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Combined pCPA and Muscarinic Antagonist Treatment Produces a Deficit in Rat Water Maze Acquisition

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HARDER, J. A., M. E. KELLY, C. H. K. CHENG AND B. COSTALL. *Combined pCPA and muscarinic antagonist treatment produces a deficit in rat water maze acquisition.* PHARMACOL BIOCHEM BEHAV **55**(1) 61–65, 1996.—A 3-day treatment with p-chlorophenylalanine (pCPA, 100 mg/kg/day) produced a significant decrease (63–89%) in 5-HT levels in both the hippocampus and the cortex of rats, while noradrenaline, adrenaline, and dopamine levels were unaffected. Treatment with pCPA alone did not affect the acquisition of a spatial learning task in the water maze. Treatment with low doses of either scopolamine (0.25 mg/kg) or atropine (10 mg/kg) was also insufficient to cause a significant impairment of water maze acquisition. However, a combined treatment of a 3-day pCPA regimen with the low dose of atropine or scopolamine produced a significant deficit in the acquisition of a water maze task.

Learning and memory Alzheimer's disease Scopolamine Atropine pCPA Water maze 5-HT Acetylcholine Spatial learning

THE cholinergic hypothesis of Alzheimer's disease has been extensively investigated using animal models such as scopolamine impairment of spatial learning in the rodent (2). However, postmortem brain tissue taken from patients diagnosed with Alzheimer's disease has shown that acetylcholine is not the only neurotransmitter depleted in the disorder. One of the many other substances affected is 5-HT, which is markedly reduced (2). It has been suggested that the 5-HT neurone degeneration in Alzheimer's disease may be linked to cognitive symptoms, having the most marked effect when there is a concurrent compromise of the cholinergic system (4,7).

The indication that 5-HT levels are altered in Alzheimer's disease has led to an increased interest in the potential role of 5-HT both in learning and memory per se, and in relation to Alzheimer's disease symptoms. Studies of the effects of 5-HT agonists and antagonists on animal learning and memory models have shown a variety of effects. For example, studies investigating the effects of 5-HT agonists suggest a generally impaired performance on two-way active avoidance, Y-maze brightness discrimination, and the water maze; whereas antagonist studies show varying effects according to whether pre- or posttrial administration was used (1) for review. More consistent results have been reported when both 5-HT and acetylcholine are concurrently affected. The combined effect of 5-HT and cholinergic manipulation has been tested using a 5-HT synthesis inhibitor and atropine treatment to produce a marked deficit in spatial learning in the rat water maze (7).

This study seeks to confirm the behavioural impairment in the Morris water maze using pCPA combined with atropine or scopolamine. A standard training schedule for a square water maze is described here. The extent and selectivity of the 5-HT depletion produced with a small dose of parachorophenylalanine (pCPA) (administered over 3 days) is assessed by HPLC with electrochemical detection to measure the levels of 5-HT, noradrenaline, dopamine, and their metabolites in the cortex and hippocampus.

METHOD

Animals

Male Lister Hooded rats (250–500 g) were used for all experiments. Rats were Bradford derived and housed in

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groups of five in exclusively male holding rooms, illuminated between 0700 and 2000 h. Food and tap water were available ad lib.

Apparatus

A square tank (122 × 122 × 54 cm) of opaque white perspex was used for all experiments. Water temperature was maintained at 27 ± 2°C, and water was rendered opaque by a white latex liquid, such that a white painted island located 1–2 cm below the water surface could not be seen. Video recording and tracking equipment (HVS Image, London) allowed latency and swim speed to be calculated for each trial on an Apple IIe microcomputer. Black curtains surrounded the pool and visual cues were provided by two boards, painted with black and white stripes, positioned within the curtains but distal to the island position. For experiments in which a visible island was required, a taller, black painted island was used, such that it was clearly visible 1–2 cm above the level of the water.

Protocol

On day 1 of training, each rat was placed onto the island for 10 s and allowed a short swim to allow habituation to the sensation of immersion in water. Animals were then placed facing the pool wall in a starting position determined by a latin square design. Timing was initiated when the rat was released in the start position, and ended when the rat had all four paws on the island, or if the island was not found when 100 s had elapsed. Any rat failing to find the island in 100 s was guided to it by hand. Each rat remained on the island for 10 s before the next trial began. On subsequent days (days 2, 3, and 4) the initial period on the island and the habituating swim were omitted. In all experiments, four trials per day were given on each of the 4 test days.

