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RESEARCH ARTICLE

Active exploration of an environment drives the activation of the hippocampus–amygdala complex of domestic chicks

Anastasia Morandi-Raikova and Uwe Mayer*

ABSTRACT

In birds, like in mammals, the hippocampus critically mediates spatial navigation through the formation of a spatial map. This study investigates the impact of active exploration of an environment on the hippocampus of young domestic chicks. Chicks that were free to actively explore the environment exhibited a significantly higher neural activation (measured by c-Fos expression) compared with those that passively observed the same environment from a restricted area. The difference was limited to the anterior and the dorsolateral parts of the intermediate hippocampus. Furthermore, the nucleus taeniae of the amygdala showed a higher c-Fos expression in the active exploration group than in the passive observation group. In both brain regions, brain activation was correlated with the number of locations that chicks visited during the test. This suggests that the increase of c-Fos expression in the hippocampus is related to increased firing rates of spatially coding neurons. Furthermore, our study indicates a functional linkage of the hippocampus and nucleus taeniae of the amygdala in processing spatial information. Overall, with the present study, we confirm that in birds, like in mammals, hippocampus and amygdala functions are linked and likely related to spatial representations.

KEY WORDS: Avian hippocampal formation, Place cells, Immediate early genes, Spatial map, c-Fos, Nucleus taeniae of the amygdala

INTRODUCTION

The neuroanatomical organization of the hippocampus homologues differs dramatically between birds and mammals (Striedter, 2016). Despite these differences, in both of these taxonomic groups, the hippocampus supports similar cognitive functions. One example is represented by navigation, which depends on the hippocampus in mammals (O′Keefe and Dostrovsky, 1971; Morris et al., 1982; Lavenex and Lavenex, 2009), birds (Fremouw et al., 1997; Bingman et al., 1984; Mayer et al., 2013; Bingman and Muzio, 2017; Morandi-Raikova and Mayer, 2021), reptiles (Day et al., 2001; López et al., 2003), amphibians (Sotelo et al., 2016) and fish (Rodrìguez et al., 2002). However, the extent to which these functions share similar neural mechanisms across vertebrate species needs further investigation.

Animal navigation is mainly based on ‘internal’ cognitive maps (Tolman, 1948). At least in mammals, the cognitive maps are formed through the critical contribution of hippocampal place cells (O′Keefe and Dostrovsky, 1971; O’Keefe and Conway, 1978). These cells exhibit spatially localized increases in firing rates when an animal occupies a specific field in an environment. Since the seminal discovery of place cells, many other spatially responsive cells have also been described in mammals (Moser et al., 2017; Poulter et al., 2018). Head direction cells (Taube et al., 1990a,b), grid cells (Sargolini et al., 2006), border cells (Sargolini et al., 2006), speed cells (Kropff et al., 2015) and vector trace cells (Poulter et al., 2021) all contribute to the formation of a cognitive map in mammals. In birds, hippocampal place cells (Payne et al., 2021) and head direction cells (Ben-Yishay et al., 2021; Takahashi et al., 2022) have also been recently found. Payne et al. (2021) found place cells in two different species of bird: tufted titmice (*Baeolophus bicolor*) and zebra finches (*Taeniopygia guttata*). Interestingly, the spatially responsive cells observed in these two bird species were located predominantly in the anterior hippocampus. The density of place cells decreased along the anterior–posterior axis. In rodents, place cells follow a similar gradient along the dorsal–ventral axis (Jung et al., 1994). The avian subregions along the anterior–posterior axis might thus be equivalent to the hippocampal regions along the dorsal–ventral axis in mammals (Smulders, 2017; Payne et al., 2021). Indeed, following the same assumption, Agarwal et al. (2021 preprint) recorded place cells within the anterior hippocampus in freely flying barn owls (for earlier studies with pigeons, see also the pioneering work by Bingman et al., 2003; Hough and Bingman, 2004; Siegel et al., 2005, 2006; Kahn et al., 2008). Overall, these studies suggest that spatial processing in birds and mammals is based on similar mechanisms. Moreover, a higher number of spatially coding cells can be expected in the anterior region of avian hippocampal formation.

The importance of the avian hippocampus for spatial navigation has often been addressed using immediate early genes (IEGs) as neural activity markers (Smulders and DeVoogd, 2000; Bischof et al., 2006; Mayer et al., 2010; Mayer and Bischof, 2012; Grella et al., 2016; Sherry et al., 2017). As an alternative to electrophysiology, the use of IEG products offers a practical approach to investigating the activation of entire neural ensembles (Lanahan and Worley, 1998). Neural IEG expression rapidly increases in response to trans-synaptic signalling between neurons, and the resulting genomic response is closely associated with neuronal plasticity (Jones et al., 2001; Guzowski, 2002; Barry and Commins, 2011). Using the IEG product c-Fos to study hippocampal activity in domestic chicks (*Gallus gallus domesticus*), it has been shown that this structure has many similar functions to its mammalian counterpart. Like in mammals, the hippocampus in chicks is sensitive to environmental boundaries. It shows high levels of c-Fos during navigation by the geometrical shape of the environment (Mayer et al., 2016) and responds to changes in the shape of the environment (Mayer et al., 2018). Furthermore, the hippocampus of chicks processes spatial relational information, showing high expression of c-Fos during navigation in

Center for Mind/Brain Sciences (CIMeC), University of Trento, Piazza Manifattura 1, I-38068, Rovereto, TN, Italy.

*Author for correspondence (uwe.mayer@unitn.it)

A.M.-R., 0000-0003-1367-9481; U.M., 0000-0001-6841-0282

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List of abbreviations

c-Fos-ir	c-Fos immunoreactive
DL	dorsolateral
DM	dorsomedial
HF	hippocampal formation
Hp	hippocampus
IEGs	immediate early genes
IMM	intermediate medial mesopallium
Int.	intermediate
PBS	phosphate buffered saline
PFA	paraformaldehyde
Post.	posterior
TnA	nucleus taeniae of the amygdala
V	ventral

relation to freestanding objects (Morandi-Raikova and Mayer, 2021).

More importantly for the present study, in chicks, like in mammals, exposure to novel environments induces high levels of c-Fos expression within the hippocampus (Morandi-Raikova and Mayer, 2020; for mammals, see Kubik et al., 2007). In a novel environment, animals need to acquire a new spatial representation. Learning of a new spatial map likely requires plastic changes of the hippocampal circuitry and thus high levels of IEG expression. However, at present it is not clear whether this activation/plasticity of the hippocampus is induced by the mere visual input from an environment or whether it requires its active exploration. If hippocampal IEG expression is related to the activation of spatially responsive cells (place cells, boundary cells, etc.; Ben-Yishay et al., 2021; Payne at al., 2021), active exploration of an environment should cause a higher number of c-Fos-expressing cells. Whenever a new location is visited during the exploration, the cells that encode the corresponding location should increase their firing rate, inducing c-Fos expression. The more locations are visited, the higher number of hippocampal cells should express c-Fos. Moreover, based on previous studies carried out in other bird species, one can expect to find a higher degree of activation in the anterior segment of the hippocampus during spatial mapping (Payne et al., 2021). However, no study so far has reported differences in activation between the anterior, intermediate and posterior hippocampal segments in domestic chicks.

