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METABOLIC ACTIVATION OF THE SEROTONERGIC NEUROTOXIN PARA-CHLOROAMPHETAMINE TO CHEMICALLY REACTIVE INTERMEDIATES BY HEPATIC AND BRAIN MICROSOMAL PREPARATIONS*

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Abstract—Para-chloroamphetamine (PCA) is selectively toxic to serotonergic neurons in laboratory animals. Acute, reversible neurotoxicity is followed by long-term effects which include inactivation of tryptophan hydroxylase and destruction of neurons. We have studied the metabolic formation of reactive intermediates that might be involved in long-term PCA neurotoxicity. Incubation of [^{3}H]PCA with rat hepatic microsomes resulted in NADPH-dependent and oxygen-dependent covalent binding of radioactivity to microsomal protein. Addition of SKF-525A and glutathione to incubation mixtures inhibited [^{3}H]PCA covalent binding 30% and 92% respectively. No inhibition of radiolabeled covalent binding was observed in an atmosphere of carbon monoxide/oxygen (80/20). 7,8-Benzoflavone was more effective than metyrapone in inhibiting [^{3}H]PCA covalent binding. The extent of [^{3}H]PCA covalent binding to microsomal protein was unchanged after phenobarbital pretreatment of rats, whereas 3-methylcholanthrene pretreatment increased [^{3}H]PCA covalent binding (175%). NADPH-dependent and oxygen-dependent covalent binding of radioactivity was also observed when [^{3}H]PCA was incubated with rat brain microsomal preparations. Addition of SKF-525A and glutathione to incubation mixtures inhibited covalent binding 10 and 40% respectively. There were no significant differences in total, NADPH-independent or NADPH-dependent covalent binding of radiolabeled *R*,*S*(±)-, *R*(−)-, or *S*(+)-PCA to rat hepatic microsomal protein. Less covalent binding was observed when [^{3}H]amphetamine was incubated with rat liver microsomal preparations as compared to results with [^{3}H]PCA. Minimal covalent binding was observed when [^{3}H]PCA was incubated with liver microsomal preparations from rabbits, a species resistant to PCA neurotoxicity. Results of these metabolism studies are consistent with the hypothesis that oxidative metabolic activation of PCA to reactive and toxic metabolites is related to the long-term neurotoxicity of this agent.

Para-chloroamphetamine (PCA) is selectively toxic to serotonergic neurons following intraperitoneal injection to rats and mice. Short-term, reversible reductions in brain concentrations of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) [1, 2] are followed by long-term (up to 4 months) effects in serotonergic neurons [3–5]. The long-term neurotoxicity is additionally characterized by loss of tryptophan hydroxylase [3] and by irreversible cytopathological damage to B-9 cell groups [3, 6–8]. Long-term neurotoxic effects differ for PCA enantiomers. *S*(+)-PCA depletes serotonin [9] and 5-HIAA [5] for longer periods of time and to a greater extent than does the *R*(−)-enantiomer. *S*(+)-PCA also reduces tryptophan hydroxylase activity to a greater extent and for longer time periods than does *R*(−)-PCA [9].

There is evidence to suggest that PCA neurotoxicity is mediated by a metabolite rather than by the parent drug. PCA does not inhibit tryptophan hydroxylase *in vitro* [10]. The half-life of the parent molecule in rat brain is 8.5 hr [5], yet radioactivity is present in the brain 800 hr after administration of radiolabeled PCA [11]. Immature rats, which are deficient in drug-metabolizing enzymes, are resistant to the long-term toxic effects of PCA [12, 13]. It has been suggested that PCA neurotoxicity may be related to formation of chemically reactive metabolites which irreversibly interact with essential proteins in serotonergic neurons [14–16]. In preliminary studies, it was determined that rat hepatic microsomal preparations convert PCA to reactive species which covalently bind to microsomal protein [14]. We have now characterized the enzymatic pathway responsible for activation of PCA in rat hepatic microsomal preparations and determined the covalent binding of tritiated *R*,*S*(±)-, *R*(−)-, and *S*(+)-PCA to rat hepatic microsomal proteins. In addition, we have shown that rat brain microsomal preparations catalyze oxygen-dependent and NADPH-dependent metabolic activation of [^{3}H]PCA to species which covalently bind to microsomal protein. These results suggest that metabolic activation may be related to the neurotoxic effects of PCA.

