Brain Struct Funct
DOI 10.1007/s00429-017-1537-5

ORIGINAL ARTICLE

Representation of environmental shape in the hippocampus of domestic chicks (*Gallus gallus*)

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Received: 1 June 2017 / Accepted: 5 October 2017

Abstract The hippocampus plays an important role in spatial encoding and memory across various vertebrate species. In rodents, hippocampal neurons are particularly sensitive to a change in environmental geometry. Given the similarities in function between the mammalian and avian hippocampi, we aimed to measure whether enclosures varying in geometric shape (square and rectangle) can differentially activate hippocampal cells in the domestic chick (*Gallus gallus domesticus*). Chicks exposed to both a square and a rectangular arena exhibited a significantly higher neural activation (as measured by c-Fos expression) than those exposed twice to just the square or just the rectangle (both of which were significantly higher in activation than a one-environment control group). For the first time in an avian species, we show that exposure to two enclosures of different geometric shape activates the hippocampus to a greater degree, suggesting a possible effect of spatial remapping.

Keywords Avian hippocampus · Immediate early genes · Boundary geometry · Remapping · Novel environment

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Introduction

Animal spatial navigation is mediated by internal 'maps' of the environment consisting of allocentric representations of locations and their spatial relationships (Tolman 1948). The hippocampus (Hp) is a phylogenetically ancient part of the vertebrate brain (Butler and Hodos 2005). In all vertebrate groups, the hippocampal pallium homologue is involved in the use of map-like, relational representations of the environment that provide stable allocentric reference for flexible navigation (Rodríguez et al. 2002; Broglio et al. 2015). These similarities suggest a common evolutionary ancestry of the functional properties of the hippocampus, which are retained through the independent evolution of vertebrate lineages. Although the spatial mapping function of the hippocampus is shared across both mammals and non-mammals, the understanding of avian hippocampus at the neuronal level is very limited. In particular, because the avian hippocampus lacks a layered structure, the anatomical subdivisions that correspond to the mammalian hippocampal regions are still highly debated (Atoji et al. 2006; Gupta et al. 2012; Herold et al. 2014; Striedter 2016). Nevertheless, the question of how such seemingly different structures can contribute to similar functions makes it worthwhile to further investigate the avian hippocampus in ways that can be directly compared to its mammalian counterpart.

In mammals, internal 'maps' of the environment are generated through a critical contribution of hippocampal place cells (O'Keefe and Dostrovsky 1971). These neurons fire when the animal passes through a specific part of its environment, a phenomenon that has been investigated in detail in hundreds of experiments over four decades (Barry and Burgess 2014; Moser et al. 2014). Spatial coding in mammals is heavily dependent on the inputs from spatial

Published online: 14 October 2017

boundaries (Solstad et al. 2008; Lever et al. 2009; Tommasi et al. 2012; Stewart et al. 2014; Lee 2017). Change in the environmental shape causes remapping of the hippocampal place cells to new preferred firing fields or activation of new cell populations (Muller and Kubie 1987; O'Keefe and Burgess 1996; Lever et al. 2002). Although the above studies on rodent measured neural activity using electrophysiology, environmental novelty can also be observed using immediate early genes (IEGs) as neuronal activity markers (Kubik et al. 2007). Imaging of IEG products offers an effective approach to studying the activation of entire neuronal ensembles with a high degree of spatial resolution. However, the expression of IEGs is more closely associated with neuronal plasticity and not necessarily indicative of the absolute amount of activity (Lanahan and Worley 1998). IEG expression is low in quiescent cells and rapidly increases in response to trans-synaptic signalling between neurons (Sheng and Greenberg 1990). The resulting genomic response is linked to long-term structural changes to the neuron by encoding transcription factors, cytoskeletal proteins, growth factors, metabolic enzymes and proteins involved in signal transduction (Lanahan and Worley 1998). For this reason, IEG expression in home cages is typically low and is often used as a control measure, whereas spatial novelty induces high level of responsiveness within the hippocampus of mammals (Kubik et al. 2007). The pattern of IEG activation is tightly linked with specific environments and appears to be involved in the subsequent consolidation of spatial information (Jones et al. 2001; Guzowski 2002; Barry and Commins 2011). The availability of this alternative measure of neural activity is especially important in the study of the avian hippocampal formation and how it represents multiple environments.

Despite the structural differences, the hippocampus is crucial to spatial navigation in birds, just as it is in mammals (Bingman and Able 2002; Smulders 2006; Mayer et al. 2013). Hippocampal lesions in birds impair large-scale navigation (Bingman et al. 1985, 2005), disrupt orientation in the 'dry Morris water maze' (Watanabe and Bischof 2004; Watanabe et al. 2008) and interfere with the use of boundary geometry of the environment (Tommasi et al. 2003; Vargas et al. 2004; Bingman et al. 2006). In pigeons, hippocampal neurons comparable to place cells were found using electrophysiology, but their responses were not as spatially selective as in rats (Hough and Bingman 2004; Siegel et al. 2005; Bingman and Sharp 2006). The importance of the avian hippocampus for spatial navigation has been further confirmed with IEG experiments (Smulders and DeVoogd 2000; Bischof et al. 2006; Mayer et al. 2010; Mayer and Bischof 2012). Our most recent study using IEGs in domestic chicks trained in a standard reference memory task (Vallortigara et al. 1990), further confirmed hippocampal involvement in goal-based navigation by the shape of a rectangular arena (Mayer et al. 2016), suggesting similar sensitivity of avian hippocampus to the boundary space. Given the fundamental importance of boundary geometry for spatial orientation abilities across vertebrates (Vallortigara 2009; Vallortigara and Chiandetti 2017), including domestic chicks (Vallortigara et al. 2009; Lee et al. 2012; Versace and Vallortigara 2015), in the present study we aimed to address whether the hippocampus of newly hatched chicks would respond to a change in environmental shape in a way that is similar to remapping effects observed in the mammalian hippocampus.

