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Hippocampus and Medial Striatum Dissociation During Goal Navigation by Geometry or Features in the Domestic Chick: An Immediate Early Gene Study

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ABSTRACT: We employed a standard reference memory task to study the involvement of the hippocampal formation (HF) of domestic chicks that used the boundary geometry of a test environment to orient to and locate a reward. Using the immediate early gene product c-Fos as a neuronal activity marker, we found enhanced HF activation in chicks that learned to locate rewarded corners using the shape of a rectangular arena compared to chicks trained to solve the task by discriminating local features in a square-shaped arena. We also analyzed neuronal activity in the medial part of the medial striatum (mMSt). Surprisingly, in mMSt we observed a reverse pattern, with higher activity in the chicks that were trained to locate the goal by local features. Our results identify two seemingly parallel, memory systems in chicks, with HF central to the processing of spatial-geometrical information and mMSt important in supporting local feature discrimination. © 2015 Wiley Periodicals, Inc.

KEY WORDS: avian hippocampus; geometric navigation; spatial cognition; feature discriminations; medial striatum

INTRODUCTION

Visual information in the form of environmental geometry or object features can guide goal navigation in many animal species. Environmental geometry can be considered a property that a surface, line or point possesses relative to the position of other objects or surfaces (Gallistel, 1990), e.g., "a short wall is located to the right of a long wall." In contrast, "non-geometric information" is a property that cannot be defined by relative position (Gallistel, 1990), e.g., an object has blue stripes. In the context of goal navigation, "non geometric information" can be provided by local features at the goal location; features that can be used as a beacon for orientation and do not require any relational information. By contrast, Cheng (1986) demonstrated that animals can also navigate by reference to the shape ("geometry") of the local environment and proposed that rats possess a geometric module, which enables a representation of the overall shape of an environment that can be used to indicate goal locations (but see Sutton and Newcombe, 2014).

The pioneering study of Cheng (1986) stimulated an industry of research in which numerous animal species have been tested in rectangular enclosures demonstrating an almost universal use of environmental geometry for locating a goal (rats: Cheng, 1986; Golob and Toube, 2002; monkeys: Gouteux et al., 2001; chicks: Vallortigara et al., 1990; Chiandetti, et al., 2007; Chiandetti and Vallortigara, 2008a,b, 2010; Pecchia and Vallortigara, 2010a,b, 2012; Lee et al., 2012a; pigeons: Vargas et al., 2004, Bingman et al., 2006; Pecchia et al., 2011; fish: Sovrano et al., 2002, 2003; Lee et al., 2012b; humans: Hermer and Spelke, 1994; Hermer and Spelke, 1996; Gouteux and Spelke, 2001; Lee et al., 2006; Lee and Spelke 2008; for reviews see Wang and Spelke, 2002; Cheng and Newcombe, 2005; Vallortigara, 2009; Tommasi et al., 2012). However, the nature of the representational strategy used to find a geometrically correct corner within a rectangular enclosure continues to be discussed. Most species tend to ignore the displacement or removal of a colored wall (Cheng, 1986; Kelly et al., 1998; Vargas et al., 2004; Lee et al., 2006, 2012a,b; Vallortigara, 2009), which would indicate use of geometric information in the sense of Cheng (1986). However, it is also possible that animals can additionally use the visual pattern created by the junction of a short and long wall (George et al., 2001; Pearce et al., 2004, 2005; Tommasi and Poli, 2004; Cheng and Gallistel, 2005; Esber et al., 2005; McGregor et al., 2006); an ability that has been termed 'feature-structure discrimination' (Pearce et al., 2005; Bingman et al., 2006). To better understand the behavioral mechanisms that can guide goal navigation based on environmental geometry or shape, it is important to investigate associated neural mechanisms because different combinations of brain structures likely support different geometric-representational strategies.

In mammals, it has long been known that the hippocampus plays an important role in spatial navigation (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978; Morris et al., 1982; Nadel, 1991;

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Schenk et al., 1995; Nadel and Hardt, 2004). The avian hippocampal formation (HF) is homologous to the mammalian hippocampus based on similarities in development, connectivity, neuromorphology, and neurochemistry (Casini et al., 1997; Colombo and Broadbent, 2000; Atoji and Wild, 2006; Watanabe, 2006). It is also similarly involved in spatial navigation (Bingman and Mench, 1990; Hampton and Shettleworth, 1996; Colombo et al., 1997; Fremouw et al., 1997; Gagliardo et al., 1999; Macphail, 2002; Shiflett et al., 2003; Watanabe and Bischof, 2004; Bischof et al., 2006; Watanabe et al., 2008; Mayer et al., 2013). Among gallinaceous birds, spatial orientation by environmental geometry has been demonstrated not only in chicks -the animal model used in the present study- (Vallortigara et al., 1990), but also in quails (Ruploh et al., 2011). Lesioning experiments with domestic chicks confirmed participation of HF in aspects of spatial cognition (Tommasi et al., 2003).

Given the importance of the avian HF for spatial cognition and the well documented sensitivity of birds to environmental geometry as a source of navigational information, research examining the relationship between HF and navigation with respect to the boundary geometry of an environment is surprisingly sparse (but see Vargas et al., 2004; Bingman et al., 2006). Although not applied to tasks involving environmental shape, the importance of the avian HF for spatial navigation has been further confirmed with experiments using immediate early genes (IEGs) as markers for neuronal activation (Smulders and DeVoogd, 2000; Bischof et al., 2006; Mayer et al., 2010; Mayer and Bischof, 2012). IEGs are rapidly activated when neuronal activity increases and they are thought to play an important role in memory consolidation (Lanahan and Worley, 1998; Jones et al., 2001; Guzowski, 2002; Kubik et al., 2007; Barry and Commins, 2011). Here we investigate the hypothesized involvement of the HF of domestic chicks in locating a rewarded corner using the boundary geometry of a rectangular arena. We do so by using the standard geometric reference memory task introduced by Cheng (1986) and later applied to chicks (Vallortigara et al., 1990). By using c-Fos as a neuronal activity marker, we compare HF activation of chicks that learned to locate the goal by use of the geometrical properties of the environment with that of chicks that learned to use local features in a square shaped arena to locate a goal. We also measure c-Fos activity in the medial part of the medial striatum (mMSt), where neuronal activity has been shown to be upregulated in pigeons navigating home from a familiar site (Shimizu et al., 2004). The medial striatum is also of interest because, in rodents, both the striatum and hippocampus contribute to experience dependent navigation, but they do so in different ways (see Mizumori et al., 2009 for a review). The two structures are believed to work in a semi-parallel manner providing independent sources of information that can guide navigation (Mizumori et al., 2004). That hippocampal and striatal representations of space are different is consistent with the common conceptualization that different types of memory are mediated by parallel operations of distinct neuronal systems (see review in Eichenbaum and Cohen, 2001; Knowlton et al., 1996). Given the hypothesis that the avian HF plays an important role in the use of environmental geometry for goal navigation, we expected that c-Fos activity in HF would be higher in chicks trained to locate the goal by geometry compared to chicks trained to use local features.