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MATERIALS AND METHODS

R,*S*(±) - 1 - (4 - Chloro)phenyl - 2 - aminopropane (PCA) hydrochloride was obtained from the Regis

Chemical Co. (Morton Grove, IL). *R*(−)-PCA and *S*(+)-PCA were resolved as diastereomeric salts of *N*-acetyl-D-leucine and *N*-acetyl-L-leucine, respectively, as described previously [17]. [^{3}H]-*R*,*S*(±)-PCA (290 mCi/mmole), [^{3}H]*S*(+)-PCA (32.0 Ci/mmole) [^{3}H]*R*(−)-PCA (19.6 Ci/mmole) and [^{3}H]-*S*(+)-amphetamine sulfate (15.1 Ci/mmole) were obtained from Dr. Stephen Hurt, New England Nuclear (Boston, MA). Chemical, radio, and optical purities of radiolabeled PCA substrates were > 98% as determined by high performance liquid chromatographic analysis using an assay for racemic PCA and an assay for PCA enantiomers [18]. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were purchased from the Boehringer Mannheim Co. (Indianapolis, IN). Glutathione, α-naphthoflavone (7,8-benzoflavone), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), trichloroacetic acid, phenobarbital and 3-methylcholanthrene were obtained from the Sigma Chemical Co. (St. Louis, MO). Proadifen hydrochloride (SKF-525A) was obtained from Smith Kline & French Laboratories (Welwyn Garden City, Herts, England).

Male Sprague–Dawley rats (200–300 g) and male New Zealand rabbits (2.0–2.5 kg) were used for all metabolism studies. Rats were killed by decapitation and rabbits were killed by carbon dioxide inhalation. For enzyme induction studies, animals were pretreated with phenobarbital (80 mg/kg, saline, three daily intraperitoneal injections) or 3-methylcholanthrene (24 mg/kg, corn oil, four daily intraperitoneal injections). Animals were killed 24 hr later. Hepatic microsomes were prepared by differential centrifugation of liver homogenates as described by Ernster *et al.* [19]. A modification of this procedure was used to prepare microsomes from whole rat brain. Each brain was quickly removed and washed with ice-cold deaerated 0.02 M Tris buffer (pH 7.4, 15% glycerol, 1.15% KCl) to remove excess blood. All subsequent operations were carried out at 0–5°. The brains were homogenized (1:4 volume) in Tris/glycerol buffer and sonicated for 10 sec. Supernatant fractions were obtained by centrifugation in a Beckman model J-21C centrifuge at 15,500 rpm (10,000 *g*) for 20 min. The supernatant fractions were transferred to new tubes, and the microsomal fraction was isolated following centrifugation in a Beckman L5-50 centrifuge at 47,000 rpm (100,000 *g*) for 45 min. The supernatant fractions were discarded. The pellets were resuspended in a small volume of 0.10 M phosphate buffer (pH 7.4). Protein concentrations were determined by the method of Lowry *et al.* [20], with crystalline bovine serum albumin as the standard.

Hepatic microsomal incubations consisted of the following components present in final concentrations as stated: microsomal protein (2 mg/ml), Tris buffer (83 mM, pH 7.4), $NADP^+$ (2.0 mM), glucose-6-phosphate (118 mM), glucose-6-phosphate dehydrogenase (0.7 units/ml), $MgCl_2$ (5.0 mM) and substrate (0.5 mM, 1500 dpm/nmole) in a final volume of 3 ml. Brain microsomal incubations consisted of the following components present in final concentrations as stated: microsomal protein (3 mg/ml), phosphate buffer (83 mM, pH 7.4), $NADP^+$ (2.0 mM), glucose-6-phosphate (118 mM), glucose-6-phosphate dehydrogenase (0.7 units/ml), $MgCl_2$ (5.0 mM) and substrate (0.5 mM, 15,000 dpm/nmole) in a final volume of 3 ml. For inhibitor experiments, 100 μl of inhibitor stock solutions was added to the microsomal incubation to yield in final concentrations: SKF-525A (0.1 mM), metyrapone (1 mM, 3 mM), 7,8-benzoflavone (0.1 mM, 0.01 mM) and glutathione (4 mM). For gas mixture experiments, ice-cold incubation vials containing all components except substrate were sealed, and appropriate gas mixtures were introduced through rubber septa alternately with vacuum (three cycles of approximately 2 min each). The carbon monoxide/oxygen (CO/O_2) gas mixture (80/20) was prepared by calibrating gas flow meters to deliver the correct mixture of gases to the vials. All incubations were begun by addition of substrate. Incubation vials were placed in a shaking water bath at 37°. The reaction was stopped at appropriate times by placing vials in an ice bath for 15 sec. Contents of the vials were immediately transferred to 12-ml glass conical tubes followed by addition of 0.3 ml of 3 M trichloroacetic acid. After 15 min at 4° sample tubes were centrifuged for 30 min at 1000 *g* in a Beckman model TJ-6 centrifuge.