Methods

Rationale of the experimental design

A standard procedure to map neuronal activity in brain sections is to quantify cells containing IEG products. The underlying assumption is usually that the experimental task should activate a greater number of cells in the region of interest of the experimental animals compared to the controls. However, the differences in neural coding associated with the differing environmental conditions can also involve variations in which neuronal populations, within the same region, are activated by two different experiences (Chaudhuri et al. 1997; Vazdarjanova and Guzowski 2004; Guzowski et al. 2005). Thus, the involvement of brain structures in which two different neural populations are activated in the control and experimental conditions might be overlooked, if the two populations are approximately of the same number.

Prior to the current study, a series of pilot experiments revealed equally high activity levels in the hippocampus of chicks that were habituated to visit a square-shaped arena over multiple days and then exposed either to a new rectangular arena or to an identical square environment to that seen in habituation. Both conditions elicited similar hippocampal activation, with no differences in activated cell counts between the two groups (unpublished results). These surprising results led us to hypothesize that distinct neural populations might be activated in the two conditions, but since they were of similar numerosity we could not detect the difference.

To overcome this limitation, the design of the present experiment involved exposing different groups of chicks, either two times to the same shape environment or to two different environmental shapes. By considering the time course of c-Fos expression (an immediate early gene product with protein peak level between 1 and 2 h after behaviourally induced activation), we expected to observe

highest number of c-Fos activated cells in the group of chicks which were exposed to two different environmental shapes, because the two different experiences within a short period would activate different (potentially overlapping) populations of neurons, both containing high level of c-Fos at 1–2 h after exposure. On the contrary, two repeated exposures to the same environmental shape within a short period would induce an overlap of neuronal populations (Guzowski et al. 2005) and, therefore, activate lower densities of cells.

Subjects

Thirty-four laboratory-hatched, male domestic chicks (*Gallus gallus domesticus*), of the Aviagen ROSS 308 strain, 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 × 40 × 30 cm^3) at room temperature of 30–32 °C with LED illumination from above at a day/night cycle of 14 h light and 10 h dark. Chicks were deprived of food in the evening before each day of training. After the training sessions, chicks received food ad libitum for at least 4 h before it was removed again in preparation for the next day of training. Water was available ad libitum during the entire training period. All experiments were carried out in accordance with ethical guidelines current to European and Italian laws. The experimental procedures were licensed by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanità Pubblica Veterinaria (permit number 25587).

Apparatus

Chicks were trained to forage for mealworms in two square-shaped chambers (60 cm × 60 cm × 60 cm) connected by a door (15 cm × 15 cm) that they had to walk through to get a mealworm (*Tenebrio molitor* larvae) (Fig. 1). Only the first chamber (base arena) contained a water dish, which was positioned in the centre, whereas the second chamber (experimental arena) was empty. The wall between the two arenas could slide vertically (max. 15 cm). This way the door appeared in the centre of the wall when it was elevated and disappeared below the floor when the wall was slid down again. All inner surfaces were white and the arenas were homogeneously illuminated through a 10-cm hole in the centre of the ceilings with a 40 W warm light bulbs. The room outside of the arenas was dark. For the test phase, the experimental arena was replaced either with a new square-shaped arena or a rectangular arena ($L \times W \times H$: 80 cm × 45 cm × 57.6 cm). All arenas had identical overall area of the inner surfaces (21,600 cm^2); however, due to the difference in shape between the rectangular- and square-shaped arenas the illumination was slightly different (∼ 10% higher in the rectangular arena). In the base arena, we measured 362 Lux in the centre and 284, 300, 292, 285 Lux in the four corners. Almost identical luminance was present in the square test arena with 366 Lux in the centre and 289, 301, 296, 288 Lux in the corners, whereas the rectangular test arena was slightly brighter with 396 Lux in the centre and 328, 318, 338, 326 Lux in the corners. The total surface areas of the floors on which chicks could move around were identical: 3600 cm^2 (square 60 cm × 60 cm; rectangle 45 cm × 80 cm).

Habituation training

On the first day, after hatching, male chicks were placed in individual home cages with food and water provided ad libitum (see "Subjects" for details). On day four, chicks were familiarized with the experimental environment. Each chick was individually placed in the base arena (which was not covered from above) with a water dish in the centre. They were allowed 5–10 min to explore the arena and find mealworms which were placed nearby. To encourage exploration, chicks were trained to walk through the door when it appeared, to the experimental arena, by placing a mealworm directly in front of the door in the experimental arena. The door was then closed and after 1 min it was opened again to allow the chick to go back in the base arena and to find the next reward. This procedure was repeated at least three times with each chick. After the habituation training chicks were moved back to their home cages where they remained until the next day. Transport from the home cage to the experimental setup and back occurred in a closed, plastic box (32 cm long, 18 cm wide, 30 cm high).

On the following 3 days, chicks underwent six daily sessions of habituation training (three in the morning and three in the afternoon). One session contained five trials, in which the chicks needed to walk through the open door to find a reward in the experimental arena and when the door was opened again after 1 min to walk back to the base arena to receive another worm. The arenas were now covered from above and the light in the experimental room was off. The worms were delivered at random positions through the central light bulb holes in the ceilings of the arenas when the chick was in the other arena. Between the sessions, the chick remained in the base arena, which contained a water dish in the centre. The intervals between each trial were 1 min, between sessions 30 min, and between morning and afternoon sessions 2 h. On the fifth day of training, the morning session was carried out as usual. After the end of the morning session, the chick