MATERIALS AND METHODS

Subjects

Eighteen laboratory-hatched, male domestic chicks (*Gallus gallus domesticus*), of the "Hybro strain" (a local variety derived from the white leghorn breed), were used. Fertilized eggs were obtained from a local commercial hatchery (Agricola Berica, Montegalda (VI), Italy) and incubated under standard conditions in darkness. After hatching, chicks were maintained individually in metal cages ($22.5 \times 40 \times 30$ cm^3) located in a temperature-controlled room (30–32°C). The day/night cycle was 12h dark and 12h light. During the light period, the cages were illuminated from above by LED lights (240 LUX; 3000K). Water was available ad libitum during the entire training period. The chicks were placed on a food deprivation regime starting the evening before the first day of training. On each subsequent training day, chicks were deprived of food until the training session, after which they received food ad libitum for at least 4 h before the food was removed again in preparation for the next day's training. All experiments were carried out in accordance with ethical guidelines current to European and Italian laws.

Apparatus

The geometry discrimination training was performed in a white *rectangular* arena, (Figs. 1a,b) 70-cm long, 35-cm wide, and 50-cm high. The arena was located on a rotatable table and was homogeneously illuminated with a 60W lamp located centrally above it (no other light source was present). Four black, cylindrical feeders (3×5 cm^2), covered with black plastic disks, were positioned in the corners. During training, only two feeders, located at diagonally opposite corners, were baited with one to two mealworms (*Tenebrio molitor* larvae), which served as reward. Because the feeders were visually similar and different feeders/covers were used in different corners on different trials, the two rewarded feeders could only be discriminated by their position with respect to the boundary geometry of the environment (the two geometrically equivalent corners with a long wall on the left and a short wall on the right or the two other geometrically equivalent corners with a short wall on the left and a long wall on the right). The rewarded corners were randomized across subjects.

At the beginning of each trial the chicks were released from a thin circular platform (22-cm diameter) located at the center of the arena. The platform and the rest of the apparatus could be rotated independently from each other (Fig. 1). After each trial,

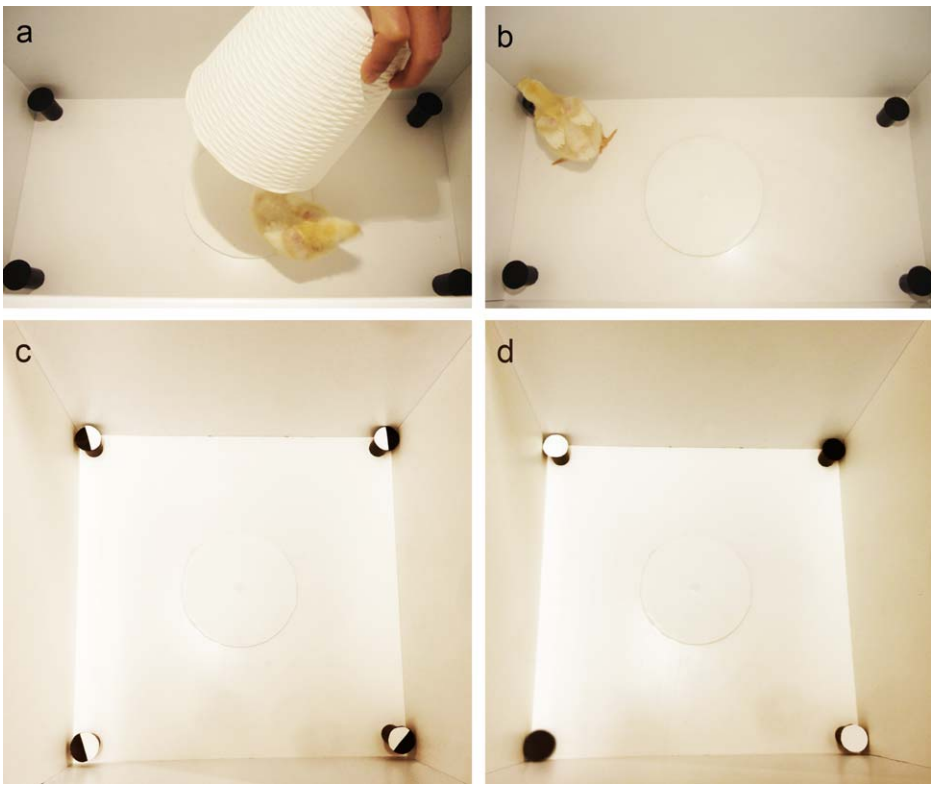

FIGURE 1. Photographs of the experimental arenas. (a) Rectangular arena with four black feeders at each corner. An experimental chick is released from the circular platform at the arena center after the arena was rotated (see methods). (b) Image of a chick choosing a correct corner after re-orienting using the shape of the rectangular enclosure. (c) Square-shaped arena with four feature-similar black-white feeder cover-disks, which were used only during habituation training in the square-feature training arena (the same black cover disks were used during habituation in the rectangular-geometry arena). (d) Square-shaped arena with two white covered and two black covered feeders, which were used during feature discrimination training. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the chick was captured with an inverted white basket (20-cm diameter and 19cm high) and positioned back on the central platform. A disorienting procedure was then administered during the inter-trial interval, while the subject was confined on the platform and covered by the basket, without access to external visual cues. The disorientation was implemented by rotating the arena and the platform (with the subject) independently in opposite directions, and changing the number of rotations across trials. The effect of the procedure was to render unreliable orientation strategies based on any possible extra-maze cue or on egocentric body position. Once the disorientation procedure was completed, the basket was lifted and the chick was allowed to search for the food in the feeders. Animals were observed on a computer screen via a digital camera suspended centrally above the arena.

The feature discrimination training was performed in a white, *square-shaped* arena 60-cm wide, 60-cm long, and 50-cm high [Figs. 1c (used only for habituation, see below), 1d). The arena dimensions were chosen to render equal the distance from the corners to the center for both the feature and geometry arenas. The feature arena contained four black feeders, one in each of the corners. Two of the feeders, located at diagonally opposite corners, were covered with black disks and contained food. The remaining two, unrewarded feeders were covered with white plastic disks (Fig. 1d). As such, the rewarded feeders could be discriminated from the unrewarded feeders by the local feature cues. The rewarded feeders occupied different paired corners on different trials and the feeders/covers were changed from trial to trial. All other characteristics of the experimental design and procedures were the same as described for the geometry condition.

Habituation Training

Nine subjects underwent habituation in the rectangular (geometry) arena and nine in the square-shaped (feature) arena. This initial training aimed to familiarize the chicks with their testing environment and handling procedures, and to train them to peck at the plastic disks that covered the feeders that allowed access to the mealworms. Habituation training for both groups started on day 3 after hatching and was completed on day 5.

On day 3 post-hatch, the chicks were individually placed in their experimental arena, which contained a single feeder baited with worms. The feeder was located at a random position in the arena and was already uncovered. Each chick was allowed

5–10 min to explore the environment and find the worms across three to four trials before being moved back to its home cage. Transport from the home cage to the experimental arena and back occurred in a closed, cardboard box (32 cm long × 21 cm wide × 13 cm high) such that the chicks could not see the surroundings. On day 4, chicks were trained in their experimental arena to open the single feeder by pecking on the associated plastic-disk cover and displacing it. This typically occurred in four to eight trials. For chicks in the geometry group, the cover disks of the feeders used during habituation were black; for the feature group, each feeder cover disk was half black and half white (Fig. 1c). The black-white cover disks were used to habituate the chicks to both colors without giving them the opportunity to learn any discrimination based on the different colors. After each habituation trial, the chicks were covered with the basket, gently guided to the center of the arena and disoriented by rotating the arena and the platform. On day 5 post-hatch, four feeders were positioned in the corners of the two arenas. For four consecutive trials, the birds were required to visit all of the feeders and eat the worms.

