the striatum, with intensely stained NADPH diapho-

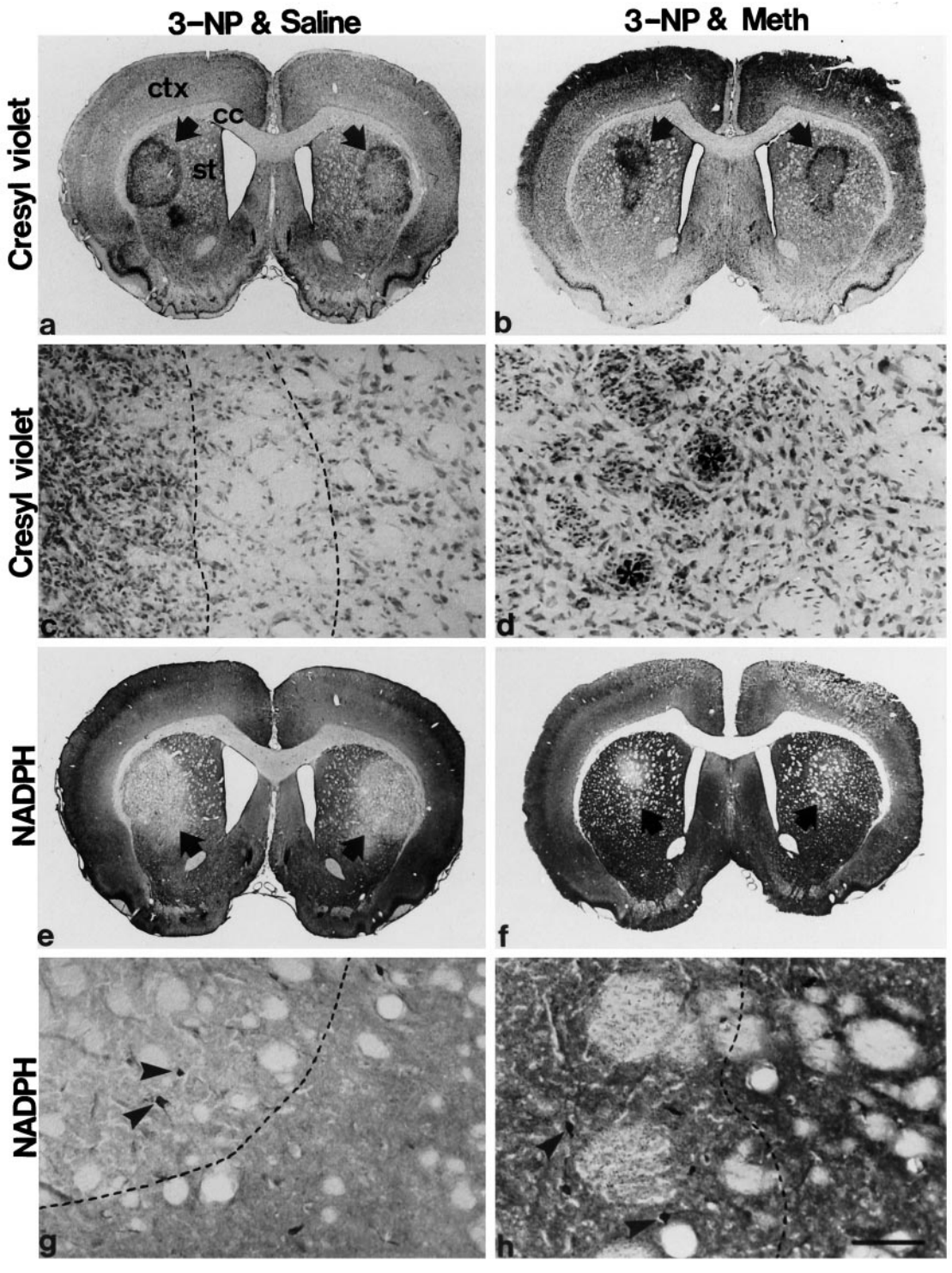

Figure 1. Histological appearance of spontaneous (*3-NP & Saline*) and methamphetamine-induced (*3-NP & Meth*) lesions in rat brain. Parallel coronal sections of rat brain were stained for cresyl violet (*a–d*) or NADPH diaphorase activity (*e–h*). Spontaneously induced lesions (*arrows, a, e*) displayed reactive gliosis, which was especially dense around the edge of the lesions (*a* and *left-hand zone* in *c*). Limited neuronal survival and diffuse gliosis were seen in the penumbral region of the lesion (*middle zone*, *c*). Methamphetamine-induced lesions (*arrows*, *b*, *f*) displayed gliosis, which was localized predominantly over the fiber bundles at the edges of the lesion (*asterisks*, *d*). In spontaneous 3-NP lesions, NADPH diaphorase-positive neuropil staining was markedly reduced in the center of the lesion, and no NADPH diaphorase-positive neurons were seen in this region. In the penumbral region (*upper left zone*, *g*) there was also reduced neuropil staining, although some surviving NADPH diaphorase-positive neurons were observed (*arrowheads*, *g*). In contrast, in 3-NP/methamphetamine-induced lesions there was stronger NADPH diaphorase neuropil staining (*arrows*, *f*), with greater sparing of positively stained neurons (*arrowheads*, *h*). Scale bars: *a, b, e, f*, 2 mm; *c, d, g, h*, 50 μm. *ctx*, Cortex; *cc*, corpus callosum; *st*, striatum.

Table 2. Neuronal counts within the lesion area

Treatment	Total number of neurons (per mm^2, $n = 5$ rats)	Number of NADPH neurons (per mm^2; $n = 5$ rats)
Saline	1092 ± 9	8.85 ± 1.19
3-NP and methamphetamine	634 ± 90[a]	3.93 ± 0.71[b]
3-NP alone	4.80 ± 2.44[c]	0.079 ± 0.061[d]

[a,b] $p < 0.05$ compared with saline; [c,d] $p < 0.001$ compared with saline.

rase interneurons scattered throughout the striatal neuropil. In rats with 3-NP lesions, NADPH diaphorase staining showed well circumscribed areas of very pale staining (Fig. 1*e*), which corresponded to the area of neuronal loss in adjacent sections stained with cresyl violet (compare Fig. 1*a*). Within these lesions there was a core region in which there was an increase in gliosis and in which neuronal loss was virtually complete (Fig. 1*a,c, left zone*), surrounded by a penumbral region in which NADPH diaphorase staining was reduced but in which the loss of neurons was less pronounced (Fig. 1*c, middle zone*). These findings are similar to those observed by others (Beal et al., 1993; Bossi et al., 1993).

