RESEARCH ARTICLE

Brain activation pattern depends on the strategy chosen by zebra finches to solve an orientation task

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SUMMARY

Zebra finches (*Taeniopygia guttata*) were trained to find food in one of four feeders on the floor of an aviary. This feeder was always in the same place during training and was additionally marked by a distinct pattern. In the test trial the distinctly patterned feeder was interchanged with one of the other feeders, so that the birds had to decide to use either the pattern or the original location for finding food. Half of the birds used one strategy and half used the other. According to the strategy applied, different brain areas were activated, as demonstrated by c-Fos immunohistochemistry. The hippocampus was activated when spatial cues were used, while in birds orienting using the pattern of the feeder, part of the collothalamic (tectofugal) visual system showed stronger activation. The visual wulst of the lemnothalamic (thalamofugal) visual system was activated with both strategies, indicating an involvement in both spatial and pattern-directed orientation. Because the experimental situation was the same for all zebra finches, the activation pattern was only dependent on the strategy that was voluntarily chosen by each of the birds.

Key words: birds, visual pathways, hippocampus, septum, c-Fos.

INTRODUCTION

The ability to retrieve food locations based on previously learned visual information from the surroundings is important for successful foraging. Cues such as the colour, shape and pattern of the food source as well as the spatial arrangement of environmental cues can be used for orientation towards food locations. The question of whether birds rely more on spatial cues or on local traits if both cue types are available has been investigated frequently. It has been shown that a preference for one of these two cue types varies across species. Food-hoarding birds, for example, prefer to use spatial cues whilst closely related non-storing species use the two cue types equally. One explanation for this difference is that spatial cues in a changing environment may generally be more reliable over longer time periods compared with local cues. Storers need to relocate caches over long periods and may therefore be under a particular selection pressure (Clayton and Krebs, 1994; Brodbeck, 1994; Herz et al., 1994; Brodbeck and Shettleworth, 1995) (but see Hodgson and Healy, 2005; LaDage et al., 2009; Feenders and Smulders, 2011).

Many studies in mammals have demonstrated the important role of the hippocampal formation for spatial orientation and learning (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978; Morris et al., 1982; Nadel, 1991; Schenk et al., 1995; Nadel and Hardt, 2004). The avian hippocampus, although quite different in shape and organization, is assumed to be homologous to the mammalian one, and is also involved in spatial tasks. The hippocampus of food-storing birds has been shown to be larger than that of non-storing species (Krebs et al., 1989; Sherry et al., 1989); it shows a greater amount of neurogenesis (Hoosholey and Sherry, 2007) and its size may vary seasonally in food-storing birds (Smulders et al., 1995) (for review, see Sherry and Hoshooley, 2010). The hippocampus is also involved in the

homing behaviour of pigeons (Bingman et al., 1988; Bingman et al., 2005) and in spatial orientation of zebra finches (Watanabe and Bischof, 2004; Bischof et al., 2006). Using lesions and the expression of immediate early genes (IEGs) as an activity marker, we have demonstrated in zebra finches that the hippocampus is involved in the acquisition as well as the retention of spatial learning tasks (Watanabe and Bischof, 2004; Bischof et al., 2006; Mayer et al., 2010).

Spatial orientation using both distant landmarks and local cues is based on visual information. Anatomical studies have favoured the lemnothalamic (thalamofugal) pathway as the source of the visual information used in spatial learning because there are direct connections between the telencephalic station of this pathway, the visual wulst and the hippocampus (Atoji et al., 2002). In contrast, the collothalamic (tectofugal) pathway has been shown in a variety of studies (for reviews, see Bischof and Watanabe, 1997; Shimizu et al., 2010) to be involved in object detection and pattern recognition. Visual information is transferred within this pathway from the eye *via* the tectum opticum and the thalamic nucleus rotundus to the entopallium, the first telencephalic station. From there, projections have been shown to the overlaying nidopallium and mesopallium (Husband and Shimizu, 1999; Krützfeldt and Wild, 2004; Krützfeldt and Wild, 2005). Using electrophysiological recordings, Schmidt and Bischof demonstrated that the nidopallium is indeed a visual area (Schmidt and Bischof, 2001). Neurons within this area differ from entopallial neurons by having a longer latency and a greater influence of ipsilateral visual stimuli. The nidopallial and mesopallial areas above the entopallium (lateral nido-mesopallium) have been shown to be involved in sexual imprinting (Lieshoff et al., 2004) and are activated by presentation of visual stimuli resembling the imprinted object (Huchzermeier et al., 2006). Similar findings have been reported in pigeons (Patton et al., 2009).

Indeed, lesions of the zebra finch entopallium (tectofugal pathway) have been shown to cause deficits in pattern discrimination but have no effect on spatial learning (Watanabe et al., 2008), while lesions of the hippocampus cause large deficits in spatial orientation (Bischof et al., 2006) but not in pattern discrimination (Watanabe et al., 2008). Lesions to the visual wulst (thalamofugal pathway) produced a clear impairment of spatial orientation but had no effect on pattern discrimination (Watanabe et al., 2011). According to these studies, the lemnothalamic visual system is the main source of visual information for the hippocampus. Based on their findings, the authors also concluded that the lemnothalamic pathway may be responsible for the analysis of the 'where' (the location) of an object, whereas the collothalamic pathway is responsible for the 'what', i.e. the visual traits of the same object.

As already mentioned above, a number of studies with immediate early genes have shown that the hippocampus is activated in situations where spatial memory is used in orientation tasks (e.g. Bischof et al., 2006; Mayer et al., 2010). No studies, however, have as yet examined the activation pattern of the two visual systems shown to be involved in spatial orientation and object identification. The present study was designed to tackle this question in combination with the initially posed one: in a situation of choice, which of the two alternatives, orientation by distant landmarks (spatial cues) or orientation using local cues (visual traits of the feeder) is preferred by the zebra finches? And might it be possible to find a relationship between the strategy that a zebra finch chooses and the activation of the brain areas involved in spatial orientation and pattern recognition; that is, the hippocampus and the two visual pathways?

MATERIALS AND METHODS

Seventeen male zebra finches (*Taeniopygia guttata castanotis*, Gould 1837), between 12 and 24 months of age, from the institute's stock were used for this study. The birds were housed in individual cages with water freely available. The light cycle was 14 h light:10 h dark. For the daily experiments, food was removed shortly before 22:00 h, when the light was switched off. The experiments started at 09:00 h, 1 h after the light was switched on. After the end of the daily experiments, food was provided *ad libitum*.

