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TRYPTOPHAN PRETREATMENT AUGMENTATION OF *p*-CHLOROAMPHETAMINE-INDUCED SEROTONIN AND DOPAMINE RELEASE AND REDUCTION OF LONG-TERM NEUROTOXICITY

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Abstract—The impact of tryptophan (TRP) pretreatment on the neurochemical effects of *p*-chloroamphetamine (PCA) was investigated. The neurotoxic effects of PCA on serotonin (5-HT) neurons, the acute effects of PCA on extracellular 5-HT and dopamine (DA), and the displacement by PCA of whole blood 5-HT were examined. TRP pretreatment (400 mg/kg of the methyl ester) significantly reduced the long-term (1 week) decrease in tissue 5-HT resulting from PCA (2 mg/kg, i.p., of the hydrochloride salt) in the prefrontal cortex and striatum, but not in the dorsal hippocampus. Microdialysis studies in awake animals showed that this pretreatment regimen resulted in augmented PCA-induced increases in extracellular 5-HT (4-fold) and DA (2-fold). TRP pretreatment also resulted in increased displacement of 5-HT from whole blood. The implications of these results toward possible mechanisms of action of PCA-induced neurotoxicity are discussed.

Key words: amphetamine; transporter; *in vivo*; microdialysis; platelets; neuronal; rat

The systemic administration of the amphetamine analogs PCA¶, MDA, and MDMA has long been known to exert multiple effects upon dopaminergic and serotonergic neurotransmitter systems in the rat brain. These include an acute release of 5-HT [1–7] and DA [6, 8–11] and an acute decrease in TRP hydroxylase activity [12–15]. These acute changes are followed by the long-term degeneration of 5-HT axon terminals [14, 16–21]. The degeneration caused by these three analogs appears to be similar, and can be evidenced by loss of tissue 5-HT content, TRP hydroxylase activity and 5-HT neuronal uptake.

While the phenomena associated with PCA/MDA/MDMA neurotoxicity are well known, an elucidation of the underlying mechanisms remains less clear [22]. The mechanisms are of interest in that they may yield insight into more general mechanisms of neurotoxicity and neurodegeneration. The most consistent line of evidence (see Discussion) points to an involvement of DA in the neurotoxicity of these compounds. However, there is also evidence indicating that endogenous 5-HT may be the source of a toxic agent formed following PCA administration [23], agreeing with the recent demonstration by extensive depletions with *p*-chlorophenylalanine and reserpine [24, 25] that a releasable pool of 5-HT is required for PCA-induced damage to 5-HT neurons. Based upon this evidence, it has been proposed that a toxic metabolite of 5-HT, either directly, or in combination with a dopaminergic mechanism, may be necessary for the expression of PCA toxicity toward 5-HT neuronal terminals [23–25]. We have demonstrated previously the ability of TRP to potentiate MDMA-induced 5-HT release from an *in vitro* midbrain slice preparation [26]. Given an ability to manipulate the extent to which amphetamine analogs release 5-HT using TRP augmentation, we posed the following questions, using PCA as the model neurotoxic agent: (1) What is the impact of TRP pretreatment upon PCA-induced degeneration of 5-HT neurons as assessed by whole tissue content of 5-HT? (2) What is the impact of TRP pretreatment upon PCA-induced 5-HT and DA release in the brain *in vivo*? (3) Does TRP pretreatment alter the ability of PCA to release 5-HT from whole blood *ex vivo*?

By addressing these questions, we hoped to increase information relevant to the mechanism of amphetamine analog neurotoxicity. Because the mechanisms proposed for the neurotoxicity induced by the amphetamine analogues have included a role for neuronal DA, it was proposed to measure the impact of TRP pretreatment on PCA-induced DA release as well as 5-HT. This would allow study of interactions between the DA and 5-HT systems in addition to an elucidation of the relevance of DA release to PCA-induced neurotoxicity.

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¶ Abbreviations: PCA, *p*-chloroamphetamine; TRP, tryptophan; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; DA, dopamine; 5-HT, serotonin; PFC, prefrontal cortex; STR, striatum; and HIP, hippocampus.