In 3-NP/methamphetamine-treated rats, the lesion area was defined by an increase in gliosis (Fig. 1*b,d*) and a loss of NADPH diaphorase staining (Fig. 1*f*). However, within these lesions there was no obvious core/penumbral division (Figure 1*d*), and neuronal loss appeared uniform throughout the lesion area. This observation was confirmed by neuron counting, and quantification of total neuronal loss showed that within the central region of the 3-NP/methamphetamine lesions neuronal loss was less severe (40% of total number of neurons) than was seen in the corresponding region of lesions from rats treated with 3-NP alone (>95% loss; $p < 0.001$) (Table 2).

The sparing of striatal NADPH diaphorase-positive interneurons is a hallmark of the striatal damage in HD and often is used as a histopathological measure of the usefulness of animal models of HD. In lesions induced by 3-NP alone, few NADPH diaphorase-positive neurons were found within the core region, although there was limited sparing of these neurons in the penumbral region (Fig. 1*g, upper left*). This is consistent with the findings of others (Beal et al., 1993; Bossi et al., 1993). In contrast, within the 3-NP/methamphetamine-induced lesion 45% of the NADPH diaphorase neurons were spared within the lesion area (Table 2; Fig. 1*h*). This suggests that, although the lesions were similar in many ways, neurotoxic damage seen after 3-NP/methamphetamine treatment is less severe than that seen with 3-NP alone.

Glial reactions associated with methamphetamine and 3-NP-induced lesions

There was a highly consistent astroglial reaction associated with both 3-NP-induced lesions and 3-NP and methamphetamine-induced lesions. All lesions, whether induced by 3-NP/methamphetamine or 3-NP alone showed a pronounced astrogliosis in the penumbra of the lesions (Fig. 2*a–d*). Although there was astrogliosis within the core of both types of lesion, this was less pronounced than in the penumbral regions. No astrogliosis was observed in the striata of rats treated with saline and methamphetamine (data not shown).

Microglia were observed in all lesions, although the distribution of the microglial reaction was very different in the two types of lesion (Fig. 2*e–h*). Lesions induced by 3-NP alone displayed intense immunoreactivity for microglial markers in neuropil and fiber bundles throughout the whole lesion (Fig. 2*e,g*), whereas the microglial reaction in lesions induced by 3-NP/methamphetamine was much less severe and was associated primarily with fiber bundles (Fig. 2*f,h*).