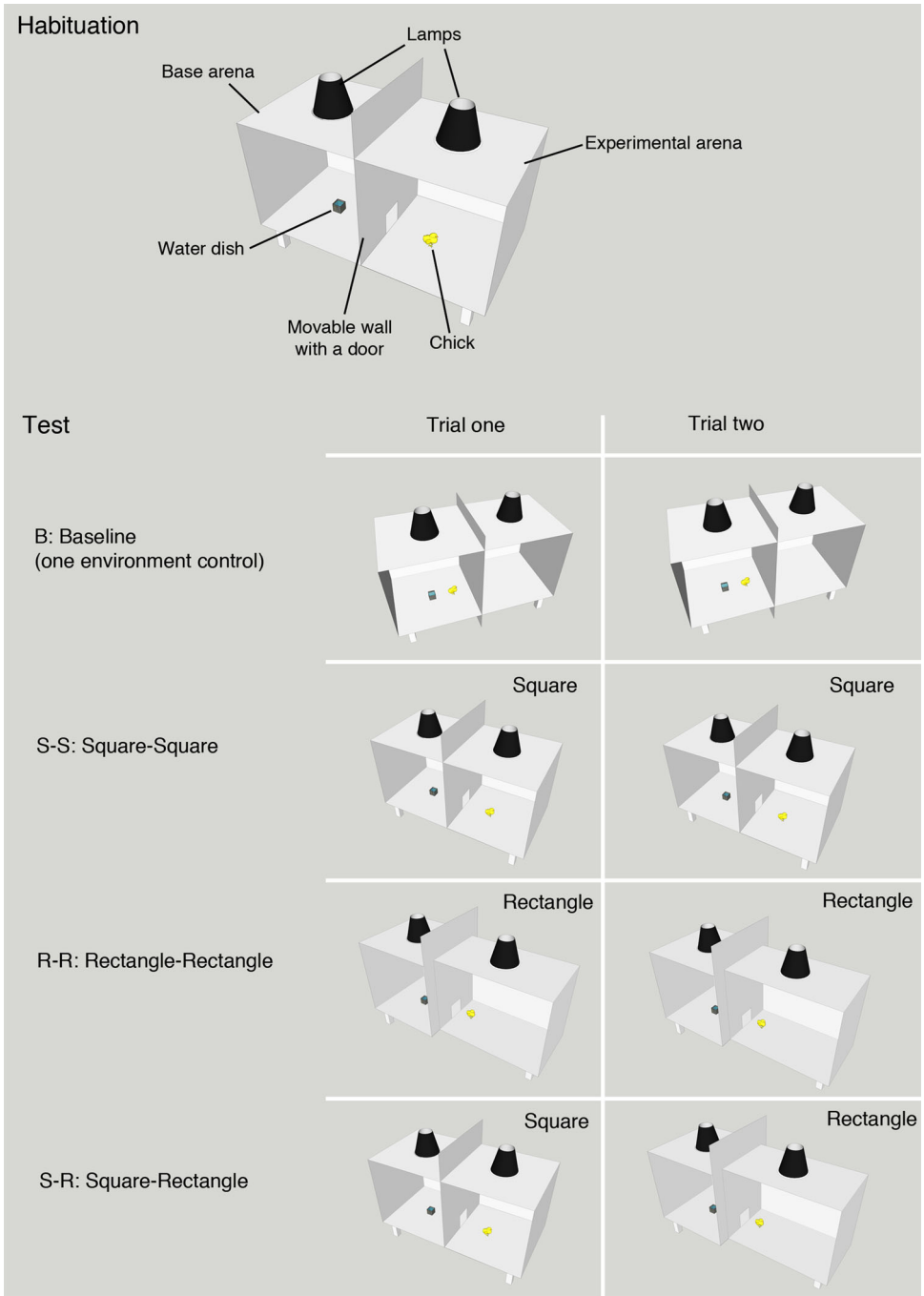

Fig. 1 Schematic representation of the experimental setup and procedures. The base arena (square shaped) contains a water dish in the centre and is connected to the experimental arena. During habituation, chicks were trained to forage for mealworms by traversing between the base arena and an identical square-shaped arena, which were connected by a door opening which appeared in the centre of the wall when it was elevated. Chicks were repeatedly habituated to this procedure multiple times over multiple days (see "Methods" for details). On the morning of testing, chicks were placed in the base arena for 5 h, prior to the test session. For the test session, chicks were divided into four groups. The baseline group stayed in the base arena, where they received four worms with an interval of 1 min. One hour after receiving the last worm, the chicks were perfused. The other three groups performed two experimental trials with the following sequence: Trial 1: chicks entered the experimental arena, found a worm and stayed there for 1 min; and then, they came back to the base arena, found another worm and stay there for 1 min. Trial 2: chicks entered the experimental arena, found a worm and stayed there for 1 min; and then, they came back to the base arena, found another worm and stayed there for 1 h until perfusion. The square–square (S–S) group visited a new square-shaped arena (identical to the one used during habituation) twice, both in trials 1 and 2. The rectangle–rectangle (R–R) group visited a rectangular arena twice, in both trials 1 and 2. The square–rectangle (S–R) group visited a square arena in the first trial and a rectangular one in the second trial. All arenas had identical inner wall and floor surface areas

remained in the base arena until the beginning of the test session, which was performed in the afternoon (no training session was performed in the afternoon of the test day). Chicks remained in the base arena continuously for 5 h after the morning training session, before the test session began. This ensured that c-Fos expression due to brain activation in response to the base arena was at baseline at the time of perfusion after the test (Zangenehpour and Chaudhuri 2002). Thus, we did not expect our measures to be influenced by any activity associated with experiencing the base arena.

Test session for c-Fos labelling

Chicks were divided into four experimental groups. The test session was performed 5 h after the morning session and consisted of two trials for each experimental group (except the baseline control group). The baseline group ($n = 10$) was a control condition to measure hippocampal baseline activity. The chicks of this group remained in the base arena where they received four worms randomly placed in the environment with a 1-min interval between each worm. For the three experimental groups, which differed only in the shape of the experimental arena used during test, the chick went from the familiar base arena to the experimental arena and back twice, receiving four worms total. The second group (square–square: $n = 8$) was exposed twice to a novel square-shaped arena (the experimental arena). The inter-trial interval was 1 min. The third group of chicks (rectangle–rectangle: $n = 8$) was exposed two times to a novel rectangular experimental arena, with the same procedure as the second group. The fourth experimental group (square–rectangle: $n = 8$) was exposed to both the square and rectangular arenas. Thus, in the first trial, the experimental arena was a square. When the chick went back to the base arena, the square arena was replaced with the rectangular one for the second trial. After the second trial, the chicks remained in the base arena until perfusion.