The protein pellet was washed in 5% trichloroacetic acid, 80% aqueous methanol, and ethanol/ether (80/20) until no further radioactivity could be detected in supernatant washes. At least two washings with trichloroacetic acid, two washes with aqueous methanol and four washes with ethanol/ether were carried out for all samples. The extracted pellet was dissolved in 0.5 ml of 1 N NaOH at 60° for 30 min. Two 10-μl aliquots were reserved for protein determination, and the remaining protein solution was placed in 22 mm plastic scintillation vials (RPI, Prospect, IL) along with a water wash (1.5 ml). Scintillant (15 ml, Beckman Ready-Solv HP) was added, and the sample was counted in a scintillation counter. Radioactivity was corrected for background quenching (external standardization) and expressed as picomoles PCA drug equivalents per milligram protein. It was assumed that radioactivity remaining in these washed preparations represented covalent binding to microsomal proteins. For all metabolism studies, at least three experiments were conducted with duplicate incubations at each recorded time point.

RESULTS

As previously reported [14], incubation of tritiated racemic PCA with rat hepatic microsomal preparations and an NADPH-generating system resulted in covalent binding of radioactivity to microsomal protein. Covalent binding was minimal in the absence of NADPH or oxygen, in the presence of glutathione, and when liver microsomes were boiled prior to incubation (Table 1). The time courses of total and NADPH-independent covalent binding of [^{3}H]PCA racemate are shown in Fig. 1. Non-specific (NADPH-independent) protein binding was 2.0 to 7.0% of the NADPH-dependent binding at all time points.

Studies were next conducted to characterize the enzymatic pathway responsible for conversion of [^{3}H]PCA to chemically reactive metabolite(s). Pretreatment of animals with phenobarbital had no effect on covalent binding, whereas pretreatment with 3-

Table 1. Characterization of covalent binding of [^{3}H]PCA to rat liver microsomal protein

Incubation conditions*	% Control binding† 60 min	240 min
Complete	100	100
−NADP$^+$	3.5	4.4
−O_2	9.0	10.5
NADPH-dependent	96.5	95.6
Boiled	3.8	8.5
Glutathione (4.0 mM)	8.5	9.2

* Substrate concentration, 0.5 mM.

† One hundred percent equals 1.41 ± 0.22 nmoles/mg protein at 60 min and 2.16 ± 0.22 nmoles/mg protein at 240 min.

Table 2. Effects of cytochrome P-450 modifiers on the covalent binding of [^{3}H]PCA to rat liver microsomal protein

Incubation conditions*	% Control binding† 60 min	240 min
Complete	100	100
−NADP$^+$	3.5	4.9
Phenobarbital pretreatment	83	100
3-Methylcholanthrene pretreatment	175	169
SKF-525A (0.1 mM)	73	68
CO/O_2 (80/20, v/v)	97	95
Metyrapone		
1 mM	84	92
3 mM	84	98
7,8-Benzoflavone		
0.01 mM	75	79
0.1 mM	64	69

* Substrate concentration, 0.5 mM.