The effect of dopamine antagonists on 3-NP/methamphetamine-induced behavior

The role of dopamine receptors in lesion formation was examined by the use of selective dopamine receptor antagonists, with SCH23390 and sulpiride used to block D_1 and D_2 receptors, respectively. Pilot studies were conducted to determine appropriate doses of the antagonists. The dose of each antagonist for these experiments was chosen so that (1) the dose was within the range at which the drug action is selective for D_1 or D_2 type dopamine receptors (Mailman et al., 1997), and (2) dopamine-mediated behaviors (e.g., hypoactivity and catalepsy) were induced in drug-naive control animals by a single dose of the antagonist.

Pretreatment of the saline-treated control animals with either sulpiride or SCH23390 resulted in a marked reduction in spontaneous movement in all animals (Table 3). Both drugs also caused catalepsy in most animals in the experimental group (Table 3). The administration of sulpiride and SCH23390 together resulted in hypoactivity and induced catalepsy in 8 of 10 rats.

The pretreatment of rats with sulpiride did not prevent methamphetamine-induced activity but prevented the development of stereotypical movements in more than one-half of the animals (Table 3). In contrast, SCH23390 had little or no effect on either methamphetamine-induced hyperactivity or the development of stereotypy. Seven 3-NP rats pretreated with sulpiride and four rats pretreated with SCH23390 developed a wobbly gait ~20 min after methamphetamine treatment; three animals in each group then went on to display hind limb recumbency. When both antagonists were administered together, methamphetamine-induced activity and stereotypy were greatly reduced. Although 4 of 10 rats pretreated with both antagonists developed a wobbly gait, only one of these became recumbent.

Effect of dopamine antagonists on methamphetamine/3-NP-induced lesion formation

Blocking D_2 receptors with sulpiride did not prevent 3-NP/methamphetamine-induced lesions. Fewer lesions were induced in rats pretreated with the D_1 antagonist SCH23390, and when both antagonists were administered simultaneously, the number of rats presenting with lesions was greatly reduced. These data suggest that, although D_1 receptor activity plays a part in 3-NP-induced lesion formation, both receptor subtypes are important for the mediating effects of dopamine.

Histopathologically, there was no difference between the neuropathological profiles of lesions induced by 3-NP/methamphetamine in the presence of dopamine receptor antagonists and

