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IMPORTANCE OF DURATION OF DRUG ACTION IN THE ANTAGONISM OF *p*-CHLOROAMPHETAMINE DEPLETION OF BRAIN SEROTONIN—COMPARISON OF FLUOXETINE AND CHLORIMIPRAMINE

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Abstract—Fluoxetine inhibited both the rapid depletion of brain serotonin by *p*-chloroamphetamine (PCA) and the ultimate irreversible effects of PCA on brain serotonin neurons in rats; the differences between fluoxetine and chlorimipramine as PCA antagonists appeared to be related to the duration of uptake inhibition by these agents. Fluoxetine given along with PCA in a single dose prevented serotonin depletion at all times after PCA. Chlorimipramine antagonized serotonin depletion initially, but at later times there was little or no protection against PCA effects. The dose-dependence of the antagonism of PCA by fluoxetine did not vary greatly with the time of serotonin measurement after PCA, but with chlorimipramine the effectiveness of a given dose depended markedly on that time interval. Lengthening the pretreatment interval prior to PCA injection from 0 to 16 hr diminished the effectiveness of chlorimipramine but not fluoxetine as antagonists of serotonin depletion. The differences between fluoxetine and chlorimipramine may arise primarily because these compounds are metabolized by *N*-demethylation. The demethylated metabolite of fluoxetine was as potent and specific as fluoxetine itself as a serotonin uptake inhibitor both *in vitro* and *in vivo*, whereas the demethylated metabolite of chlorimipramine was less active than chlorimipramine as a serotonin uptake inhibitor and more active as a norepinephrine uptake inhibitor. Chlorimipramine was a more effective PCA antagonist when injected into mice in repeated doses or when injected into rats along with an inhibitor of liver microsomal enzymes. Thus, comparison of uptake inhibitors as antagonists of PCA is strongly influenced by the pharmacokinetics of the drugs involved.

p-Chloroamphetamine (PCA) depletes brain serotonin by a mechanism that requires active transport of PCA into the serotonin neuron [1, 2], and blockade of serotonin depletion by PCA is thus a useful measure of the ability of a compound to inhibit uptake into serotonin neurons *in vivo*. In previous studies, we have reported that fluoxetine is very effective in preventing serotonin depletion by PCA, whereas chlorimipramine and some other tricyclic antidepressant drugs are much less effective [3, 4]. Part of the reason for this difference relates to the duration of uptake inhibition by these compounds. PCA has a long half-life in brain [5, 6], and continual reuptake of PCA into serotonin neurons is necessary for its effects to persist until ultimately the depletion of serotonin is irreversible [7], perhaps due to toxic destruction of the serotonin neurons [8, 9]. Thus, it appears possible for an uptake inhibitor to transiently prevent the effects of PCA on brain serotonin until the inhibitor concentration declines to ineffective levels; if PCA concentrations in brain are still adequate it could then deplete serotonin. We are reporting here a series of experiments to test this idea and to investigate the influence of pharmacokinetic factors on the action of fluoxetine and chlorimipramine as PCA antagonists.

MATERIALS AND METHODS

Male albino Wistar rats weighing about 150 g were obtained from Harlan Industries, Cumberland, IN. Male albino Cox standard mice weighing approximately 20 g were obtained from Laboratory Supply, Indianapolis, IN. All animals were given food and water *ad lib*. After treatment with *p*-chloroamphetamine hydrochloride (Regis), fluoxetine hydrochloride (Lilly 110140), or chlorimipramine (Ciba/Geigy), the animals were killed by decapitation. Serotonin in whole brain and norepinephrine in heart were measured spectrofluorometrically [10]. Mean values ± standard errors for five animals/group were calculated; comparisons between groups were made by Student's *t*-test. The uptake of radiocarbon-labeled serotonin and norepinephrine into synaptosomes of cerebral cortex from rat brain *in vitro* was measured as described previously [11].

