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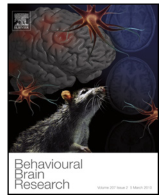

Research report

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.

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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 ($8 \times 9 \times 16$ 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 ($14.5 \times 14.5 \times 19$ 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

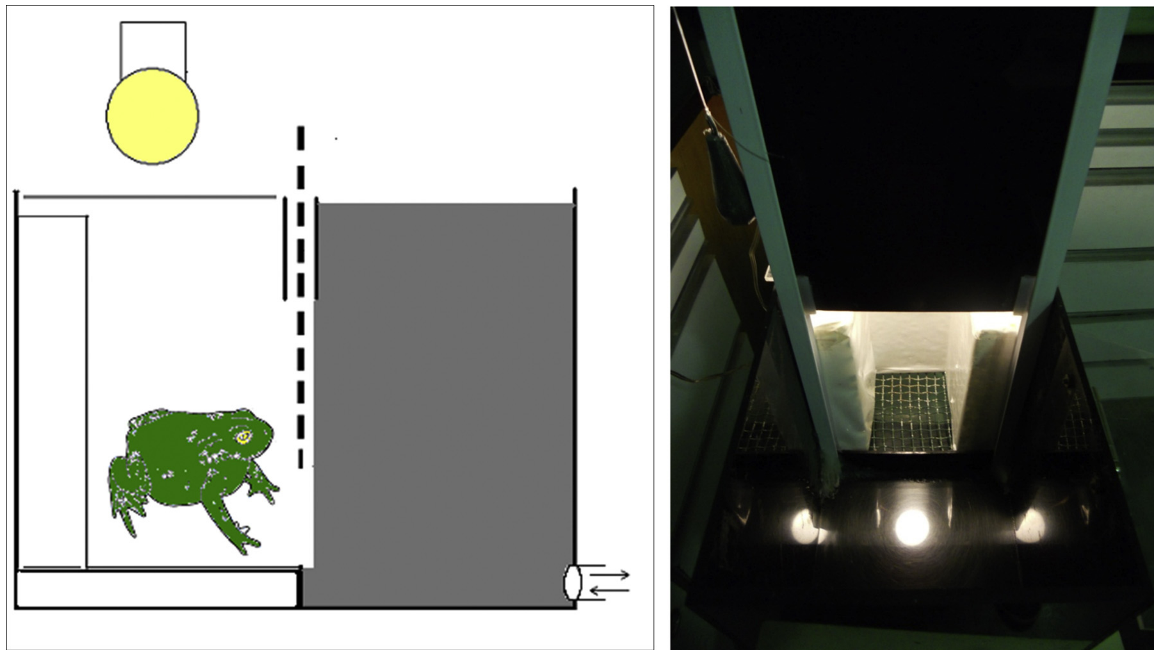

Fig. 1. Left. Schematic of one-shuttle box used to train toads. Right: Photograph from the upper side of the dark compartment.

procedure in extinction trials was implemented to allow animals to experience the lack of aversive consequences for moving into the dark compartment when exposed to the neutral 300-mM NaCl solution. In all trials, exposure to the solutions was inescapable and lasted 120 s. At the end of this period, toads were gently taken out of the shuttle box, their skin was dried with a cloth, and they were transferred to dry cages where they remained until the next day. At least 30 min after each daily trial, animals that had lost weight during the trial were supplemented with deionized water until they reached their target weight, whereas those that had gained weight during the trial were dehydrated before the next trial. This procedure ensured that animals would be at 79–81% of their standard weight at the start of each trial.

Analysis of the data was made by an analysis of variance with trials as a repeated-measure factor whenever applicable, followed by LSD pairwise comparisons derived from the main analysis. The alpha value was set to less than 0.05 for all tests. All statistics were computed with the IBM SPSS Statistics 21 package.

2.2. Results

During the two pretraining trials, the average (±SEM) running latencies for each trial were 113.9 (±22.4) and 208.3 s (±48.5) for Group 800 and 136.8 (±49.0) and 184.8 s (±36.6) for Group 300. An analysis of these pretraining scores indicated no differences between groups, trials, or their interaction, $Fs < 2.97$, $ps > 0.10$. Thus, there was no detectable pretraining bias across groups in terms of their behavior. Fig. 2 (top) shows the response latency of each group during acquisition and extinction trials. Response latencies increased during acquisition and decreased during extinction in Group 800, whereas no major changes in latency were observed in Group 300. These conclusions were supported by statistical analyses. A Group (800, 300) x Trial (1–10) analysis of acquisition data showed significant effects for groups, $F(1, 18) = 6.10$, $p < 0.03$, trials, $F(9, 162) = 2.43$, $p < 0.02$, and their interaction, $F(9, 162) = 4.18$, $p < 0.001$. Pairwise LSD comparisons indicated that the source of the interaction was group differences on trials 3, 4, 6, 8, and 10, $Fs(1, 18) > 5.07$, $ps < 0.04$. Complete avoidance performance (i.e., trials with the maximum latency possible) was observed in five animals