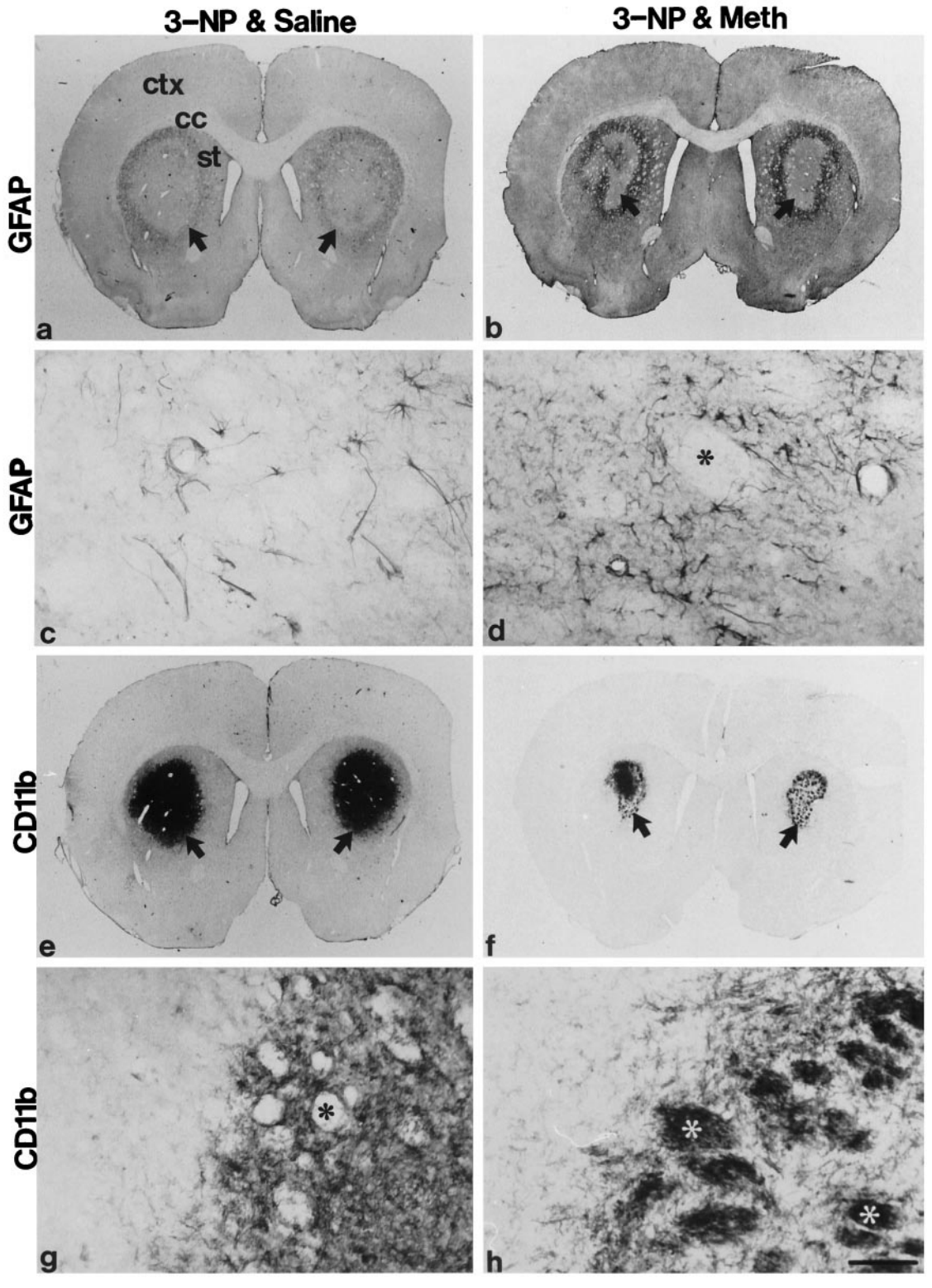

Figure 2. Glial and microglial immunoreactivity in spontaneous (*3-NP & Saline*) and methamphetamine-induced (*3-NP & Meth*) lesions in rat brain. Parallel coronal rat brain sections were stained for GFAP (*a–d*) or CD11b (*e–h*) immunoreactivity. Lesions induced by 3-NP and saline (*a, c, e, g*) show the typical pattern of gliosis associated with 3-NP lesions. Increased astrogliosis was seen in the penumbral region of the lesions (*a*, *c*), whereas microgliosis was very dense throughout the lesion (*e*, *g*). Methamphetamine-induced lesions also displayed gliosis in the penumbral region (*b*, *d*); this was not associated with fiber bundles (*asterisk*, *d*). The extent of microgliosis associated with the neuropil varied in lesions of different animals (compare *left* and *right* lesions, *f*) but was generally less intense than that seen in spontaneous lesions. Microglial staining often was strongly associated with fiber bundles (*asterisks*, *h*). Scale bars: *a, b, e, f*, 2 mm; *c, d, g, h*, 100 μm. *ctx*, Cortex; *cc*, corpus callosum; *st*, striatum.

Table 3. Effect of dopamine antagonists on methamphetamine-induced changes

Treatment	Behavioral changes (Number of rats displaying behavior/Total number of rats)				Number of rats with lesions
	Hyperactivity	Stereotype	Wobbly gait	Splayed hind legs	
Saline	14/14	14/14	14/14	10/14	14/14
Sulpiride	7/10	4/10	7/10	3/10	9/10
SCH23390	9/10	7/10	4/10	3/10	6/10[a]
SCH23390 + sulpiride	3/10	1/10	4/10	1/10	2/10[b]

[a] $p < 0.02$ compared with saline; [b] $p < 0.0001$ compared with saline (Fisher's exact test).

those induced by 3-NP/methamphetamine alone (data not shown).

The effect of 6-OHDA lesions on 3-NP-induced behavioral changes

To determine the effect of lowered dopamine levels on 3-NP lesion formation, we performed a unilateral 6-OHDA lesion of the median forebrain bundle 7 d before rats began their 3-NP treatment. Because 3-NP was administered systemically, the unlesioned contralateral striatum served as the control for 3-NP lesion formation in individual rats.

Because lesion formation induced by the use of constant low doses of 3-NP (12 mg/kg) is unpredictable, in these experiments a more severe 3-NP treatment was used, with stepwise increases in the dose of 3-NP. With the stepwise protocol the characteristic behaviors consistent with 3-NP intoxication were observed after an average of 7 or 8 d. At 1–2 hr after its final 3-NP injection the rat became ataxic, sitting in hunched position, and often displayed marked piloerection. The hind limbs usually were splayed out to either side of the body, and their movement was uncoordinated during locomotion. Rats displaying symptoms of intoxication received no further 3-NP injections and recovered functionally over the next few days.

The behavioral changes associated with 3-NP intoxication in 6-OHDA-lesioned rats were very similar to those seen in intact rats. The only difference was that some 6-OHDA-lesioned rats (4 of 10) displayed a small degree of anticlockwise rotational movement when placed in a novel environment.