The present study aimed to investigate the impact of active exploration on hippocampus of young domestic chicks. To trigger hippocampal activation, we exposed chicks to a novel environment. Using c-Fos imaging, we measured the activation of the anterior, intermediate and posterior segments of the hippocampus in chicks exploring the environment. This was compared with a control group that observed the same environment from a restricted area, without exploring it. We expected to find more c-Fos immunoreactive cells after active exploration of the environment. Moreover, we expected that this increased activity would be more visible in the anterior segment of the chicks' hippocampus, as observed in titmice, zebra finches and owls (Agarwal et al., 2021 preprint; Payne et al., 2021). We also measured the activation of the nucleus taeniae of amygdala (TnA), which is a homologue of the mammalian medial amygdala (Abellán et al., 2009). The chick's TnA responded to a novel environment in our previous study (Morandi-Raikova and Mayer, 2020). We hypothesized the presence of a functional connection between this structure and the chicks' hippocampus and thus a similar activation pattern in both structures. As a control region, we measured the activity in the intermediate medial mesopallium (IMM), which is involved in filial imprinting (Horn, 2004) and was not responsive to novel environments in our previous studies (Mayer et al., 2018; Morandi-Raikova and Mayer, 2020).

MATERIALS AND METHODS

Subjects

We used 24 male domestic chicks (*Gallus gallus domesticus*, Linnaeus 1758) of the Aviagen ROSS 308 strain. The eggs were obtained already fertilized from a commercial hatchery (CRESCENTI Società Agricola S.r.l. – Allevamento Trepola – cod. Allevamento127BS105/2). Incubation and hatching occurred in complete darkness. After hatching, chicks were individually housed in metal cages (28×32×40 cm, width×height×length) with food and water *ad libitum*, at a constant temperature of 30–32°C and variable light conditions of 14 h:10 h light:dark. Chicks were food deprived 3 h before the training on post-hatching day 4. During the training in the experimental room (28°C), chicks received mealworms (*Tenebrio molitor* larvae) as food rewards, while water was always present *ad libitum*. At the end of the training sessions, all chicks returned to the animal house and remained there until the next day with food and water *ad libitum*. On post-hatching day 5, all chicks were tested and subsequently perfused. The experiment was carried out in accordance with the ethical guidelines current to European and Italian laws. All the experimental procedures here described were licensed by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanita' Pubblica Veterinaria (permit number 560/2018-PR).

Experimental setup

The experimental apparatus was composed of two compartments. During training, the apparatus consisted of a home compartment (28×40×32 cm, width×height×length) and a habituation compartment (40×31×30 cm). The wall that divided the home compartment from the habituation compartment could slide vertically and reveal an opening (15×15 cm) in its centre. This door allowed chicks to enter the habituation compartment from the home compartment. During training, the habituation compartment was located inside an experimental environment compartment (60×60×60 cm), which was not visible to the chicks (Fig. 1A). The inner surfaces of the setup were white. The walls of the habituation compartment were composed of a metal grid. On the outer side of the grid, additional walls made of white plastic were present. These acted as visual occluders and prevented chicks from seeing the experimental environment during the training. During the test, either the visual occluders only (Fig. 1B) or the visual occluders and the grid (Fig. 1C) could be removed. The setup was equipped with a digital camera (Microsoft LifeCam Studio HD 1920×1080 pixels), placed on the top of the apparatus to record chick behaviour.

Habituation training

All chicks underwent a habituation training on post-hatching day 4. This training aimed to familiarize the animals with the experimental apparatus and to train them to enter and exit the habituation compartment. For this purpose, domestic chicks were individually placed into the home compartment for 30 min of acclimatization, where they received two to three mealworms. After the acclimatization period, chicks started the training. At the beginning of each training trial, the wall that divided the compartments was lifted, the door appeared and chicks could enter the habituation compartment that contained a mealworm. Then, the wall slid down and the door disappeared. After 1 min of remaining

Journal of Experimental Biology (2022) 225, jeb244190. doi:10.1242/jeb.244190

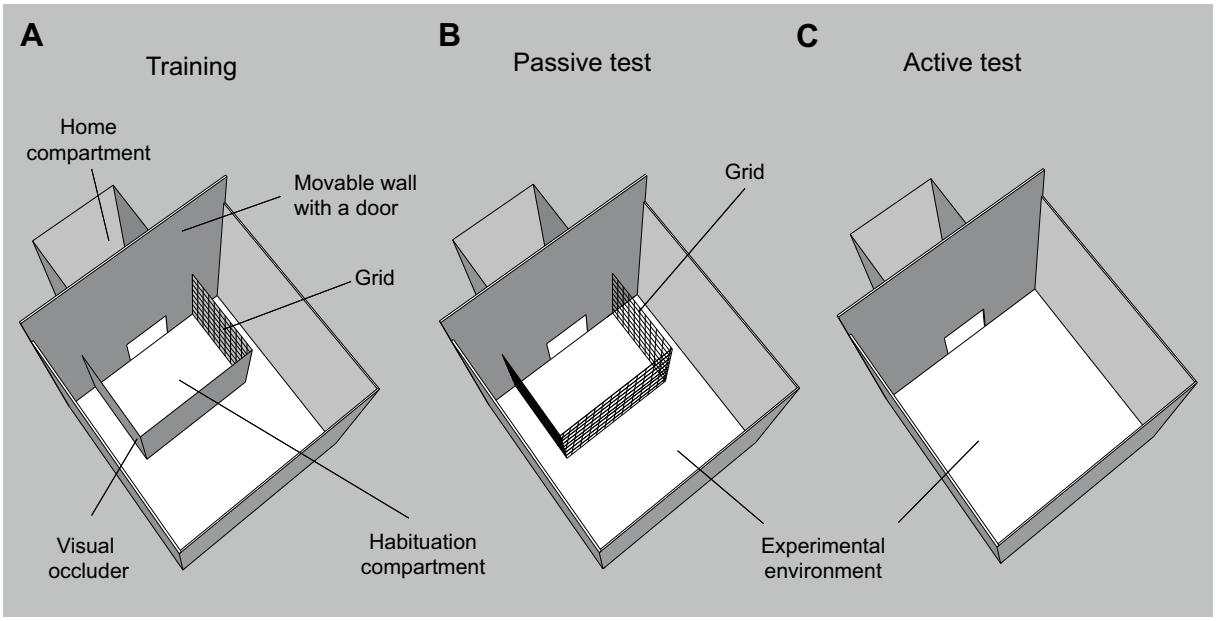

Fig. 1. Experimental setup and procedures. (A) During training, the grid with the visual occluder was placed inside the experimental environment. All chicks were trained to walk through the open door and forage for a mealworm in two distinct compartments (home compartment and habituation compartment). At test, chicks were divided into two different groups. (B) The passive exploration group was tested with the same grid available at training, but this time the visual occluder was removed. Chicks could see the experimental environment, but not explore it actively. (C) For the active exploration group, the entire habituation compartment was removed and the experimental environment was exposed. Chicks could enter the experimental environment and actively explored it.

in this compartment, the door appeared again and chicks returned to the home compartment, which contained a mealworm, remaining in it for an additional 1 min. This procedure was repeated 10 times for each training session. Each subject underwent six training sessions in total, three in the morning and three in the afternoon. During the intersession intervals (30 min), chicks remained inside the home compartment.