Immunohistochemistry

Seventy minutes after the test session, subjects were overdosed with an intramuscular injection of 0.8 ml ketamine/xylazine solution (1:1 ketamine 10 mg/ml + xylazine 2 mg/ml). The chicks were perfused transcardially with phosphate-buffered saline (PBS; 0.1 mol, pH = 7.4, 0.9% sodium chloride, 4 °C) and paraformaldehyde (4% PFA in PBS, 4 °C). The skull with the brain was post-fixed in 4% PFA/PBS solution for 7 days. For the removal of the brain from the skull, procedures described in chick's brain atlas Kuenzel and Masson (1988) were applied to ensure that the coronal brain sections of all brains had the same orientation (45°). After removing the brains from the skull, the left and the right hemispheres were separated and processed separately. Each hemisphere was embedded in gelatine (7%) containing egg yellow, post-fixed for 48 h in 4% PFA/PBS containing 20% sucrose at 4 °C, and further 48 h in 30% sucrose in 0.4% PFA/PBS. The brain hemispheres were frozen at − 80 °C covered with OCT (Tissue-Tek freezing medium). Four series of 40-µm coronal sections were cut in a Cryostat (Leica CM1850 UV) at − 20 °C. The sections were collected only from the regions of interest A(nterior) 7.8 to A 5.4 (Kuenzel and Masson 1988). The sections of the first series were used for processing and labelling. The sections of the other series were kept as backup or for testing antibody specificity (processing without the primary antibody). Washing in PBS (3 × 5 min) was performed between each of the following reaction steps. Endogenous peroxidase activity was depleted in 0.3% H_2O_2 in PBS for 20 min. After incubation with 3% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min, the sections were treated for 48 h at 4 °C with an anti-c-Fos antibody solution (1:2000; mouse monoclonal, E-8, sc-1669, Santa Cruz, CA, USA), followed by biotinylated anti-mouse in PBS (1:2000; BA-9200, made in goat; Vector Laboratories) for 60 min at room temperature. The ABC kit was used for signal amplification (Vectastain Elite ABC Kit, PK 6100; Vector Laboratories) and VIP substrate kit for peroxidase (SK-4600; Vector Laboratories) for visualization of c-Fos-immunoreactive (-ir) neurons. Sections were serially mounted on gelatin-coated slides, dried at 50 °C, counterstained with methyl green (H-3402; Vector Laboratories) and coverslipped with Eukitt (FLUKA).

Brain analysis

Brain sections were examined with a Zeiss microscope (objective: 20× with a numerical aperture of 0.5) and a digital camera (Zeiss AxioCam MRc5). Contrast and exposure time of the camera were adjusted so that the image on the screen matched the view under the microscope (eyepiece 10×, overall magnification 200×). Successful immunostaining produces dark, purple-black stained nuclei, which can easily be discerned from background and non-activated cells, which were stained light green (see Fig. 3). The imaging software ZEN (Zeiss) was used for counting of c-Fos-ir neurons on a computer screen. For this purpose, a rectangular "enclosure" (150 × 250 µm) was positioned over the different sample areas (see below). Counting was performed blind to the experimental condition. Every activated c-Fos-ir cell within each sample area was marked on the screen using the ZEN software, which computed the total counts.

To estimate labelled cell density within the hippocampus, five to eight sections of each hemisphere were selected from that part of hippocampus extending from A(nterior) 7.0 to A 6.0 [determined by the shape and anatomical organization matched to the atlas of Kuenzel and Masson (1988)]. The hippocampus of each section was parsed into three subdivisions: the ventral hippocampus (HpVM), the neighbouring dorsomedial hippocampus (HpDM) and the dorsolateral hippocampus (HpDL) (Fig. 2a). For cell counting of each subdivision across the sampled sections, the counting enclosure was placed in a way such that it covered as many activated neurons as possible while keeping a minimum distance of 20 μm to the edge of the brains section and the border of a neighbouring subdivision. Typical placements are schematically shown in Fig. 2a. Labelled cell density was estimated also in the intermediate medial mesopallium (IMM) as control region, which showed high densities of c-Fos-labelled neurons in all subjects. IMM (IMHV, Intermediate medial hyperstriatum ventrale according to the old nomenclature) is an area involved in filial imprinting (Horn 1986, 2004) and its activity has never been reported to be linked to specific environmental shapes. For the identification of IMM, we relied on previous anatomical descriptions of this region (Ambalavanar et al. 1993). Five brain slices were selected from a region where the shape of IMM corresponded to what is depicted on plate A7.6 of the Kuenzel and Masson atlas. The counting enclosure was positioned inside the IMM according to the drawings (Ambalavanar et al. 1993), in a way such that it covered as many activated neurons as possible while keeping a minimum distance of 20 μm to the borders (Fig. 2b).