6-OHDA lesions protect against 3-NP-induced striatal lesions

All intact rats treated with 3-NP developed bilateral striatal lesions. Although the lesion volume varied somewhat among animals (9.41 ± 2.76 mm^3), there was no significant difference between the left and right striatal lesions of a given animal ($p = 0.442$, paired t test). In contrast, in 6-OHDA-lesioned rats, removal of the dopaminergic input to the striatum provided significant protection against 3-NP-induced striatal toxicity. In two of the rats this protection was complete, and no lesion was seen on the dopamine-denervated side (Fig. 3*a–e*). The remaining nine animals had bilateral lesions, but in all rats the lesion in the dopamine-denervated striatum was significantly smaller than the lesion in the intact striatum ($p < 0.0002$, paired t test; denervated side $= 2.04 \pm 0.71$ mm^3 and intact side $= 4.85 \pm 0.97$ mm^3).

The histopathological characteristics of the striatal lesions in 6-OHDA-lesioned rats were very similar to those of lesions induced in intact rats, with neuronal loss and pronounced CD11b immunoreactivity throughout the region of the lesion and increased reactive gliosis in the penumbral region (Fig. 3*a–d*). Although the lesions in the intact contralateral striatum were slightly smaller than those seen in intact rats treated with 3-NP, this difference did not reach statistical significance.

Striatal lesion volume correlates with the extent of nigrostriatal dopamine input

TH immunocytochemistry confirmed the expected loss of dopaminergic neurons from the lesioned side of the brain in the 6-OHDA-lesioned animals (Fig. 3*e,f*). The extent of loss varied among animals, with loss of dopaminergic neurons from both the SNpc as well as the VTA. There was a strong correlation between the size of the striatal lesions and the extent of dopaminergic innervation from the SNpc ($r^2 = 0.66$; $p < 0.002$, linear regression) (Fig. 4), but not the VTA ($r^2 = 0.03$).

There was no effect on the total number of TH-positive neurons in the SN/VTA after 3-NP treatment or after 3-NP/methamphetamine treatment [TH cell counts in the SN/VTA were saline 219 ± 20; 3-NP alone, 234 ± 15; 3-NP/methamphetamine, 231 ± 11. Numbers are the mean number of TH-positive cells per section, five sections per animal, and five animals for each group (all sections at comparable levels)].

DISCUSSION

Current theories do not account fully for the neurotoxic action of 3-NP

One of the strengths of the energy impairment/excitotoxicity hypothesis is that it proposes a means whereby striatal damage can occur without the elevation of striatal glutamate levels usually associated with excitotoxicity. This is particularly important because glutamate levels do not appear to be increased in HD (Nicoli et al., 1993). However, the main weakness of this hypothesis is that succinate dehydrogenase (SDH) activity in all neurons in the brain is affected similarly by 3-NP (Gould et al., 1985; Brouillet et al., 1998). Thus one would expect that other neurons that are targets for glutamate input (e.g., CA1 neurons in the hippocampus and Purkinje cells in the cerebellum) also would be vulnerable to endogenous glutamate in 3-NP-intoxicated animals. This does not appear to be the case, and although lesions are seen occasionally in the hippocampus, extrastriatal regions do not appear to be vulnerable to 3-NP (Bossi et al., 1993; Wüllner et al., 1994; Fu et al., 1995a; Nishino et al., 1995; Shimano et al., 1995). Thus the current hypothesis does not account fully for the striatal selectivity of 3-NP neurotoxicity. It seems likely that another factor must be important for the induction of 3-NP lesions; we propose that this factor is dopamine.

Dopamine action underlies the development of 3-NP lesions

We used methamphetamine to increase dopamine levels *in vivo*. The increase in dopamine after methamphetamine is well described (Schmidt et al., 1985; O'Dell et al., 1993) (for other