RESULTS

Temporal relationships in PCA antagonism by fluoxetine and chlorimipramine in rats. Figure 1 shows the results of an experiment in which PCA was injected into rats alone or 30 min after fluoxetine (20 mg/kg) or chlorimipramine (40 mg/kg). After PCA alone, brain serotonin levels had declined within 3 h to 48 per cent of control, further at 8 hr to 39 per cent of control, and remained at 44 per cent of control at 24 hr. Fluoxetine completely prevented the depletion of brain serotonin by PCA at all time points. At 3 hr in chlorimipramine-treated rats, brain serotonin levels were not decreased significantly by PCA

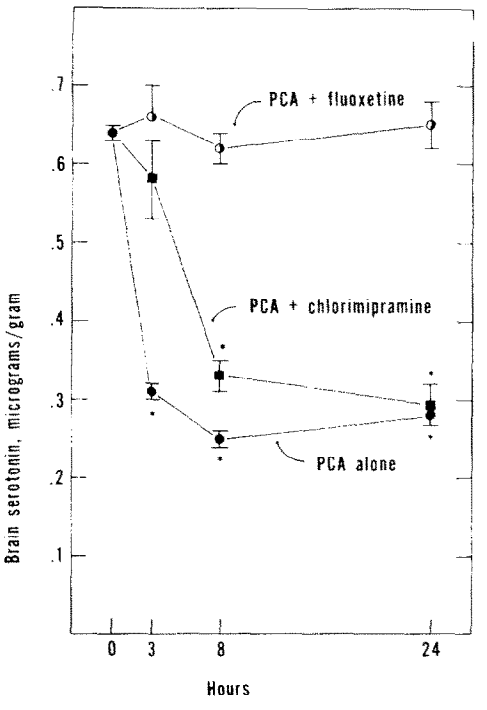

Fig. 1. Brain serotonin levels in rats after the injection of PCA alone (●) or after fluoxetine (◑) or chlorimipramine (■) pretreatment. PCA hydrochloride (20 mg/kg, i.p.) was injected at zero time alone or 30 min after either fluoxetine hydrochloride (20 mg/kg, i.p.) or chlorimipramine (40 mg/kg, i.p.). Asterisks indicate significant differences from the zero time group ($P < 0.05$).

(91 per cent of control), i.e. chlorimipramine had essentially completely prevented the action of PCA. By 8 hr, the protection by chlorimipramine was only slight though still significant, whereas at 24 hr there was no effect at all by chlorimipramine. We have shown in separate experiments that fluoxetine and chlorimipramine alone do not appreciably influence whole brain serotonin levels.

Next we measured serotonin depletion at a fixed time after PCA in rats pretreated with uptake inhibitors at different time intervals before PCA (Table 1). Fluoxetine (10 mg/kg) completely prevented serotonin depletion when given at 0, 2, 4 or 6 hr before PCA. In fact, serotonin levels tended to be increased at early times after the combination of fluoxetine and PCA; this observation has previously been made and studied with PCA or analogs of it [3, 12]. Chlorimipramine (50 mg/kg) prevented serotonin depletion when given along with PCA or only 2 hr previously, but incompletely antagonized PCA if the pretreatment interval was increased to 4 or 6 hr. At the 4- and 6-hr pretreatment intervals, the PCA group treated with fluoxetine differed significantly from those treated with chlorimipramine ($P < 0.05$). In a separate experiment, the uptake inhibitors were injected 16 hr prior to PCA. All doses were the same as in Table 1. Control levels of serotonin were depleted from 0.61 ± 0.02 down to 0.37 ± 0.02 μg/g by PCA alone. Fluoxetine completely prevented the depletion; serotonin levels were 0.58 ± 0.02 μg/g in the group receiving fluoxetine 16 hr before PCA. Chlorimipramine had no significant protecting effect; serotonin levels were 0.40 ± 0.03 μg/g in rats receiving chlorimipramine 16 hr prior to PCA.