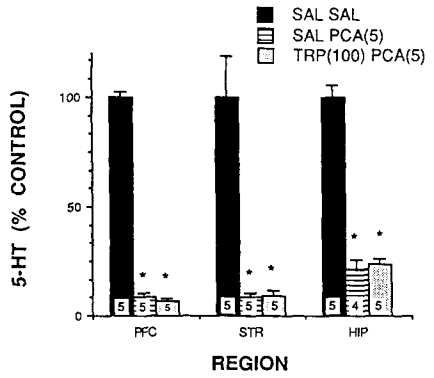

Fig. 1. Effect of TRP pretreatment on PCA (5 mg/kg, i.p.) induced decrease in tissue 5-HT, 1 week following treatment. TRP methyl ester (100 mg/kg, i.p.) was administered 20 min prior to PCA. Key: (*) $P < 0.05$ vs sal-sal. One hundred percent equals the mean (±SEM) value of 48.0 ± 1.1 pmol 5-HT/mg protein for PFC, 20.0 ± 3.7 for STR, and 8.2 ± 0.5 for HIP. The numbers of animals are shown in each bar.

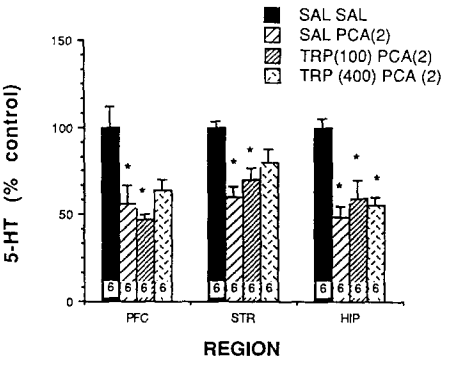

Fig. 2. Effect of TRP pretreatment on PCA (2 mg/kg, i.p.) induced decrease in tissue 5-HT, One week following treatment. TRP methyl ester (100 and 400 mg/kg) was administered as in Fig. 1. Key: (*) $P < 0.05$ vs sal-sal. One hundred percent equals the mean (±SEM) value of 24.5 ± 2.9 pmol 5-HT/mg protein for PFC, 21.5 ± 0.7 for STR, and 9.1 ± 1.2 for HIP.

MATERIALS AND METHODS

In all studies, male Sprague–Dawley rats (obtained from CAMM) were used. Rats were housed two or three per cage, given free access to standard rat chow and water, and maintained on a 12-hr light schedule in a temperature-controlled colony room. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

5-HT depletion studies. In each experiment, the animals of the different treatment and/or dosage groups were obtained at the same time and housed in the same room. Animals were pretreated with TRP or saline, followed 20 min later by PCA. Dosages were of the hydrochloride salt of PCA, and the methyl ester of TRP. Animals were decapitated 1 week following PCA treatment, and the PFC, HIP, and STR were dissected on ice. The dissected tissue samples were frozen rapidly on dry ice and stored at −80° until analysis. Tissue 5-HT and 5-HIAA (5-hydroxyindoleacetic acid) contents were determined using liquid chromatography with electrochemical detection [27]. The methods of analysis were essentially those of Reinhard and Roth [28] with minor modifications. *N*-Methylserotonin was used as the internal standard. Statistical significance for differences between treatment groups was determined by one-way ANOVA followed by a Bonferroni test for multiple comparisons.

In vivo *microdialysis studies.* Surgery was performed upon animals anesthetized with halothane. Body temperatures were maintained at 37° using a heating pad. Each rat was placed in a stereotaxic frame,the skull was exposed, and a burr hole was drilled through the cranium above the striatum. The microdialysis probe was then inserted using the following stereotaxic co-ordinates for the probe tip: 0.5 mm anterior to bregma, 2.8 mm lateral to the midline, and 8 mm below the surface of the dura. The probe was fixed in place with dental cement and the animal was allowed to recover from the procedure overnight during which period the probe was perfused at a flow rate of 0.5 μL/min. The collection of dialysates was initiated the following day.

