Social Odour Activates the Hippocampal Formation in Zebra Finches (*Taeniopygia Guttata*)

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Abstract

Experiments from our research group have demonstrated that the olfactory sense of birds, which has been considered as unimportant for a long time, plays a prominent role as communication channel in social behaviour. Odour cues are used e.g. by zebra finch chicks to recognize the mother, by adult birds to distinguish their own eggs from others, or to recognize kin. While there is quite a lot of evidence for the importance of odour for social behaviour, it is not known as yet which brain areas may be involved in the processing of socially relevant odours. We therefore compared the brain activation pattern of zebra finch males exposed to their own offspring odour with that induced by a neutral odour stimulus. By measuring head saccade changes as behavioural reaction and using the expression of the immediate early gene product c-Fos as brain activity marker, we show here that the activation pattern, namely the activity difference between the left and the right hemisphere, of several hippocampal areas in zebra finch males is altered by the presentation of the odour of their own nestlings. In contrast, the nucleus taeniae of the amygdala (TnA) exhibits a tendency of a reduction of c-Fos activation in both hemispheres as a consequence of exposure to the nestling odour. We conclude that the hippocampus is involved in odour based processing of social information, while the role of TnA remains unclear.

Highlights

- Male zebra finches were exposed to own-offspring odour or neutral odour
- Offspring odour caused changes in arousal (head saccades)
- Brain activity was measured by immediate early gene (c-Fos) expression
- Social odour activated hippocampus in a lateralised way

Keywords: avian hippocampus, lateralisation, zebra finch, c-Fos, TnA, social odours**,** parent-offspring communication

Abbreviations: HpV - ventral hippocampus; HpDM: dorsomedial hippocampus; HpD: dorsolateral hippocampus; TnA: nucleus taeniae of the amygdala; c-Fos-ir cells: c-Fos immunoreactive cells.

1. Introduction

Most birds have been considered to be ‘relatively anosmic’ for a long time, mainly because the olfactory bulb of most birds is small [1] compared to that of mammals, which are known to have an excellent sense of smell. Recent studies, however, demonstrate that birds make frequent use of olfactory cues, for example when searching for food, navigating over long distances, or in a social context (for reviews see [2–5]). Also, the number of olfactory receptor genes, which is said to correlate with the scent detection abilities of a given species [6], varies between 150 and 650 in birds. As a comparison, the amount of olfactory receptor genes found in humans, 339, is approximately in the middle of this range [7]. This indicates that the performance of the sense of smell is not uniform among birds, and it may in many species be comparable or even superior to that of humans. The zebra finch genome even comprises 479 olfactory receptor genes, including 111 pseudogenes (genes that have lost some functionality), thus slightly more than humans [8]. The zebra finch, a gregarious-living songbird from Australia, has become an important model organism for research on the songbird specific song control system and the visual and acoustic sensory systems, all involved in communication [9]. Olfaction was not presumed to be important for zebra finch communication until Caspers and Krause [10] demonstrated that zebra finches are able to recognize the odour of the nest where they had hatched. Since then, a good amount of evidence has been accumulated [5] demonstrating that odour plays an important role for the guidance of zebra finch social behaviour. It has been shown, for example, that odour cues enable zebra finches to recognize kin [11], zebra finch females can distinguish between own and other zebra finch eggs by smell [12], mate choice is affected by conspecific odours [13] and zebra finch chicks recognize the odour of their parents [14]. Research on the neural mechanisms involved in avian odour perception is comparatively sparse (for a review see [5]). In addition to the gene studies, there is only a limited number of publications concerning the processing of odour within the brain. The function of the olfactory bulb and the general organisation of the olfactory system are quite well known and very similar to those of mammals. However, there is one difference that is important for our study: Birds lack a vomeronasal system, which is supposed to be the main recipient of pheromones [15, 16]. This partly accounts for the reduced relative size of the avian olfactory bulb in comparison to other vertebrates. The main olfactory bulb of birds, however, has been

shown to process pheromones as well as other odours [17]. In addition to the anatomy and function of the olfactory bulb, the main projections and brain areas receiving olfactory information from it have also been described, but there is only sparse information on the function of these areas.