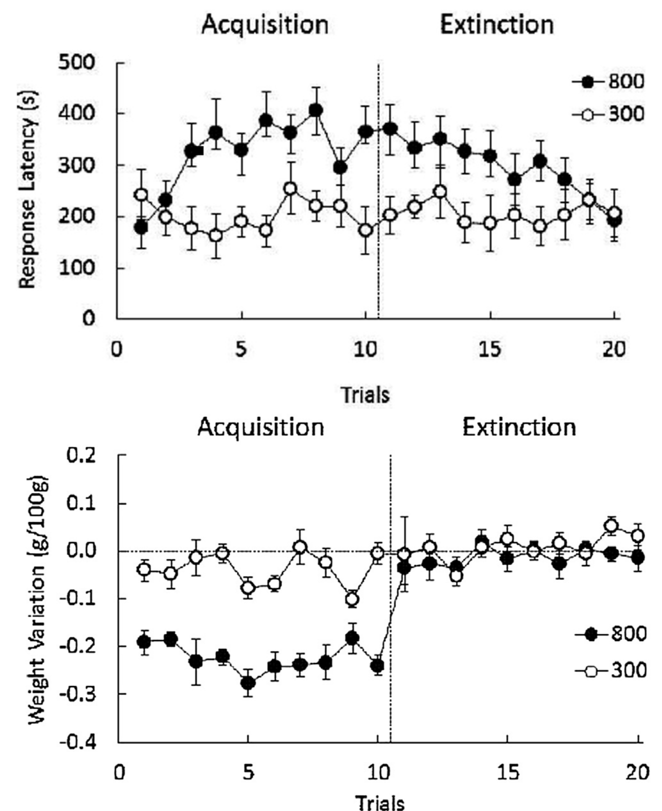

Fig. 2. Top: Mean (±SEM) of response latencies (s) during 10 daily acquisition trials and 10 daily extinction trials for both groups. Bottom: Mean (±SEM) of weight variation (g/100 g) between measurements taken before and after each daily trial, for both groups.

(in a total eight trials) in Group 800, but in one animal (two trials) in Group 300. A similar analysis of the last five acquisition trials showed only significant differences between groups, $F(1, 18) = 8.92$, $p < 0.009$, but no trial or interaction effects, $Fs < 2.24$, $ps > 0.07$. Thus, instrumental behavior was asymptotic during the second half of

acquisition. Separate analyses of latencies across acquisition trials indicated that whereas there was a significant acquisition effect in Group 800, $F(9, 81) = 4.47$, $p < 0.001$, there was no detectable acquisition effect in Group 300, $F(9, 81) = 1.42$, $p > 0.19$.

Fig. 2 (top) also shows that latencies decreased during extinction trials in Group 800, but continued without much change in Group 300. A Group x Trial (11–20) analysis showed a significant interaction, $F(9, 162) = 1.94$, $p < 0.05$), but nonsignificant effects for groups or trials, $Fs < 2.80$, $ps > 0.05$. Pairwise LSD comparisons revealed that Group 800 scored above Group 300 on trials 1 and 4, $Fs(1, 18) > 4.50$, $ps < 0.05$. Separate analyses of individual groups indicated that there was a significant extinction effect in Group 800, $F(9, 81) = 3.09$, $p < 0.004$, but no appreciable extinction in Group 300, $F < 1$.

Fig. 2 (bottom) shows weight variation data during the course of acquisition and extinction for each group. The reinforcer manipulations produced the expected results, that is, a reduction in body weight in the toads exposed to an inescapable 800 mM NaCl solution, but not much change in body weight in animals exposed to a 300 mM NaCl solution. An analysis of acquisition data confirms that there was a significant difference between groups, $F(1, 18) = 81.45$, $p < 0.001$, but no trial or interaction effect, $Fs < 1.27$, $ps > 0.26$. These group differences dissipated during extinction, when none of the effects was significant, $Fs < 1.75$, $ps > 0.08$. The mean weight variation of each group, in each phase and over the entire ten trials, was also contrasted against zero with a one-sample, two-tailed T-test. In acquisition, both groups differed from zero, $ts(9) > -3.09$, $ps < 0.02$, whereas in extinction none of the groups differed from zero, $ts(9) > -1.64$, $ps > 0.13$. The mild, but significant, loss of weight observed in the acquisition of the Group 300 may reflect the slightly hypertonic effect of a 300 mM NaCl solution in these animals during this first phase of the training. Notice that this effect was not present during extinction trials, when access to 300 mM NaCl solution was given to all the animals. Thus, factors common to both phases (e.g., evaporation due to heat in the lighted compartment) cannot account for the acquisition effect in Group 300.