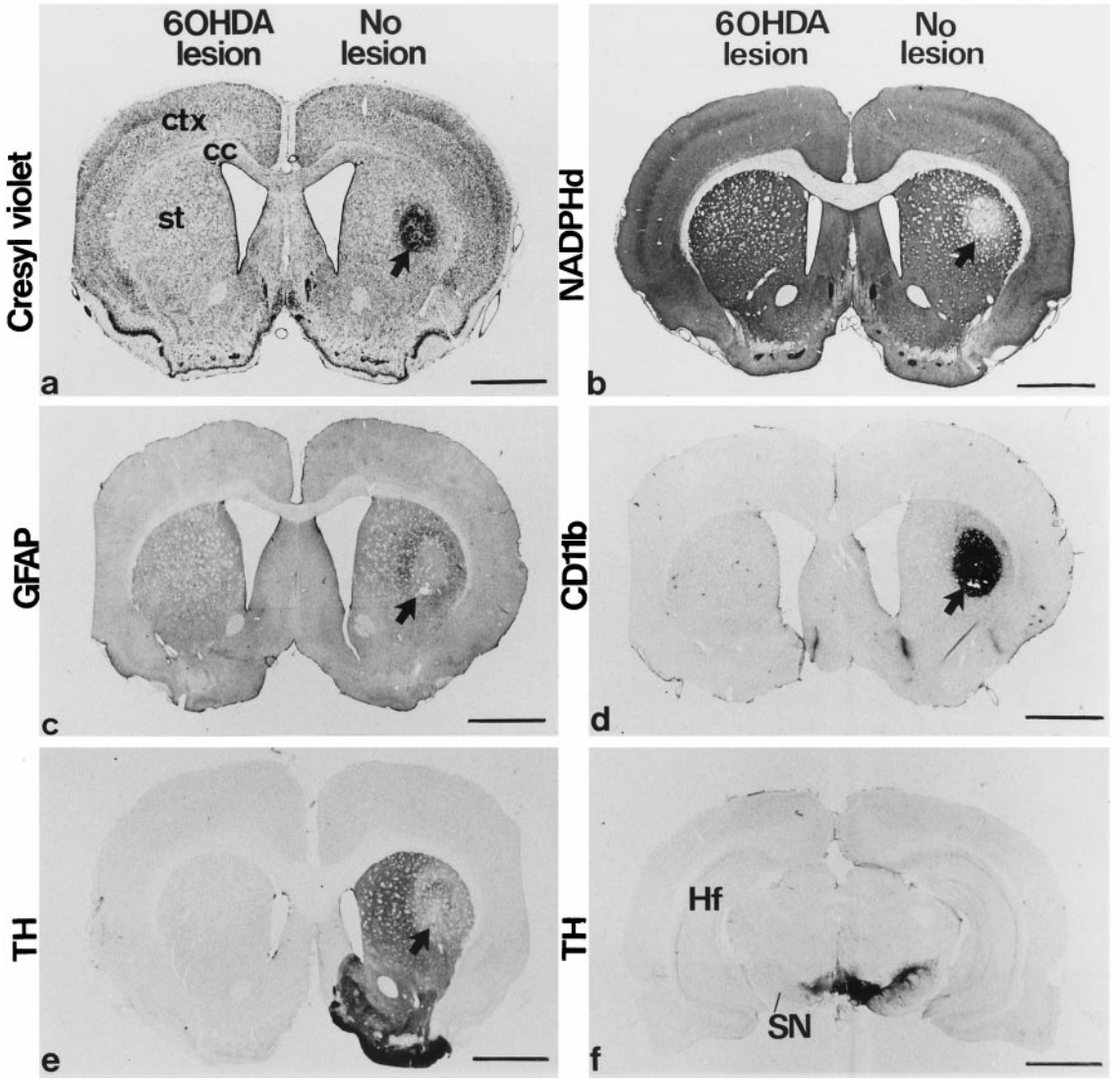

Figure 3. The effect of 6-OHDA lesions on 3-NP-induced striatal lesion formation. A striatal 3-NP lesion is observed only on the side with the intact dopamine input (*arrows*, *right side*). Cresyl violet (*a*), NADPH (*b*), GFAP (*c*), and CD11b (*d*) staining of the striatal lesion is very similar to that seen in rats with bilateral striatal 3-NP lesions (compare with Fig. 1). There is no striatal pathology visible on the lesioned side. TH staining (*e*, *f*) revealed a marked loss of dopaminergic neurons from the SNpc (*f*) on the 6-OHDA-lesioned side of the brain and complete loss of staining in the ipsilateral striatum (*e*). Scale bar, 2 mm. *ctx*, Cortex; *cc*, corpus callosum; *st*, striatum; *Hf*, hippocampus; *SN*, substantia nigra.

references, see Seiden and Ricuarte, 1987; Gibb et al., 1990). Although we did not measure striatal dopamine levels directly, blocking dopamine receptors is known to reduce methamphetamine-induced rises in dopamine (O'Dell et al., 1993) and in this study prevented the formation of methamphetamine-induced lesions in 3-NP-treated rats (Table 3).