In the present experiment, we were especially interested in evaluating the role of two brain regions, the nucleus taeniae of the amygdala (TnA) and the hippocampus, in the processing of social odours. TnA strongly influences sociosexual behaviour in zebra finches. For example, zebra finch males with TnA lesions were never chosen as sexual partners by a female in a triadic situation with another not lesioned male, suggesting that TnA plays an important role in the control of male-female and male-male interactions [18], and ZENK expression within TnA correlates with the frequency of social behaviour in females [19]. The nucleus receives a direct projection from the olfactory bulb [16] and is considered to be homologue to the mammalian subpallial medial amygdala [20–25]. Like its mammalian equivalent, it is rich in androgen and estrogen receptors [26–30]. Involvement of TnA in a wide range of sociosexual behaviours has also been shown in other bird species [20,31,32] and, due to its homology with the mammalian medial amygdala, it can be seen as a central structure of the so called "Social Decision Making Network" proposed by O´Connell and Hofmann [33]. Indeed, TnA is one of the structures connecting the two subsystems of this network evaluating stimulus salience and/or regulating social behaviour: the 'Mesolimbic Reward System' and the 'Social Behaviour Network' [34,35].

The role of the zebra finch hippocampus for visual orientation and spatial memory is quite well established (reviewed by Mayer et al. [36]). Its involvement in social odour processing, however, has not been investigated as yet. The avian hippocampal formation receives a direct projection from TnA [37,38] and by the prepiriform cortex [39]. Hippocampus is connected to the 'Mesolimbic Reward System', the neural circuit where the salience of external stimuli is evaluated and where appetitive behaviour seems to be regulated [40,41]. Because hippocampus and TnA are closely connected with each other and both are positioned within the 'Social Decision Making Network' as defined by O´Connell and Hofmann [33], we expected that we would find a change in activation of one or both of these brain areas when the experimental birds were exposed to a socially relevant stimulus like the offspring odour. This stimulus was

chosen because we, in another almost identical experiment, were able to show that zebra finch males can differentiate between odours of the own and unrelated offspring [44].

Activation of the brain areas was assessed using the expression of immediate early genes (IEG´s). IEG expression within cell nuclei increases rapidly in response to neuronal activation and also leads to long-term structural changes affecting signal transduction [43,44]. IEG products like the c-Fos protein can be detected by immunohistochemistry. The technique has been used frequently to study neuronal activation of specific brain regions in zebra finches [45–47] and in other birds [48–50]. The expression of c-Fos is involved in long-term neuronal modifications, which characterize neuronal plasticity underlying learning and memory [51–54]. Therefore, c-Fos baseline expression, e.g. at home cages, is typically low, whereas an experimental treatment induces higher expression in specific brain regions.

In addition, we measured a behavioural parameter to assess differences in odour recognition in adult males by counting head saccades of the birds in response to the odour stimulus. Experiments exposing zebra finches to a "novel object" indicated that the frequency of head saccades, which are fast horizontal movements of the head observed in many birds, correlates with arousal [55]. By measuring the frequency of these saccades in the experimental and the control odour groups, we wanted to obtain a behavioural measure of the strength of the arousal reaction of the birds exposed to the one or the other stimulus, which could then be compared with the brain activation measurements.

2. Material and Methods

For the present experiment, breeding pairs of zebra finches from the Institute´s stock at the Department of Bielefeld University were housed in separate cages (80 x 30 x 40 cm^3) until the offspring was independent. When the odour exposures were finished, males were perfused for the IEG immunohistochemistry. Females and offspring were returned to the laboratory stock aviaries when the offspring had reached independence.

2.1. Ethical Note

Housing and breeding of birds were approved by the Gesundheits-, Veterinär- und Lebensmittelüberwachungsamt der Stadt Bielefeld (#530.421630-1, 18.4.2002). Housing conditions are assumed to be superior to natural conditions because all animals had *ad libitum* access to water and food plus additional vitamins, germinated seeds and egg food (CéDé, Evergem, Belgium) which were given daily once the first chick hatched. The light cycle was 14h light: 10h dark. Chickweeds and a water bath were provided once a week. All animals and their offspring were checked daily to verify that the individuals were healthy. Experimental protocols were approved by the responsible state authority (LANUV NRW (# 84 02.05.40.17.009). Behavioural tests and perfusions were made at the Department of Animal Behaviour, Bielefeld University. Brain sectioning and immunohistochemistry was performed at the Center for Mind/Brain Sciences, University of Trento, Rovereto, Italy.

