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Telencephalic Neuronal Activation Associated with Spatial Memory in the Terrestrial Toad *Rhinella arenarum:* Participation of the Medial Pallium during Navigation by Geometry

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Key Words
Geometric and feature orientation · Spatial learning · Neuronal activation · Medial and lateral pallia · Toads

Abstract
Amphibians are central to discussions of vertebrate evolution because they represent the transition from aquatic to terrestrial life, a transition with profound consequences for the selective pressures shaping brain evolution. Spatial navigation is one class of behavior that has attracted the interest of comparative neurobiologists because of the relevance of the medial pallium/hippocampus, yet, surprisingly, in this regard amphibians have been sparsely investigated. In the current study, we trained toads to locate a water goal relying on the boundary geometry of a test environment (Geometry-Only) or boundary geometry coupled with a prominent, visual feature cue (Geometry-Feature). Once learning had been achieved, the animals were given one last training session and their telencephali were processed for c-Fos activation. Compared to control toads exposed to the test environment for the first time, geometry-only toads were found to have increased neuronal labeling in the medial pallium, the presumptive hippocampal homologue, while geometry-feature toads were found to have increased neuronal labeling in the medial, dorsal, and lateral pallia. The data indicate medial pallial participation in guiding navigation by environmental geometry and lateral, and to a lesser extent dorsal, pallial participation in guiding navigation by a prominent visual feature. As such, participation of the medial pallium/hippocampus in spatial cognition appears to be a conserved feature of terrestrial vertebrates even if their life history is still tied to water, a brain-behavior feature seemingly at least as ancient as the evolutionary transition to life on land.

Introduction

Following the seminal study of Cheng [1986] in rats, several animal species including humans have been shown to routinely orient toward goal locations by relying on the boundary geometry of an environment [Cheng and Newcombe, 2005; Sovrano and Vallortigara, 2006;

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Tommasi et al., 2012]. In addition to rats [e.g. Cheng, 1986; Gallistel, 1990], fish [e.g. López et al., 1999, 2000a, b; Sovrano et al., 2003; Vargas et al., 2004a], chicks [e.g. Vallortigara et al., 1990], pigeons [Kelly et al., 1998; Vargas et al., 2004b], and humans [e.g. Hermer and Spelke, 1994] have all been shown to employ boundary geometry to locate a goal (although it should be noted that the extent to which geometry is weighed more heavily than other sources of spatial information is variable and may depend on age [Hermer and Spelke, 1994], the size of the environmental enclosure [Learmonth et al., 2002; Sovrano and Vallortigara, 2006; Sovrano et al., 2007], and species characteristics [Gray et al., 2005]). Indeed, some evidence suggests that encoding of geometry proceeds rapidly, with little if any priming by experience [e.g. Chiandetti and Vallortigara, 2008; Chiandetti et al., 2015].

Inextricably bound to understanding the relationship between boundary geometry and goal navigation is the hippocampal formation of mammals and birds and the dorsomedial or medial forebrain of most other vertebrate classes [Tommasi et al., 2003, 2012]. In rats, the place fields of hippocampal place cells change in response to changes in the geometric-boundary properties of a recording environment [O'Keefe and Burgess, 1996]. In chicks, a recent study by Mayer et al. [2016] found enhanced hippocampal formation activation in subjects that learned to locate rewarded corners using the shape of a rectangular arena. Hippocampal formation lesions can also eliminate or interfere with geometric learning [rats, McGregor et al., 2004; pigeons Vargas et al., 2004b; Bingman et al., 2006; Nardi and Bingman, 2007] while keeping other forms of spatial learning intact. Indeed, lesions to the likely hippocampal homologue of teleost fish can disrupt navigation by geometry under some experimental conditions [Vargas et al., 2006, 2011], but not others [Vargas et al., 2011].

In the context of research on vertebrate comparative cognition, it is perhaps surprising that amphibians are so poorly studied because, as the closest extant relatives of the ancestral amphibious tetrapods that colonized land, they represent an important evolutionary transition. Nonetheless, previous work on cognition in the terrestrial toad *Rhinella arenarum* demonstrated considerable learning abilities [e.g. Muzio et al., 1992, 2011], particularly with regard to spatial learning and memory [Daneri et al., 2011, 2015]. Though less complex compared to that of amniotes [e.g. Roth et al., 2007], anatomical and histochemical evidence has identified the amphibian medial pallium (MP) as homologous to the hippocampal formation of mammals and birds [Northcutt, 1974; Angevine, 1975; Northcutt and Kicliter, 1980; Northcutt and Ronan, 1992]. Interestingly, we recently conducted a first study in toads which revealed that MP lesions impair spatial learning in a plus maze, explicitly linking the amphibian hippocampal formation to spatial cognition [Daneri et al., unpubl. obs.]. More relevant for the current study, we have previously demonstrated that the same terrestrial toad could use the boundary geometry of an enclosure to locate a reward (a pool containing water), and that boundary geometry was even preferred over a salient, visual feature cue in reaching the water [Sotelo et al., 2015]. Following up on that finding, we were naturally curious to discover whether the MP of toads was recruited in supporting navigation by boundary geometry but not other spatial memory strategies (e.g. guidance by a feature cue). Therefore, in the current study, the same toads used in the experiment by Sotelo et al. [2015], which had already successfully learned to locate a water goal, were given an additional test session to locate the goal. The toads were then sacrificed to examine the c-Fos immediate early gene (IEG) expression in various telencephalic regions, with a particular interest in the MP. Differential IEG expression has been successfully used to reveal differential telencephalic subdivision involvement when distinct spatial strategies are employed [for an elegant e.g., see Mayer et al., 2010], and we predicted that the c-Fos expression in the MP would be elevated when the toads relied on boundary geometry to locate the goal.

