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LONG-TERM EFFECTS OF CONTINUOUS EXPOSURE TO *p*-CHLOROAMPHETAMINE ON CENTRAL SEROTONERGIC MECHANISMS IN MICE*

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Abstract—Consistent with its effects in rats, the i.p. administration of 45 mg/kg of *p*-chloroamphetamine (PCA) to mice produced decreases in brain levels of 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid and tryptophan hydroxylase activity at early times after its administration. However, in contrast to the long-lasting nature of these reductions in rats, substantial recovery was evident within 2 weeks in mice. Regional analyses indicated that the hippocampus and remaining telencephalon were most sensitive to the PCA-induced decrease in 5-HT levels; however, complete recovery was observed 1 month after the administration of 45 mg/kg of PCA even in these relatively sensitive areas. These data are consistent with previous results indicating that PCA does not produce long-term, neurotoxic effects on serotonergic neurons in mice. However, the continuous release of PCA from subcutaneously implanted ALZET Osmotic Minipumps for a period of 3 days produced decreases in brain levels of 5-HT which lasted for at least 4 weeks. This finding suggests that the insensitivity of mice to the neurotoxic effects of PCA is related to its relatively short half-life in this species.

The administration of *p*-chloroamphetamine (PCA) to rats causes rapid and pronounced decreases in the brain levels of 5-hydroxytryptamine (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), as well as decreases in the activity of the rate-limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase, and in the high-affinity uptake of 5-HT [1–4]. These effects are extremely long lasting in rats and have been observed as long as 4 months after a single injection of PCA [1]. However, in mice, the actions of PCA on 5-HT mechanisms are either absent or relatively short-lived [5, 6]. Thus, the administration of PCA produces rapid decreases in the brain levels of 5-HT and 5-HIAA [6] which disappear within 1 day after moderate doses [5, 6]. These findings have generally been taken as evidence that the long-term decreases in 5-HT functions which occur in rats after PCA administration [1–4], and perhaps reflect a cytotoxic action on 5-HT neurons, are not manifested in mice. Recent data [7] from our laboratory showing that the brain levels of 5-HT and the activity of tryptophan hydroxylase are unchanged 3 weeks after very high doses (75 mg/kg) of PCA are consistent with this conclusion.

The present experiments were designed to characterize further the effects of PCA on serotonergic neurons in mice. The half-lives of PCA in mouse body and brain are approximately one-third to one-fifth those in rat tissues, and Miller *et al.* [6] proposed that the rapid disappearance of PCA in mice (perhaps reflecting a relatively rapid rate of metabolism) might explain their apparent insensitivity to the long-term effects of the drug. Experiments were designed to test this hypothesis by examining the effects of prolonged, continuous administration of PCA on brain levels of 5-HT and on the high affinity uptake of 5-HT in mice.

MATERIALS AND METHODS

Experimental animals and drugs. Male albino mice (20–25 g) were purchased from Harlan Industries, Inc. (Cumberland, IN) and housed five per cage with constant access to food and water. A 12-hr light–dark cycle was maintained in the animal quarters. (±)-*p*-Chloroamphetamine hydrochloride (PCA) was obtained from Regis Chemical Co. (Chicago, IL). The dose of intraperitoneally administered PCA is expressed as the base and was injected in a volume of 10 ml/kg.

Biochemical assay. 5-HT and 5-HIAA were assayed by fluorescence after derivation with *o*-phthalaldehyde [8] according to the method of Curzon and Green [9]. Tryptophan hydroxylase activity was measured by a modification of the method of Gal and Patterson [10] as described in detail previously [7]. The *in vitro* uptake of [^{3}H]-5-HT was measured by a modification of the method of Snyder and Coyle [11] as described previously [2]. Brain and body levels of PCA were measured by the gas chromatographic method of Sekerke *et al.* [12] as modified by Steranka and Sanders-Bush.†

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† L. R. Steranka and E. Sanders-Bush, *J. Pharmac. exp. Ther.* in press, 1978.

In the regional study, brains were dissected into four regions: brain stem, including tissue caudal to a plane extending from the anterior border of the pons to the anterior border of the superior colliculi; diencephalon, including hypothalamus, thalamus and adjacent structures; hippocampus; and remaining telencephalon, including cerebral cortex and basal ganglia.

Continuous administration of PCA. PCA was administered continuously by means of ALZET Osmotic Minipumps (ALZA, Palo Alto, CA), which are miniature, implantable pumps advertised by the manufacturer to release their contents (170 μl total volume) at a constant rate of approximately 1 μl/hr over a period of 7 days. Minipumps were filled with a solution of 60 mg PCA (expressed as the base)/ml of saline, implanted subcutaneously under the skin of the back during ether anesthesia, and removed 3 days later, again during ether anesthesia. Mice were housed individually during the 3-day treatment period to avoid injury from cage mates.