2.2. Experimental setup

The experiment took place in the same room in which the birds were housed to minimize relocation stress. The mate and the offspring of the experimental male were brought to an adjacent room for the duration of the test to avoid acoustic contact with familiar individuals. The experimental cage (Figure 1) was 28cm wide, 20cm in length and 51cm high, with two wooden perches 15cm above the floor and a feeder on the floor. The short sides and the rear of the rectangular cage were solid and wooden, the front part contained a grid to enable view to the room, like the housing cages. At the back wall a wooden nest box was attached (15 x 15 x 15cm), filled with coconut fibres and identical to the one in the breeding cages, but access was prevented by a wire mesh. The back of the nest box had a round opening (diameter 7.5cm) with a fan mounted behind (Sunon 40 x 40 x 10cm, 12 volt reduced to 9 volt). The odour stimulus sample (see below) was placed between the fan and the nest box. The fan generated a constant airflow transporting the odour across the nest box into the cage [12]. To facilitate the recording of the bird´s head saccades, a small triangle of reflectance foil was fixed on the head feathers of the male. The foil was easily removable at the end of the experiment, and did not lead to visible behavioural changes. The behaviour of the bird was recorded by a video camera (Panasonic WV-BL202-E CCTV Camera) from above, and recordings were stored on a laptop computer using an USB drive adapter (Swann 4CH SW 24I-UD4 N3960).

178 *2.3 Stimulus preparation*

179 To get an odour sample of the chicks, we used dark cotton nylon socks (nylon socks,
180 63 % polyamide, 37 % cotton, Söckchen Naturelle 60, NUR DIE, DBA Deutschland
181 GmbH, Rheine, Germany), which were regularly used as a transport bag. These nylon
182 socks were only used for these experiments and were cleaned for each experiment
183 with a perfume free soap (Eubos liquid soap). The soft material of the socks snuggles
184 closely to the chicks' body and absorbs the body odours as shown in previous
185 experiments [14,56,57]. Chicks were placed in these nylon socks for half an hour, and
186 then returned to their natal nest boxes. To standardize the amount of odour transferred
187 into the socks, the number of nestlings placed in the sock was chosen according to the
188 body weight (i.e. in case nestlings were light, two or once three offspring were used).
189 The total mass of chicks within each sock was on average 8.73±1.64g (mean±s.d.),
190 with on average 1.57±0.72 nestlings. The odour impregnated empty socks were then
191 used as odour stimuli within the next 2-5 minutes. Controls were made by placing
192 cleaned socks without a chick odour in front of the fan.

193 *2.4. Experimental procedure*

194 The experiments were executed when the brood, which was fathered by the
195 experimental male, was on average ten days of age. At this time, young zebra finches
196 are still nestlings and do not leave the nest, which happens usually around day 19.
197 Clutches were reduced early in the incubation period to two to three fertilized eggs so
198 that the females after removal of the male had to rear only a few hatchlings that
199 developed normally thereafter. On average, 2.88 ±1.08 (mean±s.d.) nestlings fledged
200 successfully and survived until independence.

201 A total of 15 male zebra finches were divided in two groups: the experimental group
202 ("own chick odour group", n=8), which received the own chick odour stimulus and the
203 "control group" (n=7), which were exposed to a neutral odour stimulus, i.e. empty sock.
204 Before the experiment, individuals of both groups were treated in exactly the same
205 way, all of them fathered and reared offspring up to the day of the experiment.

206 Prior to the test, the animals were allowed to habituate to the experimental cage for 5
207 hours. Then, the odour of the own offspring or the control stimulus was presented for

one hour, and at the end of the session, the bird was perfused and the brain processed for immunohistochemistry (Figure 2a). Both habituation and test were conducted under normal light conditions during the day. The duration of the habituation time was chosen because of the properties of c-Fos expression: Typically the c-Fos protein level reaches a peak at about 1-2 hours after neuronal activation and returns to the baseline level 6 hours later [58]. Thus, at the time the birds were perfused, 6h after being placed in the experimental cages, the c-Fos expression induced by the handling procedure was reduced to the baseline, whereas the c-Fos expression induced by the stimulus presentation (1h prior to perfusion) was at peak levels.