Table 2 shows the effects of three different dose levels of fluoxetine and chlorimipramine when serotonin depletion was measured at a short time (2 hr) or a long time (1 week) after PCA. Fluoxetine had no effect at 1 mg/kg, a slight effect at 3 mg/kg, and completely prevented serotonin depletion at 10 mg/kg when serotonin depletion was measured at 2 hr. The degree of protection was only slightly less when serotonin depletion was measured at 1 week. Thus, the ED_{50} for fluoxetine would be between 3 and 10 mg/kg irrespective of the time interval used for measuring serotonin depletion. Chlorimipramine significantly antagonized serotonin depletion by PCA at all three doses (12.5, 25 and 50 mg/kg) at 2 hr after PCA but had no significant effect at any of these doses at 1 week. Thus, the ED_{50} for chlorimipramine was between 25 and 50 mg/kg when serotonin depletion was measured at 2 hr, whereas chlorimipramine was essentially ineffective after a single dose when serotonin depletion was measured at 1 week.

Comparison of N-demethylated metabolites of chlorimipramine and fluoxetine. Metabolic N-demethylation is known to occur extensively with both fluoxetine [13] and chlorimipramine [14, 15]. Though fluoxetine and chlorimipramine are similar in potency and speci-

Table 1. Influence of pretreatment interval on PCA antagonism by fluoxetine and chlorimipramine in rats*

Group	Pretreatment interval (hr)	Brain serotonin (μg/g)	% Antagonism
Control		0.67 ± 0.01	
PCA alone		0.42 ± 0.02†	
PCA after fluoxetine	0	0.73 ± 0.03	100‡
	2	0.74 ± 0.01	100‡
	4	0.73 ± 0.01	100‡
	6	0.67 ± 0.01	100‡
PCA after chlorimipramine	0	0.75 ± 0.02	100‡
	2	0.67 ± 0.05	100‡
	4	0.57 ± 0.02†	60‡
	6	0.56 ± 0.03†	56‡

* PCA (10 mg/kg, i.p.) was injected 2 hr before the rats were killed. Designated groups received fluoxetine (10 mg/kg, i.p.) or chlorimipramine (50 mg/kg, i.p.) at the same time as PCA or 2, 4 or 6 hr previously.

† Significantly lower than control, $P < 0.01$.

‡ Different from group with PCA alone, $P < 0.05$.

Table 2. Dose-dependence in the antagonism by fluoxetine and chlorimipramine of initial or neurotoxic depletion of rat brain serotonin by PCA*

Group	Dose of uptake inhibitor (mg/kg)	Two hr after PCA		One week after PCA	
		Brain serotonin (μg/g)	% Antagonism	Brain serotonin (μg/g)	% Antagonism
Control		0.68 ± 0.02†		0.69 ± 0.03†	
PCA alone		0.36 ± 0.01‡		0.26 ± 0.02‡	
PCA + fluoxetine	1	0.36 ± 0.01‡	0	0.26 ± 0.03‡	0
	3	0.45 ± 0.01‡	27†	0.32 ± 0.02‡	14
	10	0.67 ± 0.03	97†	0.57 ± 0.03‡	72†
PCA + chlorimipramine	12.5	0.47 ± 0.01‡	33†	0.29 ± 0.02‡	7
	25	0.49 ± 0.02‡	39†	0.27 ± 0.02‡	2
	50	0.60 ± 0.04	73†	0.31 ± 0.02‡	12

* PCA (20 mg/kg, i.p.) was injected simultaneously with fluoxetine or chlorimipramine. Brain serotonin concentration was measured 2 hr or 1 week later.
† Different from group with PCA alone, $P < 0.01$.
‡ Significantly lower than control group, $P < 0.01$.

ficity as uptake inhibitors *in vitro* [16, 17], they are very different *in vivo* [3, 4, 16, 17], presumably due to differences in their metabolites which contribute substantially to activity *in vivo*. Therefore, we compared desmethylchlorimipramine and desmethylfluoxetine as PCA antagonists (and as antagonists of 6-hydroxydopamine to evaluate effects on norepinephrine neurons). Table 3 shows that the desmethyl metabo-