Test session for c-Fos labelling

On post-hatching day 5, chicks were divided into two experimental groups: an active exploration group and a passive exploration group. Before the test, chicks were taken to the experimental room and placed inside the home compartment, where they remained undisturbed for 5 h. At test, chicks of the active exploration group could enter the experimental environment and explore it for 1 h (Fig. 1C). In contrast, chicks of the passive exploration group entered the passive compartment (Fig. 1B), where they remained for 1 h. For this group, only the visual occluders were removed from the habituation compartment. The experimental environment was visible through the grid, which restricted chicks from entering it. Thus, chicks belonging to the passive exploration group could see the experimental environment, but not explore it actively.

Immunohistochemical procedure

Immediately after the test, all chicks were overdosed with an intramuscular injection of 0.4 ml of 1:1 xylazine (2 mg ml^{-1}) and ketamine (10 mg ml^{-1}) solution. For visualizing the immediate early gene product c-Fos, brains were processed blind to the experimental conditions and a standard immunohistochemical protocol adapted to chicks was used.

Chicks were perfused transcardially with cold phosphate-buffered solution (PBS; 0.1 mol, pH 7.4, 0.9% NaCl, 4°C) and paraformaldehyde (4% PFA in PBS, 4°C). The heads were severed from the body and placed for 7 days into a 4% PFA/PBS solution for post-fixation. Brains were extracted from the skulls with the use of a stereotaxic head holder (Stoelting). To ensure that the subsequent coronal brain sections would have the same orientation as described for chick's brain atlas (Kuenzel and Masson, 1988), the horizontal axis of the skulls was oriented at 45 deg in respect to the horizontal axis of the stereotaxic apparatus.

Brain hemispheres were then separated and embedded into gelatine (7%) containing egg yolk. Sections were cut after an incubation in 20% sucrose in 4% PFA/PBS for 48 h and a further 48 h in 30% sucrose in 0.4% PFA/PBS at 4°C. Brains were cut and frozen with the use of a cryostat (Leica CM1850 UV). During cutting, four series of 40 µm coronal sections containing the regions of interest (corresponding to one-third of the most posterior part of the telencephalon) were collected. Only sections of the first series were used for labelling, while the others were stored as backups.

Endogenous peroxidase activity was depleted with 0.3% peroxide in PBS for 20 min. The sections were then treated with 3% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min at room temperature to block unspecific binding sites. Anti-c-Fos antibody solution (1:1500 in PBS; rabbit polyclonal, AF-488, Santa Cruz, CA, USA) was applied for 48h at 4°C. Afterward, all brain sections were transferred into the secondary antibody solution (1:200 in PBS; biotinylated anti-rabbit made in goat, BA-1000 Vector Laboratories) for 60 min at room temperature. The ABC kit (Vectastain Elite ABC Kit, PK 6100; Vector Laboratories) was used for signal amplification; this step was followed by visualization with the VIP kit (SK-4600; Vector Laboratories). Lastly, all sections were mounted on gelatine-coated slides, dried (50°C), counterstained with Methyl Green (H-3402; Vector Laboratories) and cover-slipped with Eukitt (FLUKA).

Brain analysis

Brains were examined blind to the experimental groups and hemispheres with a Zeiss microscope (objective magnification 20×, numerical aperture 0.5; eyepiece 10×), connected to a digital camera (Zeiss AxioCam MRc5) and a computer with the imaging

Journal of Experimental Biology (2022) 225, jeb244190. doi:10.1242/jeb.244190

software ZEN. For the analysis, a standard rectangular counting area (150×250 µm) was positioned, within the regions of interest, over the spots with the highest number of c-Fos-ir cells. When positioning the counting area, a minimum distance of 20 µm to the borders was always kept. Subsequentially, every activated c-Fos-ir cell was manually marked on the computer monitor with the ZEN software, which then computed the total counts. The measured values derived from different sections were averaged for each area and subsequently standardized to cells mm^{-2}.

For the analysis of the hippocampal formation (HF), 10 to 13 sections of each brain hemisphere were used (anterior Hp: three sections; intermediate Hp: five sections; posterior Hp: five sections). Based on the anatomical landmarks and its shape, HF was divided into anterior (A 8.6 to A 8.0), intermediate (A 7.8 to A 7.0) and posterior parts (A 6.8 to A 4.6) (Kuenzel and Masson, 1988). In addition, the intermediate and the posterior parts of HF were further subdivided into ventral (V), dorsomedial (DM) and dorsolateral (DL) (see Fig. 2).

To quantify c-Fos-ir cells in the nucleus taeniae of the amygdala (TnA), five sections corresponding to the A 7.4 and A 6.4 of the brain atlas (Kuenzel and Masson, 1988) of both hemispheres were selected. Finally, for the quantification of c-Fos-ir cells contained within IMM, this brain region was outlined according to the drawings of Ambalavanar et al. (1993). The counting in this area was performed on five brain sections from A 8.6 to A 4.6.

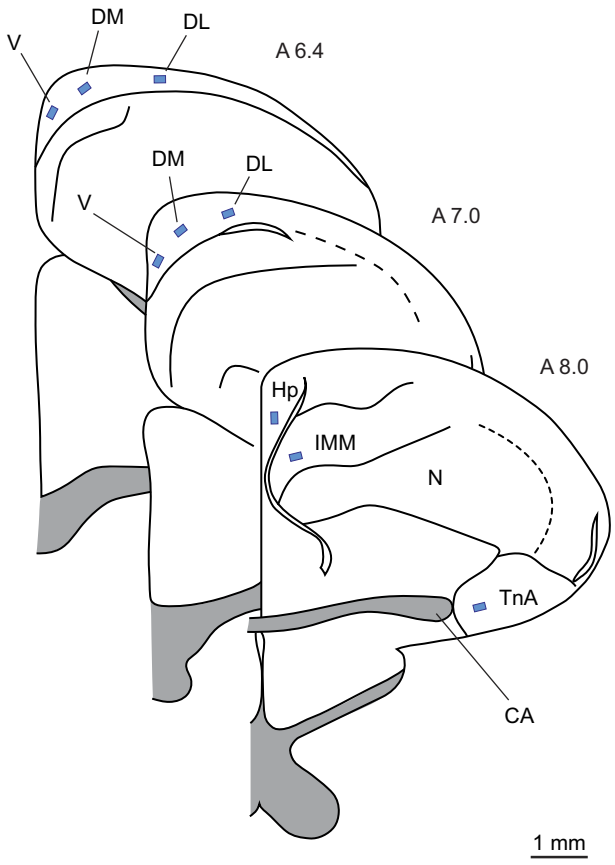

Fig. 2. Typical placements of cell counting zones (blue rectangles) in the regions of interest. The intermediate (A 7.0) and posterior (A 6.4) segments of the hippocampal formation were portioned into ventral, dorsomedial and lateral parts. Hp: hippocampus; V: ventral; DM: dorsomedial; DL: dorsolateral; IMM: intermediate medial mesopallium; TnA: nucleus taeniae of the amygdala; N: nidopallium; CA: anterior commissure.