The behaviour of the birds was observed using video recordings. Video recordings started 1h before the odour presentation and continued for 2h until the end of the experiment (Figure 2a). From the videos, head saccades were counted by a person blind to the experimental condition and to the stimulus provided. A head saccade had been defined as a rapid horizontal movement of the head to either side by more than ten degrees. A measurement of the number of head saccades without a stimulus was made directly after starting the recording session, and another one after starting the stimulus presentation. For both, baseline and stimulus, the number of head saccades was counted within ten consecutive intervals of one minute each. These ten counts were then averaged to obtain the number of head saccades per minute.

To normalise for differences of the basic activity level of the individual birds, a head saccade change score (Hs) during stimulus presentation was calculated in relation to the baseline measurement:

$$Rate\ of\ change\ in\ head\ saccades = \left(\frac{\#\ Hs\ stimulus}{\#\ Hs\ baseline} * 100\right) - 100$$

Positive values indicate an increase in head saccades after the stimulus presentation, whereas negative values represent a decrease and 0 indicates that the number of head saccades was unchanged between the two phases. The rate of change was used for the comparison of the two groups exposed to the different odour stimuli.

Furthermore, we developed a MATLAB routine to count head saccades from the video recordings, and compared the manual with the automated counting. However, only ten

of the fifteen males could be analysed by the software, as four individuals lost their tracking marker (triangular reflectance foil on the head) during the test and a fifth individual spent most of the time in the border region of the cage and was thus not trackable by the program. We used both measurements (baseline and stimulus) of each of the ten trackable birds and compared these twenty countings. Both procedures provided similar results and were significantly correlated ($R_{Pearson}$=0.66, t=3.7332, df=18, p<0.002, see supplement). The rate of change in head saccades using the MATLAB routine was on average -15.26 ± 20.6 (mean±s.d.) for the males receiving their own chick odour (n=5) and 41.81± 65.8 for the males receiving a control odour (n=5). The difference between the two treatment groups was also significant using the MATLAB based counting (U=6, n=10, p=0.017). Because the manual and the automatic counting method correlated and yielded the same results, the automated measurements are not described in the results section in detail.

2.5. Immunohistochemistry

Sixty minutes after stimulus presentation the male zebra finches were overdosed by an intramuscular injection of 0.03ml Narcoren (Rhone Merieux, Laupheim, Germany; 30% in 0.9% sodium chloride) and perfused transcardially via the left ventricle with cold (4°C) phosphate-buffered saline (PBS; 0.1mol, pH=7.4, 0.9% sodium chloride) for 7 minutes, followed by 4% paraformaldehyde (PFA) in PBS for additional 7minutes. The head was severed from the body, the skin was removed and the head was stored in 4% PFA at the refrigerator for 4-6 weeks until processing. For removal of the brains from the skulls, the heads were oriented at 45° in a stereotaxic head holder specifically designed for zebra finches [59]. The caudal part of the skull was opened and a coronal plane cut (0.5mm posterior to the Y-point) was made with a scalpel blade attached to a micromanipulator. This plane surface was used for freezing of the brains to ensure that the coronal brain sections of all brains had the same orientation and would correspond to the atlas drawings [60]. The left and the right hemispheres were then separated and processed separately. Each hemisphere was embedded in gelatine (7%) containing egg yellow, post-fixed for 48h in 4% PFA/ PBS containing 20% sucrose at 4°C, and further 48h in 30% sucrose in 0.4% PFA/ PBS. The brains were frozen in a refrigerator at -80°C covered with OCT (Tissue-Tek freezing medium) and cut at -20° in a Cryostat (Leica CM1850 UV). Three series of 40μm were cut, the sections of the first series were collected