Materials and Methods

Subjects

Terrestrial toads *(R. arenarum),* a species not listed as threatened [IUCN, 2014], were used. Animals were maintained according to the guidelines outlined by the Guide for the Care and Use of Laboratory Animals [National Research Council, 2011]. Nine of the toads used in the current study, i.e. those that underwent spatial training, were among the 19 used in the study of Sotelo et al. [2015] (in fact, the current study is effectively an extension of that study) and had navigational training. Four additional toads were also collected at the same time as the behaviorally trained animals but remained in the laboratory for the duration of the previous behavioral experiment and were used as inexperienced controls in the current study. Those control animals were maintained under the same conditions as the behaviorally trained toads.

Toads were captured in ponds near Buenos Aires, Argentina, during September 2012 and kept under standard laboratory conditions until the experiment started. Behaviorally trained subjects were divided into two groups which were trained under the same experimental procedures during November 2012 and January 2013. Standard weights (weight of the hydrated animal with its urinary bladder empty) [Ruibal, 1962] were obtained the day before pretraining began. Weights varied between 80.8 and 139.0 g (mean 109.81, SE 6.35). No statistical differences were found between the November

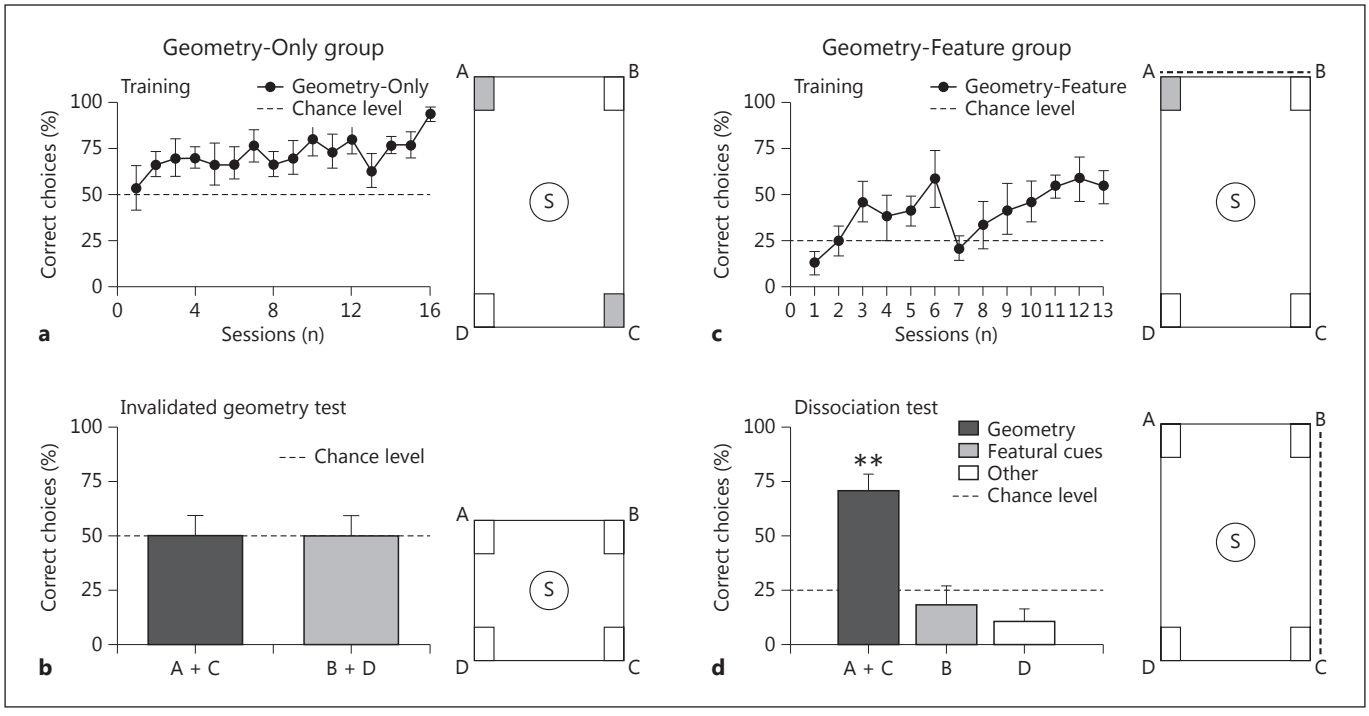


Fig. 1. a Schematic representation of the rectangular arena (right) of the geometry-only toads trained in the study of Sotelo et al. [2015]. The four plastic green water containers are located in the corners (grey would be an example of diagonally related, rewarded containers), and S refers the trial start location (toads were released from an opaque container). No visual, feature cues were present on the walls. On the left is the mean percentage of correct choices, with SE bars, across the sixteen training sessions of the Geometry-Only group (the chance is 50%). **b** Percentage choice distribution (mean percent of choices with SE bars) to the two pairs of diagonal corners on the probe trials in which boundary geometry as a navigational cue was eliminated as the arena was converted into a square (right). **c** Schematic representation of the rectangular arena (right) of the geometry-feature toads [Sotelo et al., 2015]. Now the arena had one of the short walls covered with a distinctive feature panel (dashed wall) and only one of the water containers/corners was rewarded (grey container). On the left, the mean percentage of correct choices (with SE bars) across the thirteen training sessions of the Geometry-Feature group (the chance is at 25%) are shown. **d** Percentage choice distribution (left, with SE bars) and schematic representation (right) of the conflict/dissociation test when the feature cue was rotated to a long wall creating a conflict between the geometry and feature information (** $p < 0.01$). Note: the animals were also tested on probe trials in which the feature panel was removed or the arena was converted into a square.

and January animals, and therefore data from the two experimental periods were pooled. The toads were kept at a constant temperature (24–27°C) and humidity (48–52% relative humidity) and under a 16:8-hour light/dark cycle (lights on at 6.00 h local time). Experimental subjects began their training sessions dehydrated to 80% of their standard weight, a procedure widely used to motivate toads to search for water as a primary reinforcer [e.g. Muzio et al., 2011]. The inexperienced control subjects were also dehydrated.