The experimental setup was a 'dry' version of the Morris water maze (Fremouw et al., 1997; Watanabe and Bischof, 2001), consisting of a cubic aviary made of wire mesh (180×180×180 cm) and positioned in a sound-isolated room (3×3×2.50 m, see Fig. 1A). A coloured poster on one wall, a punched board on another, the entrance door to the room and a chair in one corner provided extra-aviary cues. Each wall of the aviary included a flap door (10×7.5 cm) with a wooden platform attached to the outer side. The individual home cages (42×22×24 cm) could be placed on these platforms to enable the release of an animal into the experimental arena. Each individual home cage was equipped with a lamp at the ceiling (DC 12 V). There was one long perch (27 cm) below each flap door and seven short perches (9 cm) attached to the walls inside the aviary. The floor was covered with white paper, which was cleaned or replaced after each trial to prevent uncontrolled local cues. Food dispensers were placed on the floor. The feeders were made from the base of commercially available pet bird feeders in the shape of cylindrical white cups (3.5 cm in diameter, 3.5 cm high), each with a hollow arm (1.0 cm wide, 3.5 cm long) allowing the bird to reach the mixed grain (Fig. 1B). The cups were covered with cardboard (6×9 cm), so that the birds could not see the food inside the arm until they came closer to the feeder. Further details of the experimental setup can be found elsewhere (Watanabe and Bischof, 2001).

During habituation training, the birds had to learn to enter the aviary when the room light was switched on, then to eat the food on the floor, and to fly back to their home cage when the light for the experimental room was switched off and the ceiling lamp of their home cage was switched on. The habituation training continued for at least 5 days. On the first day, the birds were released into the aviary in groups of six for 2 h to explore the new environment and to find the feeder on the floor at the centre of the aviary. For the following days, the birds were trained individually to find food and fly back to the home cage. For this purpose, the individual home cage with the ceiling lamp on was transported in the dark into the experimental chamber and placed on the platform of the aviary. The flap door was opened by an attached string. One minute later, the light in the experimental chamber was switched on, allowing the birds to enter the setup. After reaching one of the feeders they were allowed to peck for food 5–10 times. The aviary light was then switched off, whereas the home cage light was still on. In this situation, most of the birds returned spontaneously to their illuminated home cage. If not, they were gently chased in the correct direction. After each trial, the home cage was removed and the floor of the aviary was cleaned. The releases occurred from four different positions (release sites in the aviary) in random order. The training trials of each bird were separated by at least 60 min. Visual observation was carried out through a small window in the entrance door to the chamber, which was covered with a semi-transparent window foil. The habituation training consisted of four training trials per day and continued until the birds had learned to eat food from the feeder and to return to their home cage.

The birds were then trained to discriminate a baited feeder from three non-baited ones. The four feeders were placed on the floor of the experimental aviary at a distance of 40 cm from the walls. Three of the feeders were covered with carton sheets that had a regular pattern of black stripes. These feeders contained food but the grains were inaccessible as they were covered with a transparent adhesive tape, so the birds were able to see the food when they approached the feeder but could not reach it. The fourth feeder was marked with a grid pattern and the food was accessible. All feeders remained at the same location over the training session. For a trial, the birds were released randomly from one of the flap doors at the four aviary sides. Thus, the experimental birds had the choice of learning the position either by orienting on extra-maze cues or by finding the baited feeder on the basis of its pattern (or by using both cues). A circular area of 12 cm radius around the feeder was defined as 8the 'choice area'. A choice was determined as incorrect when the bird had approached an unbaited feeder to a distance of less than 12 cm. The time to reach the correct feeder after entering the aviary and the number of incorrect visits were recorded. Training consisted of four trials per day and continued until the learning criterion of four successive correct trials was reached. The training procedure was followed by the test trial, where the location of the differently patterned baited feeder was changed (from position a to position c; Fig. 1) to examine whether the birds preferred the previously learned position in relation to the extra maze cues or the differently patterned feeder at the new location. During the test trial, food in all feeders was accessible. The approximate flight trajectories were plotted freehand in two-dimensional sketches of the aviary from above. These sketches were then analysed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) to estimate the distance that birds travelled during the test trial.

One hour after the end of the test trial, the animals were deeply anaesthetized by an intramuscular injection of 0.03 ml Narcoren
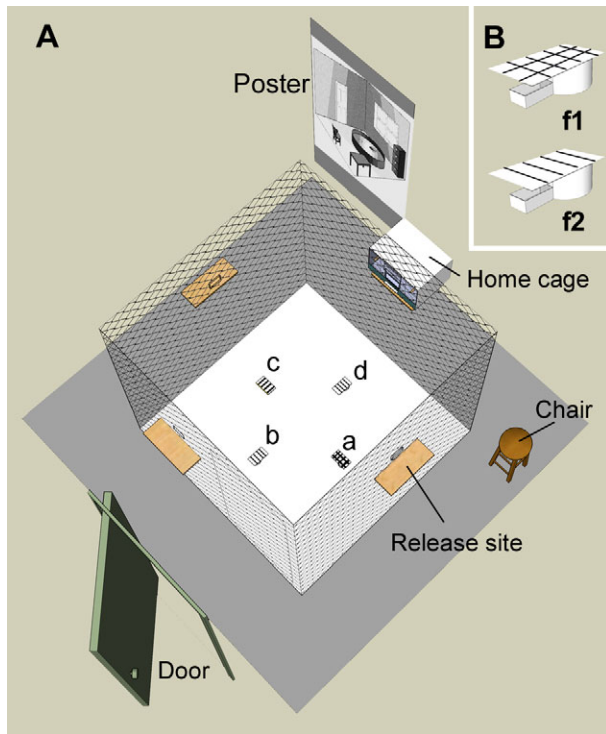
Fig. 1. (A) Sketch of the experimental setup (view from above). The experimental aviary (180×180×180 cm) was placed in the middle of a chamber (3×3×2.50 m) with extra maze cues (poster, door and chair). The home cage is placed at one of the four release sites. (B) Sketch of the feeders (f) with visual stimuli: f1, baited feeder with square pattern, which was placed at position a during the training and at position c during the test; f2, non-baited feeders with striped pattern at positions b, c and d during training and positions a, b and d during the test.