for c-Fos labelling, whereas the other series were kept as backup and for testing antibody specificity (processing without the primary antibody). Between each of the following reaction steps, washing in PBS (3 x 15 sec + 3 x 5 min) was performed. After the endogenous peroxidase activity was depleted in 0.3% H_2O_2 in PBS for 20min, blocking of unspecific binding was performed by a 30min incubation with 3% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS. The anti-c-Fos antibody solution (1:2000; made in rabbit, K-25, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 48h at 4°C, which was followed by an incubation with the biotinylated anti-rabbit in PBS (1:200; BA-1000; Vector Laboratories) for 60min at room temperature. ABC kit (Vectastain Elite ABC Kit, PK 6100; Vector Laboratories) was used for signal amplification and VIP substrate kit for peroxidase (SK-4600; Vector Laboratories) for visualization of c-Fos-immunoreactive (-ir) neurons. Sections were serially mounted on gelatine-coated slides (Gatenby slides), dried at 50°C, counterstained with methyl green (H-3402; Vector Laboratories) and cover slipped with Eukitt (FLUKA).

2.6. Brain Analysis

Brain sections were examined with a Zeiss microscope (objective: 20x with a numerical aperture of 0.5) and a digital camera (Zeiss AxioCam MRc5). Counting of the c-Fos immunoreactive neurons was performed blind to the experimental condition on the computer screen with Zeiss imaging software (ZEN). Here, after the image on the computer screen was matched in contrast, colour and cam exposure time to the view under the microscope (eyepiece 10x, overall magnification 200x), every c-Fos-ir cell within a sample area (see below) was marked with the 'event marker' of the ZEN software, which automatically provided the total counts.

To estimate labelled cell density within hippocampus, five sections of each hemisphere were selected from the part of hippocampus extending from A(nterior) 2.79 to A2.07. The sections corresponding to these coordinates have been determined under microscope by visual observation, based on the shape and anatomical organization of the hippocampal sections, which had to match those visible in the zebra finch atlas for the above mentioned coordinates [60]. The hippocampal area of each section was divided into three subdivisions: the ventral hippocampus (HpVM), the dorsomedial hippocampus (HpDM) and the dorsolateral hippocampus (HpDL) (Figure 3a). For counting, a rectangular square

of 150 x 250μm was positioned over the area comprising the highest density of c-Fos-ir neurons within a given subdivision, while keeping a minimum distance of at least 20μm from the border of a neighbouring subdivision and the edge of the brain section. The location of the area with the highest density was determined by visual inspection under the microscope by the experimenter who was blind to the experimental group. Typical placements for each of the three subdivisions are schematically shown in Figure 3a. Labelled cells in TnA were counted from three sections of each hemisphere, selected from the region corresponding according to shape and anatomical landmarks with A 1.08 of the zebra finch atlas [60]. Labelled neurons were marked within a counting square of 250 x 250μm, which was positioned over the area with the highest number of c-Fos-ir cells within the borders of TnA (see Figure 3b). After completing the cell counts, means were calculated for each brain region/subdivision in both hemispheres of all animals, and cell densities were standardized to 1 mm2. The resulting individual area means were employed for further statistical analysis.

2.7. Statistical Analysis

In the behavioural part of the experiment, every bird was tested once, in one of two different treatment groups. To compare the two groups, we used t-tests for independent data. To evaluate the significance of the head saccade changes within each group, we used one-sample t-tests. The presence of difference in the density of c-Fos-ir neurons was tested by repeated measures ANOVA, with brain area and hemisphere as within-subjects factors and group as a between-subjects factor. For post hoc analyses of the hemispheric differences within the two groups, paired t-tests were carried out for each brain area (HpV, HpDM, HpDL, TnA). To account for brain area-specific differences in lateralisation between the two groups, a lateralisation index (left minus right) was computed for each brain area. To confirm the region specific lateralisation differences between the groups, a repeated measures ANOVA, with the factors brain area and group, was performed on the lateralisation index data. For post hoc analyses of group differences, t-tests were carried out for each brain area. All statistical tests were two-tailed. Statistical analysis was performed with the software IBM SPSS Statistic for Windows (Version 22.0).

3. Results

Our analysis found that the change of head saccades of the 'own offspring odour group' was significantly lower compared to the 'control group' (independent t-test: $t(13)=-2.685$; $p=0.019$, Figure 2b). A significant decrease in number of head saccades was observed in the 'own offspring odour group' after stimulus presentation (N=8; change in head saccades: -14.92%±6.4 (mean±s.e.m.); one sample t-test against '0': $t(7)=2.4970$; $p=0.041$). Such difference was not present in the 'control group' (N=7; change in head saccades: 11.97%±7.9; one sample t-test against '0': $t(6)=1.6495$; $p=0.150$).