Table 3. Comparison of fluoxetine and its demethylated metabolite (103947) as antagonists of PCA*

Treatment group	Brain 5-HT (μg/g)	% Antagonism of PCA effect
Control	0.65 ± 0.02	
PCA alone	0.34 ± 0.02†	
PCA + fluoxetine (2.5)	0.41 ± 0.01†	23‡
PCA + fluoxetine (5)	0.58 ± 0.03	77‡
PCA + fluoxetine (10)	0.64 ± 0.04	97‡
PCA + 103947 (2.5)	0.35 ± 0.02†	3
PCA + 103947 (5)	0.57 ± 0.02†	74‡
PCA + 103947 (10)	0.62 ± 0.03	90‡

* PCA (10 mg/kg, i.p.) was injected 30 min after fluoxetine or 103947 and 4 hr before the rats were killed. The numbers in parentheses represent mg/kg doses of fluoxetine and 103947, which were injected i.p.
† Significantly lower than control, $P < 0.05$.
‡ Different from group with PCA alone, $P < 0.05$.

Table 4. Comparison of chlorimipramine (CMI) and its demethylated metabolite (DM-CMI) as antagonists of PCA*

Treatment group	Brain 5-HT (μg/g)	% Antagonism of PCA effect
Control	0.59 ± 0.03	
PCA alone	0.30 ± 0.02†	
PCA + CMI (12.5)	0.40 ± 0.03†	34‡
PCA + CMI (25)	0.46 ± 0.05	55‡
PCA + CMI (50)	0.69 ± 0.03	100‡
PCA + DM-CMI (12.5)	0.31 ± 0.02†	3
PCA + DM-CMI (25)	0.36 ± 0.02†	21
PCA + DM-CMI (50)	0.57 ± 0.04	93‡

* Conditions as in Table 3. The numbers in parentheses represent mg/kg doses of CMI and DM-CMI, which were injected i.p.
† Significantly lower than control, $P < 0.05$.
‡ Different from group with PCA alone, $P < 0.05$.

Table 5. Comparison of chlorimipramine (CMI) and its demethylated metabolite (DM-CMI) as antagonists of 6-hydroxydopamine*

Treatment group	Heart norepinephrine (μg/g)	% Antagonism of 6-hydroxydopamine effect
Control	1.08 ± 0.11	
6-OHDA alone	0.59 ± 0.10	
6-OHDA + CMI (12.5)	0.45 ± 0.06†	0
6-OHDA + CMI (25)	0.50 ± 0.08†	0
6-OHDA + CMI (50)	1.02 ± 0.04	88‡
6-OHDA + DM-CMI (12.5)	0.60 ± 0.09†	2
6-OHDA + DM-CMI (25)	0.83 ± 0.05	49
6-OHDA + DM-CMI (50)	1.00 ± 0.03	84‡

* 6-Hydroxydopamine HBr (100 mg/kg, i.p.) was injected 1 hr after CMI or DM-CMI and 16 hr before the rats were killed. The numbers in parentheses represent mg/kg doses.
† Significantly lower than control, $P < 0.05$.
‡ Different from group with PCA alone, $P < 0.05$.

Table 6. Inhibition of serotonin and norepinephrine uptake into synaptosomes from rat cerebral cortex

Inhibitor	K_i (μM) Serotonin	K_i (μM) Norepinephrine	Ratio Serotonin / Norepinephrine
Chlorimipramine	0.11	1.3	0.08
Desmethylchlorimipramine	0.23	0.06	3.8
Fluoxetine	0.17	4.5	0.04
Desmethylfluoxetine	0.22	10	0.02