In a separate experiment, which included a final day of training (day 5), a visible island was positioned in the quadrant to which each animal had been trained (using a submerged island) on the preceding days (1 to 4). Rats received two trials only with the visible island. The timing for each trial ended when the animal gripped the island with both forepaws, as the additional height of the island made it difficult for some of the animals to climb onto the surface.

Treatments

pCPA (100 mg/kg) was suspended in a 5% v/v Tween 80 vehicle and given once daily for 3 days before water maze training was initiated. Maze exposure occurred at least 4 h after the final dose. Atropine (10 mg/kg) HCl and scopolamine (0.25 mg/kg) HBr were dissolved in 0.9% w/v saline and administered on each day of training 30 or 20 min prior to maze exposure, respectively. All drug salts were supplied by Sigma. Doses are expressed as the base and were administered in a volume of 1 ml/kg IP.

Determination of Neurochemical Levels Using HPLC-ECD

Groups of vehicle and pCPA (100 mg/kg)-treated rats that did not undergo water maze training were killed by cervical dislocation and the hippocampus and cortex dissected out within 1–2 min. Samples were placed in preweighed polypropylene vials and immediately immersed in liquid nitrogen. Samples were taken on the final day of pCPA dosing (equivalent to day 1 of maze testing), on day 4 of maze testing, and on the subsequent day (day 5). All samples were weighed and homogenized for approximately 5 s with an ultrasonic homogenizer in 200 μl of 0.2 M perchloric acid containing 100 pg/μl dihydroxybenzoic acid (DHBA) as an internal standard for catecholamine analysis, and 100 pg/μl *N*-methyl-5-HT as an internal standard for indoleamine analysis. The homogenate was centrifuged at 15,000 × *g* for 2 min and the supernatant was analyzed using HPLC with electrochemical detection.

For the determination of 5-HT and 5-HIAA, the mobile phase consisted of a buffered mixture of 0.1 M citric acid, 0.2 M disodium hydrogen orthophosphate, and 2 mM tetraethylammonium bromide, 11% v/v methanol, pH 6, filtered through a 0.2 μm nylon membrane (Rainin, Wolburn, MA), and degassed under vacuum. The mobile phase was delivered at a rate of 1.4 ml/min via a model 510 solvent delivery system with a Wisp 710B automatic injector (Waters, Milford, MA). A Hypersil ODS column, 15 cm × 4.6 mm internal diameter, 5 μm particle size, was used. The electrochemical detector consisted of a TL5 glassy carbon electrode with an LC-4A amperometric detector (Bioanalytical Systems, Lafayette, USA). Amounts of 5-HT and 5-HIAA were calculated per unit weight from the fraction of the original weight injected and the wet weight of the tissue.

For the determination of noradrenaline, adrenaline, dopamine, and DOPAC 60 μl supernatant was added to activated alumina in 120 μl of 1 M Tris buffer (pH 8.6). The tubes were shaken, centrifuged, and the alumina washed three times with Milli-Q water after discarding of supernatant. One hundred twenty microliters of 0.1 M citric acid, pH 2, was added to each tube and the contents were centrifuged for 30 s. The mobile phase consisted of a buffered mixture of 0.1 M citric acid, 0.2 M disodium hydrogen orthophosphate, and 1.8 mM octane sulphonic acid, 12% v/v methanol, pH 4. The mobile phase was delivered at a rate of 1.4 ml/min via a model 510 solvent delivery system with a Wisp 710B automatic injector (Waters, Milford, USA). A Hypersil ODS column, 25 cm × 4.6 mm internal diameter, 5 μm particle size, was used. Coulochemical detection was performed using a model 5011 analytical cell with a model 5020 guard cell and a 5100A Coulochemical detector (ESA). Amounts of noradrenaline, adrenaline, and dopamine in supernatant were calculated as described above for 5-HT.