3. Experiment 2

The procedure used in Experiment 1 proved effective to induce passive avoidance learning in toads using inescapable immersion in a hypertonic NaCl solution that causes dehydration. These results extended previous research using a similar procedure [10,40] to the passive avoidance situation. In Experiment 2 we trained toads in the same situation with the goal of identifying regions in the amphibian telencephalon functionally activated during the performance of the passive avoidance task. At the end of acquisition training equal to that of Experiment 1, toads were sacrificed and their brains were prepared for histological analysis. We used the AgNOR technique to determine quantitatively the level of neuronal activity after asymptotic performance in the passive avoidance task. The nucleolus organizer region (NOR) is a compartment of the cell nucleus which main function is the rRNA synthesis and biogenesis of ribosomal subunits [7]. Nucleolus is formed by loops of DNA containing ribosomal coding sequences, and most of all proteins and ribonucleoprotein complexes with high affinity for silver ions. Nucleoli stained with silver nitrate are called AgNORs [60]. AgNOR is a histochemical procedure used to study cellular activity by comparing the size of the NOR to the size of the cell's nucleus. Since NOR's volume increases in relative size when there is an increase in protein synthesis [53], NOR's size could be used as a marker of protein synthesis activity. For example, AgNOR has been extensively used to assess tumoral cells, and has proved to be a predictor of treatment outcome [47]. As many brain functions, such as learning, require protein synthesis [44], therefore, AgNOR has also been used to study brain structures activity. In other aspect, AgNOR is also a convenient technique for studies in comparative psychology because, unlike immunohistochemical techniques, it does not require the conservation of specific peptide sequences and, therefore, it can be applied to distantly related species, such as mammals [15,17] and teleost fish [5,61]. Particularly interesting is the fact that this technique has been extensively used in fish, another heterothermic group, where has given strong evidence linking increased neural activity to increased AgNOR labeling after training animals in operant procedures [5,61].

Most studies of the neural basis of learning in amphibians involve telencephalic lesions and appetitive tasks [9,42,43,45]. In mammals, the amygdala has long been identified as one of the main structures implicated in aversive learning [12,46], including passive avoidance [20,22]. Homologous regions of the fish telencephalon have also been implicated in active avoidance learning [49,50], a finding suggesting substantial evolutionary conservation of brain function in aversive learning across vertebrates. In amphibians, several histochemical-morphological studies have proposed the ventral part of the lateral pallium of the telencephalon as homologous to the mammalian amygdalar complex [27,34,35,37]. However, there are few behavioral studies testing the functions of these regions [18,19,26].

3.1. Method

3.1.1. Subjects and apparatus

The subjects were 24 experimentally naive, adult, male toads (*R. arenarum*). They were obtained and maintained as described in Experiment 1. The training apparatus was the same described in Experiment 1. The standard weight of these animals varied between 59.6–159.5 g (Mean = 99.1 g, SEM $\pm$ 5.7).

3.1.2. Training procedure

The pretraining and acquisition training procedures were identical to the groups included in Experiment 1 (Group 800, n = 12; and Group 300, n = 12).

3.1.3. Staining procedure and AgNOR quantification

We followed the staining and quantification protocols employed previously in similar operant procedures in fish [5,61]. Twenty four hours after the last training trial toads were deeply anesthetized with tricaine methanesulfonate (MS222, Sigma USA) and perfused transcardially with saline solution followed by 10% formalin in 0.1 M phosphate buffer solution (pH 7.4). Brains were removed, immersed for at least a week in the same solution used for perfusion, and then embedded in paraffin wax. Telencephalons were cut in series of 12-μm thick coronal sections with a microtome. One of each three sections at medial level of the telencephalon was silver stained following the staining procedure described in [48].

NORs were quantified in five telencephalic regions selected according to Moreno and González [33–35] and Marín et al. [30], as shown in Fig. 3A: The medial zone of the dorsal region (medial pallium), the medial zone of ventral region (septum), the lateral zone of the subdorsal region (lateral pallium), the lateral zone of the ventral region (striatum), and the lateral zone of the medial region between the lateral pallium and the striatum (amygdala). The latter region, what we call amygdala, according to some authors refers to a subdivision of the amphibian amygdaloid complex, corresponds to the lateral and anterior amygdala [33–35].

The sections were observed with a light microscope Leica DM500 (Leica Microsystems, Buffalo Grove, IL, USA) using an oil immersion lens (1000$\times$ magnification). NORs were visualized as black or black-brown intranuclear dots, in contrast with the nuclei of neurons that appeared weakly stained in brown-golden color; these structures are shown in Fig. 3B and C. Glial cells were dis-