Behavioural analysis

Video recordings of the test session were analysed offline with EthoVision 3.1 (Noldus Information Technology, Leesburg, VA, USA; Noldus et al., 2001). Videos were analysed at a rate of 6 samples s^{-1}, and to track animals' position (x, y coordinates), the background subtraction method was used. These coordinates in pixels were converted to cm by calibrating the software to the width of the experimental compartments. Behavioural analyses were carried out for both experimental groups. The behavioural parameters that were extracted through EthoVision were: distance moved (cm), velocity (cm s^{-1}), number of visited sectors and sector change frequency. To extract these last two behavioural variables, both experimental compartments were subdivided into equal zones of 10×10 cm, to represent the various locations domestic chicks could have visited at test. Thus, the experimental environment was subdivided into 36 different sectors (6×6, rows×columns) and the passive compartment was subdivided into 12 sectors (3×4). By computing the number of sectors visited by each chick, we could measure how widely each animal explored the available space. For instance, a chick of the active exploration group that always remained motionless during the test would have visited only one sector, whereas a chick that explored all the available space would have a score of 36 visited sectors. In contrast, the frequency of sector changes and the distance moved by each animal revealed how intensively the animal moved across space, regardless of the absolute number of locations visited. Additionally, to assess whether the motivation to explore the available space differed among groups, a percentage of explored sectors was computed by dividing the number of visited sectors by the overall available sectors for each individual. Data from Ethovision were then exported into the software IBM SPSS Statistics (v. 20) and analysed.

Statistical analysis

To analyse whether there was any difference in the measured behavioural parameters between the two experimental groups, a multivariate analysis of variance (MANOVA) was conducted. To reveal differences in the activation pattern of the investigated brain areas between the two experimental groups, a repeated-measures ANOVA was performed. This analysis included a between-subject factor, group (two levels: active exploration group, passive exploration group), and two within-subject factors, area (nine levels: anterior Hp, intermediate ventral Hp, intermediate dorsomedial Hp, intermediate dorsolateral Hp, posterior ventral Hp, posterior dorsomedial Hp, posterior dorsolateral Hp, TnA, IMM) and hemisphere (two levels: left, right). Subsequently, for the *post hoc* analyses, an independent samples *t*-test was conducted. To assess whether there was any relationship between the measured behavioural parameters and the activation of the investigated brain regions, a Pearson correlation analysis was run.

The alpha level of 0.05 was considered significant; however, the obtained values were also tested against a Bonferroni-adjusted alpha level of 0.006 (0.05/9). We report *F*- and *t*-statistics, exact *P*-values, means, s.e.m. and standardized effect sizes (Cohen's *d* for *t*-tests and η_p^2 for ANOVAs). All statistical analyses were performed using SPSS and R (https://www.r-project.org/), whereas the graphs were created with the software GraphPad Prism 8 and R. Datasets used for the statistical analysis are in Table S1.

RESULTS

Behavioural results

Analyses of chick's walking tracks revealed significant differences between the groups (Fig. 3). As expected, chicks of the active

Journal of Experimental Biology (2022) 225, jeb244190. doi:10.1242/jeb.244190

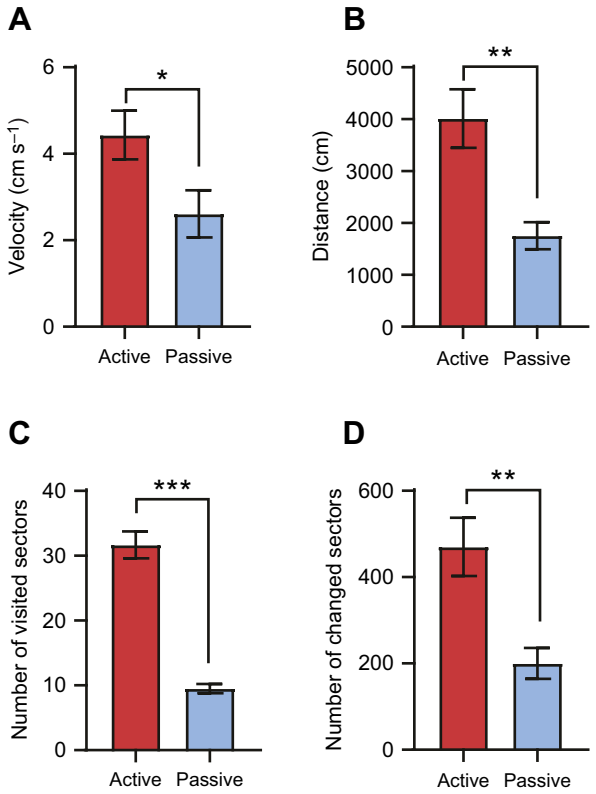

Fig. 3. Behavioural performance during test of the active exploration group in red and passive exploration group in blue. Chicks of the active exploration group (A) moved faster, (B) moved longer distances, (C) visited more sectors and (D) changed the sectors more often compared with the passive group. Bar plots show means±s.e.m. (*P<0.05, **P<0.01, ***P<0.001).

exploration group moved longer distances compared with the passive group (active: 4013.72±565 cm; passive: 1754.48±259 cm; $F_{1,22}$=13.232, P=0.001, η_p^2=0.376, mean±s.e.m. rounded numbers) and visited more sectors (active: 31.7±2.1; passive: 9.5±0.7; $F_{1,22}$=99.835, P<0.001, η_p^2=0.819). They also moved faster (active: 4.44±0.6 cm s^{-1}; passive: 2.61±0.6 cm s^{-1}; $F_{1,22}$=5.399, P=0.030, η_p^2=0.197) and changed the sectors more often during exploration of the experimental environment (active: 470.3±7; passive: 200.1±4; $F_{1,22}$=12.450, P=0.003, η_p^2=0.361). However, the percentage of available space covered by chicks at test was not different between the two groups (active: 87.96±5.8%; passive: 79.17±6.1%; $F_{1,22}$=1.102, P=0.305, η_p^2=0.048).