Discrimination Training

Geometry discrimination training started on post-hatch day 8, 2 days after the last habituation training. The birds had to learn to find either one of the two geometrically correct feeders that were located at opposite corners of one of the diagonal axes. Training consisted of 3 sessions of 10 trials each per day. For each session, the chicks were first transported from the home cage to the experimental room, and after completing 10 trials, they were placed back in their home cages for at least a 1h inter-session interval (what may be considered a consolidation opportunity). At the beginning of each trial, a chick was released from the central platform. A trial ended when the chick found the worm in one of the rewarded-correct feeders or if it pecked at one of the incorrect feeders. The chick was then gently covered with the basket and positioned back on the center platform. After cleaning the experimental arena, the chick was disoriented by rotating the arena and the platform.

Feature discrimination training started 3 days later than geometry training. The delay was introduced because we previously observed that chicks typically need only 2 days of training to learn a local-feature discrimination task, but at least 4 days to learn a geometry discrimination. To control for age and time spent in the arena, chicks in the feature condition received 3 additional habituation-training days (on days 8, 9, and 10) using the same procedure described above, but now with 3 sessions of 10 trials per day. Therefore, feature discrimination training started on day 11. Identical to the geometry training procedure, each chick completed 3 training sessions of 10 trials per day. Only the two black-covered feeders located at opposite corners contained worms, whereas the feeders covered by a white disk were not rewarded (Fig. 1d).

Individual geometric and feature learning performance of each chick was analyzed each day by calculating the number of correct choices across the cumulative 30 trials from the 3 sessions. Training continued until the chicks reached a learning criterion of 23/30 (77%) correct trials for 1 day. This level of performance is significantly higher than chance (binomial probability: $P = 0.001$). However, the adoption of this learning criterion still allowed us to end training when a subject was still learning and had not yet reached asymptotic performance, which typically can reach close to 100% correct (Vallortigara et al., 1990). This is relevant because asymptotic learning (overtraining) has been shown to correlate negatively with IEG expression in the HF of birds (see Mayer et al., 2010).

Test Session for c-Fos Labelling

The test session for c-Fos labelling was performed 1 day after a chick reached the learning criterion. The test session consisted of three consecutive unrewarded trials identical to the training trials. To advance to histological processing, a chick had to choose correctly on at least two of the three trials. The one bird that failed to get two out of three trials correct was excluded from histological processing (see Results). The test sessions were video recorded. After completing the test session, a chick was transported back to its home cage.

Immunohistochemistry

Ninety min after the end of the test session, subjects were overdosed with an intramuscular injection of 0.05 ml Ketamine/Xylazine Solution (1:1 Ketamine 10 mg ml^{-1} + Xylazine 2 mg ml^{-1}) per 10 g of body weight. After 5 min, when the animals became unresponsive (tested by gently pulling the feet and wings), they were immobilized on a plate, the thorax was opened and the heart was exposed. The chicks were perfused transcardially via the left ventricle with cold phosphate-buffered saline (PBS; 0.1 mol, pH = 7.4, 0.9% sodium chloride, 4°C) for 10 min and then fixed with 4% paraformaldehyde (PFA) in PBS for 10 min. The head was then severed from the body, the skin and the eyes were removed, and the skull was transferred to 4% PFA where it was post-fixed overnight. On the following day, the skull was secured in a stereotaxic head holder (Stoelting, using a Kopf Instruments pigeon head holder). To ensure that the subsequent coronal brain sections would have the same orientation as that depicted in the chick brain atlas of Kuenzel and Masson (1988), the horizontal axis of the skull was oriented at 45 degrees (anterior downward) with respect to the horizontal axis of the stereotaxic instrument. The caudal part of the skull was opened and the brain was exposed. A coronal-plane cut was made with a scalpel blade attached to a micro manipulator (for details see Kuenzel and Masson, 1988). The brain was then removed from the skull, post-fixed for approximately 48h in 4% PFA/PBS containing 20% sucrose at 4°C, and then transferred to 30% Sucrose/0.4%PFA/PBS for 48–72 h until it sank. The left and the right hemispheres were separated and processed independently. The brain hemispheres were frozen at −50°C in plastic molds covered with O.C.T (Tissue-Tek freezing medium) and stored at −20°C until processing.

For free-floating immunostaining, four series of 40-µm coronal sections were cut on a Cryostat (Leica CM1850 UV) at −20°C and collected in PBS. The sections of the first series were used for processing and labelling. The sections of the other series were kept in PBS at 4°C as backup or for testing antibody specificity (processing without the primary antibody). Endogenous peroxidase activity was depleted by incubation in 0.3% H_2O_2 in PBS for 20 min. After washing in PBS (3×5 min^2), the sections were treated with 3% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA) in PBS for 30 min. The sections were then transferred to the c-Fos antibody solution (c-Fos antibody, 1:2000; rabbit, polyclonal K-25, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4°C on a rotator. After several washes in PBS, the secondary antibody reaction was carried out using a biotinylated anti-rabbit solution (1:200, BA-1000, Vector Laboratories) in PBS for 75 min at room temperature. The ABC method was used for signal amplification (Vectastain Elite ABC Kit, PK 6100, Vector Laboratories). Neurons with concentrated c-Fos protein were visualized with the VIP substrate kit for peroxidase (SK-4600, Vector Laboratories). This produced a purple reaction product confined to the cell nuclei of activated neurons. Sections were then transferred to distilled water and serially mounted on gelatin-coated slides. They were dried at 50°C on a heating plate and counterstained with methyl green (H-3402, Vector Laboratories). After gradual dehydration in ethanol (70, 80, 90, and 99% EtOH for 3 min each, and then placed in Xylene) the mounted sections were cover slipped with Eukitt (FLUKA).

Brain Analysis of c-Fos Immunoreactive Neurons

Brain sections were examined with a Zeiss microscope at a magnification of 200× and a digital camera (Zeiss AxioCam MRc 5). The ZEN Imaging software (Zeiss) was used for the manual counting of immunoreactive (IR-) neurons on a computer screen. For counting, a square "enclosure," 250 × 250 µm^2, was positioned over the different sample areas (see below). Counting was performed blind to the experimental condition. Contrast and exposure time of the camera were adjusted so that the image on the screen matched the view under the microscope. Successful immunostaining produces dark, purple-black stained nuclei, which can easily be discerned from background and non-activated neurons, which were stained light green (see Fig. 5). Every activated c-Fos IR-neuron within each sample area was marked on the screen with the "event marker" of the ZEN software, which automatically computed the total number of counted IR-neurons.