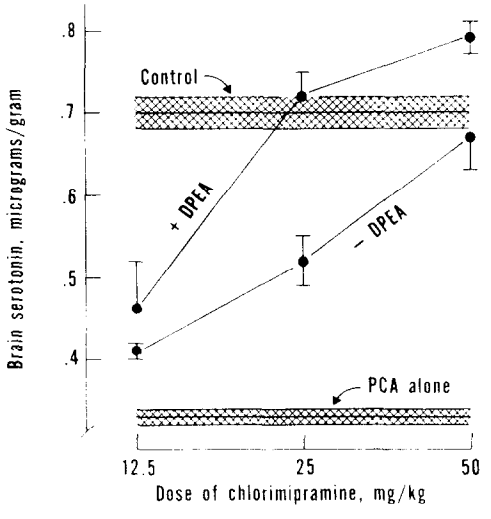

Fig. 2. Enhanced PCA antagonism by chlorimipramine in rats treated with an inhibitor of liver microsomal enzymes. PCA was injected at 10 mg/kg, i.p., 4 hr before the rats were killed and 10 min after chlorimipramine. Chlorimipramine was injected i.p. at the doses indicated along the abscissa. Some rats were pretreated with DPEA (2,4-dichloro-6-phenyl-phenoxyethylamine, an inhibitor of drug-metabolizing enzymes in hepatic microsomes [19]) at a dose of 10 mg/kg, i.p., 1 hr before chlorimipramine. Mean values ± standard errors for five rats/group are shown. The shaded areas at top and bottom represent standard error range (with the central horizontal line representing the mean) for untreated control rats or for rats treated with PCA alone. Serotonin concentration in the brain of rats treated with DPEA alone was 0.68 ± 0.01 μg/g, not significantly different from controls.

lite of fluoxetine (103947) was virtually identical to fluoxetine as a PCA antagonist. There was a greater difference between chlorimipramine and its desmethyl metabolite (Table 4). Significant antagonism of PCA occurred with 12.5 and 25 mg/kg doses of chlorimipramine but not desmethylchlorimipramine, though the latter compound was like chlorimipramine in completely preventing serotonin depletion at the highest dose (50 mg/kg). Though desmethylchlorimipramine was less effective at the two lower doses as a PCA antagonist, it was more effective in antagonizing 6-hydroxydopamine depletion of heart norepinephrine (Table 5). These results are consistent with comparison *in vitro* of chlorimipramine and fluoxetine to their desmethyl metabolites as inhibitors of serotonin and norepinephrine uptake (Table 6). The desmethyl metabolite of chlorimipramine is less active as a serotonin uptake inhibitor and more active as a norepinephrine uptake inhibitor compared to chlorimipramine [18]. Fluoxetine and desmethylfluoxetine differ less markedly. The effect that metabolic demethylation would have on uptake inhibition can be seen by comparing the ratio of K_i values for serotonin vs norepinephrine. Chlorimipramine and desmethylchlorimipramine have ratios that differ by a factor of about fifty, whereas fluoxetine and desmethylfluoxetine have essentially identical ratios.

Effect of a microsomal enzyme inhibitor on chlorimipramine effectiveness as a PCA antagonist. If the metabolic demethylation of chlorimipramine could be prevented, it ought to be a more potent antagonist of serotonin depletion by PCA. Therefore, we determined the effect of 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) on chlorimipramine action. This com-

Table 7. Antagonism of PCA-induced depletion of brain serotonin in mice by chlorimipramine*

Dose of PCA (mg/kg)	Dose of chlorimipramine (mg/kg)	Brain serotonin (μg/g)
0	0	0.89 ± 0.01†
20.6	0	0.57 ± 0.01‡
20.6	1	0.71 ± 0.02†,‡
20.6	3	0.82 ± 0.03†
20.6	10	0.96 ± 0.01†,‡
0	10	0.84 ± 0.02†,‡

* Chlorimipramine was injected i.p. 15 min before and 105 min after PCA. PCA was injected i.p. 4 hr before the mice were killed.

† Significantly different from group with PCA alone, $P < 0.05$.