(Rhone Merieux, Laupheim, Germany; 30% in 0.9% sodium chloride). They were perfused transcardially *via* the left ventricle with phosphate-buffered saline (PBS; 0.1 mol l^{-1}, pH 7.4, 0.9% sodium chloride) for 10 min and then with 2% paraformaldehyde (PFA) in PBS for 15 min. The head was then severed from the body, the skin was removed and, after the skull was partly opened, the head was transferred to 2% PFA overnight. The next day, the skull was fixed in a stereotaxic head holder specifically designed for zebra finches (Bischof, 1981). The caudal part of the skull was opened and the brain was exposed. Then, according to the stereotaxic atlas of the zebra finch brain (Nixdorf-Bergweiler and Bischof, 2007), a coronal plane cut (~0.15 mm posterior to the Y-point) was made with a scalpel blade attached to a micromanipulator to guarantee that the sections made thereafter had the same orientation as the atlas drawings. The brain was then removed from the skull, postfixed overnight in 2% PFA/PBS containing 20% sucrose at 4°C, then transferred to 30% sucrose/PBS for between 24 and 72 h. The left hemisphere of the brain was marked by a slight rostocaudal knife cut in advance of sectioning on the freezing microtome. Three series of 40 μm coronal sections were cut after freezing the brain in 5% alcohol to the microtome head. The sections were collected in PBS. Endogenous peroxidase activity was depleted by incubation in 0.3% H$_2$O$_2$ in PBS. The sections were washed in PBS (5×5 min) and treated with 3% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min. The sections of the first series were then transferred to c-Fos-antibody solution (c-Fos antibody, 1:2000; K-25, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated overnight at 4°C on a rotator. The sections of the second and the third series were kept in PBS at 4°C as a backup or for testing the antibody specificity. For this purpose, sections were treated as in the first two series, but without the primary antibody. After several washes in PBS, the secondary antibody reaction was carried out in a biotinylated anti-rabbit solution (1:200, BA-1000, Vector Laboratories) in PBS for 1 h at room temperature. The ABC method was used for signal amplification (Vectastain Elite ABC Kit, PK 6100, Vector Laboratories). Neurons expressing the c-Fos protein were visualized with VIP substrate kit for peroxidase (SK-4600, Vector Laboratories). This produced a purple reaction product confined to the cell nuclei of activated neurons. Sections were then transferred to distilled water and serially mounted on gelatine-coated slides. They were dried at 50°C on a heating plate, then counterstained with Methyl Green (Methyl Green nuclear counterstain, H-3402, Vector Laboratories), dehydrated and coverslipped with DePeX (Serva, Heidelberg, Germany).

Sections were examined by a Zeiss microscope at a magnification of ×100. Photomicrographs were made using a Zeiss microscope with a digital camera (Olympus E520). In an initial analysis, an overview of the c-Fos distribution within all available regions of all brains was made. For this purpose all sections were examined and it was noted which brain areas contained c-Fos-immunoreactive (IR) neurons independent of the number of stained neurons. On this basis it was decided which of these activated brain areas had to be quantified. Counting of labelled neurons was performed within the hippocampal formation, hyperpallium apicale, hyperpallium densocellulare, lateral nido-mesopallium (LNM), preoptic area and septum. Measurements were performed using an ocular grid with a side length of 200×800 μm, with the areas defined as detailed below.

For estimation of the cell densities within the hippocampal formation, seven serial sections (spacing 120 μm) were selected from a part of the hippocampus, extending from frontal (2.97 mm anterior to the Y-point) to caudal (2.25 mm anterior to the Y-point). The IEG labels were not congruent with the known subdivisions of the avian hippocampus, but were different within the ventral and dorsal hippocampus. To quantify these differences we decided to measure the densities within the ventral, medial and dorsal part of hippocampus, and also the parahippocampal area separately. For this purpose, the hippocampus of each section was subdivided into three equal parts starting ventrally at the height of the smallest distance between the ventricles (the border to the septum), and ending dorsally at the surface of the telencephalon. The dorsal part was divided vertically into dorsal hippocampus and parahippocampus (Fig. 2). For counting, the ocular grid was positioned in the centre of each subdivision.

Estimation of the cell densities within hyperpallium apicale and hyperpallium densocellulare was performed on seven serial sections (with an equal distance of 72 μm from each other), which were selected from an area extending from frontal (4.23 mm anterior to the Y-point) to caudal (3.69 mm anterior to the Y-point). Within this region, Keary and colleagues (Keary et al., 2010) described retinotopic maps and, according to Watanabe and colleagues (Watanabe et al., 2011), lesions impair spatial orientation. To estimate cell density, the ocular grid was positioned in a central part of hyperpallium densocellulare and hyperpallium apicale that could be identified by anatomical landmarks. The same seven serial sections were used for estimation of cell density within the rostral hippocampus.
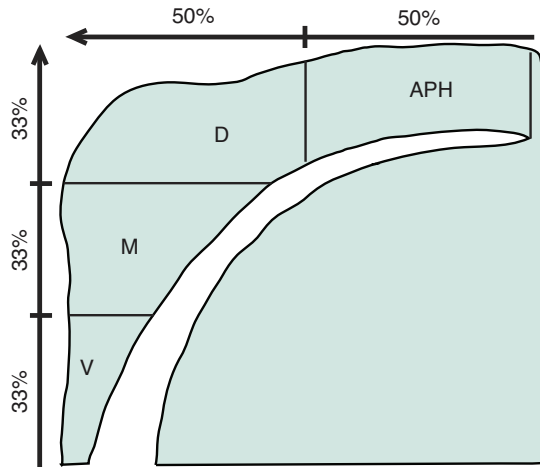
Fig. 2. Schematic view of the hippocampal subdivisions. Vertically, the hippocampus was subdivided into three equal parts: the ventral (V), medial (M) and dorsal (D) hippocampus. Horizontally, the dorsal part was then further subdivided into two equal parts: the dorsal hippocampus and the parahippocampal area (APH). For more details see Material and methods.
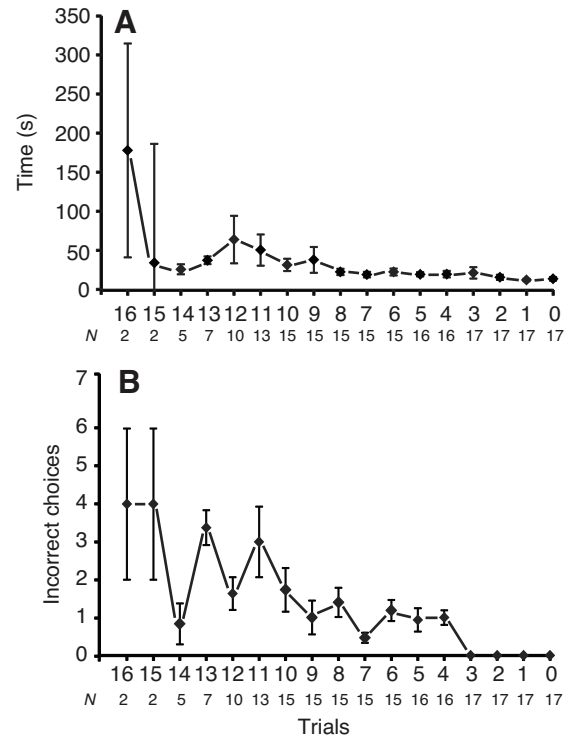