Statistical analyses. The results were analyzed by means of two-tailed *t*-tests for experiments involving single or orthogonal comparisons and Dunnett's tests [13] for experiments involving multiple comparisons with a single control mean. The half-lives of PCA in brain and body were calculated from estimates of the first-order rate constants, which were derived from least squares best-fitting straight line analyses of the natural logarithms of the tissue values.

RESULTS

Effects of a single dose of PCA on brain levels of 5-HT, 5-HIAA and tryptophan hydroxylase activity. Mice were injected i.p. with either saline or 45 mg/kg of PCA and killed at various times after injection. As shown in Table 1, the levels of 5-HT in brain were reduced at all times, although substantial recovery was evident by 14 days. The time course of the reduction of 5-HIAA levels and tryptophan hydroxylase activity was similar, except for an intermediate recovery at 30–48 hr.

Regional distribution of 5-HT levels after PCA administration. Mice were injected i.p. with either saline or 45 mg/kg of PCA and killed 1 week or 1 month later. Brains were dissected into four regions and the levels of 5-HT were determined (Table 2). One week after a single dose of PCA, 5-HT levels were markedly reduced in the hippocampus and slightly reduced in the remaining telencephalon. However, significant changes were not observed in either the brain stem or the diencephalon. After 1 month, 5-HT levels had returned to control values in all four brain regions.

Table 1. Levels of 5-HT, 5-HIAA and tryptophan hydroxylase activity in mouse brain at various times after PCA administration*

Time after injection	5-HT	5-HIAA	Tryptophan hydroxylase activity
	Per cent of control ± S. E. M.		
6 hr	69.7 ± 6.9†	62.5 ± 3.0†	51.4 ± 2.0†
18 hr	47.7 ± 3.7†	45.0 ± 8.1†	
24 hr	44.0 ± 3.2†	59.5 ± 8.0†	60.4 ± 11.2
30 hr	57.0 ± 3.2†	72.0 ± 7.0	33.3 ± 2.2†
48 hr	45.5 ± 2.0†	57.9 ± 3.9†	71.2 ± 10.2
7 days	65.8 ± 4.9†	79.8 ± 8.6†	69.5 ± 14.8
14 days	80.0 ± 3.3†	79.1 ± 5.2‡	89.4 ± 5.8

* Mice were injected i.p. with either saline or 45 mg/kg of PCA and killed at various times after injection. The values reported represent the mean per cent of controls ± S. E. M. of three to eight animals. Means ± S. E. M. for all control animals were: 5-HT, 0.92 ± 0.06 μg/g; 5-HIAA, 0.44 ± 0.02 μg/g; and tryptophan hydroxylase activity, 2.92 ± 0.22 nmoles/mg/hr.
† $P < 0.01$.
‡ $P < 0.05$.

Tissue levels and half-life of PCA after its continuous release from subcutaneously implanted minipumps. Minipumps containing 60 mg/ml of PCA in saline were implanted subcutaneously into mice under light ether anesthesia. In order to determine the brain and body levels of PCA, groups of mice were killed 1, 2 or 3 days later. As shown in Table 3, the brain levels of PCA reached a steady state concentration of 12–15 μg/g in 2 days. The concentration of PCA in the remainder of the body varied from 4 to 6 μg/g during this time.

In order to determine the half-life of PCA under these conditions, the minipumps were removed 3 days after implantation and groups of mice were killed 1, 2, 4 or 6 hr later. As shown in Fig. 1, the levels of PCA in brain and body declined in a monoexponential manner with a half-life of 5.1 and 6.2 hr respectively. Since these values were

Table 2. Regional distribution of 5-HT in mouse brain after PCA administration*

Time after injection	Telencephalon	Hippocampus	Diencephalon	Brain stem
1 week	82.1 ± 5.3†	58.7 ± 5.7‡	90.6 ± 11.5	79.6 ± 8.4
1 month	91.6 ± 4.0	89.5 ± 9.4	90.7 ± 6.7	81.5 ± 5.6

* Mice were injected i.p. with either saline or 45 mg/kg of PCA and killed either 1 week or 1 month after injection. The values shown represent the mean per cent of controls ± S. E. M. of five to eight animals. Mean values for the 1-week and 1-month controls, respectively, were: telencephalon, 0.52 ± 0.02 and 0.75 ± 0.03 μg/g; hippocampus, 0.67 ± 0.04 and 0.58 ± 0.09 μg/g; diencephalon, 0.76 ± 0.09 and 0.47 ± 0.06 μg/g; and brain stem, 0.69 ± 0.08 and 0.87 ± 0.08 μg/g.
† $P < 0.05$.
‡ $P < 0.01$.