To estimate labelled cell density within the hippocampal formation, five sections of each hemisphere were selected from that part of HF extending from A(nterior) 7.0 to A 6.0 (Fig. 2a determined by using the shape and anatomical organization of the HF sections matched to the atlas of Kuenzel and Masson, 1988). The HF of each section was parsed into four subdivisions: a ventral subdivision (HpVM) defined by the limits of two conspicuous, dense cell layers, the neighboring dorsomedial hippocampus (HpDM), dorsolateral hippocampus

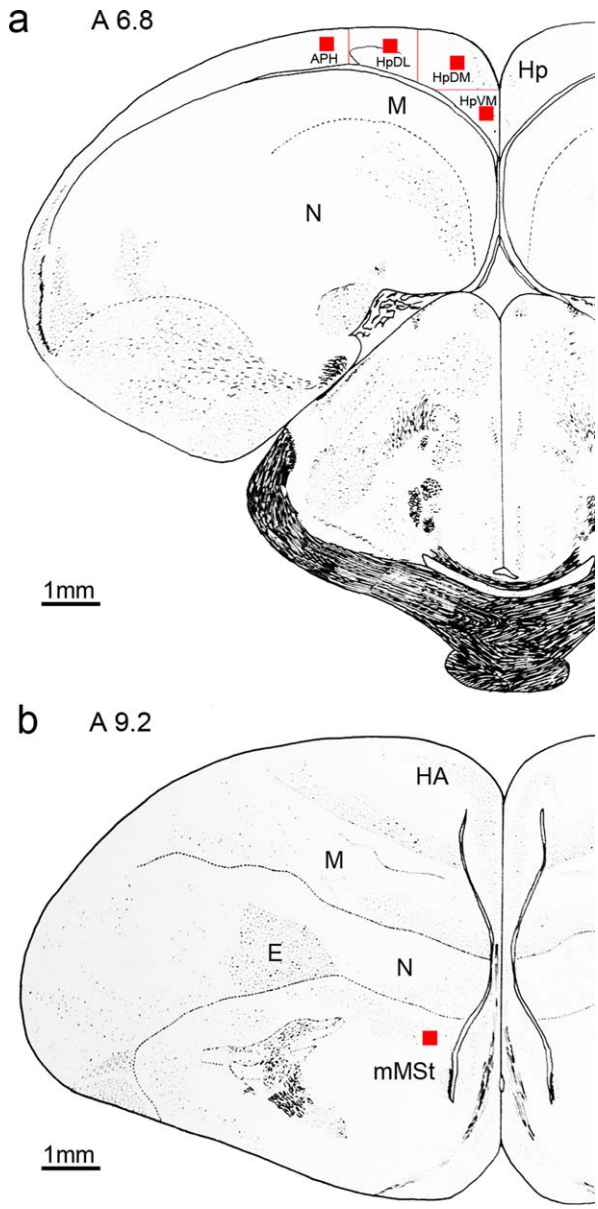

FIGURE 2. Typical placement of cell count zones (red squares) for the four HF subdivisions and mMSt. (a) Schematic view of a coronal section showing HF and its partitioning into four subdivisions (red lines are boundaries). The thin black line identifies the transition in cell density that was used to define what we considered the boundary between APH and HpDL (see also Fig. 4a). (b) Schematic view of a coronal section showing the typical placement of the cell count zone within the medial part mMSt. Drawings were adapted from the atlas of Kuenzel and Masson (1988). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(HpDL) and the most lateral parahippocampus (APH). The boundary between HpDL and APH was based on an anatomical landmark, which can be seen in Figure 4a. For cell counting of each subdivision across the sampled sections, the square enclosure was placed in a way such that it covered as many activated neurons as possible while keeping a minimum distance of at least 20 µm from the border of a neighboring

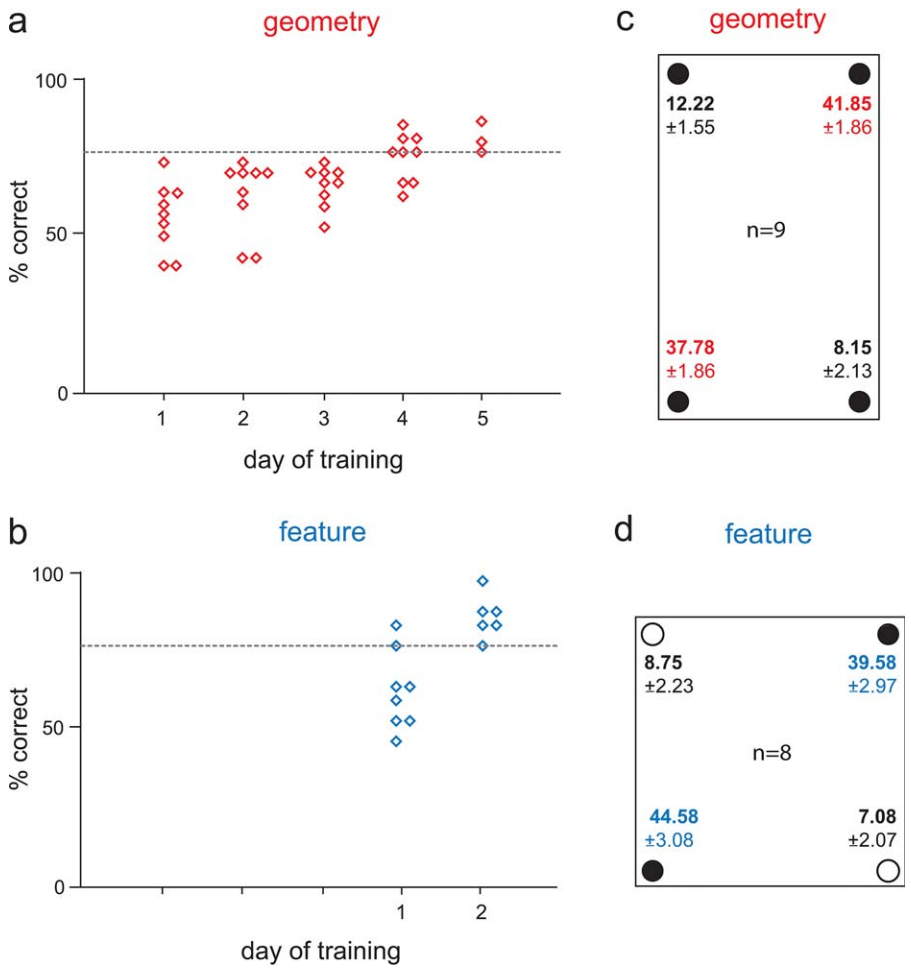

FIGURE 3. Learning performance of the individual birds across training until reaching the learning criterion of 77% correct choices for 1 day (dashed line). (a) Performance of the "geometry" chicks. (b) Performance in the "feature" chicks. Note that the "geometry" birds on Training Day 4 were the same age as the "feature" birds on Training Day 1 (see methods for more details). (c) Summary of the corner choice-distribution of the "geometry" birds on the last day of training (correct feeders are the red numbers). (d) Summary of the corner choice-distribution of the "feature" birds on the last day of training (correct feeders are the blue numbers). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

subdivision and the edge of the brain section. Typical placements for each of the four subdivisions are schematically shown in Figure 2a. In addition to HF, we also were interested in labelled cell densities within the medial striatum (mMSt). Labelled cells in mMSt were counted from five sections of each hemisphere, selected from the region extending from A 10.6 to A 9.0. One counting square was positioned dorso-medial within the mMSt of each section (see Fig. 2b).

After completing the cell counts, the mean values (derived from the five sections) for the four subdivisions were calculated for each hemisphere and cell densities were standardized to 1 mm^2. Cell counts pooled from the 4 HF subdivisions were further averaged to estimate overall HF activity. Initially, this was done for the two hemispheres separately. However, because no significant lateralization effect was found for any subdivision, the measured values from the two hemispheres were pooled for further analysis. Thus, the overall estimate of hippocampal activity of an individual bird was based on an average from all 40 counted areas (20 from each hemisphere). The calculated neuronal activity for each individual in mMst was based on 10 counted areas (5 sections, 2 hemispheres). The resulting individual bird means were considered overall indicators for the number of c-Fos IR-neurons and were employed for further statistical analysis.