† One hundred percent equals 1.05 ± 0.08 nmoles/mg protein at 60 min and 1.78 ± 0.11 nmoles/mg protein at 240 min.

methylcholanthrene significantly increased covalent binding (Table 2). Greater than 97% of the increase in covalent binding observed after 3-methylcholanthrene pretreatment was NADPH dependent in nature. Addition of SKF-525A to incubation mixtures partially inhibited NADPH-dependent covalent binding (Table 2). Covalent binding was not inhibited when hepatic microsomal incubations were carried out under an atmosphere of CO/O_2 (80/20) (Table 2). In experiments carried out simultaneously with [^{3}H]PCA incubations, and with the same incubation reagents, the CO/O_2 gas mixture inhibited N-demethylation of a test substrate (hexamethylmelamine) to the same extent as observed previously in our laboratory (~ 70%). NADPH-dependent covalent binding was inhibited when incubations contained 7,8-benzoflavone (Table 2). When metyrapone was added to incubation mixtures, the long-term (~ 4 hr) radiolabeled covalent binding of PCA was unchanged.

Since PCA neurotoxicity exhibits some stereoselectivity, covalent binding of radiolabeled PCA enantiomers to hepatic microsomal protein was compared to covalent binding observed with racemic substrate. Total, NADPH-independent, and NADPH-dependent covalent binding of [^{3}H]*S*(+)-PCA to microsomal protein were similar to that of [^{3}H]*R*(−)-PCA (Fig. 2). Covalent binding of [^{3}H]PCA racemate to microsomal protein (Fig. 1) was slightly greater than that of [^{3}H]PCA enantiomers (Fig. 2).

Covalent binding of radioactivity was also determined following incubation of [^{3}H]PCA with rat brain microsomal preparations. Significant NADPH-dependent and oxygen-dependent covalent binding was detected (Table 3). Nonspecific (NADPH-independent) binding was higher in brain preparations than in liver preparations, due to the 10-fold greater specific activity of tritiated PCA employed in the brain studies. Significantly less inhibition of NADPH-dependent covalent binding by SKF-525A and glutathione was observed in rat brain microsomal preparations (Table 3) than in rat liver preparations (Tables 1 and 2). As with liver preparations, no inhibition was observed when incubations were carried out under an atmosphere of CO/O_2 (80/20) (Table 3).

To further relate binding and neurotoxicity, covalent binding of [^{3}H]PCA to hepatic microsomal protein of the rat was compared with that of the rabbit, a species resistant to PCA neurotoxicity [21]. Total

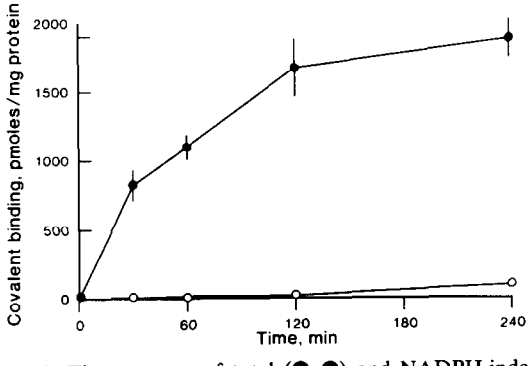

Fig. 1. Time courses of total (●–●) and NADPH-independent (○–○) covalent binding of [^{3}H]*R,S*(±)-PCA (0.5 mM, 1500 dpm/nmole) to rat liver microsomal protein.

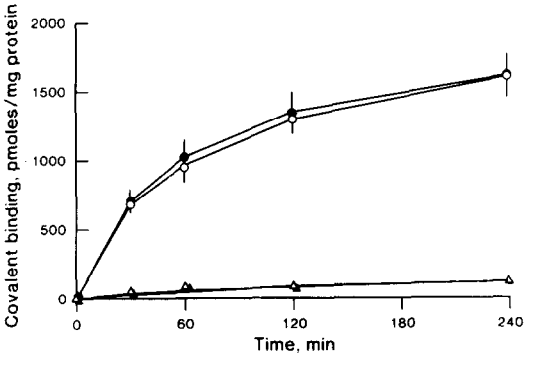

Fig. 2. Time course for total (●–●) and NADPH-independent (▲–▲) covalent binding of [^{3}H]*S*-(+)PCA (0.5 mM, 150 dpm/nmole) and time course for total (○–○) and NADPH-independent (△–△) covalent binding of [^{3}H]*R*(−)-PCA (0.5 mM, 1500 dpm/nmole) to rat liver microsomal protein.