Microdialysis probes of a concentric design, similar to others previously reported [29], were constructed as previously described [30] using Cuprophan (Enka, Germany) hollow fibers (300 μm i.d., 330 μm o.d.) housed in a section of 23-gauge (0.64 mm o.d.) hypodermic tubing. The fiber extended approximately 5.5 mm beyond the tip of the tubing, exposing an active length of about 5 mm. Perfusion buffer (in mM: KCl 2.4, NaCl 137, $CaCl_2$ 1.2, $MgCl_2$ 1.2, NaH_2PO_4 0.9, ascorbic acid 0.3; pH 7.4) was pumped through a section of vitreous silica (73 μm i.d., 140 μm o.d., Polymicro Technologies, Phoenix, AZ), which extended to the tip of the hollow fiber. Collection periods were fixed at 20-min intervals with the flow rate set at 2 μL/min. Probes were calibrated *in vitro*, and the resulting percentage recoveries were used to correct for differences between probes. Basal values of DA and 5-HT reported herein are corrected for probe recovery, but are not presumed to represent true extracellular values because of well-documented differences between *in vitro* and *in vivo* probe recoveries.

For liquid chromatographic determination of neurotransmitter levels in the brain dialysates, microbore columns (100 × 1 mm i.d. packed with 3 μm C-18 particles, Bioanalytical Systems) were used in conjunction with a thin-layer amperometric detection electrode assembly and potentiostat (E_{app}: + 0.6 V vs Ag/AgCl reference, Bioanalytical Systems). A pneumatic fluid-displacement pump [31] was used to pump the mobile phase through the column, providing extremely smooth flow and isolation from electrical noise. The composition of the mobile phase was, for DA: 0.07 M NaH_2PO_4, 440 mg/L sodium octanesulfonate, 0.1 mM disodium

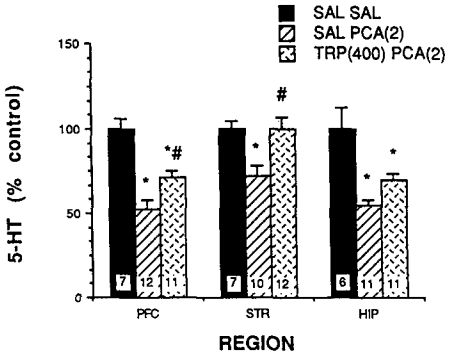

Fig. 3. Effect of 400 mg/kg TRP pretreatment on PCA (2 mg/kg) induced decrease in tissue 5-HT, 1 week following treatment. Key: (*) $P < 0.05$ vs sal-sal; and (#) $P < 0.05$ vs sal-PCA. One hundred percent equals the mean (±SEM) value of 34.2 ± 2.1 pmol 5-HT/mg protein for PFC, 30.6 ± 1.4 for STR, and 9.6 ± 1.2 for HIP.

EDTA, and 60 mL/L acetonitrile; for 5-HT: 0.07 M NaH_2PO_4, 750 mg/L sodium octane sulfonate, 0.1 mM disodium EDTA, 80 mL/L acetonitrile and 10 mL/L tetrahydrofuran. The pH was unadjusted and was 4.5 for the DA system and 4.6 for the 5-HT system. The limit of detection attainable was routinely between 2 and 5 fmol for both monoamines. Rat brain dialysates were divided into two portions for the analysis of DA and 5-HT on separate analytical columns. Statistical significance of the impact of TRP pretreatment was tested for by two-way repeated measures ANOVA.

Ex vivo *whole blood studies.* Sixty minutes following pretreatment with TRP (400 mg/kg, i.p.), animals were deeply anesthetized with halothane, and whole blood was removed by cardiac puncture. It was placed into heparinized tubes, and then transferred to microcentrifuge vials containing sufficient disodium EDTA to make the final concentration 1.5 mg/mL in order to prevent platelet aggregation. A microdialysis probe constructed as described above, but with 1 cm exposed length, was placed in the blood and perfused for two 10-min collection periods to determine basal free 5-HT concentration. Any blood samples with free 5-HT concentrations (based on *in vitro* probe calibration) greater than 4 nM were not used because of concerns that platelet aggregation might have been triggered by the collection procedure prior to the addition of EDTA.

RESULTS

TRP impact on PCA-induced decrease in tissue 5-HT. Our initial study utilized a dose of 5 mg/kg PCA, i.p., combined with pretreatment doses of 100 and 400 mg/kg TRP given i.p. 20 min prior to PCA. Under these conditions, TRP enhanced the lethality of PCA in that five of the six animals receiving the 400 mg/kg TRP pretreatment did not survive the 7-day period prior to being killed. The results from the other three groups are presented in Fig. 1. As can be seen, there was greater than 90% depletion of 5-HT in the STR and PFC, while in HIP there was an approximately 80% depletion. In each region, there was no impact of 100 mg/kg TRP pretreatment. In a separate study, 5-HT levels in animals that received only TRP at 400 mg/kg were not impacted significantly at the 1-week time point (data not shown).