Fig. 3. Learning performances of the zebra finches. (A) Time curve. (B) Incorrect choices across trials. Individual birds needed different numbers of trials to reach the learning criteria; therefore, the trials on the x-axis are plotted backwards from the first point of the four trials defining the learning criterion. Because some birds learned faster than others, the N values decrease from the initial trials.

Counting of c-Fos IR neurons within the LNM was done within an area extending from frontal (3.69 mm anterior to the Y-point) to caudal (3.15 mm anterior to the Y-point). The lamina mesopallii and entopallium were used as anatomical landmarks. The ocular grid was positioned at the lamina mesopallii above the entopallium covering the mesopallium and nidopallium in equal parts. Seven consecutive sections (intersection interval 72 µm) were used for this counting. More details about the definition of the LNM are given elsewhere (Lieshoff et al., 2004; Huchzermeier et al., 2006). For counting of labelled neurons within the septum, seven consecutive sections were selected from an area extending from frontal (3.69 mm anterior to the Y-point) to caudal (2.43 mm anterior to the Y-point). The shorter side of the ocular grid (200×800 µm) was positioned dorsally within the septum as far as possible between the lateral ventricle and the midline of the brain.

As a control, the IEG-expressing neurons were examined within the preoptic area, which has been shown to express quite constant IEG labels. For this purpose, seven serial sections at the level of the nucleus rotundus were selected (rostrocaudally from 2.79 mm to 2.25 mm anterior to the Y-point). The ocular grid was positioned at the bottom of the brain section so that it was in contact with the medial boundary of the hemispheres. In addition, we tried another method of quantification within the hippocampus, using electronic counting of c-Fos IR neurons with help of the computer program ImageJ. Because of the necessity for high-resolution photomicrographs of relatively big areas with constant lighting conditions, this method was much more time consuming than the manual counting and revealed comparable results (see also Lieshoff et al., 2004). Therefore we decided to count manually.

After counting the IR neurons within all areas of interest, mean densities from all seven sections were calculated and cell densities were standardized to 1 mm². Initially this was done for the two hemispheres separately, but because the means were almost identical for the two hemispheres, the measured values were lumped together for further analysis. The individual bird means were considered as overall ratings for the number of IEG IR neurons in the corresponding areas and were taken for further statistical analysis. The differences of the IEG-expressing neurons were tested by two-

way ANOVA with 'brain area' and 'preference group' as factors. Statistical analysis was performed separately for the hippocampal areas (ventral hippocampus, medial hippocampus, dorsal hippocampus, parahippocampus and rostral hippocampus), the visual areas (hyperpallium densocellulare, hyperpallium apicale, LNM) and the remaining areas (preoptic area and septum). Correlations between covered flight distances and expression of c-Fos were tested with Pearson correlation test; the P-values calculated were Bonferroni corrected. Learning performance of the two groups was compared by a Mann–Whitney U-test (MWU). Statistical analyses were performed by the computer programs SPSS 17.0 and Statistica 6.1.

RESULTS

All birds learned to find the baited feeder during training and improved their performance across consecutive entries to the arena. The time to reach the food and the number of errors decreased from trial to trial until the learning criterion (correct choices in four consecutive trials) was reached (Fig. 3). The training procedure was followed by the test trial, where the location of the differently patterned feeder was changed and food in all feeders was accessible. In this situation, eight of the birds preferred the previously learned position ('position' choice), whereas nine of the birds chose to orient according to the patterned feeder at the new position ('pattern' choice). The two remaining positions were not chosen by any of the birds.

In the test trial, not all birds flew directly to the selected goal. Some flew around within the aviary for a while until a choice was
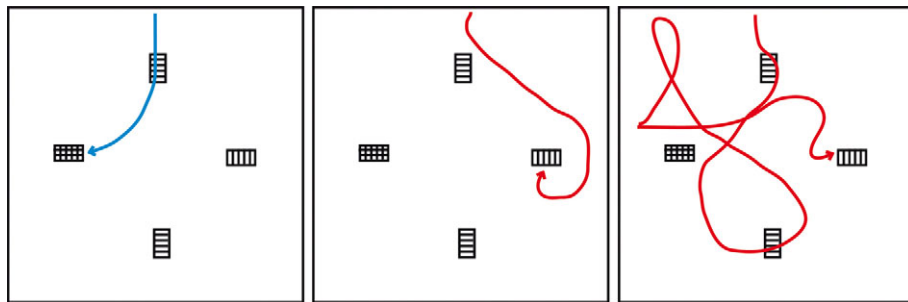
Fig. 4. Three example flight trajectories during the test trial. Left, direct pattern choice. Middle, direct position choice. Right, this bird was undecided and needed some time until the choice was made.