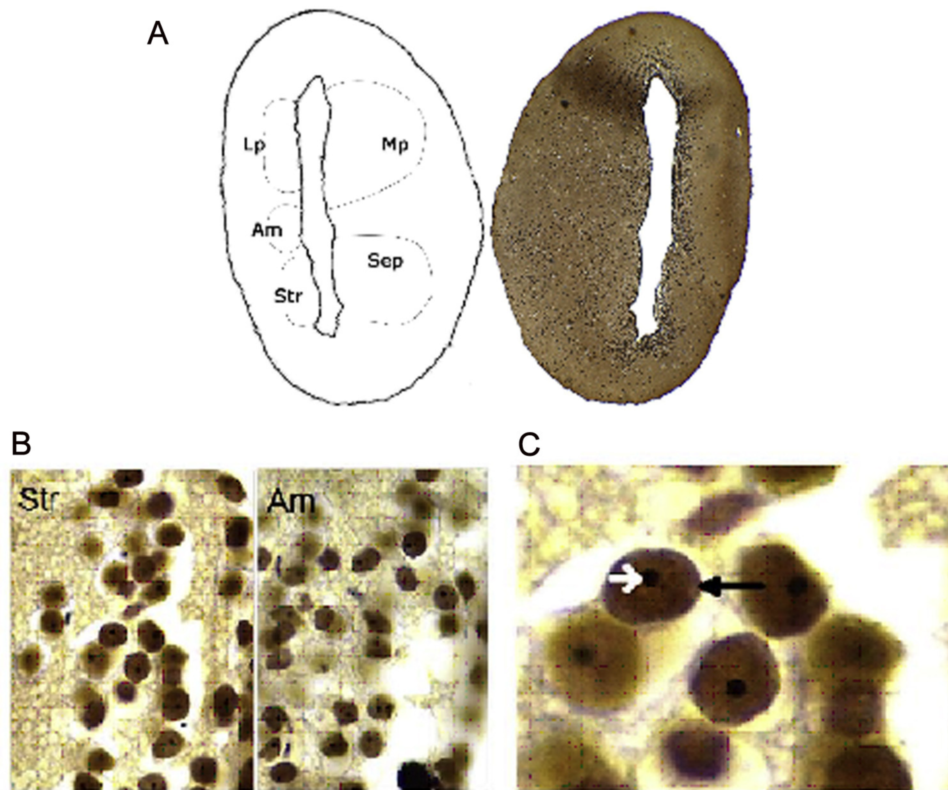

Fig. 3. A. Diagram (left) and representative AgNOR-stained coronal section (×40, right) at the medial telecephalic level of the anterior-posterior axis in the toad *Rhinella arenarum* showing the five regions where the analysis was conducted (MP: medial pallium; LP: lateral pallium; Sep: septum; Str: striatum; Am: amygdala). B. Examples of AgNOR staining in neurons of the striatum and amygdala (×1000). C. Enlargement of striatum image showing a nucleus (black arrow) and a nucleolus (white arrow).

tinguished from neurons on the basis of their smaller size and the clearly visible nuclear border. A computer-assisted interactive image analyzer (Leica, Application Suite version 2.0.0) and software (Adobe Photoshop CS5) were used to measure cellular parameters in each selected region. The parameters evaluated were the number of NORs per neuron, the total nuclear area, the absolute NOR area, and the relative NOR area, defined as (absolute NOR area/total nuclear area)*100.

Quantifications were made in 3–5 consecutive sections selected from the total of serial sections which contained the relevant regions. A total of 80 nuclei were sampled in each region. To obtain an unbiased sampling, we performed a systematic scanning of each telencephalic region using four microscopical fields within each section, avoiding repetition of the measures. About 20 cells were chosen from each brain region and section. Multiple focal planes were evaluated to avoid erroneous measurements of counting of overlapping nuclei. The sections were analyzed by an observer blind to the animal's training condition.

3.2. Results

3.2.1. Behavioural performance

The mean (±SEM) running latencies for each pretraining trial were 149.8 (±43.7) and 198.0 s (±41.2) for Group 800 and 179.8 (±49.0) and 202.3 s (±34.0) for Group 300. An analysis of these pretraining scores indicated no differences between groups, trials, or their interaction before the start of training, $Fs < 1.49$, $ps > 0.23$. Fig. 4 (top) shows the running latency for each group during acquisition trials. Animals showed a learning profile similar to that observed in the previous experiment. Running latencies increased across acquisition trials in Group 800, but showed little change in Group 300. A Group x Trial analysis yielded a significant group effect, $F(1, 22) = 7.38$, $p < 0.02$) and a significant acquisition effect, $F(9198) = 5.42$, $p < 0.001$, but the interaction failed to reach significance, $F(9198) = 1.83$, $p > 0.06$. Five toads in Group 800 received the maximum latency in a total of eleven trials, whereas two animals in Group 300 failed to enter into the dark compartment in a total of three trials. Avoidance performance was asymptotic during the second half of training (trials 6–10), as inferred from nonsignificant trial or interaction effects, $Fs < 2.25$, $ps > 0.07$, combined with a significant group difference, $F(1, 22) = 8.95$, $p < 0.008$. Separate analyses for each group confirmed that whereas Group 800 exhibit a significant increase in latencies across trials, $F(9, 99) = 5.30$, $p < 0.001$, the performance of Group 300 did not vary significantly across trials, $F(9, 99) = 1.09$, $p > 0.37$.

Weight variation data during the course of acquisition are shown in Fig. 4 (bottom) for each group. The two reinforcers used had the intended effects, with the 800 mM NaCl solution resulting in weight loss and the 300 mM solution in lower, but still noticeable weight loss. However, unlike in Experiment 1 (see Fig. 2, bottom), there was greater variability in these values. Still, an analysis of the weight variation during acquisition indicated a highly significant difference between the groups, $F(1, 22) = 32.13$, $p < 0.001$. The trial and interaction effects were nonsignificant, $Fs < 1$. The overall performance of each group averaged across the ten acquisition trials was also compared to zero using a one-sample *t*-test. Group 800 was clearly different from zero, $t(11) = -9.05$, $p < 0.001$, but Group 300′s weight variation was not different from zero, $t(11) = -1.49$, $p > 0.16$.

3.2.2. AgNOR assessment

No differences between groups were found in terms of absolute NOR area, absolute nucleus area, or number of NORs per nucleus for any of the five telencephalic regions studied, $ts(22) < 1.90$, $ps > 0.14$.