In subsequent experiments, a lower dose of PCA (2 mg/kg, i.p.) was administered. Figure 2 presents the results from an experiment containing both 100 and 400 mg/kg TRP pretreatment groups. There was significantly less neurotoxicity resulting from the lower dose of PCA, and the 400 mg/kg pretreatment did not induce any lethality. In both the PFC and STR, the 400 mg/kg pretreated group showed

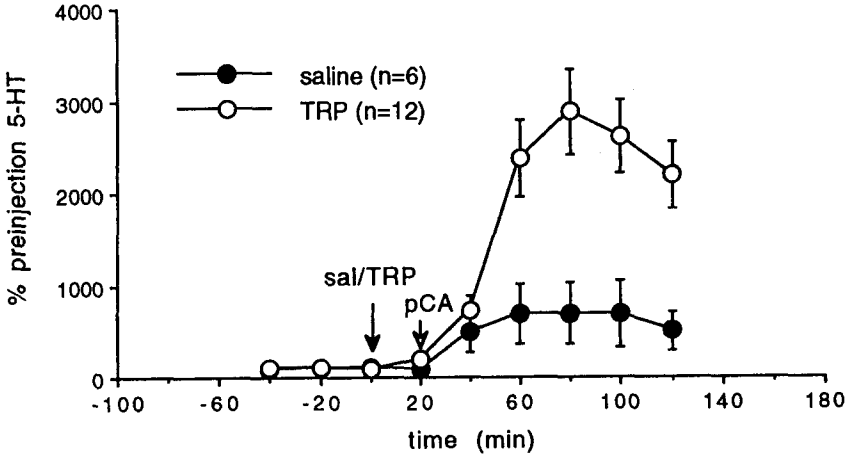

Fig. 4. Impact of TRP pretreatment (400 mg/kg, i.p.) on PCA (2 mg/kg, i.p.) induced increases in striatal extracellular 5-HT. Mean (±SEM) basal level of 5-HT was 0.82 ± 0.16 fmol/μL (corrected for probe recovery).

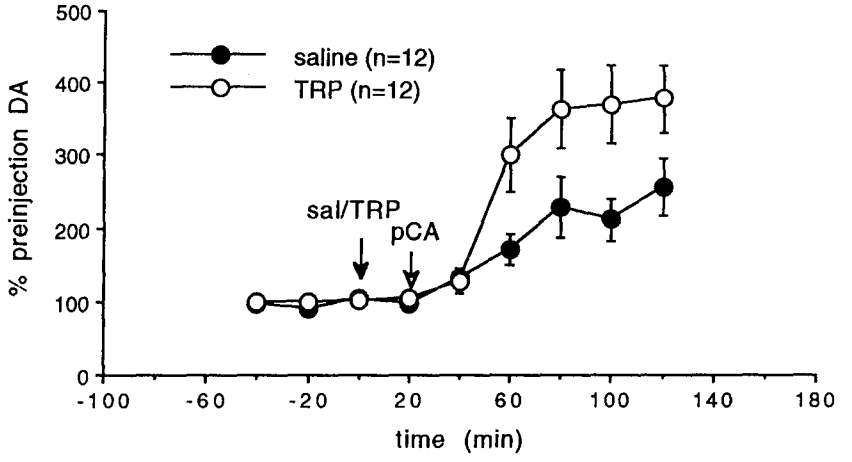

Fig. 5. Impact of TRP pretreatment (400 mg/kg, i.p.) on PCA (2 mg/kg, i.p.) induced increases in striatal extracellular DA. Mean (±SEM) basal level of DA was 23.7 ± 4.4 fmol/μL (corrected for probe recovery).