Experimental Environment and Behavioral Procedures

The experimental environment and behavioral procedures for the behaviorally trained toads are described in detail in the paper by Sotelo et al. [2015]. As such, we provide here only the essential details of the behavioral protocol. The experimental environment consisted of a rectangular white Plexiglas arena (90 cm long × 45 cm wide × 60 cm high), surrounded by white curtains (fig. 1). A green plastic water container, covered with a metal mesh, was placed in each corner of the arena. The animals were subjected to one of two experimental procedures: Geometry-Only (n = 10) or Geometry-Feature (n = 9).

The Geometry-Only group (fig. 1a) was trained using the rectangular arena without any additional features. Of the four water containers, only two, located in diagonally opposite corners (balanced across subjects), had a water level high enough (reward) for the toads to make contact. The experiment was designed to determine whether the toads could rely on the boundary geometry of the environment to locate one of the two correct corners. The animals were trained until they collectively reached a performance of >75% correct choices. A probe test, in which the boundary geometry was turned into a square, resulted in random choice behavior by the toads (fig. 1b), demonstrating that the successful training performance was based on the boundary geometry of the rectangular environment [Sotelo et al., 2015].

The Geometry-Feature group (fig. 1c) was trained under the same conditions as the geometry-only group, except a feature panel was attached to one of the short walls of the test arena. The fea-

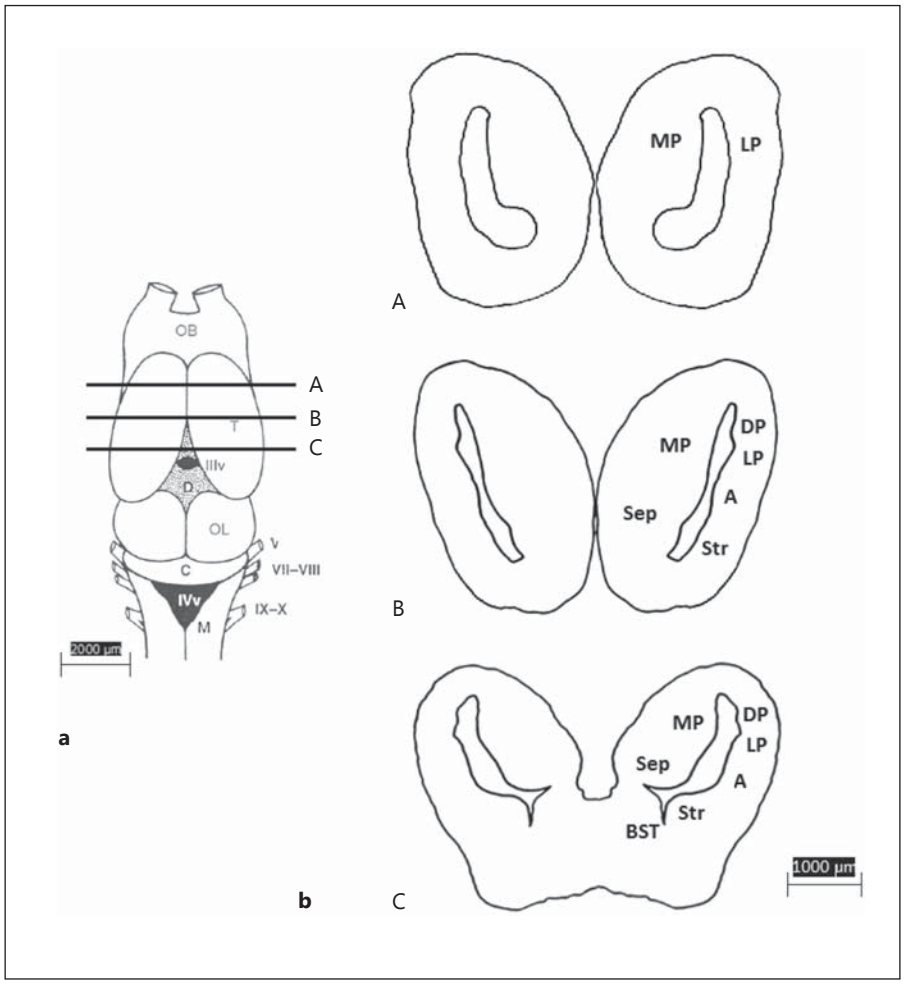


Fig. 2. a Diagram of the toad brain (dorsal view) showing the approximate locations of the three transverse sections (A, B, and C) of the telencephalon of the toad *R. arenarum* used to count labeled cells. C = Cerebellum; D = diencephalon; M = medulla; OB = olfactory bulb; OL = optic lobe; T = telencephalon; IIIv = third ventricle; IVv = fourth ventricle; V–X = positions of some cranial nerves. **b** The three rostrocaudal sections A, B and C refer to the anterior, mid-telencephalic, and posterior sections of the telencephalon, respectively. A = Amygdala; BST = bed nucleus of the stria terminalis; DP = dorsal pallium; LP = lateral pallium; MP = medial pallium; Sep = Septum; Str = striatum.

ture panel was composed of alternating red and blue horizontal stripes. Therefore, the geometry-feature subjects had two sources of information to locate the goal: environmental geometry and the feature cue. Here, only one corner location was correct (accessible water); the correct water container was balanced across the four possible locations. After the collective performance of the animals exceeded 50% correct (the chance was 25%), three types of probe tests were conducted: (i) a geometry test, when the feature panel was removed from the arena; (ii) a feature test, when the arena was turned into a square, eliminating geometric information but preserving the feature on one of the walls, and (iii) a dissociation test (fig. 1d), when the geometric and feature information were set in conflict by moving the feature cue to a long wall. Collectively, the results of the probe tests indicated that both sources of information could be used independently to locate the goal, but geometry was preferred (fig. 1d) [Sotelo et al., 2015].

In summary, the animals from the two groups entered the c-Fos stage of the study after they had successfully learned to locate the goal location relying on the boundary geometry of the test environment (Geometry-Only group) or both (independently) the geometry of the test environment and the feature panel (Geometry-Feature group) [Sotelo et al., 2015].