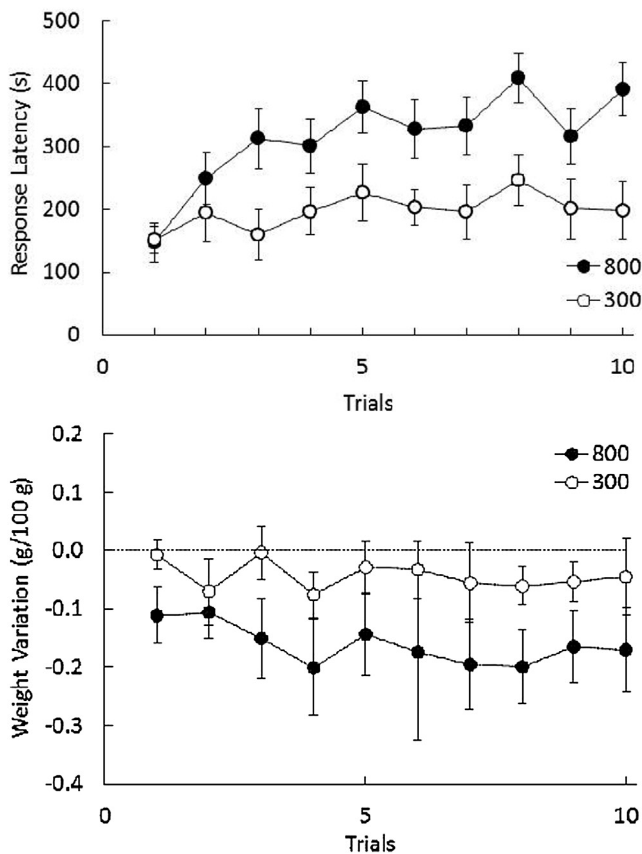

Fig. 4. Top: Mean (±SEM) of response latencies (s) during 10 daily acquisition trials for both groups. All animals were sacrificed immediately at the end of trial 10 and their brains prepared for staining. Bottom: Mean (±SEM) of weight variation (g/100 g) during the trial.

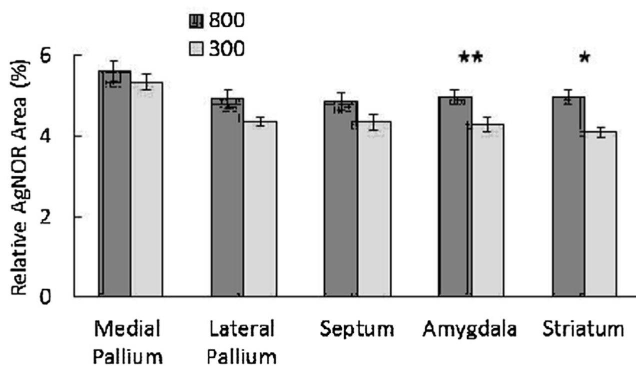

Fig. 5. Mean (±SEM) of relative size of the AgNORs in the five telencephalic regions studied. Asterisks denote significance level (*: $p < 0.05$; **: $p < 0.01$).

Variation in nucleus and NORs areas were in opposite directions with treatment, but this tendency never reached significant differences. Group differences were observed only in terms of a measure of NOR area relative to nucleus area and only in two of the brain regions. Fig. 5 shows these results for both groups in the five telencephalic regions studied: medial pallium, lateral pallium, septum, amygdala, and striatum. In general terms, all of the five telencephalic studied regions exhibited a tendency to a major relative AgNOR area in animals from Group 800 than in toads from Group 300. However, statistical analyses only showed between groups differences for the ventrolateral regions of the telencephalon: amygdala, $F(1, 22) = 8.13$, $p < 0.01$, and striatum, $F(1, 22) = 7.59$, $p < 0.02$. For two other regions, lateral pallium and septum, the results were nonsignificant, but borderline, $Fs(1, 22) < 4.03$, $ps > 0.056$. Finally, there was no difference in relative NOR area between groups in the medial pallium, $F < 1$.

4. Discussion

Toads exposed to an aversive hypertonic saline solution (800 mM NaCl) showed an increased latency of response in both experiments. This change in behavior was fast, as it started after the first or second acquisition trial, and significant differences in responses compared to the control group developed from the third trial. To the best of our knowledge, our study is the first to describe the presence of passive avoidance learning in amphibians using a highly hypertonic saline solution as reinforcer. These results confirm a previous observation that hypertonic NaCl solutions can be used to motivate aversive responses in anurans [10,40]. By contrast, toads exposed to a slightly hypertonic saline solution (300 mM NaCl) exhibited a stable behavior with minimal signs of aversive learning. The 300 mM solution provides an appropriate control condition because it involves an analogous immersion in the solution without inducing either an appetitive or an aversive response [28,40]. Therefore, behavioral differences during training can be safely attributed to the degree of aversiveness induced by each solution. The avoidance response developing during acquisition trials was reversed during the extinction trials of Experiment 1. Such a gradual reduction in response latencies is consistent with the conclusion that the hypertonic solution induced a process of aversive associative learning.