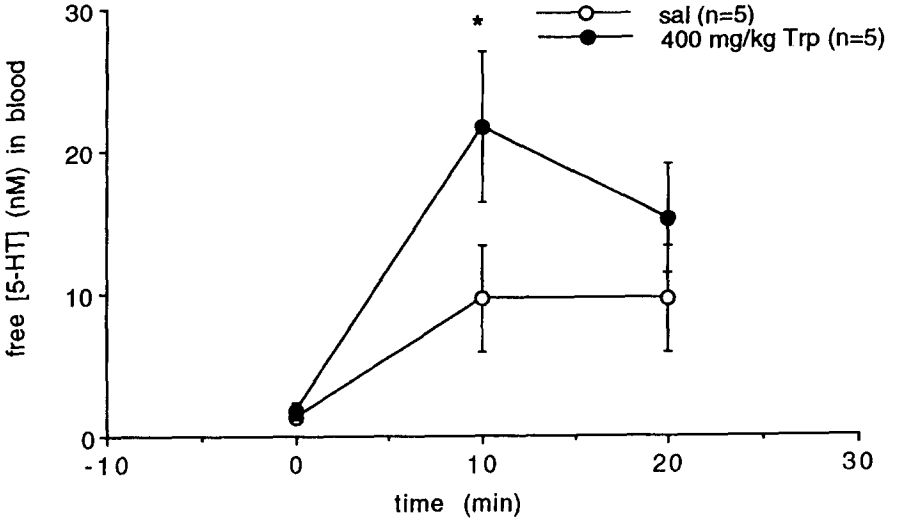

Fig. 6. Effect of TRP methyl ester pretreatment (400 mg/kg, i.p., 60 min prior to blood collection) on PCA (1.0 μM) induced displacement of 5-HT from whole blood. Values are means ± SEM. Key: (*) $P < 0.05$ (one-tailed t-test).

reduced 5-HT depletion, in that the remaining 5-HT levels did not differ from the control (saline-pretreated) values.

To clarify the effects of TRP treatment, a subsequent experiment with an increased number of animals was conducted, omitting the 100 mg/kg pretreatment dose. The results are presented in Fig. 3. The trends seen in the previous experiment were reproduced in a more clearly defined manner. The 400 mg/kg pretreatment group was significantly different from the saline-pretreated group in the PFC and HIP, with a complete reversal of toxicity in STR. As in the previous experiment, there was no reversal of toxicity in the HIP by TRP pretreatment.

TRP impact on PCA-induced release of DA and 5-HT in vivo. Figures 4 and 5 illustrate the effect of TRP pretreatment (400 mg/kg, i.p.), 20 min before the administration of PCA (2 mg/kg, i.p.), on the release of striatal 5-HT and DA induced by PCA. As can be seen in Fig. 4, TRP pretreatment significantly enhanced the ability of PCA to induce 5-HT release. Release caused by PCA was increased from 7 to 28 times over baseline by TRP pretreatment, a 4-fold increase. In addition, a comparatively smaller increase (almost a doubling) was observed in the PCA-induced increase in extracellular DA (Fig. 5). In each case, the increase induced by TRP was statistically significant ($P < 0.05$ by two-way repeated measures ANOVA). Maximal release of

neurotransmitter was observed in 80 min for 5-HT, whereas DA was still increasing 100 min following the administration of PCA.

TRP impact of PCA-induced release of 5-HT from whole blood. TRP pretreatment 60 min prior to blood collection moderately increased the ability of PCA (1.0 μM) to release 5-HT from whole blood ($P < 0.05$ by one-tailed t-test). Release was maximal in the first 10-min sample after adding PCA, as can be seen in Fig. 6.

DISCUSSION

There are a number of potential mechanisms of amphetamine analog neurotoxicity toward 5-HT neuronal terminals. The effects of PCA, MDA, and MDMA are very similar, and appear to operate through similar mechanisms [6, 14, 32]; thus, in the discussion to follow, evidence of mechanisms of toxicity obtained from each of these compounds will be referred to. It has been thought for some time (see Ref. 22 for review) that the metabolism of PCA to a toxic metabolite might occur because direct intracerebral injections fail to produce degeneration of 5-HT axons [33]. Efforts to identify toxic metabolites of PCA have not been successful [22]. The trihydroxylated metabolites of MDA [34] and MDMA [35, 36] have been shown to cause damage to 5-HT neurons. However, they also appear to cause the degeneration of catecholamine neurons, thus lacking the specificity of the parent amphetamine analogs. It has also been proposed [23–25] that systemically administered PCA may induce the formation of a toxic metabolite from an endogenous substance. In particular, this hypothesis suggests 5-HT and/or a metabolite of the neurotransmitter as a likely candidate for this endogenous substance in that extensive depletion of central and peripheral stores of 5-HT prior to the administration of PCA blocked the neurotoxicity induced by PCA.