Statistical Analysis

Water maze data (latencies and swim speeds) were analyzed using single-factorial ANOVA for each day of testing, with treatment as the dependent variable; followed by Dunnett's *t*-test to compare treatment groups to vehicle controls, and Scheffe's test to compare the treatment groups with one another. HPLC data were analyzed using Student's unpaired *t*-test.

RESULTS

In all experiments, vehicle-treated control animals showed a significant decrease in latency to find the island over the 4 training days, demonstrating acquisition of the water maze task.

Preliminary dose ranging studies showed that doses of atropine above 10 mg/kg and doses of scopolamine above 0.25 mg/kg caused significant increases in latency ($p < 0.05$). Scopolamine, at a dose of 0.5 mg/kg, caused latencies of 87.2 ± 9.2, 85.2 ± 9.6, 77.1 ± 8.4, and 50.1 ± 11.2 s, on days 1, 2, 3, and 4, respectively, compared with vehicle control values of

48.3 ± 9, 23.4 ± 9.1, 19.1 ± 5.1, and 18.2 ± 7.6 s; $p < 0.05$. Scopolamine (0.25 mg/kg) had no effect on latency on any of the 4 training days (79 ± 9.2, 44.9 ± 13.6, 29.9 ± 4.9, and 20.1 ± 5.1 s on days 1, 2, 3, and 4) ($p > 0.05$). Thus, 0.25 mg/kg was selected as a subthreshold dose for subsequent experiments.

Atropine, at doses of 20 and 50 mg/kg, caused latencies of 98.6 ± 1.4, 75 ± 9.6, 60.8 ± 8.1, and 61 ± 1.4 s (for 20 mg/kg) and 94.7 ± 3.4, 78.1 ± 5.4, 73.5 ± 8.8, and 63.3 ± 8.9 s (for 50 mg/kg); again, significantly higher than saline-treated controls (at 64.7 ± 5.8, 41 ± 6.8, 25.6 ± 5.3, and 23.7 ± 7.6 s on days 1, 2, 3, and 4, respectively) ($p < 0.05$). Atropine (10 mg/kg) caused limited impairment in acquisition, with significantly higher latencies than vehicle controls on day 2 of testing only ($p < 0.05$). Atropine (10 mg/kg) mean latencies were 80.7 ± 11.1, 65.1 ± 8, 47.1 ± 12.3, and 29.1 ± 6.7 s. This dose was selected for subsequent experiments.

Doses of scopolamine and atropine were, thus, selected from these preliminary experiments as having little or no effect when administered alone on the performance of rats in the water maze task. However, in subsequent experiments utilizing combination treatments, atropine alone at 10 mg/kg caused significantly higher latencies than vehicle control treatment on days 1, 3, and 4 of training ($p < 0.05$), as shown in Fig. 1, suggesting results with atropine can show a degree of variation. Latencies on days 1, 2, 3, and 4, respectively, were 86.7 ± 7.9, 53.8 ± 7, 51.3 ± 11, and 48.4 ± 12.5 s. Scopolamine alone (0.25 mg/kg) did not affect the latency values ($p > 0.05$, compared with vehicle controls), as shown in Fig. 2.

pCPA treatment alone had no effect on water maze acquisition, with no significant differences seen between pCPA and vehicle treated rats ($p > 0.05$) (Figs. 1 and 2).

In combined treatment groups (pCPA and atropine, pCPA and scopolamine) a severe water maze acquisition deficit was observed. With pCPA and scopolamine treatment, latencies were significantly higher than vehicle control groups on days 1, 2, and 3 ($p < 0.005$); in addition, latencies were significantly higher than scopolamine treatment alone (0.25 mg/kg) on these 3 days ($p < 0.05$) (see Fig. 2). With combined pCPA and atropine treatment, latencies were again increased when compared to vehicle controls ($p < 0.005$, all 4 days). The impairment in acquisition was also significantly larger than that seen with atropine (10 mg/kg) alone on days 2 and 3 ($p < 0.05$) (see Fig. 1).

In a separate experiment that included a final day of visible island training (day 5), it was notable that while both combination treatments (pCPA and atropine; pCPA and scopolamine) caused impaired acquisition with the submerged island on days 1 to 4 ($p < 0.05$), there were no significant differences from vehicle controls when the island was visible ($p > 0.05$) (see Fig. 3).