One reason why these saline solutions are effective reinforcers lies in some adaptations present in the skin of toads. Previous studies in our laboratory showed that the skin of the terrestrial toad *Rhinella arenarum* is sensitive to electrolytes in solution [55]; in particular to saline solutions, being reported escape behaviors from highly hypertonic NaCl solutions (400 mM or more). Thus, toads might have evolved sensory mechanisms in the skin capable of detecting hypertonic environments. Such ability would help toads to prevent dehydration by water loss through the skin, a major selective pressure for animals dependent on daily access to water for survival [58]. However, although the hypertonic, 800 mM NaCl solution supported passive avoidance, it did not completely prevent animals from entering into the goal compartment, except in some isolated trials. Unsystematic observations of these animals during training trials suggest a possible explanation. The toads in Group 300 stayed still in the illuminated compartment for a variable amount of time and then moved slowly to the dark compartment to sit on the solution. By contrast, after a few trials, the toads in Group 800 were agitated and reared against the walls of the illuminated box, even before the guillotine door was raised (see Fig. 1). In some trials, a toad engaged in what appeared to be attempts to exit the apparatus would fall into the dark compartment, therefore fulfilling the criterion for ending the latency measure.

Experiment 2 is a preliminary attempt at understanding the neural basis of passive avoidance in toads using the AgNOR technique. This technique assesses cell activation in terms of nucleolus area and number. The data presented here suggests that passive avoidance learning induces a relative increase in nucleolus area relative to nucleus area in the amygdala and the striatum, but not in other telencephalic regions. Thus, that increased relative NOR area might reflects previous changes in brain activity occurred in the amygdala and in the striatum. In anuran amphibians, the amygdala occupies the ventrolateral quadrant of the caudal telencephalon and extends rostrally into the ventral part of the dorsolateral pallium. Therefore, the amygdala region assessed in Experiment 2 corresponds to the lateral and anterior amygdala [33]. In mammals, the basolateral amygdala has been postulated to be the storage

site for Pavlovian fear conditioning [46]. Significant protein synthesis occurs in the basolateral amygdala during fear conditioning, a process that seems to be regulated by monoamine input [23]. The striatum, part of the dopaminergic system, has been proposed to mediate conditioned motor responses in mammals [25]. For example, during fear conditioning in rats, freezing is impaired by lesions of the dorsal striatum [14], but cardiovascular responses are not affected by similar lesions [56]. The present results suggest that the importance of striatal-amygdala connections may be a conserved pathway regulating fear conditioning in vertebrates.

Although interesting, there are several limitations of these neural results. First, the procedure used in Experiment 2 involved exposing animals to the solutions during the last training trial, before the animals were sacrificed for AgNOR assessment. It is possible that the observed activation is more related to the degree of aversiveness experienced in the dark compartment than to the degree of passive avoidance learning. An indirect test of this possibility would be to look for a correlation between response latencies during the final trial and relative AgNOR area. This does not directly speaks to the relationship between immersion in the solution (whether 800 or 300 mM NaCl) and relative AgNOR area in the amygdala and striatum, but it is a way of assessing a possible relationship between the degree of aversiveness (albeit only anticipatory in terms of response latency) and relative AgNOR area. Pearson's coefficients of correlation were calculated for each group separately and also for all the animals pooled, and for the striatum and the amygdala. The correlations were all rather low, r^2s < 0.14, and nonsignificant, providing no evidence for a relationship between aversiveness and relative AgNOR area in either the striatum or the amygdala. Second, the observed changes in neural activity were only observed in a relative measure, but failed to occur when absolute values of the structures involved in the relative transformation were used. One interpretation, of course, suggests that individual differences masked the effects on absolute values. Additional research would be valuable to determine the replicability of the current findings under similar conditions and with alternative techniques assessing neural activity. Finally, that a telencephalic region shows changes during conditioning does not necessarily imply that its functional integrity is necessary for learning to occur. For example, fear conditioning induces cortical changes associated to the presentation of the conditioned stimulus in rats, and yet complete decortication does not eliminate their ability to exhibit such conditioning [16].

With these cautions in mind, we suggest that striatal-amygdala activation is a key component of the brain circuit controlling passive avoidance learning in amphibians. This hypothesis implies a substantial degree of evolutionary conservation in brain circuitry underlying fear conditioning among vertebrates.