Brain results

All 24 brains (n=12 per each experimental group) were successfully stained for c-Fos. The nuclei of c-Fos-ir cells were stained black and were easily discernible from the other cells counterstained with Methyl Green (Fig. 4). Measured c-Fos-ir cell densities are summarized in Table 1.

The repeated-measures ANOVA revealed a significant interaction of area and group (P=0.001). This indicates that c-Fos-ir cell densities were different between the two experimental groups, in an area-specific fashion. However, there was no effect of hemisphere nor any interaction between hemisphere and area or group (see Table 2 for all ANOVA results). Therefore, for the *post hoc* analysis, the measures for the left and right hemispheres were pooled together.

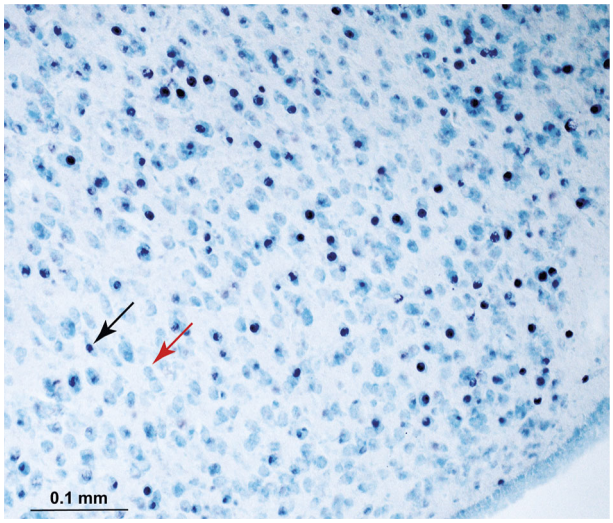

Fig. 4. Example photo of a hippocampal section of an experimental chick. c-Fos-ir (immunoreactive) cells are stained black after a successful immunohistochemical procedure (black arrow) and are easily discernible from the Methyl Green counterstained cells (red arrow).

The *post hoc* analysis revealed significantly higher c-Fos-ir cell densities for the active exploration group compared with the passive exploration group in the anterior Hp (P=0.005, also significant after the Bonferroni correction: P=0.045), in the intermediate DL Hp (P=0.020) and in TnA (P=0.003, also significant after the Bonferroni correction: P=0.027). No significant differences between the two experimental groups were found in the other hippocampal subdivisions and in the IMM (Fig. 5, Table 2).

Correlations

A significant correlation was found between the number of visited sectors and the density of c-Fos-ir cells in the anterior Hp (r=0.489, P=0.016) (Fig. 6B). Furthermore, the density of c-Fos-ir cells in the TnA was also correlated with the number of visited sectors (r=0.447, P=0.030) (Fig. 6C). No correlation between the number of visited sectors and c-Fos-ir cell density was found in the other hippocampal subdivisions nor in the IMM. Moreover, no correlation was found between the other behavioural parameters (velocity, distance moved and sector change frequency) and the density of c-Fos expression in any of the investigated brain areas (see Fig. 6A for all Pearson correlation results).

DISCUSSION

Domestic chicks that could actively explore an environment had a higher c-Fos expression in the anterior hippocampus and in the dorsolateral parts of the intermediate hippocampus. Furthermore, a higher c-Fos expression was observed in TnA of the active exploration group compared with the group confined to a restricted area. The difference was region-specific. In IMM and in the posterior hippocampal segment, c-Fos activity did not differ between the active and passive exploration groups.

Here, we show for the first time that hippocampus activation is higher only in chicks that actively explored a novel environment. Thus, a purely visual input is not sufficient to trigger the full extent of the hippocampal activation in response to the novel environment observed in our previous studies (Mayer et al., 2018; Morandi-Raikova and Mayer, 2020). Because both groups were exposed to a novel environment, we cannot assess whether novelty per se activated the hippocampus. However, as we predicted, physical movement

Journal of Experimental Biology (2022) 225, jeb244190. doi:10.1242/jeb.244190

Table 1. Measured cell densities (c-Fos-ir cells mm^{-2}) across brain areas of the left and right hemispheres and total values after the two hemispheres were lumped together (means±s.e.m., rounded numbers)

	Active exploration group			Passive exploration group		
	Left	Right	Total	Left	Right	Total
Anterior Hp	1788.9±185	1670.4±197	1729.6±164	998.5±148	1094±188	1046.3±142
Int. V Hp	320.2±57	408.6±65	364.4±46	296.8±65	274.1±45	285.4±44
Int. DM Hp	688.3±127	802.3±126	745.3±106	606.6±104	538.1±97	572.4±90
Int. DL Hp	1937±181	2054.4±177	1995.7±139	1372.3±221	1554.4±211	1463.4±162
Post. V Hp	233.3±41	313.3±44	273.3±38	273±53	252.6±59	262.8±50
Post. DM Hp	492.6±64	544.8±72	518.7±61	484.1±67	518.2±123	501.1±79
Post. DL Hp	1927±294	2304.8±212	2115.9±213	1828.9±235	1828.9±251	1828.9±168
TnA	1534.4±155	1561.1±201	1547.8±160	921.1±100	953.3±82	937.2±68
IMM	1165.8±157	1020±142	1092.9±137	1024.5±149	1048±117	1036.2±105

Hp, hippocampus; Int., intermediate; V, ventral; DM, dorsomedial; DL, dorsolateral; Post., posterior; TnA, nucleus taeniae of the amygdala; IMM, intermediate medial mesopallium.

across different locations crucially contributed to hippocampal activation. Indeed, the number of visited sectors positively correlated with the activation in the anterior hippocampus. Overall, chicks of the active exploration group moved more and faster, they changed the sectors more frequently and explored more sectors than the chicks of the passive exploration group. These results are not surprising, as chicks of the active exploration group could move within a larger area compared with the chicks of the passive exploration group. However, both groups covered a similar percentage of available space overall, meaning that their motivation to explore did not differ. Furthermore, the behavioural parameters distance moved, velocity and the frequency of sector changes did not correlate with brain activity in any of the regions of interest. Thus, the brain activity in the anterior, dorsolateral intermediate Hp and in the TnA was not influenced by how much or how fast the animals moved, but only by how many spatial locations they visited overall. As a note of caution, we would like to point out that given the relatively high number of correlations performed, false positives may have emerged. However, it is noteworthy that the only significant correlation was the one we predicted based on our *a priori* expectations. Moreover, one could argue that this correlation could be driven only by the between-group difference. However, this was not the case for any of the other behavioural parameters measured. Future studies could be conducted to clarify this issue by using a larger number of subjects exploring a larger environment to reduce ceiling effects (see Fig. 6B,C).