In these experiments, few significant swim speed differences between treatment groups were observed (see Figs. 1b and 2b).

The HPLC results demonstrated that 5-HT was depleted to a significant extent ($p < 0.01$) in both hippocampus (64% decrease) and cortex (71% decrease), as compared to vehicle controls (see Table 1) on day 3 of pCPA dosing (equivalent to training day 1 of behaving animals). The depletion increased over the following days such that 3 days after the final dose of pCPA (equivalent to day 4 of training) hippocampal 5-HT was decreased by 83% compared to vehicle controls, and cortical 5-HT was decreased by 89%, compared to vehicle controls.

Furthermore, the 3-day regimen of treatment with pCPA (100 mg/kg) was ineffective in altering noradrenaline, adrenaline, or dopamine levels over the training period used, with the catecholamine content of hippocampus and cortex not significantly different from vehicle-treated animals on day 1 or day 4 of training. On day 5, however, there was a significant reduction (41%) in the mean hippocampal noradrenaline level of pCPA treated animals ($p < 0.05$) (see Table 2).

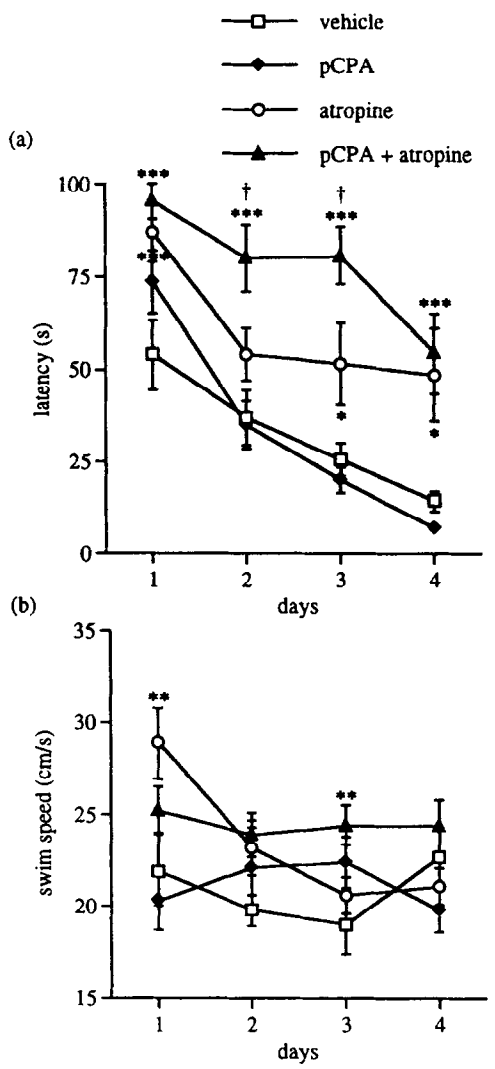

FIG. 1. Graphs show (a) time taken to reach island (s) and (b) swim speed (cm/s) following treatment with pCPA (100 mg/kg/day × 3, IP) and/or atropine (10 mg/kg, IP). Significant differences as shown using ANOVA followed by Dunnett's t-test are illustrated as $*p < 0.05$; $**p < 0.01$; $***p < 0.005$ (differences from vehicle). Significant differences as shown using ANOVA followed by Scheffe's test are illustrated as $\dagger p < 0.05$ (different from atropine treatment alone).

DISCUSSION

The decrease in latency to find the submerged island with training represents acquisition of the water maze task. Where swim speed is not affected by treatments, as in these experiments, it may be assumed that the decrease in the latency over the period of the trials is due to spatial learning, as the location of the hidden platform becomes memorized by the rats (3,6).