Statistical Analysis

A possible difference in the density of IEG-expressing neurons was tested by a mixed design ANOVA, with a between subject factor of "treatment group" and a within subject factor "area" as repeated measure. For post-hoc analyses, pairwize *t* tests (two tailed) were carried out. Alpha was set at 0.05 for all analyses. Behavioral/performance differences were tested with independent two-tailed *t*-tests. All statistical analyses were performed with the software IBM SPSS Statistics (v. 20).

TABLE 1.

Measured Densities of c-Fos, IR-neurons (Mean ± S.E.M) Within all HF Subdivisions and mMSt

	Geometry		Feature	
	Left hemisphere	Right hemisphere	Left hemisphere	Right hemisphere
APH	408.9 ± 80.7	275.2 ± 55.7	190.8 ± 48.4	175.6 ± 35.2
HpDL	1170.5 ± 125.5	877.5 ± 96.7	871.2 ± 104.3	801.9 ± 81.48
HpDM	610.84 ± 140.8	394.7 ± 72	352 ± 60.8	302.9 ± 77.2
HpVM	295.11 ± 125.8	149.3 ± 40.3	107.2 ± 19.9	110.4 ± 27.3
mMSt	567.1 ± 105.4	467.6 ± 89.9	934.8 ± 108.7	670.4 ± 151.4
total HF	522.8 ± 47.7		364 ± 34.2	
total mMSt	517.3 ± 62.3		802.6 ± 49.9	

Summary of all estimated cell densities (c-Fos positive neurons/mm^2) across the HF subdivisions, total HF and mMSt for the "geometry" and "feature" trained chicks.

RESULTS

Behavior/Learning

In the "geometry" condition, six chicks reached the learning criterion on the 4th day of training and three chicks on day 5 (Fig. 3a). In the "feature" condition, three chicks reached the criterion on the 1st day of training (which would be at the same age of the day 4 geometry chicks because of the delay in starting the feature group) and the remaining six chicks completed training on the day after (Fig. 3b). In both conditions, on the last day of training the chicks had learned to preferentially choose the correct/rewarded feeders (Figs. 3c,d). In the "geometry" condition on the last day of training, the correct feeders were chosen on 79.7% ± 3.7% (mean ± s.e.m.) of all trials. The difference between correct and incorrect choices was significant (paired sample t test: $t(8) = 25.298$, $P < 0.001$), whereas no significant difference in the number of choices was observed between the two correct feeders (41.6% ± 1.9% vs. 37.8% ± 1.7%; $t(8) = -1.19$, $P = 0.27$). Also the choices to the two incorrect feeders did not differ (12.2% ± 1.6% vs. 8.2% ± 2.2%; $t(8) = -1.23$, $P = 0.26$). Together, the results demonstrate that on the last day of training the chicks showed a strong preference for the geometrically correct feeders while showing no preference for any one feeder or corner.

In the "feature" condition, on the last day of training the correct feeders were chosen in 84.4% ± 5.9% of all trials, yielding a significant preference for the correct feature feeders ($t(8) = 17.196$, $P < 0.01$), but no preference for any one of the correct feeders (40.4% ± 2.9% vs. 44.1% ± 2.9%; $t(8) = 0.778$, $P < 0.46$) or any one of the two incorrect feeders (8.2% ± 2.1% vs. 7.4% ± 2.0%; $t(8) = -0.24$, $P = 0.82$). It is important to note that birds of both the geometry and feature groups were trained to a comparable learning performance; on the last day of training, just prior to the c-Fos test session, no significant difference in performance was present between the geometry-trained and feature-trained groups ("geometry": 79.7% ± 3.7%; "feature": 84.4% ± 5.7%; $t(15) = -1.85$, $P = 0.08$).

Behavioral Performance on c-Fos Test Day

All of the "geometry" birds chose correctly on at least two out of the three trials. In the "feature" group, one bird failed to make a correct choice and was excluded from the rest of the experiment as well as from any analyses. The use of this criterion ensured the presence of a continued, significant preference for the correct feeders for both the "geometry" ($n = 9$: 74.07% ± 4.9% correct choices, $t(8) = 4.91$ $P < 0.01$) and the "feature" ($n = 8$: 87.5% ± 6.1% correct choices; $t(7) = 6.15$ $P < 0.01$) conditions. Similar to what was observed during training, the birds did not prefer any one of the two correct feeders ("geometry": $t(8) = -0.406$, $P = 0.7$; "feature": $t(7) = -610$, $P = 0.56$) or incorrect feeders ("geometry": $t(8) = -0.359$, $P = 0.73$; "feature": $t(7) = 2.00$, $P = 0.08$). Also, during the test session, no significant difference in correct choices was present between the "geometry" and "feature" groups ($t(15) = -1.73$, $P = 0.1$), indicating that the chicks from both groups were performing at comparable levels with respect to the progression of learning.

During the search for the correct feeder, animals of both groups traveled comparable distances ("geometry" ($n = 9$) mean ± s.e.m: 114.63 ± 17.87 cm; "feature" ($n = 8$) mean ± s.e.m: 156 ± 41.08 cm; $t(15) = 0.976$, $P = 0.34$). The activity in Hp and mMSt was also not correlated with distance traveled (Pearson's correlation analysis, "geometry" HF: $r = 0.284$; $P = 0.46$; "geometry" mMSt: $r = -0.394$, $P = 0.29$; "feature" HF: $r = -0.335$, $P = 0.43$; "feature" mMSt: $r = -0.064$, $P = 0.88$).

Immunohistochemistry

Table 1 summarizes the intra-hemispheric, labelled-cell density distributions across the HF subdivisions, total HF and mMSt for the "geometry" and "feature" trained chicks. Our statistical analyses below are limited to total HF and mMSt cell densities because all higher resolution hemispheric and subdivisional comparisons did not yield a significant difference between the "geometry" and "feature" chicks. This is at least in part because of a lack of sufficient statistical power needed for