Our findings suggest that in birds, like in mammals, the increase in hippocampal c-Fos expression during exploration of an environment reflects the increased firing rates of spatially coding neurons. In mammals, different hippocampal cells encode different locations and other properties of the allocentric environment (Poulter et al., 2018). The more place fields the animal crosses, the larger the number of different place cells that are activated (O'Keefe and Nadel, 1978; Poulter et al., 2018). Given that neural activation induces immediate early gene expression, we predicted that the number of explored fields would positively correlate with the density of c-Fos expressing cells, which we indeed found in our study.

The increased c-Fos expression in the anterior and the intermediate dorsolateral hippocampal parts of the active exploration group further supports the idea that spatially coding cells exist in domestic chicks' hippocampi. Our findings are particularly interesting in the light of the divergent results coming from studies on single-unit activity in the hippocampus of different bird species. The pioneering study by Hough and Bingman (2004) already showed some spatially responsive cells within the pigeon's hippocampal formation and, together with the recent discovery of place cells in the anterior hippocampi of two Neoaves, titmice and zebra finches (Payne et al., 2021), may indicate that this function was probably already present in the common ancestor of sauropsids and mammals. However, a recent study of hippocampal activity in a galliform species, the quail (*Coturnix coturnix*), failed to identify place cells. In that study,

Table 2. Results of the repeated-measures ANOVA and the corresponding *post hoc* analysis

	Test statistic	*P*	Effect size
Main effects and interactions			
Hemisphere	$F_{1,22}$=0.351	0.560	η_p^2=0.016
Hemisphere×Group	$F_{1,68.420}$=0.055	0.816	η_p^2=0.003
Area	$F_{4.077,89.688}$=105.209	<0.001	η_p^2=0.827
Area×Group	$F_{4.077,89.688}$=5.010	0.001	η_p^2=0.185
Hemisphere×Area	$F_{8,68.420}$=0.585	0.633	η_p^2=0.026
Hemisphere×Area×Group	$F_{1,22}$=0.820	0.491	η_p^2=0.036
Post hoc analysis between groups			
Anterior Hp	t_{22}=3.148	0.005*	Cohen's *d*=513.8
Int. V Hp	t_{22}=1.243	0.227	Cohen's *d*=155.6
Int. DM Hp	t_{22}=1.246	0.226	Cohen's *d*=340
Int. DL Hp	t_{22}=2.497	0.020	Cohen's *d*=522.2
Post. V Hp	t_{22}=0.168	0.868	Cohen's *d*=154.2
Post. DM Hp	t_{22}=0.176	0.862	Cohen's *d*=244.9
Post. DL Hp	t_{22}=1.056	0.302	Cohen's *d*=665.8
TnA	t_{22}=3.503	0.003*	Cohen's *d*=427
IMM	t_{22}=0.328	0.746	Cohen's *d*=423.3

*Significant also after a Bonferroni adjustment for multiple comparisons.

Journal of Experimental Biology (2022) 225, jeb244190. doi:10.1242/jeb.244190

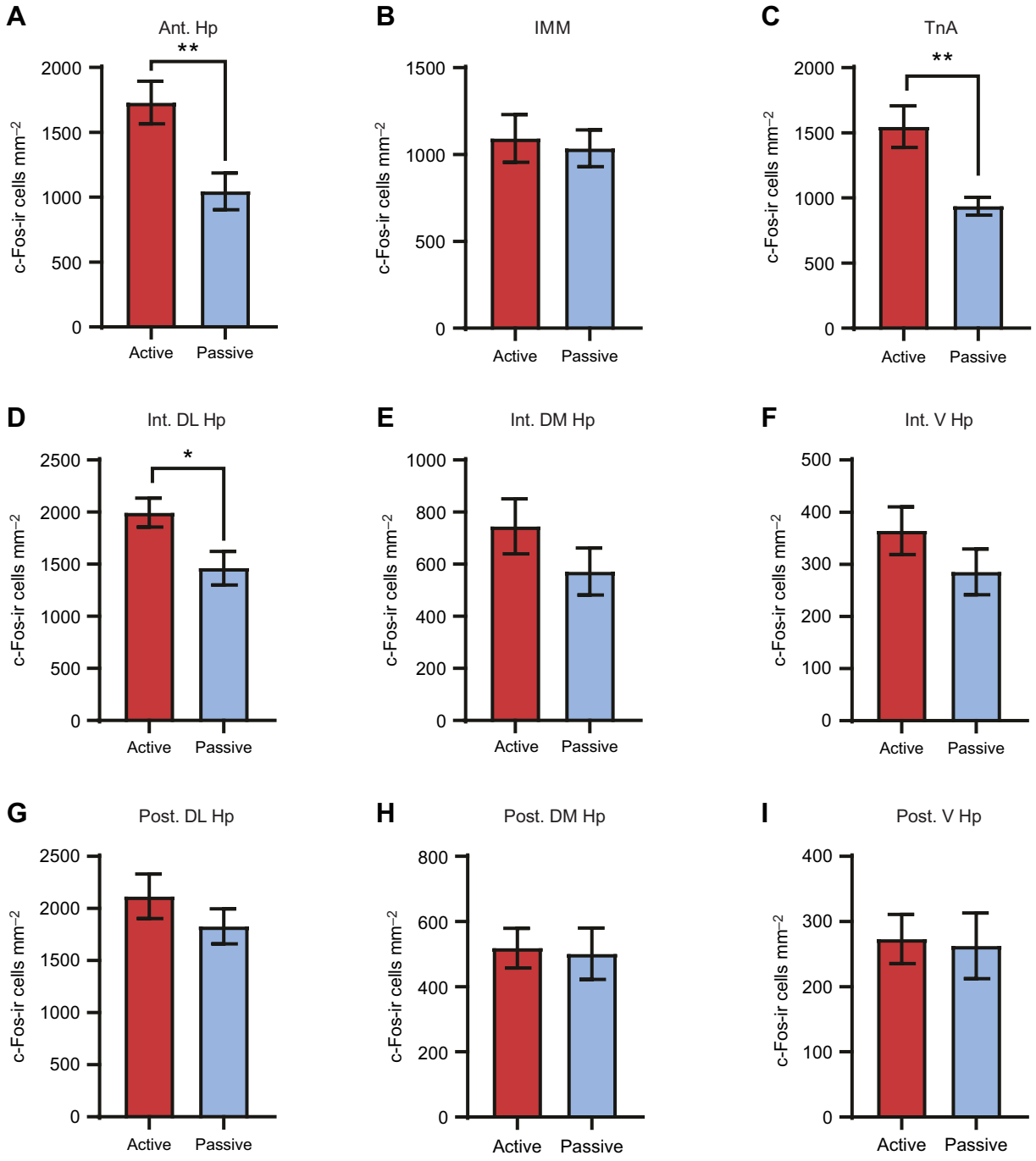

Fig. 5. Measured c-Fos-ir (immunoreactive cell densities) across brain regions of the two experimental groups. (A) Anterior hippocampus (Ant. Hp). (B) Intermediate medial mesopallium (IMM). (C) Nucleus taeniae of the amygdala (TnA). (D) Intermediate ventral hippocampus (Int. V Hp). (E) Intermediate dorsomedial hippocampus (Int. DM Hp). (F) Intermediate dorsolateral hippocampus (Int. DL Hp). (G) Posterior ventral hippocampus (Post. V Hp). (H) Posterior dorsomedial hippocampus (Post. DM Hp). (I) Posterior dorsolateral hippocampus (Post. DL Hp). Bar plots show means±s.e.m. (*P<0.05, **P<0.01).