Table 3. Levels of PCA during 3 days of continuous administration*

Time (days)	Brain PCA (μg/g)	Body PCA (μg/g)
1	4.23 ± 0.85†	2.63 ± 0.23†
2	12.0 ± 1.7	6.05 ± 0.69‡
3	15.5 ± 1.61	3.84 ± 0.35

* Minipumps containing 60 mg/ml of PCA in saline were implanted subcutaneously, and groups of four mice were killed either 1, 2 or 3 days later. The results are reported as the means ± S. E. M.
† $P < 0.01$, compared with 2-day group.
‡ $P < 0.01$, compared with 3-day group.

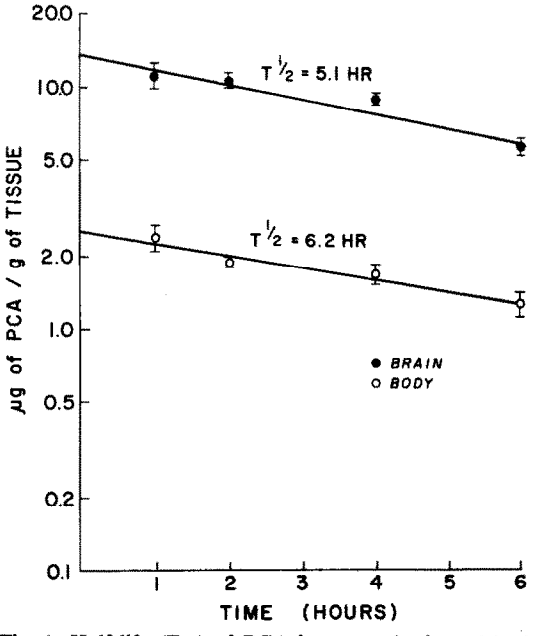

Fig. 1. Half-life ($T_{1/2}$) of PCA in mouse brain and body after 3 days of continuous administration. Minipumps containing 60 mg/ml of PCA in saline were implanted subcutaneously, removed after 3 days, and groups of four mice were killed 1, 2, 4 or 6 hr later. The values shown are the means ± S. E. M.

substantially longer than reported previously [6], the half-life of PCA was determined in similar animals after its intravenous administration. Mice were injected i.v. with 7.5 mg/kg of PCA and killed 1, 2, 4 or 6 hr later. Consistent with previous data [6], the half-life of PCA was 2.5 and 2.6 hr in brain and body respectively (Fig. 2).

Long-term effects of the continuous administration of PCA on 5-HT levels and synaptosomal uptake activity. As described above, PCA was administered by means of continuous, subcutaneous release from the minipumps for a period of 3 days. The pumps were removed and groups of mice were killed 1, 14, 28 or 56 days later for the determination of either 5-HT levels or 5-HT uptake capacity (Table 4). In contrast to the small, often insignificant effects of single doses of PCA 2 or 3 weeks later (Ref. 7 and present results), the brain levels of 5-HT were maximally reduced 28 days after the removal of the minipumps.

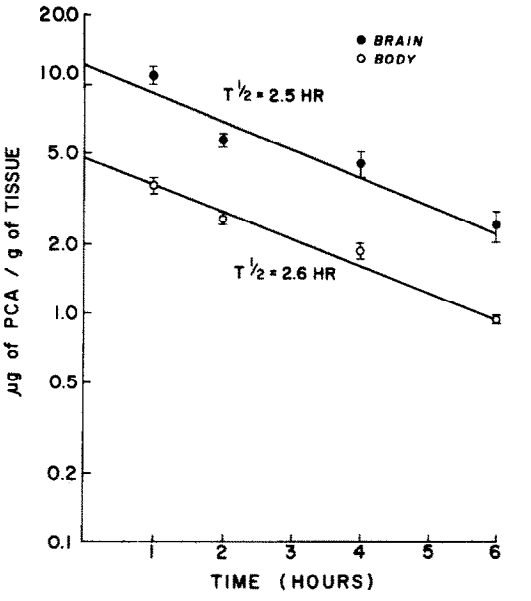

Fig. 2. Half-life ($T_{1/2}$) of PCA in mouse brain and body after its acute administration. Groups of four mice were injected intravenously (tail vein) with 7.5 mg/kg of PCA and killed 1, 2, 4 or 6 hr later. The values shown are the means ± S. E. M.

Table 4. Effects of the continuous administration of PCA on brain levels of 5-HT and 5-HT uptake activity in mice*

Time after minipump removal (days)	5-HT	5-HT uptake
	Per cent of control	
1	41.7 ± 2.4†	
14	44.5 ± 1.7†	50.9 ± 3.5†
28	50.9 ± 1.3†	
56	78.7 ± 2.5†	

* Minipumps containing 60 mg/ml of PCA in saline were implanted subcutaneously, removed after 3 days, and groups of mice (five to six) were killed 1, 14, 28 or 56 days later. The results are expressed as the mean per cent of control animals killed at the same time. The mean ± S. E. M. of control mice was 0.74 ± 0.3 μg/g for 5-HT and 0.836 ± 0.043 pmoles 5-HT/mg of protein/5 min for 5-HT uptake.
† $P < 0.01$.