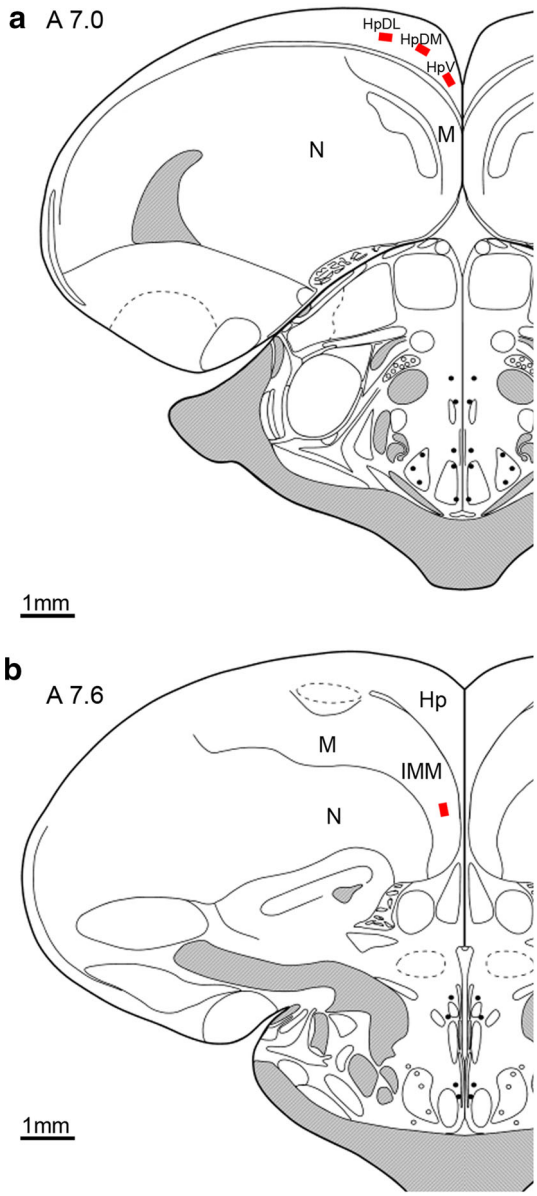

Fig. 2 Typical placements of counting enclosures (red rectangles) inside the three subdivision of hippocampus (**a**) and the intermediate medial mesopallium (**b**). Those drawings of the coronal sections were adapted from Kuenzel and Masson (1988). *Hp* hippocampus, *HpVM* ventro-medial hippocampus, *HpDM* dorso-medial hippocampus, *HpDL* dorso-lateral hippocampus, *M* Mesopallium, *IMM* intermediate medial mesopallium, *N* nidopallium

After completing the cell counts, the mean values (derived from the five sections) for the three subdivisions were calculated for each hemisphere and cell densities were standardized to 1 mm^2. Because no significant lateralization effect was found for any subdivision of the experimental groups, the measured values from the two hemispheres were pooled for further analysis. Cell counts pooled from the three Hp subdivisions were further averaged to estimate overall Hp activity. Thus, the overall estimate of hippocampal activity of an individual bird was based on an average from all 30 to 48 counted areas (15–24 from each hemisphere). The calculated neuronal activity in IMM for each individual chick was based on ten counted areas (five sections, two 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

Differences between groups in their Hp and IMM activation were tested with two independent univariate ANOVAs. Because the Levene's test for the Hp analysis revealed a significant violation of equality of variances and Kolmogorov–Smirnov test (K–S) showed that the distribution of residuals was significantly different from normality, a logarithmic transformation ($\log(x + 10)$) was applied before running the statistical analysis. This procedure increased the equality of variances and normality of the residuals as follows: Levene's test before log-

transformation: $F(3.29) = 7.022$, $p < 0.01$; Levene's test after log-transformation: $F(3.29) = 1.139$, $p = 0.35$; K–S before log-transformation: $D(33) = 0.164$, $p < 0.03$; K–S after log-transformation: $D(33) = 0.137$, $p = 0.122$. For the IMM violation of variances was not present [Levene's test: $F(3.29 = 0.574$, $p = 0.64)$ and the residuals were normally distributed [K–S: $D(33) = 0.089$, $p = 0.2$]; therefore, no transformation was required. The post hoc comparisons were carefully planned based on the a priori expectations derived from the pilot studies (see the "Rationale of the experimental design"). For analyses of Hp activation, three independent t tests (two-tailed) were carried out. First, based on our pilot study, we expected no significant differences to emerge from the second comparison between the two same-shape conditions, the square–square and rectangle–rectangle groups. Second, we expected lowest levels of activity in the baseline condition and planned to compare the baseline condition with one of the same-shape groups, the one which would show the second lowest activity level. The third planned comparison was between the square–rectangle group (in which we expected highest levels of c-Fos-ir neurons) with one of the other groups that would show the second highest levels of activity.

Results

We processed all 34 brains; however, during the staining procedure the hippocampus of one brain was damaged and could not be used for counting. This brain was excluded from further analysis resulting in $n = 7$ for the square–rectangle group. In all animals, the hippocampal slices contained high numbers of stained nuclei with exception of those from the baseline group in which hippocampus was often devoid of activation (Fig. 3). The brains also showed some individual variability of c-Fos-ir cell distribution, although in most of the cases high number of activated cells were visible in the dorso-lateral Hp. Counts in the subdivisions confirmed this observation, showing highest numbers of c-Fos-ir neurons in the dorsal region; the trends between the groups, however, were similar in all subdivisions (Table 1). In all subdivisions, the highest densities of c-Fos-ir cells were present in the square–rectangle group, intermediate densities in the square–square and rectangle–rectangle group and the lowest in the baseline condition. For statistical analysis, the Hp subdivisions were lumped together (Fig. 4a).

The density of c-Fos-ir cells within Hp of the baseline group (mean ± sem: 161.6 ± 42.4 cells/mm^2; $n = 10$) was approximately 38% lower than in the square–square group (mean ± sem: 427.2 ± 85.9 cells/mm^2; $n = 8$). The density of c-Fos-ir cells was almost identical in the rectangle–rectangle group (mean ± sem: 433.9 ± 77 cells/mm^2; $n = 8$) compared to the square–square. The density of c-Fos-ir cells was highest in the square–rectangle group (mean ± sem: 865.9 ± 188.9 cells/mm^2; $n = 7$) which was approximately 50% higher than in the rectangle–rectangle group (Fig. 4a). Statistical analysis revealed significant between-group differences in the number c-Fos-ir cells in the Hp [ANOVA: $F(3.29) = 10{,}892$, $p < 0.01$]. T test planned comparisons revealed a significant difference between the baseline and the square–square condition [$T(16) = -3151$; $p < 0.01$], no significant difference between the square–square and the rectangle–rectangle group [$T(14) = -0.179$; $p = 0.86$] and a significant difference between the rectangle–rectangle group and the square–rectangle group [$T(13) = -2189$; $p < 0.05$]. Differences between the groups were not present in the IMM: ANOVA: $F(3.29) = 0.051$, $p = 0.98$.