Analysis of the IEG Neural Activity Associated with Geometry and Geometry-Feature Learning

After completing the behavioral experiment, animals from the Geometry-Only and Geometry-Feature groups were given their group-specific training for an additional day. The 4 inexperienced control animals were placed in the experimental arena for four exposure sessions (1 per day for 4 days) of 10 min each. The same experimental arena used for the trained groups was used for the control toads, except all four containers had accessible water. Like for the geometry-only toads, there was no visual feature available. Approximately 60–75 min after completing the last training session, or, for the control animals, after completing the last exposure session in the arena, the toads were sacrificed and their brains processed for c-Fos immunoreactivity. The objective of the procedure was to evaluate differential neuronal activation across a number of telencephalic subdivisions dependent on cue use/behavioral strategies to locate the goal. The technique basically consists of immunostaining of the nuclear protein c-Fos, an early expressed gene (IEG) activated by recent neural activity [reviewed in Clayton, 1997, and Ball and Gentner, 1998]. The five telencephalic areas of the *R. arenarum* brain investigated are: the MP, the dorsal pallium (DP), the lateral pallium (LP), the septum (Sep), and the striatum

(Str). These areas were chosen because of their potential relevance based on previous studies [see Muzio et al., 1993, 1994; Papini et al., 1995]. Their anatomical boundaries were defined using the anatomical scheme of Moreno and Gonzalez [2004] for the ventral areas (Sep and Str) and the scheme of Westhoff and Roth [2002] for the dorsal areas (MP, DP, and LP), adapted to *R. arenarum* (fig. 2).

One hour after conclusion of the behavioral procedures, the toads were anesthetized with MS222 (tricaine methanesulfonate) injected subcutaneously (0.3 g/kg). After approximately 5 min, when the subjects became unresponsive (tested by gently pulling their feet), they were immobilized on a plate, the thorax was opened, and the heart was exposed. Toads were perfused transcardially via their single ventricle with 60 ml of 10% phosphate-buffered saline (PBS) and then fixed with 60 ml of 4% paraformaldehyde (PFA). The brains were extracted and stored in the same PFA solution. Two weeks after perfusion, the brains were transferred to a 30% sucrose solution (PBS) overnight, and on the next day 40-μm coronal sections of telencephalon were cut with a cryostat, slide-mounted, and stored at 4°C. For histological brain analysis, 4 toads from the Geometry-Only group, 5 toads from the Geometry-Feature group, and the 4 inexperienced control toads were successfully processed. The learning performance of all of the behaviorally trained toads taken for histological processing was representative of their respective treatment groups. Eight telencephalic coronal sections (two anterior, four mid-telencephalic, and two posterior), spanning the entire telencephalon, were taken from each animal and then used for counting of c-Fos-positive cells (A–C in fig. 2). Labelled cell counts were taken from both hemispheres.