Table 3. Characterization of covalent binding of [^{3}H]PCA to rat brain microsomal protein

Incubation conditions*	% Control binding†	
	60 min	240 min
Complete	100	100
−NADP$^+$	14.2	13.9
$-O_2$	14.1	7.6
NADPH-dependent	85.8	86.1
Boiled	7.9	8.1
SKF-525A (0.1 mM)	89.2	95.4
CO/O_2 (80/20, v/v)	106.0	109.6
Glutathione (4.0 mM)	62.5	58.0

* Substrate concentration, 0.5 mM.

† One hundred percent equals 0.379 ± 0.043 nmoles/mg protein at 60 min and 0.908 ± 0.077 nmoles/mg protein at 240 min.

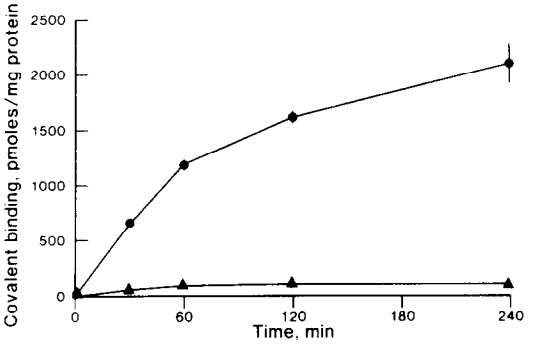

Fig. 3. Time course for NADPH-dependent covalent binding of [^{3}H]PCA to rat liver (●–●) and rabbit liver (▲–▲) microsomal protein.

(data not shown) and NADPH-dependent (Fig. 3) binding of radioactivity to rabbit liver microsomal protein were less than 10% of the values obtained with rat liver microsomal protein under identical incubation conditions. Administration of amphetamine to rats has been reported to produce long-term, possibly neurotoxic effects in dopamine nerve terminals

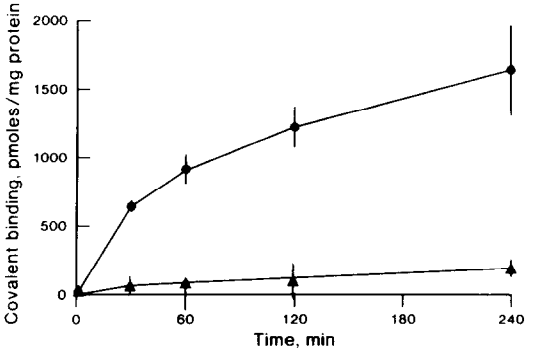

Fig. 4. Time course of NADPH-dependent covalent binding of [^{3}H]PCA (●–●) and [^{3}H]amphetamine (▲–▲) to rat liver microsomal protein.

[22–24]. We compared rat liver microsomal covalent binding of [^{3}H]amphetamine with binding of [^{3}H]PCA. Total (data not shown) and NADPH-dependent covalent binding of radioactivity to microsomal protein observed with [^{3}H]amphetamine were significantly less than observed for [^{3}H]PCA under identical incubation conditions (Fig. 4).

DISCUSSION

The serotonergic neurotoxin PCA was converted by rat hepatic and brain microsomal enzymes to chemically reactive species which covalently bind to microsomal protein. Glutathione markedly reduced covalent binding, consistent with the formation of electrophilic species which can subsequently interact with nucleophilic moieties of microsomal protein and/or small molecules. The requirement for oxygen and NADPH, and inhibition by SKF-525A suggest that cytochrome monooxygenases catalyze the activation reactions.

Inducing agents are often employed to characterize particular cytochromes involved in metabolic pathways. Results with liver preparations suggests that 3-methylcholanthrene-induced cytochrome monooxygenases (often referred to as P-448 or P_1-450) catalyzed PCA metabolic activation rather than phenobarbital-induced cytochrome P-450. Greater inhibition by benzoflavone than metyrapone also implicates cytochrome P-448 rather than cytochrome P-450 in the activation pathway [25, 26]. CO, another inhibitor of cytochrome P-450 enzymes, did not inhibit PCA metabolic activation in liver or brain preparations. However, the failure of CO to inhibit PCA covalent binding under conditions which inhibited cytochrome P-450 catalyzed N-demethylation was not entirely unexpected. Similar results were obtained in preliminary PCA metabolic activation studies [14]. Varied results relating to CO inhibition of rat microsomal metabolism of amphetamines have been reported. Para-hydroxylation of benzamphetamine is inhibited by CO [27], whereas oxidative metabolism of *N*-hydroxyamphetamine is only minimally inhibited by CO [28]. Rat microsomal catalyzed formaldehyde release from methamphetamine is inhibited by CO to a much lesser extent than aminopyrine *N*-demethylase activity in the same preparations [29]. Variations in substrate structure and the multiplicity of enzymes appear to produce a range of CO inhibition of cytochrome monooxygenases which mediate metabolism of amphetamines [29–31].