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References

[1] W.V. Bechterev, La psychologie objective (1913), Pp.iii+473, Alcan; Paris.
[2] J. Bentley, T. Yorio, Do frogs drink? J. Exp. Biol. 79 (1979) 41–46.
[3] S.D. Bilbo, L.B. Day, W. Wilczynski, Anticholinergic effects in frogs in a Morris water maze analog, Physiol. Behav. 69 (2000) 351–357.
[4] R. Boice, Avoidance learning in active and passive frogs and toads, J. Comp. Physiol. Psychol. 70 (1970) 154–156.
[5] C. Broglio, F. Rodríguez, A. Gómez, J.L. Arias, C. Salas, Selective involvement of the goldfish lateral pallium in spatial memory, Behav. Brain Res. 210 (2010) 191–201.
[6] Punishment and Aversive Behavior, in: B.A. Campbell, B.A. Church (Eds.), Appleton-Century-Crofts, New York, 1969.
[7] D. Cmarko, J. Smigova, L. Minichova, Popov A. Nucleolus, The ribosome factory, Histol. Histopathol. 23 (2008) 1291–1298.
[8] F.T. Crawford, J.W. Langdon, Escape and avoidance responding in the toad, Psychon. Sci. 6 (1966) 115–116.
[9] M.F. Daneri, E.B. Casanave, R.N. Muzio Medial Pallium lesion affects both turn and cue spatial learning in toads (Rhinella arenarum) Manuscript in preparation.
[10] M.F. Daneri, M.R. Papini, R.N. Muzio, Common toads (*Bufo arenarum*) learn to anticipate and avoid hypertonic saline solutions, J. Comp. Psychol. 121 (2007) 419–427.
[11] U. Dicke, A. Heidorn, G. Roth, Aversive and non-reward learning in the fire-bellied toad using familiar and unfamiliar prey stimuli, Curr. Zool 57 (2011) 709–716.
[12] S. Duvarci, D. Pare, Amygdala microcircuits controlling learned fear, Neuron 82 (2014) 966–980.
[13] H.G. Ferreira, C.H. Jesus, Salt adaptation in *Bufo bufo*, J. Physiol. 228 (1973) 583–600.
[14] T.L. Ferreira, K.M. Moreira, D.C. Ikeda, O.F. Bueno, M.G. Oliveira, Effects of dorsal striatum lesions in tone fear conditioning and contextual fear conditioning, Brain Res. 987 (2003) 17–24.
[15] L.M. García-Moreno, N.M. Conejo, H.G. Pardo, M. Gómez, F.R. Martin, M.J. Alonso, J.L. Arias, Hippocampal AgNOR activity after chronic alcohol consumption and alcohol deprivation in rats, Physiol. Behav. 72 (2001) 115–121.
[16] F. Gonzalez-Lima, H. Scheich, Neural substrates for tone-conditioned bradycardia demonstrated with 2-deoxyglucose: II. Auditory cortex plasticity, Behav. Brain Res. 20 (1986) 281–293.
[17] T. Gos, D. Krell, H. Bielau, J. Steiner, K. Trübner, R. Brisch, H.G. Bernstein, Z. Jankowski, B. Bogerts, Demonstration of disturbed activity of external globus pallidus projecting neurons in depressed patients by the AgNOR staining method, J. Affect Dis. 119 (2009) 149–155.
[18] I.C. Hall, I.H. Ballagh, D.B. Kelley, The Xenopus amygdala mediates socially appropriate vocal communication signals, J. Neurosci. 33 (2013) 14534–14548.
[19] A. Hoffman, M.C. Lico, *Autonomic* and *motor responses* to *electrical* stimulation of the toad's brain, Physiol. Behav. 8 (1972) 1039–1043.
[20] A.C.W. Huang, B.-C. Shyu, S. Hsiao, T.-C. Chen, A.B.-H. He, Neural substrates of fear conditioning, extinction, and spontaneous recovery in passive avoidance learning: a c-Fos study in rats, Behav. Brain Res. 237 (2013) 23–31.
[21] IUCN, 2010. IUCN red list of threatened species. Version 2010.4.
[22] F.K. Jellestad, H.K. Bakke, Passive avoidance after ibotenic acid and radio frequency lesions in the rat amygdala, Physiol. Behav. 34 (1984) 299–305.
[23] J.P. Johansen, C.K. Cain, L. Ostroff, J.E. LeDoux, Molecular mechanisms of fear learning and memory, Cell 147 (2011) 509–524.
[24] I. Karplus, D. Algom, D. Samuel, Acquisition and retention of dark avoidance by the toad, *Xenopus laevis* (Daudin), Anim. Learn. Behav. 9 (1981) 45–49.
[25] A.V. Kravitz, A.C. Kreitzer, Striatal mechanisms underlying movement, reinforcement, and punishment, Physiology 27 (2012) 167–177.
[26] F. Laberge, R.C. Feldhoff, P.W. Feldhoff, L.D. Houck, Courtship pheromone-induced c-Fos-like immunolabeling in the female salamander brain, Neuroscience 151 (2008) 329–339.
[27] F. Laberge, S. Mühlenbrock-Lenter, W. Grunwald, G. Roth, Evolution of the Amygdala: new insights from studies in amphibians, Brain Behav. Evol. 67 (2006) 177–187.
[28] M.A. Loza-Coll, R.N. Muzio. Hypertonic NaCl solutions as aversive stimuli in terrestrial toads Manuscript in preparation.
[29] E.M. Macphail, Brain and Intelligence in Vertebrates, Clarendon Press, Oxford, UK, 2016, pp. 1982.
[30] O. Marín, W.J.A.J. Smeets, A. González, Basal ganglia organization in amphibians: chemoarchitecture, J. Comp. Neurol. 392 (1998) 285–312.
[31] T.E. McGill, Response of the leopard frog to electric shock in an escape-learning situation, J. Comp. Physiol. Psychol. 53 (1960) 443–445.
[32] N.E. Miller, Learnable drives and rewards, in: S.S. Stevens (Ed.), Handbook of Experimental Psychology, Wiley, New York, 1951, pp. 435–472.
[33] N. Moreno, A. González, Localization and connectivity of the lateral amygdala in anuran amphibians, J Comp. Neurol. 479 (2004) 130–148.
[34] N. Moreno, A. González, The common organization of the amygdaloid complex in tetrapods: new concepts based on developmental, hodological and neurochemical data in anuran amphibians, Prog. Neurobiol. 78 (2006) 61–90.
[35] N. Moreno, A. González, Evolution of the amygdaloid complex in vertebrates, with special reference to the anamnio-amniotic transition, J. Anat. 211 (2007) 151–163.
[36] O.H. Mowrer, On the dual nature of learning: a reinterpretation of conditioning and problem-solving, Harv. Educ. Rev. 17 (1947) 102–148.
[37] J. Mühlenbrock-Lenter, H. Endepols, G. Roth, W. Walkowiak, Immunohistological characterization of striatal and amygdalar structures in the telencephalon of the fire-bellied toad *Bombina orientalis*, Neuroscience 134 (2005) 705–719.
[38] R.N. Muzio, Aprendizaje instrumental en anfibios, Rev. Latinoam. Psicol. 31 (1999) 35–47.
[39] R.N. Muzio, Aprendizaje en anfibios, el eslabón perdido: un modelo simple cerebral en el estudio de conductas complejas, Cuad. Herpetol. 27 (2013) 87–100.