Discussion

The key finding of our study is that even in the earliest stages of life, hippocampal response of domestic chicks is strongly influenced by the geometric layout of environmental boundaries. The number of activated cells in the hippocampus did not differ between chicks exposed twice to a novel arena having a familiar square shape or a novel rectangular shape (both had higher activation than a control group exposed only to the base arena). However, if chicks were exposed to both environmental shapes in two consecutive trials, the number of c-Fos-ir cells was doubled, showing that the avian hippocampus encodes the shape of the environment. The effect was region specific: no differences were present in IMM.

A possible explanation of the increased number of cells in the square–rectangle group is that different populations of cells represented the environments of two shapes. Although our method does not allow us to distinguish whether a given c-Fos-ir neuron was activated two times by two events occurring close together in time or only once. Presumably, two repeated exposures to same-shape arenas would induce highly overlapping activity of the neuronal network representing the shape. On the contrary, exposures to two test arenas differing in shape would cause mostly non-overlapping activation. Thus, the results confirmed this hypothesis by showing that the density of c-Fos-ir neurons in the square–rectangle group is almost twice as high compared to rectangle–rectangle group. This finding is consistent with studies of the mammalian hippocampus. IEG expression in CA1 of rats exposed either to two different environments or twice to the same environment revealed that the repeated exposure group had a higher number of double-

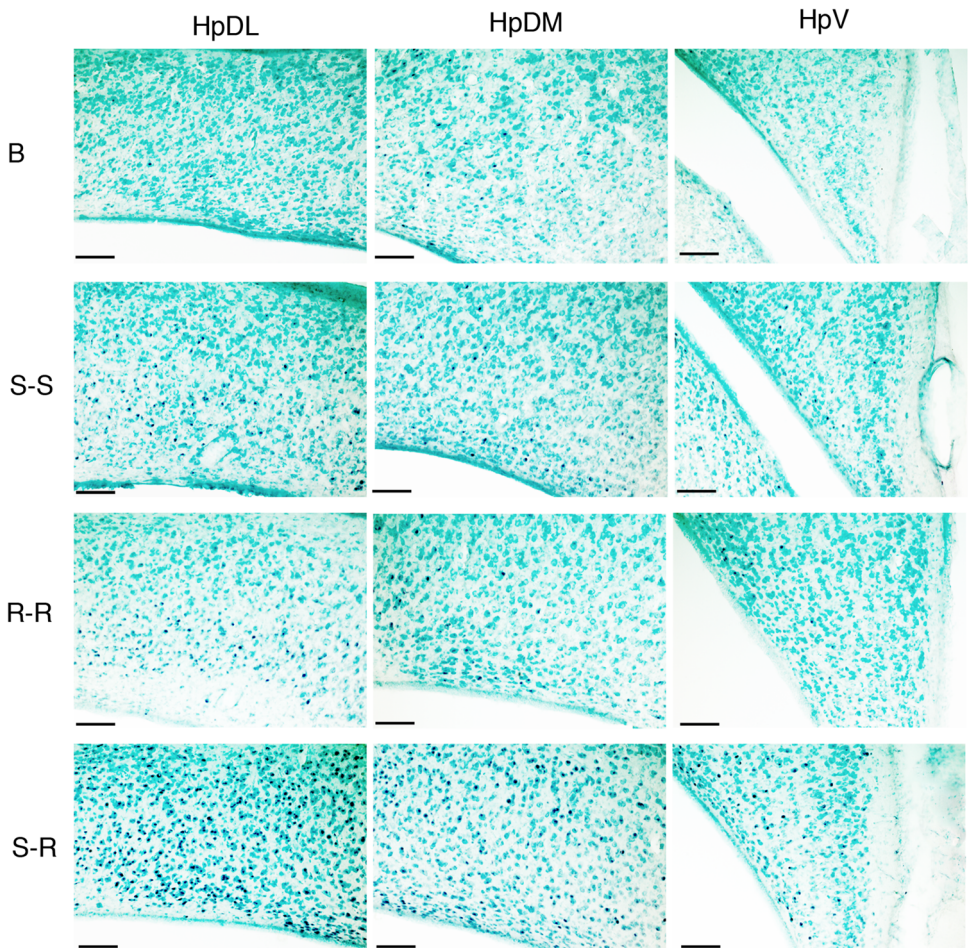

Fig. 3 Photomicrographs of hippocampal activations, showing the dorso-lateral (HpDL), dorso-medial (HpDM) and ventral (HpV) parts of one exemplary coronal section from each group of chicks. c-Fos-ir cells are stained black after the immunohistochemical procedure. The non-activated cells are counterstained in green. B (base-arena control group), S–S (square–square group), R–R (rectangle–rectangle group), S–R (square–rectangle group)

Table 1 Measured c-Fos-ir cell densities observed in all three hippocampal subdivisions (HpVM—ventro-medial hippocampus; HpDM—dorso-medial hippocampus; HpDL—dorso-lateral hippocampus) and in the intermediate medial mesopallium (IMM) for the different groups of chicks: B (base-arena control group), S–S (square–square group), R–R (rectangle–rectangle group), S–R (square–rectangle group)