‡ Significantly different from group that received neither PCA nor chlorimipramine, $P < 0.05$.

pound has previously been shown to be a potent inhibitor of demethylation and other hepatic microsomal oxygenation reactions [19]. Figure 2 shows that indeed the activity of chlorimipramine was increased by pretreatment with this microsomal inhibitor. We cannot be sure that this enhancement of chlorimipramine activity was due entirely to prevention of *N*-demethylation. These results are in agreement with the report by Ross and Renyi [20] that another microsomal inhibitor, SKF-525A, increased the ability of chlorimipramine to inhibit serotonin uptake more than its ability to inhibit norepinephrine uptake *in vivo*.

PCA antagonism in mice. Recently Von Voigtlander and Losey [21] reported data on chlorimipramine and several other uptake inhibitors as antagonists of *p*-chloromethamphetamine-induced depletion of brain serotonin in mice. They reported that chlorimipramine was effective at doses of less than 1 mg/kg in a protocol that involved giving the uptake inhibitor in two doses, one 15 min before *p*-chloromethamphetamine and the second 105 min after *p*-chloromethamphetamine (mice were killed 4 hr after *p*-chloromethamphetamine injection). *p*-Chloromethamphetamine is extensively metabolized by *N*-demethylation to PCA (R. W. Fuller and J. C. Baker, manuscript submitted for publication); to avoid the complicating possibility that chlorimipramine might inhibit this *N*-demethylation, we studied the ability of chlorimipramine to antagonize the effects of PCA itself in mice in a protocol like that of Von Voigtlander and Losey [21]. Table 7 shows that chlorimipramine antagonized PCA action when given in two doses as low as 1 mg/kg each. The effective dose of chlorimipramine was much lower than that in a protocol we used earlier [4], giving PCA 1 hr after a single dose of chlorimipramine and 6 hr before the mice were killed.

DISCUSSION

The long duration of uptake inhibition after fluoxetine administration, reported previously [3, 17] and illustrated by the data in this paper, is largely due to the persistence of the demethylated metabolite of fluoxetine in tissues for a very long time [13]. Thus, fluoxetine is an effective antagonist of the neurotoxic effects of PCA [3, 17] despite the fact that PCA itself persists in brain with a long half-life. Though the depletion of brain serotonin by PCA occurs rapidly, the effect is reversible at early times [7]. Continual uptake of PCA into serotonin neurons apparently occurs and is required for the ultimate toxic effect of PCA on serotonin neurons. Thus, a short-lasting uptake inhibitor such as chlorimipramine is an effective blocker of the early lowering of serotonin by PCA ([1, 20], Fig. 1) but comparatively ineffective (when given as a single dose) as a blocker of the neurotoxic effects of PCA ([3, 22], Table 2).

Comparison of the demethylated metabolite of fluoxetine and chlorimipramine reveals the probable basis for the difference of these two drugs *in vivo* despite their similarity *in vitro*. The desmethyl metabolite of fluoxetine is, like fluoxetine, a selective inhibitor of serotonin uptake. However, the desmethyl metabolite of chlorimipramine preferentially inhibits norepinephrine uptake ([18], Table 6). Thus, metabolic transformation does not alter the specificity of fluoxetine but reverses the specificity of chlorimipramine.

Although the antagonism of the effects of PCA (either the rapid initial lowering of tryptophan hydroxylase or 5-hydroxyindole levels or the long-lasting reduction in tryptophan hydroxylase, 5-hydroxyindole and serotonin uptake) is a highly useful index of inhibition of uptake into serotonin neurons *in vivo*, one should be aware of the dynamics of PCA so that proper interpretation of experimental data can be made. For example, an effective but short-lasting uptake inhibitor could be judged to be almost ineffective if blockade of neurotoxic effects of PCA on serotonin neurons were measured after a single dose of the uptake inhibitor. On the other hand, by measuring serotonin levels over a wide range of times after PCA injection, one can get an indication not only of the efficacy but also of the duration of action of an uptake inhibitor (Fig. 1).

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