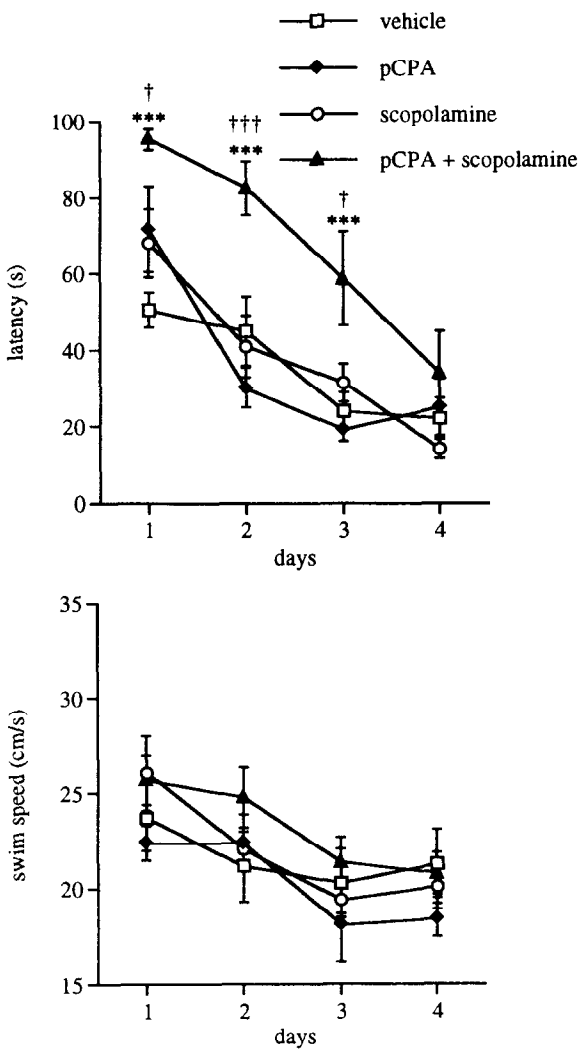

FIG. 2. Graphs show (a) time taken to reach island (s) and (b) swim speed (cm/s) following treatment with pCPA (100 mg/kg/day × 3, IP) and/or scopolamine (0.25 mg/kg, IP). Significant differences as shown using ANOVA followed by Dunnett's t-test are illustrated as $^{***}p < 0.005$ (differences from vehicle). Significant differences as shown using ANOVA followed by Scheffe's test are illustrated as $^{\dagger}p < 0.05$; $^{\dagger\dagger\dagger}p < 0.005$ (differences from scopolamine treatment alone).

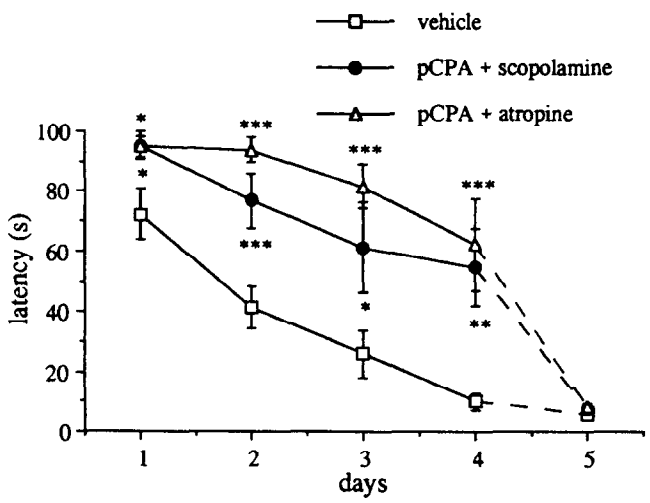

FIG. 3. Graph shows the time taken to reach island (s) following treatment with pCPA and either atropine or scopolamine. Day 5 was a day of visible island training and data represent the mean of two trials per rat, whereas on days 1 to 4 data represent the mean of four trials per day. Significant differences as shown using ANOVA followed by Dunnett's t-test are illustrated as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$ (differences from vehicle).

Possible interactions between cholinergic and other neurotransmitter systems in learning and memory are currently attracting substantial research interest (4). It is known that noradrenergic projections, for example, are also affected early in Alzheimer's disease (2), and cholinergic/adrenergic interactions may be linked to cognition (4). The evidence provided here by combined pCPA and muscarinic antagonist treatment supports the hypothesis that combined transmitter interactions are important for the formation of a memory trace (4).