There was no significant difference in the covalent binding of radioactivity to hepatic microsomal protein between the PCA enantiomers, or between the PCA racemate and enantiomers. The stereoselective nature of the neurotoxicity may therefore be related to stereospecificity in the uptake of PCA enantiomers or PCA enantiomeric metabolites into serotonergic neurons.

While the PCA metabolite responsible for covalent binding to protein is not known, the reactive species may be an electrophilic epoxide formed during aromatic hydroxylation [14], the major route of amphetamine metabolism in the rat [32]. The "NIH-shift" metabolite of PCA, 3-chloro-4-hydroxyamphetamine, has been identified in rat urine

following administration of PCA [33]. Formation of such "NIH-shift" metabolites is considered strong evidence for epoxide formation during aromatic hydroxylation reactions [34]. 3-Methylcholanthrene-inducible monooxygenases, implicated in activation of PCA, are known to convert aromatic compounds to epoxides, some of which form covalent bonds with protein and/or DNA [35]. In contrast to rats, rabbits (which do not exhibit PCA neurotoxicity) only minimally metabolize amphetamines by aromatic hydroxylation [32], and very little covalent binding of [^{3}H]PCA was observed with microsomal preparations from this species. Much less NADPH dependent covalent binding was observed when [^{3}H]amphetamine was incubated with microsomal preparations. This is also consistent with involvement of an epoxide intermediate in the activation pathway. Epoxides formed during hydroxylation of non-halogenated aromatic moieties (such as amphetamine) are highly reactive and rapidly rearrange to phenol metabolites, reducing availability of electrophile for reaction with tissue nucleophiles [34, 35].

The importance of PCA metabolic activation lies in the possible involvement of this pathway in long-term PCA neurotoxicity. Evidence that metabolites may be involved in PCA neurotoxicity includes the lack of *in vitro* enzyme inhibition [10], lack of effects on immature animals [12, 13], and rapid clearance of parent drug from brain [11]. There are additional data in the literature to support involvement of a reactive intermediate in PCA neurotoxicity, and such a mechanism has been suggested by several investigators [14–16]. PCA-induced damage to serotonergic neurons is similar to the damage observed in adrenergic neurons following 6-hydroxydopamine (6-OHDA) administration to rats [7]. There is strong evidence that 6-OHDA neurotoxicity is mediated by oxidation of parent compound and subsequent interaction of reactive oxygen species and/or reactive metabolites with critical proteins in the adrenergic neuron [36, 37]. Similar arguments have been proposed as the basis for the adrenergic and serotonergic neurotoxicity of 5,6-dihydroxyltryptamine [38]. More recently, conversion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to a reactive pyridinium species by brain mitochondrial preparations has been implicated in the nigrostriatal toxicity of this compound [39, 40]. All of these agents, including PCA, are metabolized to reactive intermediates, and all induce long-term, selective neurotoxicity in certain species.

Administration of amphetamine to rats produces long-lasting reductions of dopamine and other indications of neurotoxicity in the striatum [22–24]. Under similar incubation conditions, little NADPH-dependent covalent binding to protein was observed with amphetamine as compared to the NADPH-dependent covalent binding of PCA. The reduced covalent binding may be related to observations that amphetamine neurotoxicity is observed only after continuous infusion to rats for several days, or single injections of large doses of amphetamine in iprindole-treated animals (iprindole prolongs amphetamine exposure after bolus administration). Alternatively, amphetamine neurotoxicity may be mediated by an activation pathway not related to microsomal metabolism, as appears to be the case for MPTP [39, 40].

Additional brain metabolism studies to further characterize its enzymatic pathway are currently underway. *In vivo* studies are being conducted to correlate the radiolabeled covalent binding in brain and long-term neurotoxicity observed in treated animals.

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