Irrespective of what the toxic species is (PCA, a metabolite, or an endogenously derived agent), there is a large body of evidence for the involvement of DA in the neurotoxicity toward 5-HT neurons. Inhibitors of DA uptake prevent carrier-mediated release by amphetamine analogs [37–40], and Stone *et al.* [15] showed that pretreatment with the specific DA uptake inhibitor GBR-12909 blocks the long-term MDMA-induced toxicity. DA synthesis inhibition (the DA released comes primarily from the newly synthesized pool) also blocks the toxicity [15, 41], as does depletion of endogenous DA by lesion of the cell bodies [15]. Augmenting DA synthesis with L-DOPA increases the toxicity [42]. Comparisons of toxic and nontoxic amphetamine analogs suggest that a key to toxicity lies in the ability to interact with both 5-HT and DA transporters [43]. Thus, it appears that DA must increase in the extracellular space for damage to occur, as those treatments that block PCA-induced increases in extracellular DA also block the toxicity. The most significant shortcoming of this hypothesis is that the degree of depletion by PCA is not related to the density of dopamine innervation to particular brain regions. However, regional specificity is seen in the ability of DA lesions to prevent toxicity [15]. Substantia nigra 6-hydroxydopamine lesions prevent toxicity in the striatum, but not in the hippocampus or cortex, consistent with the projection fields of the substantia nigra DA cell bodies.

It has long been known that the administration of 5-HT uptake blockers [6, 44–46], even at times significantly after PCA, can also block the toxicity toward 5-HT terminals. The uptake blockers also inhibit the release of 5-HT [6, 26], a process that appears to be a carrier-mediated one [47]. These effects do not appear to be due to the prevention of entry of the amphetamine analog into the neuron [6, 47]. It has been proposed [48] that the manner in which the 5-HT uptake blockers prevent the toxicity is by preventing the entry of DA into the 5-HT terminal via the 5-HT uptake carrier. Thus, despite a PCA-induced increase in extracellular DA, it is unable to enter the 5-HT neurons and cause damage, perhaps through a destructive oxidation process (discussed in Ref. 15).

TRP, a 5-HT precursor, is known to increase the synthesis, storage and metabolism of 5-HT [49]. Our previous *in vitro* studies using MDMA [26, 50] suggested the potential capability of TRP to alter PCA-induced release *in vivo*. Given the hypothesis linking the neurotoxic effects of amphetamine analogs to the release of 5-HT [23–25, 51], we determined the impact of TRP pretreatment upon the neurotoxicity of PCA, and on extracellular striatal DA and 5-HT, as well as the ability of PCA to displace 5-HT from whole blood.

The results of our studies indicate that TRP pretreatment is able to prevent some of the toxicity of PCA toward 5-HT neurons in certain brain regions (Figs. 2 and 3). Under an administration regimen that was protective of 5-HT neurons, there was a 4-fold increase in PCA-induced increases in extracellular 5-HT (Fig. 4). There was also almost a doubling of PCA-induced increases in extracellular DA (Fig. 5). We believe that this TRP-induced increase in DA release by PCA is an indirect result of the potentiation of 5-HT release, as there was no impact of TRP at all on basal DA at the time point immediately following TRP (prior to PCA). It has been shown by us [52] and others [53–55] that local infusion of 5-HT into the striatum or nucleus accumbens causes an increase in extracellular DA. Thus, the very significant increase in extracellular 5-HT seen with TRP pretreatment is probably mediating the DA increase either through receptor [54, 55] or reuptake site [56] mechanisms. We also saw a significant increase in PCA-induced displacement of 5-HT from whole blood obtained from animals 60 min after administration of 400 mg/kg TRP (Fig. 6). It has been suggested that the release of peripheral 5-HT stores may be involved in generating a toxic agent [24, 25]. Others have also shown that PCA is able to release 5-HT from whole blood [57].