Thus, the results obtained in these studies confirm the increase in latency to complete a water maze task produced by combined pCPA and atropine treatment (7). The findings are here extended to show a similar effect with scopolamine, thus demonstrating that a comparable learning impairment can be produced with two different muscarinic antagonists when brain 5-HT is depleted.

The use of a low dose of pCPA repeated over 3 days produced a selective 5-HT depletion in both cortex and hippocampus, two areas implicated in spatial learning, and possible sites for interaction of cholinergic and serotonergic neurones

TABLE 1

MEAN QUANTITY OF 5-HT

Brain Area	Treatment	Day 1	Day 4	Day 5
Hippocampus	Vehicle	172.5 ± 28.2	89.6 ± 22.7	67.8 ± 9.5
	pCPA	63.0 ± 21.6*	15.5 ± 2.1*	11.7 ± 1.9*
Cortex	Vehicle	249.0 ± 23.6	247.5 ± 15.2	264.7 ± 28.0
	pCPA	71.3 ± 17.2*	27.8 ± 6.0*	31.3 ± 7.9*

(pg/mg) ± SEM, $n = 7$. Unpaired Student's t-test (two-tailed); $^{*}p < 0.01$, differences from vehicle control.

TABLE 2

MEAN CATECHOLAMINE CONTENT OF HIPPOCAMPUS AND CORTEX

Transmitter	Brain Area	Treatment	Day 1	Day 4	Day 5
Noradrenaline		Vehicle	154.6 ± 18.9	85.9 ± 15.8	90.2 ± 11.7
	Hippocampus	pCPA	120.9 ± 16.1	71.9 ± 9.3	52.9 ± 11.2*
Noradrenaline		Vehicle	125.5 ± 12.7	121.2 ± 10.0	117.3 ± 13.1
	Cortex	pCPA	93.7 ± 9.9	116.8 ± 12.6	89.0 ± 10.7
Adrenaline		Vehicle	10.0 ± 1.6	8.77 ± 1.2	10.9 ± 1.6
	Cortex	pCPA	8.3 ± 1.0	12.2 ± 1.9	11.0 ± 1.4
Dopamine		Vehicle	106.1 ± 25.6	348.1 ± 145.4	147.3 ± 39.9
	Cortex	pCPA	56.2 ± 10.2	182.1 ± 86.0	69.4 ± 19.6

(pg/mg) ± SEM, $n = 7$, unpaired Student's t-test (two-tailed); $^*p < 0.05$ significant difference from vehicle control.

(8). At the moderate dose of pCPA utilised, 5-HT levels were markedly reduced while noradrenaline, adrenaline, and dopamine levels remained essentially unaffected. This is critical, as pCPA is known to deplete catecholamine as well as indoleamine transmitters at higher doses (5). HPLC data allow us to conclude that in the experiments reported here the learning deficit induced by combined pCPA and muscarinic antagonist treatment was due to serotonergic and cholinergic interaction; as opposed to a possible interaction between catecholaminergic and cholinergic systems.

The visible island experiment was designed to control for motivation and sensorimotor effects of the compounds used to produce the cognitive deficit (6). In the present experiments, the similar latencies to vehicle control groups produced by combined pCPA and muscarinic antagonist treatment when the island was visible indicate no confounding motivational or visual effects of the combined treatment. As the swim speed was also unaffected by the combined treatments, it may be assumed that any motor effects are insufficient to affect performance of the water maze task. A report in the literature suggested that combined pCPA and scopolamine treatment could produce total abolition of learning in a type of visible island paradigm (9). However, the results may not be directly comparable to the present study, because an extremely large dose of pCPA (500 mg/kg daily for 3 days) was administered, and the apparatus used was not a standard water maze (9). Therefore, the abolition of visible platform learning may have been attributable to mechanisms other than serotonergic and cholinergic with such large quantities of pCPA. With the selective depletion of 5-HT produced in the present study, visible island trials show no such impairment.

In summary, the data demonstrate that even low dose cholinergic antagonist treatments, which alone produce little impairment of spatial learning, can produce a severe deficit when administered to a rat with extensive but selective depletions of brain 5-HT. Combination approaches such as this may be one important route in the search for the biological basis of learning and memory.

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