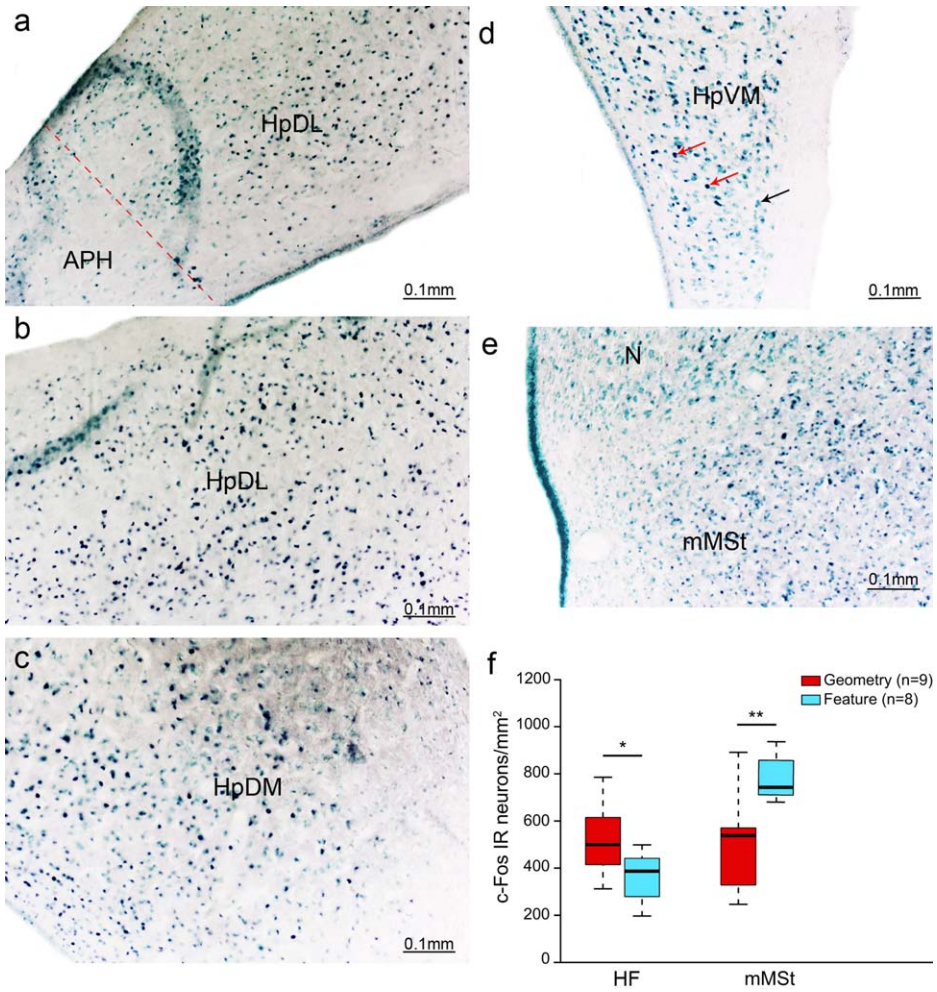

FIGURE 4. Labelled neuronal nuclei in the sampled subdivisions of the left HF of a "geometry" bird (a-d) and in the medial portion of the right medial striatum (mMSt) of a "feature" bird (e). (a) Red dashed line represents the border between the dorsolateral hippocampus (HpDL) and the parahippocampal area (APH). Note the distinct (and reproducible) change in cell density across the transition between HpDL and APH, and that the red dashed line is placed at the apex of the cell density transition. (b) High number of labelled (black) IR nuclei within HpDL. (c) High number of labelled nuclei within HpDM. (d) Labelled nuclei within the HpVM. IR-neurons stain black (red arrows) and can easily be distinguished from the c-Fos-negative, green-stained neuronal nuclei (black arrow). (e) Labelled nuclei within mMSt. (f) Summary of the quantitative analyses. Significant labelled-neuron-density differences between groups were present within HF and mMSt, revealing an unexpected double dissociation (significance levels: $*<0.05$; $**<0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

multiple comparisons. However, it is noteworthy that for every HF subdivision comparison (eight total) the density of labelled neurons was larger in the "geometry" trained birds compared to the "feature" trained birds, particularly among the left HF subdivisions. Of perhaps more interest, within the "geometry" trained group, substantially more labelled neurons were found in every left-HF subdivision compared to the right HF.

Sample photomicrographs of IR-neurons in the brain areas examined can be found in Figures 4a–e. Importantly, both HF and mMSt showed significant differences in the density of c-Fos labeled neurons when the two training groups are compared (Fig. 4f). Compared to the HF of the feature-trained chicks ($n = 8$ mean $\pm$ s.e.m: 364 ± 34.2 IR-neurons/mm^2), the density of labelled neurons was substantially higher in the HF of birds that used geometry to locate the rewarded goals ($n = 9$ mean $\pm$ s.e.m: 522.8 ± 47.7 IR-neurons/mm^2). Revealing an unexpected double-dissociation, activation in the mMSt showed the reverse pattern with respect to training condition. Chicks trained on the geometry discrimination ($n = 9$) were found on average to have 517.3 ± 62.3 IR-neurons/mm^2, whereas chicks trained on the feature discrimination had substantially more labeled neurons ($n = 8$ mean $\pm$ s.e.m:802.6 ± 49.9 IR-neurons/mm^2). The difference between the geometry and feature groups was significant for both HF and mMST (ANOVA *group*area*: $F(1,15) = 27.914$, $P \leq 0.001$; post hoc t test: HF: $t(15) = 2.381$, $P = 0.03$; mMSt: $t(15) = -3.303$, $P = 0.005$).

We also carried out correlation analyses between the number of IR-neurons/mm^2 in HF and mMSt, and the performance of each bird on the last day of training. The analyses were carried out to control for possible differences in performance (correct