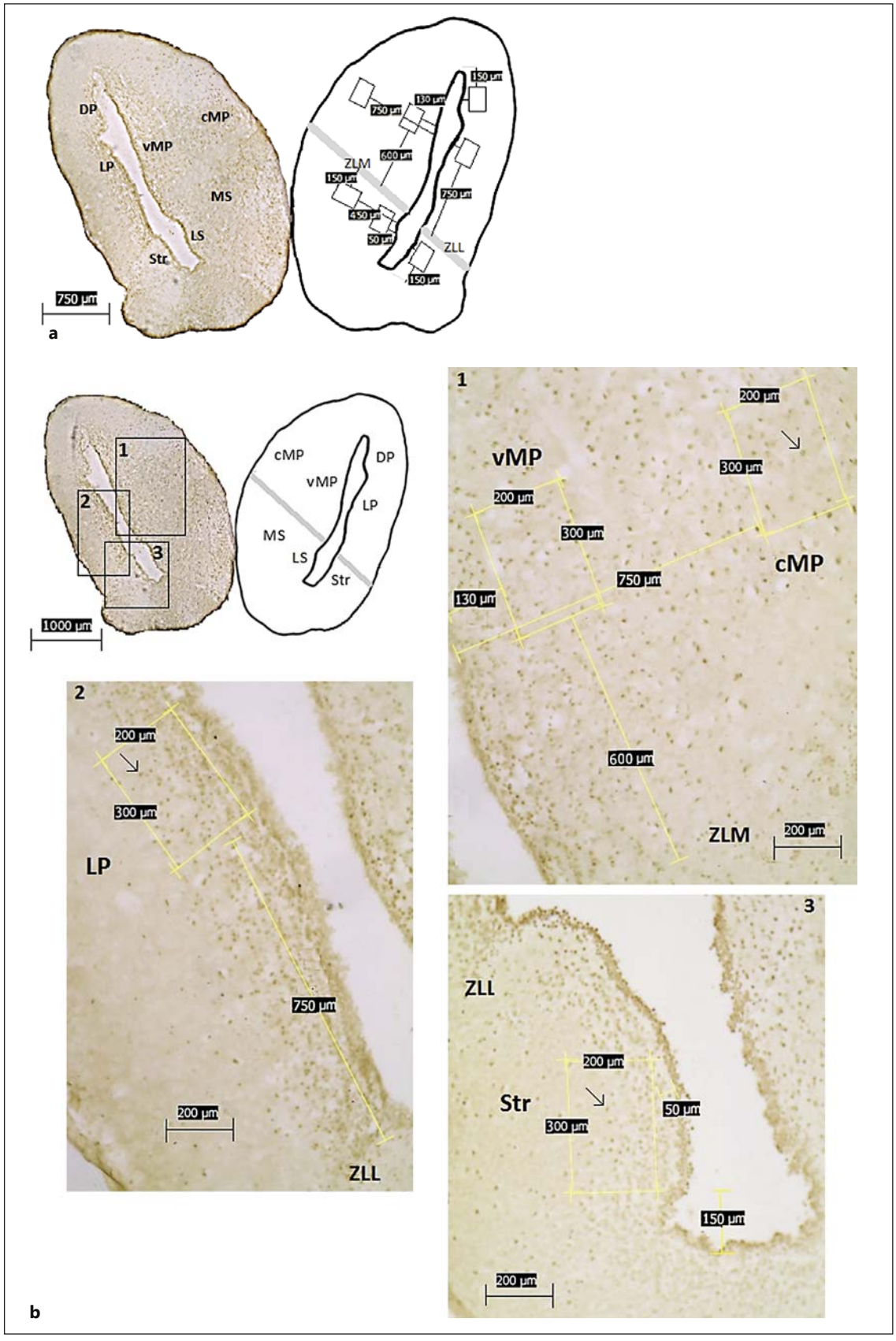
c-Fos Immunocytochemistry

c-Fos immunocytochemistry as a marker for neuronal activity has been successfully used in numerous vertebrate species, including mammals, birds, and reptiles [e.g. Bailey et al., 2002; Neal and Wade, 2007; Huang et al., 2013], as well as other amphibians (i.e. the salamander *Plethodon shermani* [Laberge et al., 2008] and *Xenopus laevis* [Calle et al., 2006]). Here we used a similar but somewhat modified procedure suitable for our study species [Daneri et al., unpubl. data]. The slide-mounted brain sections were initially washed 3× for 10 min in 10% PBS, transferred for 10 min in 1% hydrogen peroxide, and then washed again 3× for 10 min in 10% PBS. The sections were then incubated for 45 min in 5% normal goat serum and washed 3× for 10 min in 10% PBS, which was then followed by overnight incubation in a solution containing a 1:1,000 dilution of the primary antibody, i.e. rabbit anti-c-Fos (sc-253; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in 0.3% Triton X-100 (PBS). The next day, the mounted sections were washed 3× for 10 min in 10% PBS before a 5-hour incubation in a solution containing a 1:2,000 dilution of the biotynilated secondary antibody, i.e. goat anti-rabbit IgG (Vectastain Rabbit PK-4001 Kit; Vector Laboratories, Burlingame, Calif., USA), in 0,3% Triton X-100 (PBS). The sections were washed again 3× for 10 min in 10% PBS before incubation in the avidin-biotin peroxidase complex (prepared according to instructions in the PK-4001 Kit; Vector Laboratories) diluted in 0.3 Triton X-100 (PBS) and then washed again in 10% PBS. The antibody-peroxidase complex was visualized using diaminobenzidine (Sigma Aldrich, St. Louis, Mo., USA) diluted in 10% PBS and in the presence of 0.3% hydrogen peroxide. The sections were dehydrated in ascending ethanol concentrations and then cleared in xylene. Control immunocytochemical procedures, with the primary antibody omitted, were performed in parallel to the normal c-Fos assay in selected sections. The immunostained sections were then analyzed and photomicrographed using a light microscope (Leica DM500).

c-Fos-Positive Neuron Counting

c-Fos-immunoreactive neuronal nuclei were counted visually under light microscopy in rectangular boxes (or sample areas) of 200 × 300 μm. These boxes were placed in the same defined locations, relying on anatomical landmarks and topological positions, in each of the eight brain sections of each animal analyzed (i.e. 2 anterior, 4 mid-telencephalic, and 2 posterior sections per subject). Photomicrographs of each section were taken and processed using computer software (Leica Adobe Photoshop CS5, Portable Edition; Adobe Systems Inc.). As noted above, the following five areas from the telencephalon were chosen for analysis: MP, DP, LP, Sep, and Str (fig. 2, 3). We also examined the amygdala and the bed nucleus of the stria terminalis, both of which were characterized by low numbers of labeled cells (not reported) that visually did not vary across the three treatment groups. Only complete diameter and clearly stained nuclei were counted, following closely the procedures of Neal and Wade [2007].

There is considerable heterogeneity in the distribution of cells in the telencephalon of anurans. Cells are densely packed along the periventricular zone but become more scattered as one approaches more cortical areas closer to the surface of the brain [Northcutt and Royce, 1975; Northcutt and Kicliter, 1980]. For that reason, we located our cell count boxes taking into account the shape and cytoarchitectural organization of each sampled subdivision. In the MP, a large area, we placed two boxes. The first one, in what we call the ventricular MP (vMP), had one long side approximately 130 μm lateral from the lateral ventricle and one short side 600 μm dorsal to the zona limitans medialis (ZLM) (fig. 3a, b). The second sample box, placed in an area we call the cortical MP (cMP), was located parallel to the first one, with one long side approximately 750 μm lateral from the ventricle and one short side 300 μm dorsal to the ZLM. In the DP and the LP, the boxes were both located with one long side approximately 50 μm lateral from the lateral ventricle, but the DP had its upper short side 150 μm ventral to the dorsal pole of the lateral ventricle, while the LP had its lower short side 750 μm dorsal to the zona limitans lateralis (ZLL) (fig. 3a, b). In the Sep, another large region, sample boxes were located in two places: one was located with a long side approximately 50 μm lateral from the lateral ventricle and a short side 300 μm ventral to the ZLM (i.e. the lateral septum; LS). Another box was located more medially, with a long side parallel and approximately 150 μm ventral to the ZLM and a short side 450 μm lateral from the lateral ventricle (i.e. the medial septum; MS). Finally, for the Str, one sample box was located approximately 150 μm dorsal from the ventral pole of the lateral ventricle, with a long side 50 μm lateral to it. Importantly, the researcher counting the immunoreactive nuclei was completely blind to treatment. Labeled nuclei were counted in both hemispheres to the extent possible, but in a few cases, because of lost or damaged tissue, only one hemisphere was counted. The number of c-Fos immunostained nuclei per 200 × 300 μm box area was counted for each sampled box. The number of labeled nuclei in the boxes of each area were averaged and compared across the anterior, mid-telencephalic, and posterior sections, but no statistical differences were found within any sampled brain region ($p > 0.05$). As such, data from all of the boxes/sections of each sampled brain area



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were pooled and averaged for each animal. The same data pooling procedure was applied to vMP/cMP and LS/MS because no statistical differences were found in each case ($p > 0.05$). Data from each telencephalic region was compiled and compared across the three groups. The colors in the photomicrographs in figures 3 and 4 were smoothed by trying to match the different background tones obtained from different sections across subjects using the image editing software Photoshop (Adobe Photoshop CS5, Portable Edition; Adobe Systems Inc.).