The increase in lesion frequency seen after methamphetamine was very interesting, because although methamphetamine and amphetamine are both toxic, large or repeated doses of these drugs usually are required to cause damage in normal rats (Ellison et al., 1978; O'Dell et al., 1993, 1994; Bowyer et al., 1994; Finnegan and Taraska, 1996). The dose we chose is used to test rotational behavior (Ungerstedt and Arbuthnott, 1970; Dunnett et al., 1987, 1988) and does not cause neuronal or terminal damage (Ungerstedt and Arbuthnott, 1970). Thus it appears that 3-NP increased the vulnerability of striatal neurons to methamphetamine. The toxicity of methamphetamine was more pronounced when administered 4 hr, rather than 24 hr, after the last 3-NP injection. This supports the suggestion that the increased vulnerability is a consequence of energy impairment rather than a change in the efficacy of methamphetamine. [Several studies have shown that SDH inhibition is maintained for at least 24 hr after 3-NP treatment (Palfi et al., 1996; Nony et al., 1997; Brouillet et al., 1998), but partial recovery of SDH activity by *de novo* synthesis of the enzyme would be expected during this time. Hence, increased SDH activity during the recovery period may restore energy levels sufficiently to prevent methamphetamine toxicity.]

Development of spontaneous 3-NP lesions depends on an intact nigrostriatal dopamine input

Because the appearance of spontaneous lesions after 3-NP treatment is unpredictable, it was not practical to use dopamine receptor antagonists to block their development. Instead, we examined the role of dopamine by removing the nigrostriatal

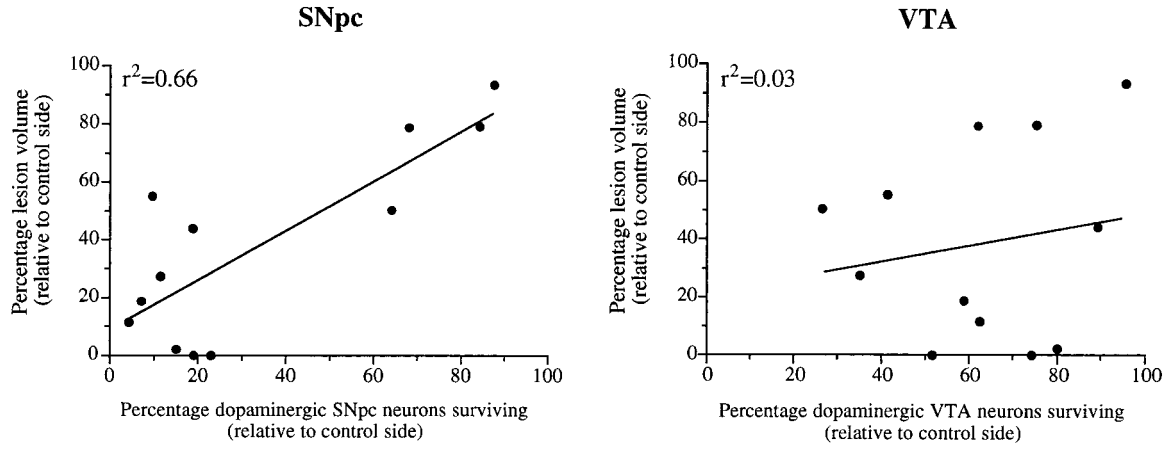

Figure 4. Correlation of relative striatal lesion volume with the number of SNpc or VTA dopaminergic neurons surviving on the 6-OHDA-lesioned side. There was a strong correlation between lesion volume and the number of dopamine neurons in the SNpc ($r^2 = 0.662$; $p < 0.002$), but not in the VTA ($r^2 = 0.03$). The lesion volume on the denervated side was expressed as a percentage of that seen on the intact side. The number of dopaminergic neurons on the lesioned side was expressed as a percentage of the number of those on the contralateral side.

input to one striatum, allowing the intact side to serve as the control. Complete removal of dopamine input prevented the formation of 3-NP lesions in the denervated striatum while having no effect on the formation of the lesion in the contralateral striatum. In animals in which partial 6-OHDA lesions were seen, there was a strong correlation between the number of dopamine neurons in the SNpc and the size of the 3-NP-induced striatal lesion. In contrast, there was a poor correlation between lesion size and dopaminergic neuron survival in the VTA. This is consistent with the idea that dopamine increases the vulnerability of striatal neurons to 3-NP, because the neurons surviving in the VTA were found in the medial areas that project to cortex rather than to striatum (Fallon and Loughlin, 1995).