Moreover, although some recovery was evident, 5-HT levels remained significantly reduced 56 days later. In addition, 5-HT uptake capacity was dramatically decreased 2 weeks after the 3-day exposure to PCA.

DISCUSSION

The present results confirm and extend those of previous studies [6, 7] of the effects of PCA on brain levels of 5-HT and 5-HIAA in mice. In addition to decreases in these parameters, the administration of 45 mg/kg of PCA also caused a large decrease in the activity of cerebral tryp-

tophan hydroxylase. However, in contrast to the long-lasting nature of these reductions in rats [1–4], the present results demonstrate substantial recovery in these parameters within 1–2 weeks after the administration of PCA to mice. Moreover, complete recovery of 5-HT levels and tryptophan hydroxylase activity were observed within 3 weeks after a dose of 75 mg/kg of PCA [7]. Analyses of specific regions of mice brains failed to reveal a long-term effect. Thus, as in rats, the hippocampus and remaining telencephalon, which contain mainly nerve terminal 5-HT, were most sensitive to the acute effects of PCA; however, 5-HT levels returned to control values within 1 month after PCA administration even in these highly sensitive areas.

Interestingly, however, the continuous administration of PCA for a period of 3 days led to pronounced decreases in the brain levels of 5-HT and 5-HT uptake capacity, which lasted for at least 2 months. The level of PCA in brain during the treatment period was constant for the last 2 days and was similar to the peak level observed after a single moderate dose of 7.5 mg/kg, which produces maximal long-term reductions in rats [2] and only short-lasting effects in mice [6]. These findings provide strong support for the hypothesis that the rapid disappearance of PCA in mice might explain their apparent insensitivity to the long-term neurotoxic effects of the drug. Although the mechanism of PCA's neurotoxicity is unknown, recent results from our laboratory* suggest that the neurotoxic effects in rats are mediated by a minor metabolite of the drug. Thus, the prolonged, continuous presence of PCA in mice may allow the formation of a sufficient amount of neurotoxic substance via a minor metabolic route by increasing the availability of substrate to that pathway. The possibility of this metabolism occurring in the brain is provocative, particularly in light of recent demonstrations of cytochrome P-450-dependent metabolism of estrogens in brain [14]. Interestingly, cerebral metabolism of PCA has been recently demonstrated, but the identified products are apparently not involved in the long-term effects of PCA [15, 16].

The half-life of PCA in mice exposed to the drug continuously for 3 days was markedly longer than in mice given a single i.v. dose of the drug. Although the half-life values were determined in independent experiments, these data suggest that the continuous administration of PCA resulted in an alteration in either its rate of metabolism or elimination. The extent to which the long-term reductions in 5-HT levels and 5-HT uptake capacity are related to this apparent alteration in the half-life of PCA is not clear.

Regardless of the mechanism, the present results indicate that PCA-induced neurotoxicity is dependent upon a relatively long duration of exposure to the drug. In rats, a species in which the major pathway for the metabolism of amphetamine involves *p*-hydroxylation [17], the addition of the chlorine atom in the para position of the aromatic ring of amphetamine results in a marked increase in the half-life of the compound and the occurrence of neurotoxic activity. In mice, in which the deamination and *p*-hydroxylation of amphetamine are of equal importance, the substitution of a chlorine atom in the para position results in only a slight increase in the half-life of the compound without neurotoxic activity. However, the present data demonstrate that the prolonged maintenance of moderate levels of PCA by means of its continuous administration produces neurotoxic effects in mice as well. This finding raises an important question regarding the extent to which the unique long-term effects of PCA in rats are due to the presence of the chlorine atom *per se* as opposed to a reduced rate of metabolism resulting from a blockade of the para-hydroxylation. Studies with *m*-chloroamphetamine agree with the latter interpretation since this compound is toxic to rats only after pretreatment with desmethylimipramine, which prevents its rapid *p*-hydroxylation [18]. We are currently investigating the possibility that other halogenated amphetamines, such as *p*-fluoroamphetamine and β,β-difluoro-*p*-chloroamphetamine, and even amphetamine itself, all of which are nontoxic after a single dose [19, 20], may induce long-term biochemical effects after continuous exposure to moderate levels released from osmotic minipumps.

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* L. R. Steranka and E. Sanders-Bush, *J. Pharmac. exp. Ther.*, in press, 1978.

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