[40] R.N. Muzio, V. Creydt, V. Pistone Iurman, M. Rinaldi, M. Sirani, M.R. Papini, Incentive or habit learning in amphibians? PLoS One (2011) 6:e25798, http://dx.doi.org/10.1371/journal.pone.0025798.
[41] R.N. Muzio, E.T. Segura, M.R. Papini, Effect of schedule and magnitude of reinforcement on instrumental acquisition and extinction in the toad, *Bufo arenarum*, Learn. Motiv. 23 (1992) 406–429.
[42] R.N. Muzio, E.T. Segura, M.R. Papini, Effects of lesions in the medial pallium on instrumental learning in the toad *Bufo arenarum*, Physiol. Behav. 54 (1993) 185–188.
[43] R.N. Muzio, E.T. Segura, M.R. Papini, Learning under partial reinforcement in the toad (*Bufo arenarum*): Effects of lesions in the medial pallium, Behav. Neural. Biol. 61 (1994) 36–46.
[44] K. Nader, G.E. Schafe, J.E. Le Doux, Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval, Nature 406 (6797) (2000) 722–726.
[45] M.R. Papini, R.N. Muzio, E.T. Segura, Instrumental learning in toads (*Bufo arenarum*): Reinforcer magnitude and the medial pallium, Brain Behav. Evol. 46 (1995) 61–71.
[46] E.A. Phelps, J.E. LeDoux, Contributions of the amygdala to emotion processing: from animal models to human behavior, Neuron 48 (2005) 175–187.
[47] A. Pich, L. Chiusa, E. Margaria, Prognostic relevance of AgNORs in tumor pathology, Micron 31 (2) (2000) 133–141.
[48] D. Ploton, M. Menager, P. Jeannesson, G. Himber, F. Pigeon, J.J. Adnet, Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level, Histochem. J. 18 (1986) 5–14.
[49] M. Portavella, B. Torres, C. Salas, M.R. Papini, Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (*Carassius auratus*), Neurosci. Lett. 362 (2004) 75–78.
[50] M. Portavella, C. Salas, J.P. Vargas, M.R. Papini, Involvement of the telencephalon in spaced-trial avoidance learning in the goldfish (*Carassius auratus*), Physiol. Behav. 80 (2003) 49–56.
[51] M.M. Puddington, R.N. Muzio, Análisis comparado del aprendizaje aversivo, estudios en anfibios, Rev. Arg. Cs. Comp. 5 (2013) 50–63.
[52] M.M. Puddington, M.R. Papini, R.N. Muzio, Vulnerability of long-term memory to temporal delays in amphibians, Behav. Proc. 99 (2013) 7–11.
[53] I. Raška, K. Koberna, J. Malínský, H. Fidlerová, M. Mašata, The nucleolus and transcription of ribosomal genes, Biol. Cell 96 (2004) 579–594.
[54] J.C. Reboreda, R.N. Muzio, M.C. Viñas, E.T. Segura, ß-adrenergic control of the water permeability of the skin during rehydration in the toad *Bufo arenarum*, Comp. Biochem. Physiol. 100C (1991) 433–437.
[55] J.C. Reboreda, E.T. Segura, Electrolyte sensivity of the skin in the toad, *Bufo arenarum* Hensel, Acta Physiol. Pharmacol. Latinoam. 34 (1984) 37–43.
[56] C. Reyes-Vazquez, T. Ibarra, H. Brust-Carmona, Persistence of classical conditioned heart rate after extensive lesions of the striatum in rats, Physiol. Behav. 22 (1979) 1101–1105.
[57] R. Ruibal, The adaptative value of bladder water in the toad, *Bufo cognatus*, Phys. Zool 35 (1962) 218–223.
[58] R.C. Stebbins, N.W. Cohen, A Natural History of Amphibians, Princeton University Press, Princeton, NJ, 1995.
[59] M.D. Suboski, Releaser-induced recognition learning by amphibians and reptiles, Anim. Learn Behav. 20 (1992) 63–82.
[60] D. Trere, AgNOR staining and quantification, Micron 31 (2) (2000) 127–131.
[61] J.P. Vargas, F. Rodríguez, J.C. López, J.L. Arias, C. Salas, Spatial learning-induced increase in the argyrophilic nucleolar organizer region of dorsolateral telencephalic neurons in goldfish, Brain Res. 865 (2000) 77–84.