Mechanism of action of dopamine in striatal 3-NP toxicity

Dopamine plays a central role in methamphetamine toxicity and is likely to be similarly important in the development of the 3-NP/methamphetamine lesions. Methamphetamine toxicity is mediated via a number of mechanisms, both direct [via the auto-oxidation of dopamine and methamphetamine to reactive oxygen species (ROS) (Breese and Traylor, 1970; Cohen and Heikkila, 1974; Graham, 1978; Seiden and Vosmer, 1984; Giovanni et al., 1995)] and indirect [via excitotoxic routes, because increased extracellular dopamine potentiates glutamate release (Nash and Yamamoto, 1993; Hu and White, 1997) and NMDA receptor antagonists block methamphetamine toxicity (Sonsalla et al., 1989, 1991; Green et al., 1992; Baldwin et al., 1993; Finnegan and Taraska, 1996) or via interactions with NO (Sheng et al., 1996; Ali and Itzhak, 1998)]. Interestingly, similar hypotheses have been proposed to account for the neurotoxicity of 3-NP. For example, it has been suggested that ROS generation/NO are important (Fu et al., 1995b; Galpern et al., 1996; Schulz et al., 1996; Tsai et al., 1997), and NO synthase inhibitors (Schulz et al., 1995) and certain free radical scavengers (Nakao and Brundin, 1997) can protect against 3-NP-induced damage. Furthermore, glutamate excitotoxicity is central to the energy depletion/excitotoxicity hypothesis (Novelli et al., 1988; Simpson and Isacson, 1993). The possibility that a similar mechanism accounts for the action of both methamphetamine and 3-NP therefore is consistent with the possibility that dopamine plays an important role in 3-NP toxicity.

Although the current hypotheses aimed at explaining 3-NP toxicity *in vivo* do not include a role for dopamine, such a role fits very well with other data. As well as having modulatory effects on striatal glutamate release, dopamine itself can be neurotoxic (Filloux and Townsend, 1993; Ben-Shachar et al., 1995; Cheng et al., 1996; Hastings et al., 1996). Although to our knowledge striatal dopamine levels have not been measured during chronic 3-NP treatment, large changes in dopamine levels have been observed under conditions in which neurotoxic damage results, e.g., anoxia and transient ischemia (Globus et al., 1988; Slivka et al., 1988). Furthermore, endogenous dopamine exacerbates the striatal toxicity of glutamate receptor agonists (Chapman et al., 1989; Filloux and Wamsley, 1991; Garside et al., 1996). Finally, it recently has been shown *in vitro* that mitochondrial inhibition potentiates dopamine toxicity in striatal cultures (McLaughlin et al., 1998).

We suggest that the current energy/excitotoxic hypothesis for 3-NP toxicity should be expanded to include a role for dopamine, because the vulnerability of striatal neurons to 3-NP depends on an intact dopamine input. It seems likely that the striatal selectivity of 3-NP lesions is attributable to the striatum being a major target for both dopaminergic and glutamatergic inputs, making it the most vulnerable region in the 3-NP-intoxicated brain.

A role for dopamine in 3-NP lesion formation: Implications for the treatment of HD

Although direct evidence for an involvement of dopamine in HD pathology is sparse, there is considerable evidence suggesting that changes in the dopaminergic system occur in HD. Dopamine receptors are decreased in HD patients (Sedvall et al., 1994; Antonini et al., 1996; Weeks et al., 1996; Ginovart et al., 1997), in asymptomatic HD gene carriers (Antonini et al., 1996; Weeks et al., 1996), and in transgenic HD mice (Cha et al., 1998). The function of the decrease in dopamine receptors is not known, but one of the consequences of these changes may be an increase in dopamine release (via a decrease in presynaptic autoinhibitory D_2 action). Our experiments suggest that an increase in dopamine would potentiate striatal neurotoxicity, particularly if there were an underlying energy deficit in HD. 3-NP is an irreversible inhibitor of SDH of Complex II in the mitochondrial chain, and the striatal toxicity associated with 3-NP intoxication suggests that a metabolic deficit might be important in the mechanism

underlying neurodegeneration in HD (Ludolph et al., 1992; Erecinska and Nelson, 1994; Koroshetz et al., 1994; Tsai et al., 1997). This suggestion is supported by a growing body of evidence, including nuclear magnetic resonance studies (Jenkins et al., 1993; Antonini et al., 1996; Harms et al., 1997) and biochemical studies (Brennan et al., 1985; Butterworth et al., 1985; Parker et al., 1990; Koroshetz et al., 1993, 1997; Gu et al., 1996).

We provide evidence for a direct role of dopamine in striatal 3-NP toxicity. This may be of particular importance for understanding the early pathology of HD, where despite ubiquitous expression of the defective HD gene and its protein product in the CNS (Sharp et al., 1995; Ferrante et al., 1997), neurodegeneration is primarily striatal. There is no treatment for HD, and although antidopaminergic therapy has been considered (Tyler et al., 1996), D_2 blockers do not appear to affect the long-term progression of HD. Nevertheless, we suggest that dopamine is important in the neurodegenerative processes underlying HD and that its role in HD needs to be reevaluated, because modulation of the dopaminergic system might provide a target for therapy for this devastating disease.

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