made (Fig. 4). Such direct and indirect approaches, however, occurred in the two groups at a similar rate and were not related to the choice of pattern or position as orientation cue. There was no interaction between group and distance travelled (pattern choice birds 426.33±149.62 cm; position choice birds 319.5±79.67 cm; MWU z=0.09, P=0.92) and we were also not able to identify any other behavioural traits significantly related to either a pattern or a position choice. Likewise, the analysis of learning performance did not reveal any difference between the two choice modes. Birds from the two groups needed a similar number of trials to reach the criterion (pattern choice birds 12.78±1.1; position choice birds 13.3±1.1, MWU z=0.29, P=0.77) and an equal overall time to learn the task (pattern choice birds 343.7±85.9 s; position choice birds 289.1±57.1 s, MWU z=–0.29, P=0.77), they made similar numbers of errors (pattern choice birds 0.97±0.22; position choice birds 1.11±0.13, MWU z=1.34, P=0.18), and the time to enter the aviary was not different (pattern choice birds 10.4±3.26 s; position choice birds 8.79±1.91 s, MWU z=0.38, P=0.7). This was true for the entire learning period, and also if the beginning and the end of learning were analysed separately. If only the first three trials were taken into account, the mean time to find the feeder was 52.33±19.21 s for the pattern group and 51.96±15.72 s for the position group (MWU z=0.38, P=0.7). The number of errors for the pattern group was 2.44±0.67 and for the position group it was 2.67±0.24 (MWU z=1.49, P=0.14). The time to enter the aviary was 11.33±4.01 s for the pattern group and 10.29±4.17 s for the position group (MWU z=0.38, P=0.7). For the last three trials, the mean time to find the feeder was 16.48±2.82 s for the pattern group and 13.08±2.56 s for the position group (MWU z=–0.52, P=0.59). The time to enter the aviary was 10.56±4.21 s for the pattern group and 9.75±3.09 s for the position group (MWU z=0.09, P=0.92). According to the learning criteria, the number of errors was 0 for all birds in the last three trials.

When the behavioural experiments were finished, the brains of all birds were processed for visualization of IEG expression. Two brains were not sufficiently stained for proper quantitative analysis; thus, only six brains from the position group, but all nine brains from the group with pattern preference could be used for further estimation. The nuclei of c-Fos IR neurons were stained dark purple. Background staining was minimal. Thus, IR neurons could easily be discerned from non-IR neurons, which were stained light green (Fig. 5).

Table 1 comprises an overview of the brain areas that showed c-Fos expression in the course of the experiment. Counting of labelled neurons was then performed within the hippocampal formation, hyperpallium apicale, hyperpallium densocellulare, LNM, preoptic area and septum. Estimation of the density of c-Fos IR neurons revealed considerable differences in the number of the hippocampal and LNM IR neurons between the groups (Fig. 6). Birds with position preference showed higher number within the medial hippocampus, the dorsal hippocampus and the parahippocampus, whereas birds with pattern preference showed more IR neurons within the LNM if compared with those with position preference. No differences between the groups were present within the rostral hippocampus, the ventral hippocampus, the hyperpallium apicale, the hyperpallium densocellulare, septum and preoptic areas (Fig. 6).

The exact values for the measurements presented in Fig. 6 are given in this paragraph. The mean of the c-Fos IR neurons within the rostral hippocampus of the position group was 194.42±73.67 neurons mm^{-2} (mean ± s.e.m.) and that of the pattern group was 252.48±48.31 neurons mm^{-2}. The mean within the ventral hippocampus of the position birds was 23.64±4.80 neurons mm^{-2} and that of the pattern birds was 34.31±8.51 neurons mm^{-2}. Medial hippocampus means were 195.83±26.03 neurons mm^{-2} for the position group and 74.02±19.07 neurons mm^{-2} for the pattern group. The position group mean within the dorsal hippocampus was 526.92±82.13 neurons mm^{-2} and that of the pattern group was 149.74±33.92 neurons mm^{-2}. The parahippocampus of the position birds had 418.98±59.05 neurons mm^{-2} for the position group and 135.11±34.02 neurons mm^{-2} for the pattern group. LNM means were 49.21±16.73 and 269.54±63.55 neurons mm^{-2} for the position and pattern group, respectively. Hyperpallium apicale means were 159.23±45.11 and 201.79±41.26 neurons mm^{-2} for the positon and the pattern group, respectively. Hyperpallium densocellulare means were 171.58±55.29 and 349.21±91.69 neurons mm^{-2} for the position and pattern group, respectively. Means of the position and pattern group within the septum amounted to 458.95±66.58 and 475.52±41.92 neurons mm^{-2}, respectively. Preoptic area means were 272.32±51.75 and 262.90±36.68 neurons mm^{-2} for the position and pattern group, respectively.

The residuals were normally distributed (Kolmogorov–Smirnov test: D=0.08, P>0.2, N=150). Statistical analysis of the hippocampal areas revealed significant differences between the two groups ($F_{1,65}$=27.869; P<0.01). Post hoc LSD tests showed significant differences between the pattern and the position groups for the medial hippocampus (P=0.048), dorsal hippocampus (P<0.01) and parahippocampus (P<0.01) but no differences between the two groups for the ventral (P=0.86) and the rostral hippocampus (P=0.34). The group differences were also significant for the visual areas ($F_{1,39}$=7.69765; P<0.01). Post hoc LSD tests showed significant differences for LNM of the two groups (P=0.02), but not for hyperpallium apicale (P=0.65) and hyperpallium densocellulare (P=0.059). The differences were also not significant within the septum and preoptic area ($F_{1,26}$=0.0055; P=0.94).