We processed the brains of all 'own offspring odour group' (n=8) and 'control group' (n=7) zebra finch males. The nuclei of the c-Fos-immunoreactive (c-Fos-ir) cells were coloured in black by the VIP stain and could thus easily be distinguished from the non-activated cells, which were stained light green due to the methyl-green counterstaining (Figure 3). We quantified the density of c-Fos-ir cells in three subdivision of the hippocampus and also in the nucleus taeniae of the amygdala for the two hemispheres separately (Table 1). In TnA, there was no difference of c-Fos labelled neurons between hemispheres in either experimental group. Within hippocampus, the highest densities were present in the dorsolateral region (HpDL), slightly lower counts were obtained in the dorsomedial hippocampus (HpDM) and the lowest densities were measured in the ventral hippocampus (HpV). More importantly, the hippocampus in the control group, i.e. of birds perceiving no odour stimulus, showed a lateralized expression of c-Fos: The densities of the labelled cells within the hippocampus of the control group were between 33–75 % lower in the right hippocampus compared to that of the left hemisphere. However, in the experimental group exposed to the own offspring odour this hemisphere difference disappeared (Table 1).

Repeated measures ANOVA revealed a significant interaction of *Brain Area* × *Hemisphere* × *Group*: $F_{(1.494,19.425)} = 4.333$; $p = 0.037$ (Greenhouse-Geisser correction was applied, because Mauchly test revealed a significant violation of sphericity $p=0.001$). In the control group, post hoc t-tests revealed significant density differences between the right and left hemisphere in the HpDM ($T_{(6)}=3.175$, $p=0.019$) and in the HpV ($T_{(6)}=3.108$, $p=0.021$), and non-significant trends within HpDL ($T_{(6)}=2.283$, $p=0.06$) and TnA ($T_{(6)}=2.017$, $p=0.09$). Such differences between the hemispheres were not present in the odour stimulus group (HpDL $T_{(7)}=-0.450$, $p=0.667$; HpDM $T_{(7)}=-1.349$,

p=0.219; HpV $T_{(7)}$=0.934, p=0.381; TnA $T_{(7)}$=1.303, p=0.234). Also, the differences between the two groups within either hemisphere were not significant (Left hemisphere: HpDL T(13)=-0.728, p=0.479; HpDM T(13)=-0.074, p=0.942; HpV T(13)=0.274, p=0.788; TnA T(13)=-0.567, p=0.580; Right hemisphere: HpDL T(13)=-1.116, p=0.285; HpDM T(13)=1.335, p=0.205; HpV T(13)=1.266, p=0.235; TnA T(13)=-0.431, p=0.674)

To compare the differences in lateralization between the groups, a lateralization index (number of activated neurons in the left hemisphere minus those of the right hemisphere) was calculated (Figure 3). A repeated measures ANOVA with these data confirmed that the groups were different in a region-specific manner by showing a significant interaction of *Brain Area* × *Group* ($F_{(1.494, 19.425)}$=4.3333; p=0.037). Post hoc analysis revealed a significant difference between the groups in the HpDM ($T_{(13)}$=-3.069; p=0.01, figure 3c), as well as marginally non-significant trends in the HpDL ($T_{(13)}$=-2.283; p=0.052, figure 3d) and in the HpV ($T_{(13)}$=-2.018; p=0.065, figure 3b). In all these three areas, the lateralization index was smaller in the treatment group receiving the own offspring odour, due to an increase in activation of the right hemisphere after stimulus exposure (Figure 3b,c,d).

In contrast, no differences between the groups were present in TnA ($T_{(13)}$=-0.243; p=0.812, Figure 3e). However, there was a correlation of the number of c-Fos-ir cells in TnA and the number of saccadic head movements in the control group ($R_{Pearson}$=0.85, T=3.66, p=0.015), but not in the group exposed to the own offspring odour ($R_{Pearson}$=0.41, T=1.13, p=0.30).