Statistics

Differences in the density of c-Fos-immunostained nuclei (c-Fos+ cells per mm^2) from each of the three experimental groups for each telencephalic region were analyzed using INFOSTAT (Infostat; UNC, Córdoba, Argentina). For each experimental group, equality of variance was tested using the Bartlett test and Q-Q plots, and no significant differences were found ($p > 0.05$). Significant ANOVA test results ($p < 0.05$) were followed by a Tukey-Kramer post hoc test to determine which of the groups (Control, Geometry-Only, or Geometry-Feature) was/were responsible for any observed between-group differences. A multivariate discriminant analysis was also performed to assay for differences among the treatment groups across the brain region, activational variables studied.

Results

Figure 1 provides a summary of the learning performance and some probe-test results of Sotelo et al. [2015]. It was this behavioral training that preceded the c-Fos training session of the geometry-only and geometry-feature toads of the current study. The data in figure 1 show that both groups learned to locate a goal water container based on geometry and, though not shown in figure 1, the geometry-feature toads could also locate the goal based on the feature information. In the additional training session preceding sacrifice, the geometry-only animals performed relatively poorly (collectively 53.3% correct), while the geometry-feature toads performed well (collectively 60% correct, with the chance being 25%).

All five analyzed brain regions contained c-Fos-immunoreactive nuclei (table 1; fig. 2, 3), but only in the MP, the DP, and the LP were differences found among the treatment groups. Compared to controls, the MP con-

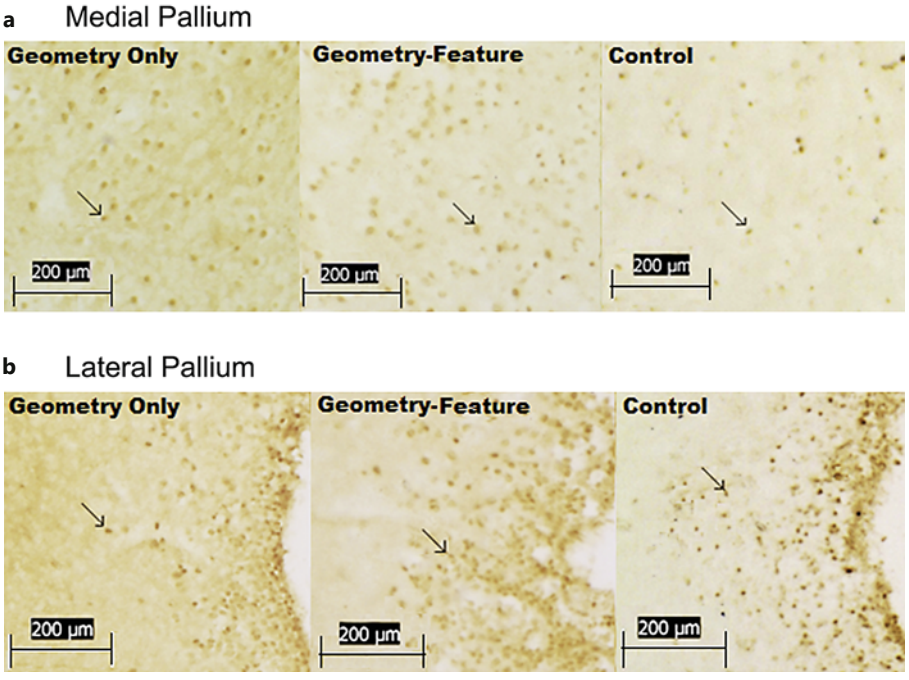

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Fig. 4. a Higher-power photomicrographs of the MP. Displayed are representative transverse, mid-telencephalic sections taken from 3 toads, 1 each from the Geometry-Only, Geometry-Feature, and Control groups. The Geometry-Only and Geometry-Feature groups both show a higher density of c-Fos-immunoreactive cells compared to the control animals (** $p < 0.01$ for both comparisons). **b** Higher-power photomicrographs of the LP. Displayed are representative transverse, mid-telencephalic sections taken from 3 toads, 1 each from the Geometry-Only, Geometry-Feature, and Control groups. The Geometry-Feature group shows a higher density of c-Fos-immunoreactive cells compared to both the Control group and the Geometry-Only group (* $p < 0.05$ for both comparisons). The arrows indicate examplar immunoreactive nuclei.

Fig. 3. a Mid-telencephalic transverse section of the telencephalon of *R. arenarum*. The left hemisphere is a sample photomicrograph with c-Fos immunostaining. The right hemisphere shows the position of the sample boxes (200 × 300 µm). The measures (in µm) taken to position the boxes in each cerebral hemisphere are shown. DP = Dorsal pallium; LP = lateral pallium; LS = lateral septum; cMP = cortical medial pallium; vMP = ventricular medial pallium; MS = medial septum; Str = striatum; ZLL = zona limitans lateralis; ZLM = zona limitans medialis. **b** Top left: mid-telencephalic transverse section of the telencephalon of *R. arenarum* (photomicrograph of the left hemisphere and diagram of the right hemisphere). Rectangles identify the location of the sampled areas of the adjacent photomicrographs 1, 2, and 3. Representative photomicrographs of the MP (1, top right), the LP (2, bottom left), and the Str (3, bottom right) at the same mid-telencephalic level including the boundaries of the sample boxes. The arrows indicate exemplar c-Fos-immunoreactive nuclei.