	B	S–S	R–R	S–R
HpVM	58.2 ± 18.6	216.2 ± 63.7	167.2 ± 30.3	308.6 ± 104.9
HpDM	123.7 ± 39.1	321.0 ± 73.4	322.3 ± 84.3	735.0 ± 184.7
HpDL	330.5 ± 88.8	713.9 ± 174	812.2 ± 141.6	1554.2 ± 322.9
IMM	208.1 ± 62.6	202.9 ± 52.3	226.7 ± 49.1	228.9 ± 55.3

labelled cells expressing two activations at two different time points (Nakamura et al. 2010). Moreover, when mRNA of different IEGs visualized hippocampal activation at two time points, different environments induced responses in different populations of CA3 neurons (Vazdarjanova and Guzowski 2004). After two exploration sessions across three conditions for which the second environment was unchanged, slightly modified, or novel, the highest degree of overlap in activated neurons in the two sessions was found in animals exposed to the same environment twice; animals exposed to two different environments exhibited a low degree of overlap, and an intermediate degree of overlap was observed for the slightly modified environment. The recruitment of an entirely new ensemble in area CA3 of the hippocampus suggests that at least this subfield clearly delineates between different environments. Future studies should capitalize on the evidence obtained here and further investigate this hypothesis, adapting for chicks the protocols developed for discriminating two time points in immediate early gene induction (Guzowski et al. 1999, 2005), as well as applying to mice

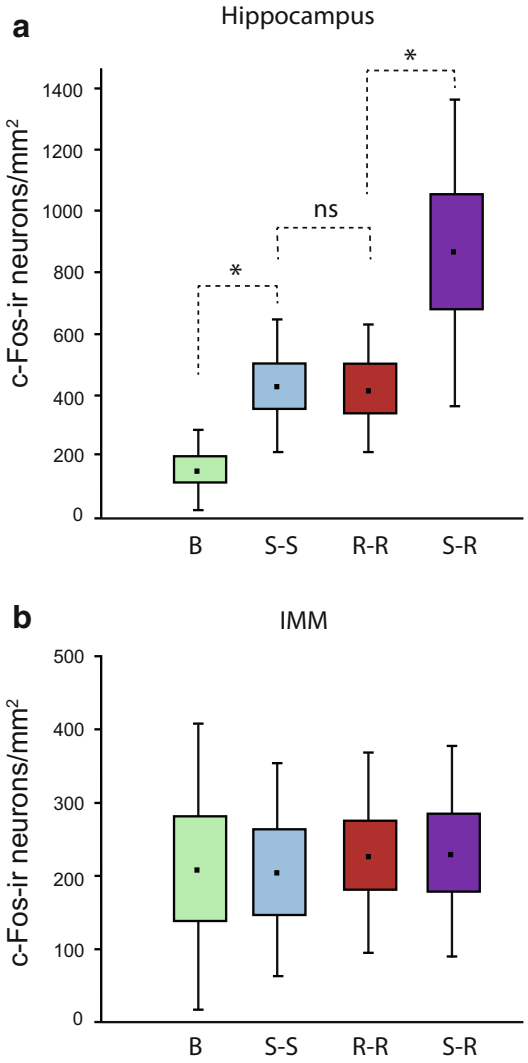

Fig. 4 Measured c-Fos-ir cell densities in hippocampus (**a**) and intermediate medial mesopallium (**b**) in four groups of chicks: B (base-arena control group), S–S (square–square group), R–R (rectangle–rectangle group), S–R (square–rectangle group). Graph-plot: mean (black square), SEM (box) and SD (whisker) (*$p < 0.05$; **$p < 0.01$). Densities of c-Fos-ir neurons per mm^2 are represented on the Y-axis

the same procedure that we used here, for the maximum comparability of results.

In the present study, we did not find functional variations within the measured subdivisions of chick hippocampi. In all three hippocampal regions (ventral, dorsomedial and dorso-lateral) similar differences among the groups were present (Table 1), suggesting that the neuronal representation of environmental shape involves the entire hippocampus in chicks. At this point, however, it is important to mention that subdivisional comparison between the mammalian and avian hippocampus is complicated. Although in both classes of vertebrates the hippocampus is crucially involved in processing spatial memory (Colombo and Broadbent 2000; Bingman et al. 2005; Mayer et al. 2013), the anatomical structures show significant differences which are not yet fully understood. For instance, in contrast to mammals and other reptiles, the avian hippocampus lacks a layered structure and, instead, consists of densely packed heterogeneous populations of neurons. Studies of the morphology, biochemistry and the projections of hippocampal neurons have led to varying views on the location and number of subdivisions of the avian hippocampus (Watanabe 2006). Remarkably, the number of suggested subdivisions ranges from two to twelve depending on the methods used (Karten and Hodos 1967; Casini et al. 1986; Erichsen et al. 1991; Krebs et al. 1991; Székely 1999; Hough et al. 2002; Kahn et al. 2003; Atoji and Wild 2004; Atoji et al. 2006). Fundamental questions regarding the position, or even the existence, of structures comparable to the mammalian dentate gyrus and cornu ammonis regions within the avian hippocampus remain unanswered (Striedter 2016; Bingman and Muzio 2017). While some authors propose the correlate of the dentate gyrus to be in the ventral parts of the avian hippocampus and the dorsal parts to be comparable to the CA regions (Atoji and Wild 2004; Herold et al. 2014; Medina et al. 2017), other authors claim the opposite pattern (Székely and Krebs 1996; Székely 1999). Thus, given these differences between birds and mammals, we do not know to what extent we can assume that the activated hippocampal neurons in birds and mammals reflect the same network mechanism.

The lack of differences between the square–square and rectangle–rectangle group might seem surprising, in that the chicks were familiar with the square but unfamiliar with the rectangular shape. Repeated exposure to the same stimuli is known to reduce IEG expression (Mello et al. 1995; Huchzermeyer et al. 2006; Stacho et al. 2016). It is highest during early learning (Anokhin and Rose 1991) and diminishes following extended training (Kelly and Deadwyler 2002), and this is also the case for birds engaged in spatial learning tasks (Mayer et al. 2010). Thus, one might expect hippocampal c-Fos production in response to square (but not rectangular) arenas, to be reduced to a minimum after repeated habituation in the square. This was not the case; however, both square–square and rectangle–rectangle groups showed higher c-Fos-ir neurons density than the one-environment control group, but there was no difference in activation between them. Thus, the activity enhancement of the hippocampus was triggered by the change from the base arena (for which c-Fos expression was at baseline, see "Methods") to the experimental arena, regardless of whether the shape of the experimental arena was familiar or novel. This would suggest two things: that the habituation to the square experimental arena during habituation training over multiple days did not reduce IEG expression and