many hippocampal neurons were successfully recorded, but only head direction cells were identified among them (Ben-Yishay et al., 2021). This evidence may be used to argue that place cells would exist only in some bird species but not in others (Damphousse et al., 2022). For instance, one could hypothesize that place cells represent a case of convergent evolution between mammals and the most cognitively advanced birds, such as Neoaves, absent in Galliformes. In this view, indeed, it has been argued that Galliformes, such as quails and chickens, retained more ancestral traits compared with Neoaves (Prum et al., 2015). In contrast, our results with c-Fos indirectly suggest that place cells (and other spatially coding cells) may exist also in Galliformes. It is likely that the density of spatially coding cells is much lower in Galliformes compared with Neoaves, making them more difficult to detect electrophysiologically. Indeed, the number of spatially coding neurons vary in different bird species, probably owing to divergent ecological adaptations. For example, fewer place neurons were found in the hippocampus of a non-food-hoarding species (zebra finch) compared with the food-hoarding titmice (Payne et al., 2021). Even fewer of such neurons may exist in domestic chicks and quails, in line

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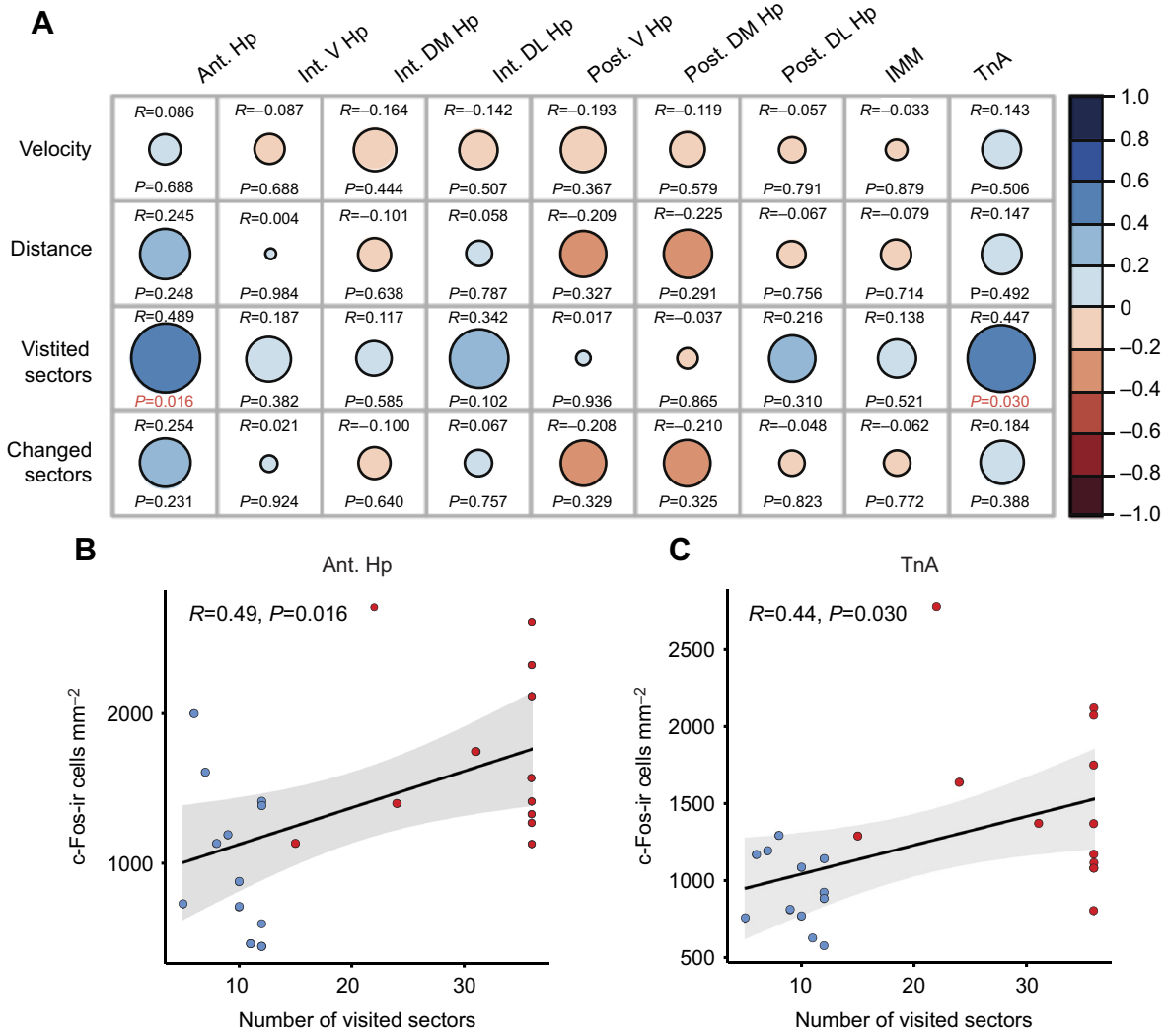

Fig. 6. Brain–behaviour correlations. (A) Correlation matrix of all brain regions and behavioural parameters. The size of the dots corresponds to the strength of correlation, while the colour corresponds to the direction (blue, positive; red, negative). (B,C) Significant correlations between visited sectors and density of c-Fos-ir cells within the anterior hippocampus (Ant. Hp) and nucleus taeniae of the amygdala (TnA). The dots represent the individuals of the two experimental groups with the active exploration group in red and passive exploration group in blue.

with the presence of more ancestral traits in these birds (Prum et al., 2015).

It is also important to consider that our current results do not provide information on the specific neural populations that were activated. In addition to place cells and head direction cells, other spatially coding cells may have contributed to the effect in the active group (e.g. border cells or grid cells, if existent in birds' hippocampi). Electrophysiological confirmation studies with domestic chicks are urgently needed at this point. Our results indicate that, in such studies, recordings should be performed in the anterior hippocampus (in line with Payne et al., 2021). A potential limitation of our study for the identification of functional hippocampal subdivisions might be related to the method used to estimate densities of c-Fos labelled cells. Here, we positioned a relatively small counting area over the spots with the highest number of c-Fos-ir cells within the anatomically defined regions of interest. This standard approach is often used to estimate cell densities in relatively large brain areas (e.g. Shimizu et al., 2004; Hicks et al., 2012; Kovács et al., 2018; Coppola and Bingman, 2020). Although this is a conservative approach, it might have masked subtle differences in very specific sub-regions. In our case, it was sufficient to reveal that the stronger spatial processing occurred in the anterior and intermediate dorsolateral portions. However, we still lack more precise information on the location of the spatially processing cells and their anatomical organization within these macro regions. It is also worth mentioning that subdivisional differences in c-Fos expression in the chick hippocampus have also been shown in our earlier studies. For instance, social novelties activated the ventral and dorsomedial parts, but not the dorsolateral parts, of the intermediate hippocampus (Corrales-Parada et al., 2021). On the contrary, here we found significant activation of the dorsolateral intermediate hippocampus and the anterior hippocampus. This highlights a potential segregation of social and spatial functions between areas of the ventral and dorsal avian hippocampus, which needs further investigation.

