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Serotonin Synthesis Inhibition by Para-Chlorophenylalanine Impairs Defensive Reactions of Aversive Learning and Long-term Sensitization in Terrestrial Snails

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Abstract
In this paper, we present a study of the effects of reducing serotonin levels by para-chlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase, the limiting enzyme in serotonin biosynthesis, on two forms of behavioral plasticity in the terrestrial snail, *Helix lucorum*: the conditioned defensive reflex food aversion and long-term sensitization. We found that the snails injected with PCPA did not learn the conditioned reflex food aversion, while saline solution injection did not affect the learning process. The latency of consuming one kind of food in these snails was significantly reduced compared to the active control. We also found that PCPA injection prevents the formation of long-term sensitization.

Keywords Serotonin · Para-chlorophenylalanine · Aversive learning · Long-term sensitization · Land snail

1 Introduction

Initially serotonin, which is chemically identified as 5-hydroxytryptamine (5-HT), was isolated in 1947 from the serum of mammals [1]. Since then, intensive studies of its function have begun. These studies have shown the important role of 5-HT in the activities of the central nervous system and in the mechanisms of learning and memory [1–3]. 5-HT is the main mediator which defensive reflex-based defensive behavior relays on in mollusks [4–7]. A large number of experiments were performed using 5-HT application to obtain cellular training analogues [8–12]. Furthermore, injection of 5-HT or its precursor 5-HTP into the snail's body accelerated the learning process [13, 14].

Another alternative approach to study the role of the serotonergic system in learning and behavior is to deplete the 5-HT using its neurotoxic analogues of 5,6- and 5,7-dihydroxytryptamine (5,6- and 5,7-DHT) [15–19]. It was found that the injection of this neurotoxin led to a disruption in the formation of long-term sensitization (LTS) in *Aplysia californica* [16] and terrestrial snail *Helix lucorum* [18], to a blockade of heterosynaptic facilitation and a disturbance in the production of conditioned reflexes [15, 19]. Reduction of 5-HT can also be achieved by using para-chlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT biosynthesis, as it was shown that PCPA reduces 5-HT to 10% of the baseline [20, 21]. The effects of 5,7-DHT and PCPA on 5-HT and catecholamine levels were similar [20, 22]. Recently, we have shown that PCPA, by decreasing the level of the 5-HT, affects significantly the process of the reconsolidation of the long-term contextual memory [23, 24].

Based on these outcomes, we conducted a study of the effect of reducing 5-HT levels by PCPA on the formation of the conditioned defensive reflex and LTS in the terrestrial snail.

2 Methods

Experiments were performed on adult *Helix lucorum* (Gastropoda, Pulmonata) land snails weighing about 25 g. At least 2 weeks prior to the experiments, the snails were kept in terrarium and received fresh raw carrots. The snails were deprived of food for 3 days before training.

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Two series of experiments were conducted to investigate the effect of the inhibition of 5-HT synthesis by PCPA on two forms of neural plasticity: the formation of the conditioned reflex food aversion and the formation of LTS. We used the saline solution (SS) of the following composition: NaCl, 80 mM; KCl, 4 mM; $CaCl_2$, 10 mM; $MgCl_2$, 6 mM; $NaHCO_3$, 5 mM (or Tris, 5 mM); and pH , 7.6 7.8 [25]. In each series, animals were divided into 2 groups: aversive learning + PCPA ($n = 12$) and its control aversive learning + saline solution ($n = 10$) in the first series (PCPA group and SS group accordingly) and LTS + PCPA ($n = 6$) and its control LTS + SS ($n = 6$) in the second (LTS + PCPA group and LTS + SS group accordingly). The experiments of the first series were performed 2 times, the data results were similar, and therefore, the figures are given for the first training.

The substance was diluted in saline solution and was injected 3 days prior to the training using a syringe (0.50 ml / snail) through the skin of the insensitive region under the mantle roll. The dose of 0.2 mg/g (about 5 mg per snail) was given before training. This dose was chosen based on the results of a previous study, where 200 mg/kg of PCPA reduced the level of 5-HT in the cerebral cortex by 8 times, while doses of 50 mg/kg and 100 mg/kg slightly changed the content of 5-HT. [21]. This dose has also been shown previously to effectively influence memory training in our experiments on the terrestrial snail *Helix lucorum* [23, 24]. The saline solution was administered to SS animals at the same time in the same amount (0.50 ml/snail).

Classical conditioned food aversion training [26] was performed with the snails attached to brackets by their shells in a way that allowed them to crawl freely on a ball floating in water-filled Becher. The food was presented 0.5 cm from the snail's mouth. Fresh pieces of cucumber were used as the conditioned stimulus (CS), and fresh pieces of carrot were used as differentiating stimulus (DS). An electric shock (50 Hz, 300 ms, 1.2 mA) served as the unconditioned stimulus (US). The electric shock was applied to the food and body of the snail after the first bite (consummatory reaction); one electrode was inserted into the food, while the other was used to shock the animal. The used current did not cause damage to the skin of animals, which can occur as pigmented areas formed after stimulation with larger current values [27]. Electrical stimulation led to the suppression of the feeding behavior and induced a withdrawal reaction (retraction of the head and body into the shell). A complete refusal of the CS during the 2-min test period served as the conditioned food aversion criteria. Two training sessions were performed daily for 5 days with half an hour between them. In each session, the CS was presented 10 times with 5 min intervals. Every time the animal refused to eat was recorded, and a percentage of the CS refusal was calculated for each session. The DS was presented two times for each animal: one before and one after the training. The latent period of the consummatory reaction to the CS was measured, from the moment of the presentation to the first bite. This training model is widely used in studies on mollusks [28].

LTS [29, 30] was produced by four applications of electrical stimuli (6 8 mA, 50 Hz, duration 1 s) per day (separated by 1.5 2 h), for 4 days, to the head area. The criterion for the acquisition of LTS was a significant increase in the duration in which the respiratory aperture was closed in response to the presentation of test tactile stimulation of the mantle, as compared with the duration of the initial response [31].

The paired Student *t* test was used for comparison within one group. The unpaired Student's t-tests and Mann-Whitney *U* test were used for comparison between two groups. The statistical software SPSS was used. The results are shown as mean ± SEM. The statistical significance criterion was $p < 0.05$.

Comparisons between groups in our work included:

1. Comparisons between 2 different group, for which we used *t* test or Mann Whitney *U* test based on whether the distribution was normal or not. These tests are used usually to compare 2 groups with different samples in each group, which applies in our case, while ANOVA repeated measures test is used to determine whether three or more group means are different where the samples are the same in each group (groups are usually determined by time lags).
2. Comparison within one group (1 variable): where we compared consecutive values of the same variable, in this case, ANOVA repeated measures test can be used; however, conducting multiple paired *t* test between consecutive sessions is more convenient as it points to when the significant change has happened (without the needless all possible pairwise comparisons that ANOVA test includes when it is coupled with post hoc test)
3. Comparison with initial value within one group: conducting paired *t* test makes more sense as there is no need to the all possible pairwise comparisons in ANOVA test.

3 Results and Discussion

In the first series of experiments, the SS group started to form the reflex in the 6th session and its CS refusal rate increased sharply since then until the end of the training. The group required a total of (65 ± 3) US-CS coupled reactions to reach a CS refusal rate of 100% at the end of the training. The PCPA group on the other hand was presented with more coupled