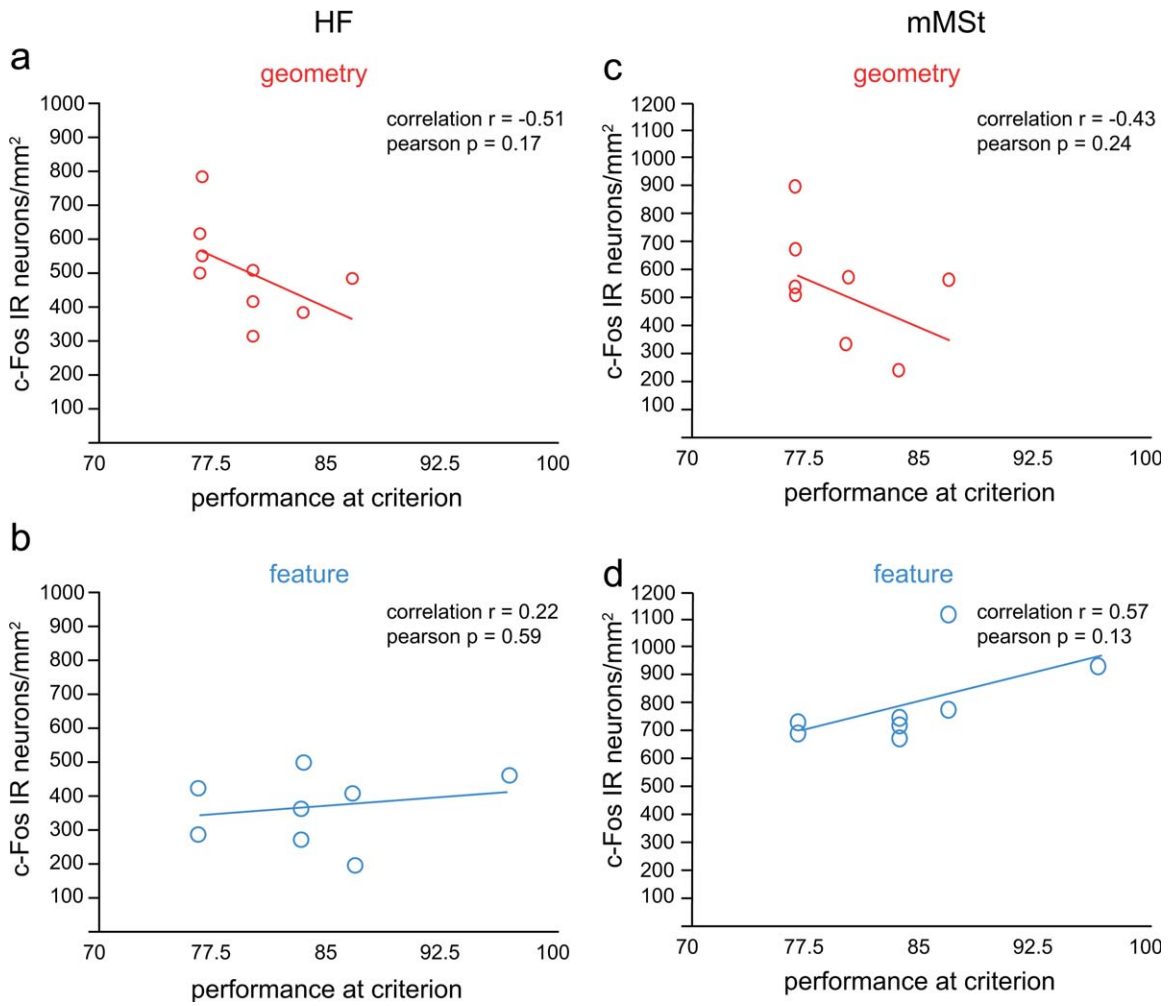

FIGURE 5. Correlation analyses between measured brain activities (IR-neurons/mm^2) and performance levels (percent correct choices) on the last day of training. None of the correlations is significant. HF-IR neuronal densities in the (a) "geometry" birds, and (b) "feature" birds. mMSt-IR neuronal densities in the (c) "geometry" birds, and (d) "feature" birds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

choices) as a contributing explanation for the differences in labelled neurons observed between the geometry- and featured-trained chicks. None of the correlations (Fig. 5) was significant (Pearson: $P > 0.10$ for each area/condition). Moreover, at the same performance level of 77.5% correct choices, the activity in the HF of the "geometry" birds was, as seen overall, higher than in the "feature" birds (Figs. 5a,b). The correlation results show that the activity differences in HF and mMSt were principally due to the differences in the representational strategies associated with the two tasks.

DISCUSSION

The present study demonstrates involvement of the HF of chicks in goal navigation by environmental geometry. We found an up-regulation of c-Fos in the HF in animals trained to orient by the geometry of a rectangular arena when compared to controls trained to discriminate local features in a square-shaped arena. We also analyzed c-Fos expression in mMSt, whose neuronal activity has been shown to be upregulated in pigeons navigating home from a familiar site (Shimizu et al., 2004; it should be noted that HF also experienced an up-regulation in neuronal activity in the same pigeons). Surprisingly, in mMSt we observed a reversed pattern compared to HF, with higher activity in chicks that were trained to discriminate local features. In the following paragraphs we will first discuss how the activity in HF might be linked to relational computations of geometrical properties. We will then provide some thoughts on how the unexpected double-dissociation can be interpreted and which task-specific parameters could have influenced the activity in mMSt.

Our results confirm those of previous studies with pigeons (Vargas et al., 2004), in which lesions of HF disrupted goal navigation in a rectangular arena. It is, however, important to

consider the nature of the geometric representation that enables goal navigation. One candidate representational strategy could be based on encoding the relative position of a goal with respect to global geometry of the long and short walls. Such a strategy is comparable to that implied in the classical studies with rats (Morris, 1981, 1984). In the Morris water maze, the position of the goal is encoded in relation to the distal visual cues outside the arena (Morris et al., 1982) and requires an intact hippocampus. By contrast, damage of the hippocampus does not affect orientation towards visible, local cues (Morris et al., 1982). Similar results have been obtained for zebra finches in a 'dry version of the Morris water maze' (Bischof et al., 2006; Watanabe et al., 2008; Mayer et al., 2010). The data from these studies allow one to infer that the avian HF, like the mammalian hippocampus, encodes positions in relation to the distal visual cues of the surroundings, but not orientation to local features. Such an encoding of distal, visual cues would result in an "allocentric" representation of space aligned with the properties of a theoretical "cognitive map" (Tolman, 1948).

It has been proposed for mammals that the hippocampus may participate in the representation of three different "allocentric" maps (Jacobs and Schenk, 2003). The so-called "bearing map" is constructed from compass information provided by directional cues that in nature could be the sun or stars. The so-called "sketch map" is constructed from the relative position of fixed positional landmarks. The so called "integrated map" would emerge from the integration of the bearing and sketch maps. The neuronal processing associated with a higher order, "integrated map" would involve the integration of head direction (bearing map) and place (sketch map) information, which in mammals would come together in the CA1 region (see Leutgeb et al., 2000). The involvement of avian HF, in this case homing pigeons, in the processing of "bearing" and "sketch map" components has been shown by Bingman et al. (2006) and Gagliardo et al. (2009). In our present experiment, orientation by compass information outside the arena was disrupted because the arena was rotated and randomly oriented after each trial. The chicks had to learn to locate the target in relation to one of the cues inside the arena, e.g. correct orientation in relation to one of the long (or short) walls or the relative position of the correct corner with respect to the relational joining of a long and short wall. It is worth noting, that both of these representational strategies would require "relational computations" either between multiple landmarks (walls) or in relation to one directional landmark (wall). It may be that it is this "relational computing" that is dependent on the hippocampal participation; i.e., what HF does. Indeed, it has been proposed that spatial processing may be a special example of a more abstract hippocampal function, such as 'relational computations' or relational learning (Cohen and Eichenbaum, 1993; Eichenbaum et al., 1999; Day, 2003). If true, spatial representations would not necessarily constitute maps of space, but instead would contribute to a more general "linking events within episodes" (Eichenbaum et al., 1999). As a consequence, hippocampal "memory space" (Eichenbaum et al., 1999) would code for both spatial and non-spatial relations among events in addition to processing spatial relations for navigation. It may be, therefore, that "relational computations" caused the upregulation of c-Fos in the HF of chicks trained in the rectangular environment. By contrast, the control chicks that oriented by a local feature of the feeder do not need to perform relational computations and should therefore not rely on hippocampal involvement. However, we should note that a functional hypothesis of "relational computations" for the hippocampus has been developed from research in mammals, and it has been argued that the avian HF may be a more dedicated spatial processing structure, whose functional properties resemble more the idealized cognitive map of O'Keefe and Nadel (1978; see Coppola et al., 2014).

Other explanations for the enhanced activity in the HF of the geometry-trained chicks seem unlikely. Could the upregulation in HF have been a consequence of the chicks learning to discriminate based on the visual pattern provided by the junction of a short and a long wall, a form of "feature-structure discrimination" ability (Pearce et al., 2005; Bingman et al., 2006)? Interestingly, lesions to the HF do not impair the ability of pigeons to solve "feature-structure discriminations" (Pearce et al., 2005; Bingman et al., 2006). Similar to "visual pattern discriminations" (e.g., Watanabe et al., 2011), HF independent feature-structure discrimination is probably processed via the entopallium and should not induce neuronal activity in HF. Alternatively, it has been discussed whether small scale orientation could be based on stable panoramic views (Pecchia et al., 2010, 2011), which would require 'snap-shot-like' memories of a visual scene, thus resembling some insect behavior (Wehner and Räber 1979; Cartwright and Collett, 1983; Wystrach and Beugnon, 2009). However, such a strategy would be another form of visual discrimination, and therefore, would not be expected to induce c-Fos activity in HF.