To control whether motor activity has any effect on the expression of c-Fos, we tested for correlation between the flight distance travelled during the test trial and the number of IR neurons within the areas investigated. There was a slight tendency for a positive correlation visible in the graphs. However, none of the correlations
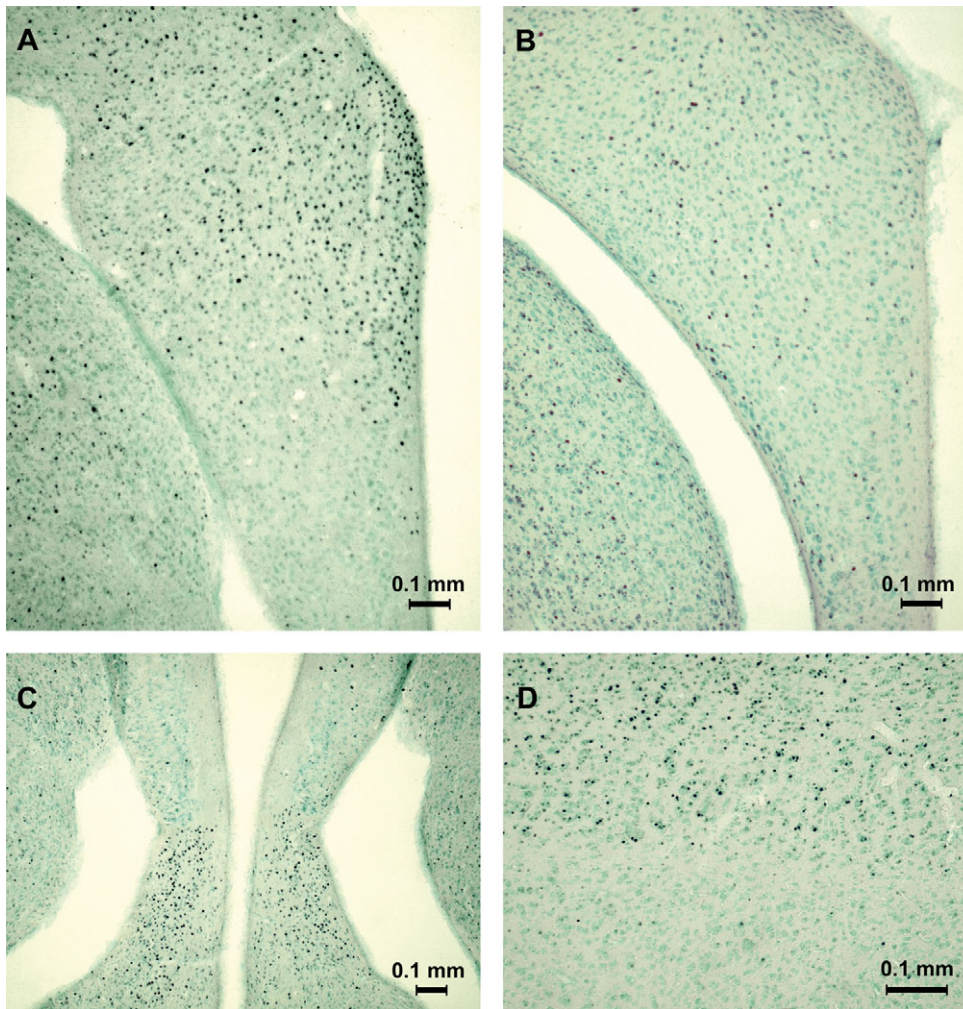
Fig. 5. Photomicrographs of c-Fos immunoreactive (IR) neurons within different brain regions. Coronal sections were counterstained with Methyl Green. (A) Right hippocampus of a bird that preferred position during the test. Labelled nuclei are mainly found within the dorsomedial parts of the hippocampus and are almost absent within the ventral areas. (B) Right hippocampus of a bird with pattern preference, which contains only a few c-Fos IR neurons. (C) Ventral hippocampus and dorsal septum of both hemispheres of a bird with pattern preference. c-Fos IR neurons are restricted to the septum and are independent of hippocampal activation. (D) Lateral nido-mesopallium of a bird with pattern preference. c-Fos IR neurons are dense at the mesopallial part (dorsally) and the nidopallium (ventrally). The two areas are separated by the lamina mesopallii.

was significant (Pearson: $N=15$; $P>0.05$ for every area). Correlation coefficients (r) ranged from 0.278 to 0.696. Three examples are shown in Fig. 7. It can easily be seen that the overall activation of the brain areas increased with longer flight distances, but there is also no doubt that the data points for the position birds were in any case different from those of the pattern group. This, however, was not seen in the hyperpallium apicale. Here, the differences between the two groups were not significant. Consequently, there is also no differentiation in the correlation graph.

DISCUSSION

Our present study demonstrates that zebra finches are able to use both local cues, like the pattern of the feeder, and spatial orientation by using distant landmarks for finding a food location. In a food-finding task where the two cue types marked the same place during learning but were dissociated during the test, half of the birds chose the feeder at the test session using extra-maze cues, while the other half followed the learned pattern, which was now at a new location. Depending on the decision to use local or distant cues, one of two distinct brain areas expressed more c-Fos-activated neurons. The distance the birds travelled to reach the correct location had some effect on the activation of the brain but it did not cause the group differences. On the basis of these results we shall consider in the course of this Discussion whether the birds learned only the one cue that they were using during the test trial, how the pattern of brain activation can be interpreted, and which task-specific

parameters could have affected or contaminated the results. In addition, we provide some thoughts about comparative aspects of the results.

All birds, irrespective of whether they chose pattern or position in the test trial, had learned the location of the feeder reliably, as all of them reached the learning criterion by about the same number of trials in the learning phase (Fig. 3). The displacement of the distinct pattern led to a dissociation of choices towards either the pattern or the location, but neither of the two other feeders was chosen. Similar results have also been obtained in other studies. Some food-storing birds such as marsh tits (*Parus palustris*), black-capped chickadees (*Parus atricapillus*) and jays (*Garrulus glandarius*), have been shown to prefer distant over local cues, while non-storing birds like the blue tit (*Parus caeruleus*), jackdaw (*Corvus monedula*) or the dark-eyed junco (*Junco hyemalis*) showed choices similar to those in the present study (Clayton and Krebs, 1994; Brodbeck, 1994; Herz et al., 1994; Brodbeck and Shettleworth, 1995). Comparison with the literature also indicates that the percentage use of one or other cue may depend on a variety of factors favouring one or the other alternative and can vary quite widely (Herborn et al., 2011). The 50/50 choice in our experiment was thus accidental, although very advantageous for our purpose.