Functional differences between the hippocampi of the two hemispheres have been reported in earlier studies with chicks (Tommasi et al., 2003; Corrales-Parada et al., 2021; Morandi-Raikova and Mayer, 2021). For instance, when we trained chicks to navigate in a large arena in relation to freestanding objects, only the right hippocampus showed c-Fos upregulation (Morandi-Raikova and Mayer, 2021). In contrast, no lateralization was found in the present study. This discrepancy may be explained by the different

nature of the two tasks. In our previous study, chicks were specifically trained to orient using the relational information provided by freestanding objects (Morandi-Raikova and Mayer, 2021). The results suggested that spatial-relational computations are predominantly processed by the hippocampus of the right hemisphere. On the contrary, in the present study, chicks had to explore an environment and to acquire a new spatial map based on the geometrical shape of the environmental layout. This task is less specific, as multiple strategies can be used to orient in this situation. Thus, the task may have activated multiple hippocampal functions, which are processed in parallel in both hemispheres during the acquisition of a spatial map. This lack of lateralization is in line with our previous studies, where chicks had to orient by the geometrical layout of the environment (Mayer et al., 2016), or were simply exposed to novel environmental shapes (Mayer et al., 2018; Morandi-Raikova and Mayer, 2020). In none of these studies were we able to detect clear lateralization. More research using different orientation tasks is needed to characterize the functional specializations of the left and the right hippocampi in birds. An alternative explanation for the lack of lateralization might be the absence of light stimulation during embryonic incubation (Rogers and Bolden, 1991; Rogers and Deng, 1999; Rogers et al., 2013). However, please note that instances of hippocampal lateralization in dark incubated chicks have been already reported (e.g. in our previous studies Morandi-Raikova and Mayer, 2021; Corrales-Parada et al., 2021; for other instances of brain and behavioural lateralization in dark incubated chicks, see also Deng and Rogers, 2002; Costalunga et al., 2022; Morandi-Raikova et al., 2021; Lorenzi et al., 2019).

The present study furthermore suggests that in birds, like in mammals, exploration of an environment induces hippocampal activation, which is likely related to the formation or updating of spatial representations (Mayer et al., 2010). To the best of our knowledge, so far only three other studies have investigated the involvement of the avian hippocampus in environmental exploration (Mayer et al., 2018; Morandi-Raikova and Mayer, 2020; Damphousse et al., 2022). Altogether, these studies contribute to the vast literature showing that, despite the fundamental differences in the structure of the hippocampal formation in birds and mammals (Striedter, 2016), its involvement in spatial function is similar among the two clades (Bingman et al., 2005; Siegel et al., 2005; Hough and Bingman, 2008; Kahn and Bingman, 2009; Mayer et al., 2010; 2013; 2016; Mayer and Bischof, 2013; Coppola et al., 2015, 2016; Sherry et al., 2017; Lormant et al., 2020; Morandi-Raikova and Mayer, 2021; Payne et al., 2021).

Finally, in the present study, we also found higher expression of c-Fos in the TnA of the active exploration group compared with the passive exploration group. This is in line with our previous finding that TnA and the hippocampus activate in chicks exposed to a novel environment (Morandi-Raikova and Mayer, 2021). In addition, in the present study, the activation of both taeniae and the hippocampus were positively correlated with the number of explored sectors. This suggests a functional linkage between these two brain regions, which may play a role for spatial memory formation. This is not unlikely, given that the nucleus taeniae of the amygdala is anatomically interconnected with the hippocampal formation (Casini et al., 1986; Cheng et al., 1999). Furthermore, a functional linkage between the amygdala and the hippocampus has also been reported in mammals (Sheth et al., 2008). Disruption of amygdala activity prevents the increase of hippocampal Fos expression in response to novel environments (Sheth et al., 2008). Based on these findings, it has been proposed that the amygdala may affect hippocampal encoding of specific environmental features. Our findings suggest that in birds, like in mammals, the amygdala may modulate spatial information processing in the hippocampus, which may be a conserved function of the hippocampus–amygdala complex in vertebrates. Whether the role of the amygdala is to encode specific environmental features remains undetermined with the current data. Also, the presence of place cells in the amygdala would be a rather surprising trait, which, as far as we know, has never been investigated in any vertebrate species. An alternative possibility would be to consider that the activity of taeniae might reflect neophobia induced by the novelty of the experimental environment, as suggested by other studies (Morandi-Raikova and Mayer, 2021; Perez et al., 2020). The more sectors that are explored, the more the animal was in the open field, which may have induced a stronger neophobic reaction. Alternatively, one could speculate on the role of taeniae in processing reward information during processing of spatial memories. A reward function of the mammalian medial amygdala (homologue of bird taeniae) has been reported in mice, at least in a social context (Hu et al., 2021), and in birds in the context of taste perception (Protti-Sánchez et al., 2022). In summary, the role of taeniae in processing spatial information remains speculative at this stage. However, the present study and our previous work (Morandi-Raikova and Mayer, 2021) strongly support the idea that the hippocampus–amygdala complex also exists in birds. Moreover, like in mammals, in avian species, both structures might play a role in spatial function.

To conclude, our findings suggest the existence of spatially coding neurons (such as place cells) in the domestic chicken's anterior hippocampus. IEG products do not represent a direct approach to investigate place cells. However, they provide a great opportunity to image the activation of large populations of cells, which can be aligned with anatomical subdivisions of the hippocampus. Future electrophysiological studies should target the anterior and the dorsolateral portion of the intermediate hippocampus, which increased the density of c-Fos-ir cells after active exploration of a novel environment. Finally, our study suggests a functional linkage of the hippocampus and nucleus taeniae of the amygdala for the processing of spatial information. Many unanswered questions still remain, but the present study opens new doors to study the evolution of the neural circuits behind animal navigation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.M.-R., U.M.; Methodology: A.M.-R., U.M.; Formal analysis: A.M.-R., U.M.; Investigation: A.M.-R.; Resources: U.M.; Data curation: A.M.-R.; Writing - original draft: A.M.-R.; Writing - review & editing: U.M.; Visualization: A.M.-R.; Supervision: U.M.; Project administration: U.M.

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