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Research report

Telencephalic neural activation following passive avoidance learning in a terrestrial toad

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HIGHLIGHTS

- Passive avoidance learning (PAL) is shown in toads using aversive saline solutions.
- PAL neural basis in toads is explored with a comparative approach.
- Amygdala and striatum displayed higher activation than other brain areas.
- Thus, these areas could be key components of the brain circuit of PAL in amphibians.

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ABSTRACT

The present study explores passive avoidance learning and its neural basis in toads (*Rhinella arenarum*). In Experiment 1, two groups of toads learned to move from a lighted compartment into a dark compartment. After responding, animals in the experimental condition were exposed to an 800-mM strongly hypertonic NaCl solution that leads to weight loss. Control animals received exposure to a 300-mM slightly hypertonic NaCl solution that leads to neither weight gain nor loss. After 10 daily acquisition trials, animals in the experimental group showed significantly longer latency to enter the dark compartment. Additionally, 10 daily trials in which both groups received the 300-mM NaCl solution after responding eliminated this group effect. Thus, experimental animals showed gradual acquisition and extinction of a passive avoidance respond. Experiment 2 replicated the gradual acquisition effect, but, after the last trial, animals were sacrificed and neural activation was assessed in five brain regions using AgNOR staining for nucleoli—an index of brain activity. Higher activation in the experimental animals, relative to controls, was observed in the amygdala and striatum. Group differences in two other regions, lateral pallium and septum, were borderline, but nonsignificant, whereas group differences in the medial pallium were nonsignificant. These preliminary results suggest that a striatal-amygdala activation could be a key component of the brain circuit controlling passive avoidance learning in amphibians. The results are discussed in relation to the results of analogous experiments with other vertebrates.

1. Introduction

Aversive conditioning in amphibians is poorly understood mainly due to difficulties finding suitable conditions to motivate defensive behavior in these animals [29,38,39,51,59]. In mammals, aversive conditioning typically involves peripheral pain induced by the administration of electric shocks [1,6,32,36]. In amphibians, however, experiments using shock-induced pain have produced inconsistent results. For example McGill [31], observed an increase in escape latency of leopard frogs (*Rana pipiens*) trained in a shuttle box, instead of the typical decrease in latency, and a high mortality. Crawford and Langdon [8] did observe a decrease in latency, but only within training sessions; retention between sessions was not observed. Using four anuran species, Boice [4] reported no evidence of avoidance learning in two of them (*Scaphiopus hammondi* and *Rana pipiens*) and modest levels of avoidance in the other two species (*Rana clamitans* and *Bufo woodhousei,* responses in 20 and 100 trials out of a total of 200 trials). Karplus et al. [24] trained aquatic frogs (*Xenopus laevis*) in a passive avoidance situation. Animals placed in a square aquatic tank were shocked whenever they

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entered the dark side of the tank. Animals successfully avoided the dark side after a few training trials. By contrast, Dicke et al. [11] using a terrestrial toad (*Bombina orientalis*), reported limited success in their attempt at punishing the prey-capture response by shocking their animals on a leg contingent with tongue extension.

The failure of extrapolating training techniques from mammals to amphibians has led more recently to alternative procedures. For example, Bilbo et al. [3] used warm water to elicit escape behavior in frogs. In anuran amphibians, another option is to exploit their skin sensitivity to hypertonic saline solutions. In toads and frogs, rehydration occurs mostly through a pelvic patch of highly vascularized skin, rather than by drinking [2]. The properties of this pelvic patch have been studied in detail in the terrestrial toad *Rhinella arenarum* (formely *Bufo arenarum*).This previous studies have shown that toads have a plasma concentration that is approximately 245 mOsm/kg, which is isotonic to a 115 mM NaCl solution [13,54]. Thus, these toads are capable of rehydrating from saline NaCl solutions with a concentration lower than 250 mM [28]. Thus, toads can rehydrate exposed both to hypotonic saline solutions (0–115 mM) as to slightly hypertonic saline solutions (115–250 mM), wherein are also able to actively rehydrate. By contrast, concentrations highly hypertonic (above 350 mM) result in dehydration and elicit escape behavior [10,40]. Exposure to a 300-mM solution leads to no net weight gain or loss; therefore, this solution is assumed to be neutral [28]. This suggests that a single continuum of salinity may yield appetitive (below 250 mM), neutral (300 mM), or aversive (above 350 mM) reinforcers [10,28].