Because we had to restrict our experiment to one test trial, we were not able to decide whether the birds learned only one orientation cue or both. In the test trial, some of the birds were dithering in the choice test and were obviously disoriented for some

Table 1. Brain areas that were activated in the course of the experiment in at least one of the groups

Brain area	Abbreviation
Hippocampus rostrale	Hp R
Hippocampus ventrale	Hp V
Hippocampus mediale	Hp M
Hippocampus dorsale	Hp D
Parahippocampus	APH
Hyperpallium apicale rostrale	HA R
Hyperpallium densocellulare rostrale	HD R
Hyperpallium apicale	HA
Hyperpallium densocellulare	HD
Lamina frontalis superior	LFS*
Mesopallium dorsale	M dorsale
Mesopallium ventrale	M ventrale
Nidopallium	N
Striatum laterale	StL
Striatum mediale	StM
Medial nido-mesopallium	MNM
Lateral nido-mesopallium	LNM
Tractus septopalliomesencephalicus	TrSM[†]
Septum	S
Preoptic area	POA
Nucleus marginalis tractus optici	nMOT
Nucleus geniculatus lateralis, pars dorsalis	GLd
Nucleus geniculatus lateralis, pars ventralis	GLv
Nucleus ventrolateralis thalami	VLT
Tectum opticum	TeO

GLd consist of six components in most birds but these are lumped together in the list.
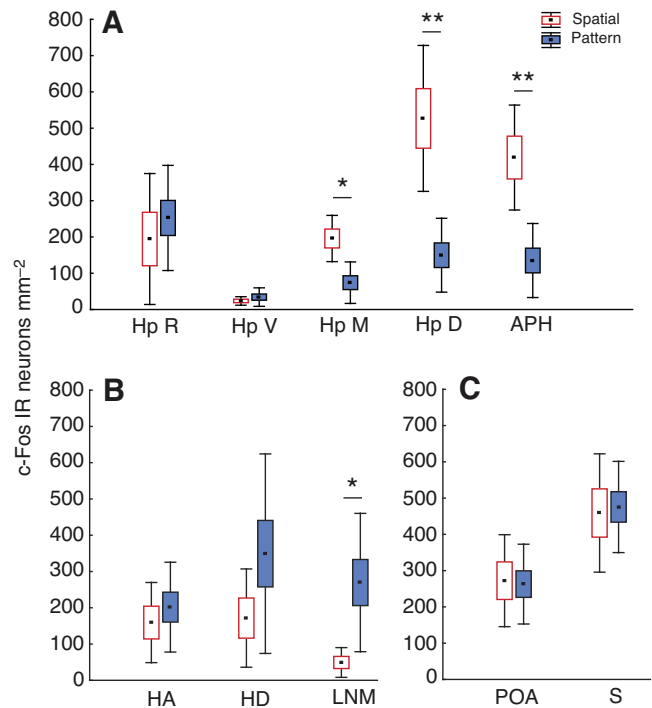*Area around LFS; [†]area around TrSM.

Fig. 6. c-Fos densities. (A) The hippocampal areas. Significant differences between groups were present within the medial hippocampus (Hp M), dorsal hippocampus (Hp D) and parahippocampus (APH). No significant differences where found within the rostral hippocampus (Hp R) and ventral hippocampus (Hp V). (B) The visual areas hyperpallium apicale (HA), hyperpallium densocellulare (HD) and lateral nido-mesopallium (LNM). Only LNM has significantly higher numbers of IR neurons within the birds that preferred pattern. (C) The preoptic area (POA), as a control region, and septum (S) with no significant differences between groups.

time, which could be interpreted as showing that both alternatives had been learnt and the birds had noticed the discrepancy. However, this has to be confirmed by an experiment with more than one test trial, or in an experiment where the initially chosen stimulus is altered. More experiments are also necessary to find out why the birds used one rather than the other strategy. As we mentioned above, we did not find any behavioural indicators allowing prediction of the choice. One may also ask whether the brain activation was caused by the behaviour, or whether the brain activation caused the behaviour. Because it is not possible to record brain activation pattern in advance of the decision, this question may never be answered.

The main aim of this study, however, was to determine whether in a situation of free choice the brain area assumed to be involved in the processing of the chosen cue was activated. Indeed, in the case in which birds chose to orient after spatial landmarks, the hippocampus was activated as predicted. Our use of the 'dry version of the Morris watermaze' (Watanabe and Bischof, 2001) guaranteed that the birds had to use allocentric cues (independent of their own position) for spatial orientation, a prerequisite that has been shown in mammals to be essential for hippocampal involvement (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978; Morris et al., 1982; Nadel, 1991; Schenk et al., 1995; Nadel and Hardt, 2004). In the group that had chosen the local cue, hippocampal activation was much smaller, as would be expected if the birds were not using spatial cues. Instead, the LNM, an area receiving input from the entopallium (Shimizu et al., 2010; Krützfeldt and Wild, 2004; Krützfeldt and Wild, 2005), the first telencephalic station of the tectofugal pathway, was strongly activated and thus obviously involved in the recognition of the local traits of the feeder. In contrast, in spatially orienting birds, this area was not active and

thus was not involved in the orienting process. This result nicely fits with data from the literature indicating that the tectofugal pathway is involved in, among other functions, the identification of objects. The LNM, in particular, has been found to be involved in the context of visual imprinting where the visual traits of female zebra finches are learnt (Lieshoff et al., 2004; Huchzermeier et al., 2006). This study is the first to show an involvement of LNM in pattern recognition in the context of orientation and suggests that the area may be generally involved in the recognition of visual object traits.