The mechanism of TRP enhancement of PCA-induced 5-HT release is unclear, but does not appear to be due to a direct effect of TRP on 5-HT release. Our previous *in vitro* studies in the raphe slice showed that TRP was able to increase MDMA-induced 5-HT release without altering basal 5-HT release. Also, while some workers have shown that

TRP administration *in vivo* (up to 200 mg/kg) causes a small increase in 5-HT release [58–60], these increases are on the order of a 50–100% increase over baseline, as opposed to the effect here in which PCA-induced release went from 700% of baseline to 2800% of baseline. Other workers have not been able to detect a TRP-induced increase in 5-HT release [61, 62]. In the one sample post TRP (prior to PCA) in the present study, we saw a slight increase in 5-HT, which was non-significant. TRP has been shown to increase whole tissue levels of 5-HT [49], though the observed increase is not as dramatic as the increase in PCA-induced 5-HT release. However, TRP administration may cause a large increase in the newly synthesized non-vesicular pool of 5-HT from which PCA induces release [63], and this relatively larger increase is masked by the large bound stores of 5-HT that make up the bulk of the total tissue content. One of the acute effects of PCA is to cause a significant decrease in TRP hydroxylase activity [12, 17]. Thus, one would not expect a great deal of 5-HT synthesis to be occurring after PCA administration. It would be of interest to know what impact the TRP pretreatment has on the hydroxylase activity following PCA. PCA also is known to inhibit monoamine oxidase (MAO). Previous studies [62] have indicated that complete inhibition of MAO A and B is able to increase extracellular concentrations of 5-HT 4- to 5-fold. In one instance, coadministration of 50 mg/kg TRP (with 20 mg/kg tranylcypromine) had no effect on the increase in extracellular 5-HT, while that dose in combination with a different MAO inhibitor (2 mg/kg MDL 72394) substantially augmented the increased extracellular 5-HT resulting from MAO inhibition [62]. Thus, it is possible that the TRP enhancement of PCA-induced 5-HT release may relate to an action on MAO.

The mechanism(s) through which TRP ameliorates PCA neurotoxicity is also unclear. On face value, previous hypotheses of DA and/or 5-HT dependent mechanisms would suggest that TRP pretreatment should increase the damage to 5-HT neurons, rather than decrease it. Not only did the TRP pretreatment increase release of 5-HT from central stores, but also from peripheral stores as indicated by the whole blood study. It also increased the release of DA in the striatum *in vivo*. Antioxidant or radical scavenging properties of TRP, itself, or its many metabolites [64–66] are one possible mechanism of protection. The long-lasting depletions of DA and 5-HT produced by methamphetamine can be attenuated by pretreatment with antioxidants such as mannitol, ascorbic acid and vitamin E [67]. In addition, the same study reports an exacerbation of the toxic effect as a result of pretreatment with the superoxide dismutase inhibitor diethyldithiocarbamate. There is also a report of cysteine inhibition of PCA toxicity [68], though a later study suggested that the inhibition of toxicity by cysteine was due to altered pharmacokinetics [69].

Another potential protective mechanism of TRP pretreatment is competition between DA and 5-HT for entry into the 5-HT terminal via the 5-HT uptake carrier. It has been proposed [48] that the manner in which the 5-HT uptake blockers prevent PCA toxicity is by preventing the entry of DA into the 5-HT terminal via the 5-HT uptake carrier. Thus, despite a PCA-induced increase in extracellular DA, it is unable to enter the 5-HT neurons and cause damage, perhaps through a destructive oxidation process [15, 48, 70]. As a result of TRP pretreatment, the 5-HT/DA extracellular ratio following PCA is increased by a factor of 2.8, as estimated from the differences in percent increase of each compound caused by PCA with and without TRP. Thus, despite the fact that TRP pretreatment causes an enhancement of PCA-induced DA release, it increases 5-HT release even more, with the result that DA may be entering the 5-HT terminals in smaller amounts than when PCA is administered without TRP. It is not possible to predict precisely what ratio of 5-HT/DA would be necessary to block the uptake of a given concentration of DA by 5-HT neurons, because the measured levels in microdialysates are not synaptic concentrations, and exact affinity constants of the 5-HT uptake carrier for DA have not been determined.

In summary, we have shown that TRP pretreatment (400 mg/kg, 20 min prior) is able to attenuate the toxicity of 2 mg/kg PCA. Under these conditions, there was a significant enhancement of PCA-induced release of 5-HT and DA in the striatum and in the displacement of 5-HT from whole blood. The mechanism of protection may involve the antioxidant activity of TRP, 5-HT or other metabolites of TRP. Another possible mechanism is a competition between 5-HT and DA for entry, via uptake sites, into 5-HT neurons.

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