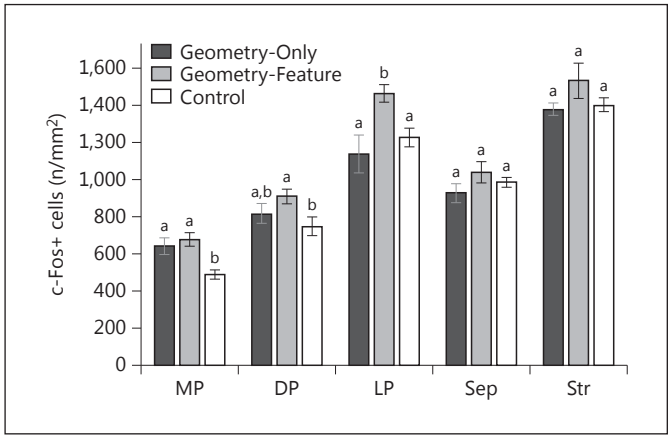

Fig. 5. Densities of c-Fos-labeled neuronal nuclei (mean number of c-Fos+ cells/mm^2 ± SE; α = 0.05) for the Geometry-Only, Geometry-Feature, and Control groups in the five brain areas analyzed. The vertical bars at the top of each column identify SE. Different letters denote significant differences between groups. For the MP, the Geometry-Only and Geometry-Feature groups both differed from the Control group ($p < 0.001$); for the DP, the Geometry-Feature group differed from the Control group ($p < 0.05$) but not from the Geometry-Only group ($p > 0.05$), and for the LP, the Geometry-Feature group differed from both the Geometry-Only group and the Control group ($p < 0.05$).

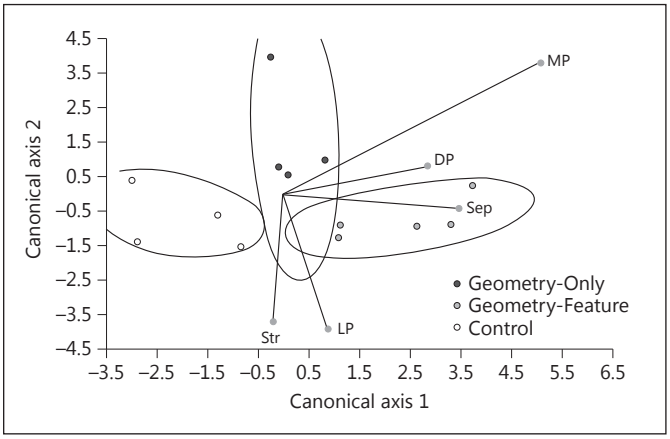

Fig. 6. Biplot graphic derived from the multivariate discriminant analysis contrasting the Geometry-Only, Geometry-Feature, and Control groups in the five areas studied (DP, LP, MP, Sep, and Str). The dots represent the toads from each group. The variables that contributed most to the separation of the treatment groups were labeled neuron density in the MP, the LP, and the DP (associated with higher coefficients in the discriminant functions). Ninety-five percent confidence ellipses are plotted.

Table 1. Densities of c-Fos-labeled neuronal nuclei for each of the five sampled regions for each experimental group

	Geometry-Only	Geometry-Feature	Control
MP	644±44***	681±37***	440±25
DP	820±53*	914±40*	750±51
LP	1,143±10	1,465±49**	1,230±48
Sep	930±50	1,039±55	988±26
Str	1,378±33	1,536±97	1,404±36

Values are presented as mean numbers of c-Fos+ cells/mm^2 ± SE. Asterisks denote significant differences between experimental groups and the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

tained more immunoreactive nuclei for both the Geometry-Only and the Geometry-Feature groups ($p < 0.001$; table 1; fig. 4a, 5). By contrast, compared to controls and the Geometry-Only group, the LP contained more immunoreactive nuclei in the Geometry-Feature group ($p < 0.01$; table 1; fig. 4b, 5). In the DP, differences were found between the Geometry-Feature group and the Control group ($p < 0.05$) but not between the Geometry-Feature group and the Geometry-Only group or between the Geometry-Only group and Control group ($p > 0.05$ for both; table 1; fig. 5). The Str showed a tendency toward increased activation in the Geometry-Feature group, but it was not significant ($p > 0.05$; fig. 5).

Figure 6 displays a biplot graphic derived from a multivariate discriminant analysis of the Geometry-Feature, Geometry-Only, and Control groups with respect to the five brain areas analyzed (i.e. MP, DP, LP, Sep, and Str). The analysis enables separation of animals using their individual values for each of the variables, while at the same time it analyzes all variables together (in this way it reduces the global error, thus enabling easier detection of significant differences). The two canonical axes extracted from the analysis explain the variation found among the treatment groups and are linear combinations of the density of the labeled neurons in the five brain areas analyzed. The graph analysis shows that animals in each group can be distinctly separated by a combination of the original variables (represented by vectors in the two-dimensional space). The brain area variables that contribute most to the separation of groups are the density of labeled neurons in the MP and the LP (higher coefficients in the discriminant function analysis) and

secondly the DP and the Str. The contours plotted for each group represent the confidence ellipses (at a 95% level).

Discussion

We began this study already having demonstrated that the terrestrial toad *R. arenarum* could exploit the boundary geometry of an environment to locate a goal and, under the behavioral conditions used here, preferentially rely on boundary geometry over the use of a prominent, visual feature cue [Sotelo et al., 2015]. Given the well-known relationship between the hippocampus and the representation of boundary geometry in birds and mammals [Tommasi et al., 2003, 2012], we were interested in determining whether the MP of our amphibian toad, the homologue of the hippocampus [Northcutt, 1974; Angevine, 1975; Northcutt and Kicliter, 1980; Northcutt and Ronan, 1992], also participates in navigation by boundary geometry. Indeed, we showed that there is a pronounced increase in neuronal activation in the MP when toads are challenged to locate a goal after they have already successfully learned to locate the goal based on boundary geometry alone or together with feature information. The observed neuronal activation, therefore, then likely reflects in part activation associated with the recall of spatial information. However, we need to acknowledge that we were unable to rely on stereological procedures to carry out the cell counts, rendering our findings susceptible to the criticism of not having an overall (labeled and unlabeled cells) cell count baseline. However, the general comparison approach we employed has been shown to be reliable in many other IEG studies [e.g. Mayer et al., 2016], but we do agree that the use of stereological procedures is generally more desirable.