Interestingly, the telencephalic station of the thalamofugal system, the visual wulst (hyperpallium apicale/densocellulare), was activated in both groups. This activation was specific to the present experiment. We examined sections of a previous experiment (Mayer et al., 2010) where birds in the same environment as used here had to fly down to the floor for feeding without any orientation task. In these birds, hyperpallium apicale/hyperpallium densocellulare activation was negligible. As yet, we can only speculate on the role of this area in the course of our experiment. Watanabe and colleagues (Watanabe et al., 2011), on the basis of a lesion study in zebra finches, attributed the role of the tectofugal pathway and especially the entopallium in orientation tasks as processing the 'what' of a stimulus, i.e. its visual traits, while the thalamofugal pathway processes the 'where', i.e. information related to the position of the object (the feeder). Consequently, in our experiment, when the birds did not use the 'what' information, the LNM, getting
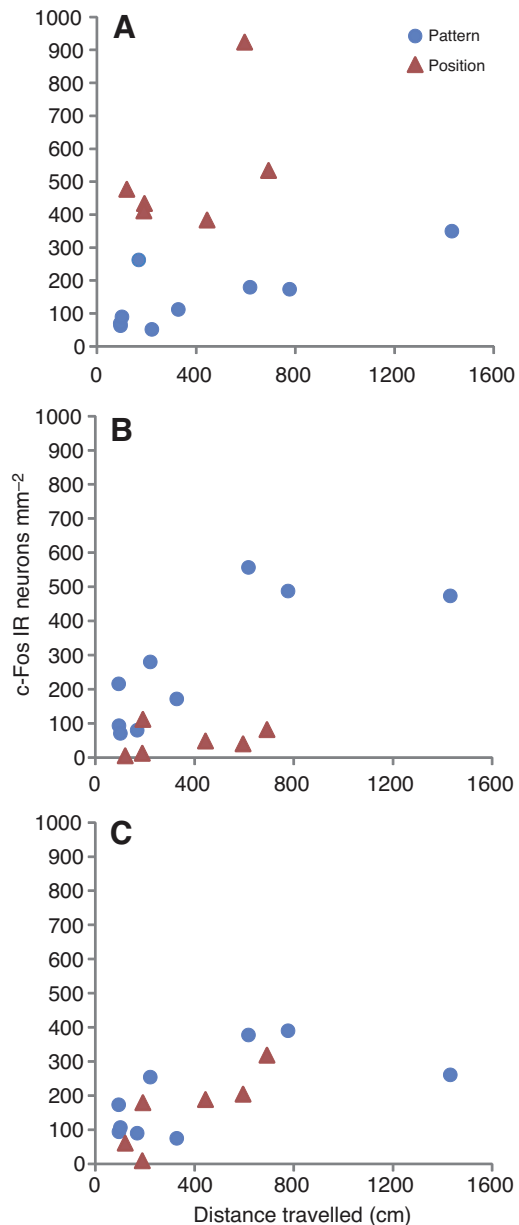
Fig. 7. Correlation between distance travelled and brain activity. (A) Dorsal hippocampus (Pearson: N=15, r=0.278, P>0.3). (B) Lateral nido-mesopallium (Pearson: N=15, r=0.621, P=0.13). (C) Hyperpallium apicale (Pearson: N=15, r=0.640, P=0.1). For further explanation, see Results.

egocentric cues in the case of the 'pattern' group. One could speculate that this differential origin of the 'where' information caused the somewhat higher activation of hyperpallium apicale in the pattern group. However, the difference was not significant in our experiment, and there is as yet no theory that calls for differential processing of egocentric and allocentric information.

Another interesting finding was the lack of activation differences within the septum. In both groups, the dorsal part of the septum was activated quite strongly, and c-Fos expression ended abruptly at the border to the hippocampus (Fig. 5). This is an unexpected finding because it was speculated that the septum could also contribute to spatial orientation on the basis of experiments in food-storing parids (Shiflett et al., 2002) showing an enhancement of septal volume in the autumn, the season in which food-storing behaviour reaches its peak. Our results, in contrast, indicate that hippocampal and septal activation may be independent of each other. This could be due to the substantial differences between the experiments, but it could also be that the volume of a brain area is not in any case a reliable indicator of its activity or involvement in a behavioural task.

In addition to the effect of the decision to use one or the other orientation cue, there was also a slight (non-significant) influence of the (flight) activity of the zebra finches in the course of the test trial. Zebra finches travelling longer distances tended to show higher brain activation. Under the areas measured, this was most obvious in hyperpallium apicale. This could be interpreted as an effect of the motor activity on the brain metabolism (by enhancement of the blood circulation, etc.). However, we prefer to interpret it as indicating that the strongest effect was within a visual area, as an enhancement of retinal activity by extended visual flow due to the longer travel times, which then in turn might also affect the activation of higher brain areas.

In any case, the weak effect of travel distance on c-Fos activation does not explain the differences between the two groups, as can easily be seen in Fig. 7, where the data points of the two groups are quite distinct. Also, the group effects cannot be due to an enhancement by general arousal because this should have led to a uniform enhancement of activation in all areas examined. We have instead observed a reciprocal activation within the hippocampus/parahippocampus and the LMN, and there was no activity difference between the groups in our control area, the preoptic area, which plays a role in controlling sexual behaviour (e.g. Balthazart and Ball, 2007; Panzica et al., 1996) but has not as yet been shown to be involved in orientation tasks.

If the hippocampus was activated, the activation was mainly restricted to the parahippocampus and the dorsal part of hippocampus proper (Fig. 5). There was no correlation to the subdivisions of the avian hippocampus described to date (reviewed by Watanabe, 2006). It may nonetheless be possible to speculate about the impact of our results on proposed homologies of avian and mammalian hippocampal subdivisions. For mammals, a widely accepted model relates the dentate gyrus to acquisition of information in learning of spatial tasks, while CA3 and, more especially, CA1 are thought to be involved in the recall of previously learned information (Jarrad et al., 1984; McClelland and Goddard, 1996; Lassalle et al., 2000; Daumas et al., 2005; Rolls and Kesner, 2006; Vago et al., 2007). In birds, there are two contrasting opinions concerning the position of the dentate gyrus within the hippocampal formation. From the results of tracing experiments and area identification attempts by (immuno)histochemical staining, Kahn and colleagues (Kahn et al., 2003) in the pigeon and Szekely and colleagues (Szekely and Krebs, 1996) in the zebra finch presumed the dentate gyrus to be located

information from the entopallium, was not activated. It should be noted that the activation of the entopallium for as yet unknown reasons cannot be determined by the expression of c-Fos or other immediate early genes. We have shown previously, using the ^{14}C–2-deoxyglucose technique, that the entopallium is active for example during sexual imprinting (e.g. Bischof and Herrmann, 1986), while it does not show any c-Fos or ZENK labelling in identical experiments (Lieshoff et al., 2004; Huchzermeier et al., 2006). 'Where' information had to be used in both choice groups to find the feeder and, accordingly, the system providing the 'where' information, hyperpallium apicale/hyperpallium densocellulare, was active in both situations. Most probably, the 'where' information originated from allocentric cues in the position group, and from

in the dorsomedial hippocampus, while Atoji and Wild (Atoji and Wild, 2004) saw the mammalian dentate gyrus as the homologue of the ventral part of the avian hippocampus, and CA1/CA3 as the homologue of the dorsal part. Our experiment is a recall task, and, according to the theory mentioned above, this activates the CA1/CA3 region in the mammalian cortex. This would favour the second theory (Atoji and Wild, 2004) locating the dentate gyrus within the ventral avian hippocampus and CA1/CA3 in the dorsomedial part. If one could show, in addition, that the ventral part is the more active in a learning situation, this would be an additional point in favour of the second theory.

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