that the novelty of being in a rectangular environment for the first time did not induce hippocampal activation to a greater degree than to the square environment. At least one study with rats found no difference in the proportion of hippocampal cells displaying Arc mRNA between animals exposed to an environment for the first time or after nine daily sessions (Guzowski et al. 2006). Future studies are needed to understand the effect of habituation over multiple days of exposure to the same environment. However, the lack of difference between the square–square and rectangle–rectangle group invites the interpretation of the higher activity in the square–rectangle group as a consequence of two different neural representations based on environmental shape, rather than simply on novelty. Another related point is to consider the possibility that repeated exposure to the same stimuli within a short period of time reduced IEG expression (e.g., in song-related experiments, Mello et al. 1995). In the present experiments, chicks were exposed two times to the same or different environments within only few minutes. Thus, in principal, the activity in the same shape conditions might have been reduced by the two exposures to the same stimuli, compared to the square–rectangle group, exposed to two different stimuli. However, also in this case, the reduction of c-Fos expression would only occur in neurons which were activated twice by same sensory stimulation, whereas single activations within non-overlapping populations of neurons would continue to induce c-Fos expression. Therefore, the higher density of c-Fos-ir cells, in the square–rectangle group would suggest that different neurons were activated by the two different environmental shapes. Moreover, reduced activity in the same shape conditions is not very likely because chicks of these groups had a higher number of c-Fos-ir cells compared to baseline. The question of whether repeated exposures of more than two times to the same environment in a short amount of time would reduce hippocampal c-Fos expression to a baseline level needs to be further investigated in future studies.

The lack of difference between the square–square and rectangle–rectangle group provides additional controls, showing that potential differences between the two conditions had no effect on the hippocampal c-Fos expression. For instance, although the floor areas were balanced in all arenas, the distances from the door to the other side of the experimental arenas were different, which could potentially induce different patterns of locomotor activity in the different shaped environments. Although we did not analyse behavioural data, if such effects were present, the square–square and rectangle–rectangle group would have shown differences in c-Fos expression; such was not the case. As mentioned in the methods, the illumination was slightly different between the rectangular and square arenas, which was probably due to reflections from the longer walls, which were closer to the light source in the rectangular arena. However, if this would be a factor for hippocampal activity there would have been differences between the square–square and rectangle–rectangle groups. Moreover, the fact that hippocampal activation was independent of luminosity is further confirmed by the difference between the baseline and square–square groups. Although lighting conditions in the base arena and the square arenas were essentially identical, hippocampal activity in the square–square group was higher and thus not induced by light. Thus, it is very likely that the observed difference between the groups was induced by differences in visual properties between the two arenas.

Why is hippocampal c-Fos expression increased when chicks visit experimental arenas of different shapes? Here we would like to consider if this activity can be related to remapping mechanisms comparable to those known in mammals (Fyhn et al. 2007; Moser et al. 2008; Barry et al. 2012). Remapping in mammals was first studied in electrophysiological recordings of hippocampal neurons. Hippocampal place cells 'remap' and alter, activate, or inactivate their preferred firing fields after changes of environmental shape (Muller and Kubie 1987; O'Keefe and Burgess 1996; Lever et al. 2002). Such changes in the firing of a single neuron are of course dependent on the inputs that it receives within a given neuronal network, which are provided by different neuronal populations. Therefore, given that at least a part of this network is within hippocampus, this area should contain different, potentially partially overlapping, populations of cells that respond to the different shapes. Indeed, results from studies with mammals using IEGs to map neuronal activities are in line with this hypothesis, showing that different hippocampal cells are activated by different environments (Vazdarjanova and Guzowski 2004; Guzowski et al. 2005; Barry and Commins 2011). Also our findings are consistent with these studies. Although we did not measure two time points independently, the twofold increase in hippocampal activity in the square–rectangle group suggests a largely non-overlapping representation of the two shapes by two neuronal populations of cells within the hippocampus of chicks. Here it is important to mention that contrary to the immediate early gene studies with mammals, the present study with chicks attempted to limit the difference between the different enclosures only by their geometric shape, to make it even more comparable to the electrophysiological investigations of place cell remapping specifically in response to the changes in environmental shape. Notably, the activity of neurons in the chick hippocampus in the present study was sensitive to changes in shape. Thus, it is possible that remapping-like mechanisms may exist also in birds, at least to the extent that environmental differences

induce a new pattern of neural activity in the hippocampus. Unfortunately, only a few studies have reported hippocampal place coding at the level of single cells in freely moving birds. Bingman and collaborators studied place-related responses in pigeons finding different location-related activities, such as location cells, grid-like cells, arena-off cells and path cells, which were not as specific as in mammals (Bingman and Sharp 2006). These effects are not necessarily a reflection of variations in biological organization, but may emerge as a consequence of testing procedures and behavioural differences (Bingman and Sharp 2006). The existence of some forms of location-specific response in pigeons, together with the results from the present study, suggests that also remapping related activities might be present in bids. Further electrophysiological investigations of hippocampal activity in freely moving birds are needed to confirm this hypothesis.

In conclusion, here we present the first evidence of hippocampal representation of environmental shape in birds. Our study adds to a series of experiments showing astonishing functional similarities between the mammalian and avian hippocampi (Colombo and Broadbent 2000; Vargas et al. 2004; Bingman and Sharp 2006; Mayer et al. 2013, 2016). The involvement of different neuronal populations should be verified with procedures discriminating two time points in IEG induction; however, the results presented here suggest that functional similarities exist not only at the anatomical level but also at the neural level. Such techniques can be used to also investigate hippocampal representation of non-spatial stimuli (e.g., if two different sounds or two odours would produce similar hippocampal increase in labelled neurons). Many questions remain, but we hope that the present study opens new doors for expanding our understanding of the hippocampus across evolution.

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