Experiments based on this continuum show that, when used as a reinforcer, an 800 mM NaCl solution supports aversive heart-rate conditioning and active avoidance learning [10]. Moreover, the runway performance of toads improved when reinforced with distilled water, but did not change when reinforced with the neutral 300 mM solution, and deteriorated when reinforced with the aversive 800 mM solution [40]. The present experiments were aimed at (1) extending this training protocol to the passive avoidance situation, which requires animals to learn to suppress a dominant response after pairings with an aversive reinforce, and (2) exploring the neural basis of passive avoidance learning by assessing neural activity in several brain regions.

2. Experiment 1

Two groups of animals were trained in a passive avoidance situation using a two-compartment shuttle box, one of them illuminated and the other not (dark). As toads normally tend to seek and stay in the dark part of an enclosure (Muzio, unpublished observation), animals were placed in the illuminated compartment and the time to enter the dark compartment was recorded. Entrance into the dark compartment was paired with forced immersion in an aversive 800-mM NaCl solution in experimental animals, but with forced immersion in a neutral 300-mM NaCl solution in controls. It was expected that animals in the experimental condition would acquire a reluctance to enter the dark compartment, as measured in terms of response latency. After 10 daily trials, all animals received another 10 daily trials in which crossing to the dark compartment resulted in immersion in the 300-mM NaCl solution. This measured the extent of extinction of passive avoidance in experimental animals.

2.1. Method

2.1.1. Subjects

Twenty naive adult male terrestrial toads (*R. arenarum*, previously *Bufo arenarum*) collected in pounds around Buenos Aires, Argentina, served as subjects. This species is not listed as threatened [21]. Animals were maintained according to the NIH Guide for Care and Use of Laboratory Animals. Upon arrival, toads were treated for a week with antibiotics and anthelmintics to control bacterial and parasitic infections, and kept in group cages with running water during at least two weeks. Before the start of the experiment, animals were fed once a week with dog chow and were subjected to a morphological examination to assess body symmetry and reflexes. Standard weights (weight of the hydrated animal with its urinary bladder empty; [57]) were obtained the day before the start of pre-training. The mean standard weight for this sample was 90.4 g (range: 72.7–123.0 g). The vivarium was kept at 24–25 °C and 40–50% humidity, and subjected to 16:8 h light:dark cycle (lights on at 04:00 h). Toads were trained between 8:00–13:00 h. Animals were kept at 80% of their standard weight during the entire experiment. This procedure successfully results in toads motivated to search for water [41,52].

2.1.2. Apparatus

We used a one-way shuttle box built with black Plexiglas and divided into two compartments separated by a guillotine door (Fig. 1). The shuttle box was covered with translucent Plexiglas lids that allowed constant observation of the animals through a mirror. One of the compartments (8 × 9 × 16 cm, LxWxH) was illuminated by a 40-W bulb over the translucent lid, had white walls, and a plastic grid in the floor. The other compartment was larger (14.5 × 14.5 × 19 cm, LxWxH), had no illumination (dark compartment), and had black walls. The dark compartment was connected through a cannula to a bottle containing NaCl solutions. Thus, this compartment could be rapidly flooded with the appropriate saline solution by simply rising the bottle.

2.1.3. Training procedure

Toads were randomly assigned to one of two groups: Group 800 ($n = 10$, exposed to an aversive 800 mM highly hypertonic NaCl solution, leading to weight loss) and Group 300 ($n = 10$, exposed to a neutral/nonaversive 300 mM slightly hypertonic NaCl solution, leading to no net weight gain or loss).

Animals received two pretraining trials, one per day, in which they were placed in the illuminated compartment with the guillotine door closed for 30 s. Then, the door was opened and they could freely move about the shuttle box for 10 min. During these two trials, the box was dry, but animals were still weighted before and after each trial.

Training involved 10 acquisition trials, followed by 10 extinction trials, at a rate of one trial per day. In each trial, toads were gently placed in the illuminated compartment for 30 s and then the guillotine door was raised. Two dependent variables were registered: (1) *Response latency* (in seconds): Time from the moment in which the animal was placed in the illuminated compartment to the moment it entered the dark compartment with its four legs. (2) *Weight variation*: The weight of each animal (in grams) was registered before and after each trial to estimate water consumption. The difference between these two weights was divided by the standard weight and multiplied by 100 to provide a relative measure of water uptake corrected for individual differences in body weight.

During acquisition trials, the goal compartment was flooded with the appropriated saline solution (800 or 300 mM of NaCl, depending on the group) as soon as the toad entered the compartment. Extinction trials were identical to acquisition trials, except that all animals were exposed to the neutral 300-mM NaCl solution in the goal compartment. In acquisition trials, if an animal did not enter into the dark compartment within 10 min of trial onset, it was removed and a latency of 600 s was recorded for that animal on that trial. In extinction trials, animals that did not respond after 10 min were guided to the dark compartment, the guillotine door was closed, and a latency of 600 s was recorded. This guidance