It is also very unlikely that the geometry chicks in our experiment used a purely egocentric orientation strategy, such as turning left or right in relation to their own body axis independent of any external visual information. Theoretically, such a strategy could lead to successful goal orientation if an animal develops a turn bias after reaching a wall. This could direct the animal to the goal location more often than to the unrewarded one because the probability of reaching a long wall is higher compared to that of reaching a short wall. However, such a strategy would likely require a chick to first approach a wall before turning toward a corner, a behavior easily detectable. However, we rarely observed a chick making a sharp turn after first approaching the center of a wall. Other purely egocentric strategies were prevented by the disorientation procedure. Therefore, regardless of the presence of any "egocentric turn bias," a chick would still have to re-orient in relation to the boundary visual information if it is to locate a goal. This would involve again encoding position in relation to the "allocentric" environment, which is, in terms of HF involvement, indistinguishable from other strategies that require relational or geometrical computations.

Consistent with studies showing that HF is not required for locating a goal based on feature properties, the density of c-Fos IR-neurons was lower in the HF of chicks trained to feature compared to chicks trained to geometry. Previously, it has been demonstrated in zebra finches that local feature discrimination is neither disrupted by HF lesions (Watanabe et al., 2008), nor results in an upregulation of HF c-Fos expression (Mayer and Bischof, 2012). This is because processing of local features in birds involves the tectofugal visual pathway, which projects to the telencephalic entopallium (Shimizu et al., 2010). Lesioning of the entopallium has been shown to impair local feature discrimination (Watanabe et al., 2008), but unfortunately, this primary sensory area almost never expresses c-Fos (Horita et al., 2010). Instead, an up-regulation of c-Fos associated with local feature discrimination has been found in the lateral nido-mesopallium (Mayer and Bischof, 2012), which receives inputs from the entopallium (Krutzfeldt and Wild, 2004) and is involved in visual imprinting in zebra finches (Lieshoff et al., 2004; Huchzermeyer et al., 2006). Functionally, the lateral nido-mesopallium probably corresponds to the intermediate nidopallium (NIL) of pigeons, which plays a similar role in the visual recognition of potential mates (Patton et al., 2009). Nonetheless, in the present study we did not observe any substantial expression of c-Fos within the lateral nido-mesopallium of chicks that learned the local feature discrimination. However, it is important to mention that immediate early gene expression such as c-Fos does not reflect every form of neuronal activity, but is probably more related to experience dependent, plastic changes (Lanahan and Worley 1998; Jones et al. 2001; Guzowski 2002). Therefore, not finding c-Fos activity in a given brain area does not necessarily mean that task relevant neuronal activity was absent in this area.

Although not statistically demonstrable, it is noteworthy that in the geometry-trained chicks the density of c-Fos labelled neurons was greater in every subdivision of the left HF compared to the right HF (Table 1). In chicks, hemispheric differences in geometric information processing were found in monocular occlusion studies (Rashid and Andrew, 1989; Tommasi and Vallortigara, 2001). While both telencephalic hemispheres contribute to orientation, the left hemisphere (served by the right eye) seem to encode absolute metric distances, while the right hemisphere (left eye) is more concerned with relational spatial information (Tommasi and Vallortigara, 2001). This has been confirmed by lesion studies: chicks with an intact right HF and sham-operated controls rely on large-scale, relative information provided by an enclosure. By contrast, after right-hemisphere or bilateral HF lesions, birds are disoriented or rely only on available local cues (Tommasi et al., 2003). Also, in a study of working memory, both object- and position-specific informations were available to both eye systems (Regolin et al., 2005). However, when a conflict between cues arose, the right and left hemispheres preferentially attended to position- and object-specific cues, respectively. Chicks also showed an increased reliance on positional information in strongly lateralized individuals [chicks that were exposed to light during embryonic development, causing asymmetric development of thalamofugal visual projections (Chiandetti et al., 2005; see Rogers 1990; Rogers and Deng, 1999)]. Overall, the trend of a left HF advantage for geometry observed in the present study, based on higher neuronal activation, seems to be in contrast with much of the chick literature. However, it needs to be considered that our chicks were dark incubated and tested binocularly. The discrepancy with previous chick findings may also be related to the different stages or types of learning, because previous investigations were based on recall of learned information or working memory.

In pigeons, the situation is somewhat different compared to chicks and, surprisingly, more in line with our data. For example, in pigeons the left HF appears to be the more important for geometry-based navigation (Nardi and Bingman, 2007) and is characterized by a more extensive functional connectivity network (in both pigeons and starlings, *Sturnus vulgaris*; Jonckers et al., in press). Unfortunately, no other study has measured neuronal activity within the HF of chicks or pigeons trained on a similar geometry-orientation task. Therefore, the trend for a greater involvement of the left HF in our geometry-trained chicks is of substantial interest for the future investigation of brain lateralization and geometry-based navigation.

Use of geometric information explains the up-regulated c-Fos activity within the HF of geometry-trained chicks, but what could be the reason of the higher activity found in the mMSt of birds that learned to orient by local features? Anatomically, mMSt resembles the mammalian ventral striatum, including the nucleus accumbens (Mezey and Csillag, 2002; Izawa et al., 2003). Although a full functional description of mMSt remains elusive, it has been reported to participate in a number of important behaviors, such as motoric aspects of song-learning (Luo, Ding, and Perkel, 2001). In pigeons, mMSt has been connected to homing behavior (Shimizu et al., 2004), which was associated with higher activity of the immediate early gene Zenk compared to control pigeons that did not home. It is noteworthy that a projection from the hippocampal formation into the MSt was reported in zebra finches (Szekely and Krebs, 1996) and in pigeons (Veenman et al. 1995; Atoji et al., 2002; Husband and Shimizu, 2011). Also, mMSt lesions disrupt the acquisition of both spatial discrimination and color discrimination (Watanabe, 2001), indicating that this structure participates in a range of learning processes. In gallinaceous birds, the medial part of the telencephalic basal ganglia was suggested to be the storage site of memory traces for passive avoidance (Rose, 1991). Interestingly, in a passive avoidance task, chicks learn to associate a bitter taste with a color that they will avoid pecking at in the future. In fact, it has been proposed that memorized color cues are represented in the mMSt (Yanagihara et al., 2001). By contrast, other studies employing chemical lesions suggested that mMSt is more involved in acquisition than memory recall: bilateral ablation did not interfere with selective pecking based on memorized color cues, but did impair subsequent novel learning (Izawa et al., 2002). Overall, it seems that parts of the medial striatum are involved in processing of object feature information and this might be specifically important for the acquisition of

associated memory traces. In our experiment we found significantly increased activation of mMSt in chicks that were in the later stages of learning a "local feature discrimination," compared with geometry-trained birds. The mMSt activation seemed to be higher in the left hemisphere and did not change as a function of performance level (Fig. 5). Our results, therefore, support the hypothesis that mMSt plays a role during recall of memorized feature associations. More specific studies are needed, however, in order to understand what aspects of information processing engage mMSt. The current results indicate that c-Fos measurements could provide a useful tool in this regard.

In conclusion, the HF of chicks seems to be recruited to support geometrical computations needed for goal orientation in a rectangular environment. By contrast, mMSt is involved in learning 'non-geometrical' information provided by local features. However, these two brain areas, and the neural systems they are a part of, should not be viewed as operating independent of each other. The mMSt not only receives projections from the HF (Veenman et al., 1995; Szekely and Krebs, 1996; Atoji et al., 2002; Husband and Shimizu, 2011), but has also been found to be involved in both spatial navigation and local feature discriminations (Watanabe, 2001; Shimizu et al., 2004). We believe that a goal of future studies should be to better understand the interactions between the hippocampal and striatal systems that enable in part the high functioning cognitive systems of birds (see Shanahan et al., 2013).

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