There is considerable discussion in the literature regarding what properties of boundary geometry may be used to guide navigation [for reviews, see Sovrano and Vallortigara, 2006; Vallortigara, 2009; Pecchia and Vallortigara, 2010; Cheng et al., 2013]. However, the experimental design used in the current study does not allow us to identify what geometric properties were used by the toads [for a fuller discussion, see Sotelo et al., 2015]. As such, we are comfortable only stating that the MP of *R. arenarum* is in involved in representing in memory some aspect of boundary geometry, which can be exploited to navigate to a goal location.

Although modern toads vastly differ from the first amphibians that colonized land [Estes and Reig, 1973; Panchen and Smithson, 1988], the toad telencephalon has nonetheless a relatively simple organization [Ebbesson, 1980; Butler and Hodos, 2005; Smeets and González, 2006; Wilczynski, 2009; Muzio, 2013]. Therefore, the organization of the toad telencephalon can be assumed to resemble, more than that of any other extant vertebrate (other than other amphibians), the telencephalon of the first partially land vertebrates, thus potentially offering insight into a more ancestral condition of vertebrate brain-behavior relations. The data of the current study reveal an MP of toads that plays a role in spatial cognition in a geometric learning task resembling those known to involve the hippocampus of mammals and birds [Tommasi et al., 2003, 2012; Vargas et al., 2004b; Bingman et al., 2006; Nardi and Bingman, 2007]. As such, a relationship between the medial or dorsomedial telencephalon of vertebrates and spatial cognition appears as ancient as the transition to life on land. In fact, evidence from fish [e.g. Rodríguez et al., 2002] suggests that an even more ancestral, candidate homologue of the dorsomedial forebrain of other vertebrate groups is also involved in spatial cognition.

Previous behavioral experiments in *R. arenarum* have shown that terrestrial toads are able to learn complex tasks such as spatial navigation using various types of information [Daneri et al., 2011, 2015]. In addition, our first data on spatial learning in toads also revealed that MP lesions impair spatial learning in a plus maze task [Daneri et al., unpubl. obs.]. With respect to the MP outside the domain of spatial navigation, we have also shown that lesions of the MP do not affect acquisition but do increase perseveration after a shift in the conditions of reinforcement in a runway task [for e.g., see Muzio et al., 1993]. The latter study provided evidence that the MP is also involved in the inhibition of a previously dominant response and/or the acquisition of alternative responses [Muzio et al., 1993, 1994; Papini et al., 1995].

The observation that the LP displayed increased activation in the Geometry-Feature group but not in the Geometry-Only group nurtures the hypothesis that the LP is important when spatial navigation is guided by responses to a prominent visual feature. This is perhaps surprising because in mammals [e.g. Packard et al., 1989] and birds [Mayer and Bischof, 2012; see also Shimizu et al., 2004] such guidance strategies typically recruit the Str or some other telencephalic structure. In this context, we were therefore not surprised by the trend of greater activation in the Str in the Geometry-Feature group. Clearly more studies are needed to assess the involvement of brain areas outside of the MP with respect to the control of spatial behavior guided by different strategies.

As noted above, the introduction of a feature-discriminative element into our task led to a higher activation in the LP, indicating an LP role in feature learning. However, this interpretation must be taken cautiously because anatomical understanding of the amphibian LP is still limited. Anatomically, the amphibian LP has some resemblance to the piriform cortex of mammals and the lateral cortex of reptiles because of similar connections to the olfactory bulb and the accessory olfactory bulb [Northcutt and Kicliter, 1980; Moreno and Gonzalez, 2004]. However, it is unknown whether the lateral cortex receives other sensory inputs. The LP is also connected to the MP via the dorsal association bundle [Kokoros, 1973; Scalia, 1976] and the amygdala [Northcutt and Kicliter, 1980]. Nevertheless, as most of the anatomical brain research in amphibians has been done on species other than *R. arenarum,* we cannot be sure how well the findings described above generalize to *R. arenarum*. In fact, to define the boundaries of the telencephalic subdivisions examined, we used an anatomical classification from Moreno and Gonzalez [2004], which was carried out in the frog *Rana perezi,* to identify the ventral areas of the telencephalon (i.e. Sep and Str). For the dorsal telencephalic areas (i.e. MP, DP, and LP), we used the scheme of Westhoff and Roth [2002], carried out in the frog *Discoglossus pictus*. We also consulted other authors [Northcutt and Royce, 1975; Northcutt and Kicliter, 1980; Neary, 1984; Roth et al., 2003] to identify the boundary between the DP and the LP. The decision to use this combined boundary information was based on our own neurohistological data derived from the terrestrial toad *R. arenarum* (unpubl. obs.). What is clear is that the higher activation in the LP of the Geometry-Feature toads cannot be explained by olfactory input.

With regard to the DP, which is also poorly studied in amphibians, it is known that this area is connected to the LP and the MP, and it has thalamic and nonolfactory inputs including direct input from the retina [Neary, 1984]. The increased activation of this area in the Geometry-Feature group could then be related to the visual processing related to both feature and geometric inputs, but more studies are needed to test this idea. In summary, our data plainly reveal a dissociation between MP activation associated with navigation by boundary geometry and LP activation associated with navigation by visual features. The data from the DP are less easy to interpret, but here general visual processing may be relevant. We therefore hypothesize that the LP, and to a lesser extent the DP, region in the terrestrial toad *R. arenarum* is in some way involved in spatial guidance by visual cues resembling the telencephalic-tectofugal activation in zebra finches *(Taeniopygia guttata)* associated with locating a goal by visual features [Mayer and Bischof, 2012].

In conclusion, in the terrestrial toad the use of a previously learned representation of environmental geometry to locate a goal is associated with enhanced activation of neurons in the MP, the presumptive homologue of the hippocampus of mammals and birds. The additional opportunity to use a prominent visual feature to locate a goal leads to enhanced activation of the LP and, to lesser extent, the DP. As such, toads, and more generally amphibians, are able to employ parallel brain mechanisms for the purpose of goal navigation, parallel systems that recall the cognitive map and taxon guidance systems proposed by O'Keefe and Nadel [1978] in the context of the mammalian hippocampus.

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