4. Discussion

The results of the present study demonstrate that zebra finch males are able to differentiate offspring odour from other non-social odour cues. Head saccade frequency (reflecting arousal) was significantly affected by the presentation of a social odour, such as that obtained from the ‘own chicks’ bodies. On the contrary, the non-social odour provided by the empty sock to the control group did not elicit significant changes in the head saccades. Thus, we can conclude that social odours specifically affect zebra finches’ arousal, which is not the case for non-social odours. Our experiment also shows that offspring odour cues induce changes of the hippocampal

activation patterns and, at least tentatively, that of TnA. As mentioned above, TnA receives direct input from the olfactory bulb [16] and has a central position within the 'Social Decision Network'. As in the other brain areas investigated here, the number of c-Fos-ir cells within the TnA was lateralised in the control group, i.e. higher in the left than in the right hemisphere. In contrast to the other regions, this TnA lateralisation was also present in the birds exposed to the offspring odour (Figure 3e). Regardless of lateralization, in the 'own offspring odour group' in both hemispheres there was a slight, non-significant, reduction of the density of activated cells (Table 1). Moreover, the correlation between head saccades and IEG expression, which could be shown in the control group, but not in the birds exposed to the offspring odour, suggests that TnA activation was in some way affected by the own offspring odour and that TnA could be involved in odour-induced brain activation. However, it should be kept in mind that, due to a lack of significant group differences, considerations on the role of TnA are at present quite speculative. Additional experiments may clarify the as yet not fully understood role of this nucleus.

More specific effects were present in all three subdivision of the hippocampus. The significant differences in the lateralisation pattern of hippocampal activation between the two odour groups confirm that male zebra finches were able to recognise the presence of the offspring odour. At present, we can only speculate about the interpretation of this result. There is quite a lot of evidence for a participation of olfactory cues in pigeon navigation [61,62], and there is no doubt that the hippocampus is important for spatial memory and navigation in all vertebrates [63-64]. Moreover, in rodents odour information contributes to the response properties of place cells in the hippocampus [66], but see [67]. The role of hippocampus for spatial orientation has been demonstrated also in zebra finches [36]. In a series of experiments using immediate early genes as neuronal activity markers, these authors showed that the avian hippocampal formation is not only involved in spatial information learning, but also in the recall of spatial memory [46,47].

However, the present experiment did not involve a spatial orientation component. The zebra finch males were exposed to the familiar own offspring odour or to a neutral odour within exactly the same environment. Therefore, the difference in hippocampal activation pattern may reflect the recall of previously stored memories. The results from

the present study seem to support the idea that hippocampus is not only the location where cognitive maps are built and processed, but is also involved in the formation and recall of declarative or episodic memory [65]. This type of memory is said to store information about what has happened at a certain time at a certain place. It is conceivable that olfactory information could be an important aspect to describe such episodes, as it has been suggested for rats [66]. Probably, the role of the hippocampus for the construction of these episodes might be to encode the spatial context associated with the actual socially arousing odours. Such a view is supported by the connectivity pattern of the hippocampus, suggesting that it is strongly involved in social behaviour control. The hippocampus is connected with the core regions of the reward system [33] and also receives direct input from the TnA, which connects the two subsystems of the 'Social Decision Making System' [33], 'The Mesolimbic Reward System' and the 'Social Behaviour Network' and sends several afferents to the Septum, another very important node of the same network [33,34]. The involvement of the hippocampus in social control has also been shown with immediate early gene studies in zebra finches [69-71]. Hippocampal c-Fos expression was upregulated after presentation of conspecific song in female zebra finches [69,70]. Also, in the course of sexual imprinting, the dorsomedial part of the left hippocampus showed strong c-Fos activity, while the right one was almost inactive [71].

Lateralisation of brain activation in birds has been demonstrated frequently [72–83]. For instance, a rightward bias in the integration of visual and olfactory non-social cues has been found in chicks [84]. In zebra finches, several cases of a lateralization pattern have been demonstrated by both, behavioural and IEG-expression studies, which are compatible with that found in other species (reviewed in [85]). This includes a right hemisphere specialisation for monitoring threatening stimuli [85], a left hemisphere specialisation for controlling foraging behaviour [86] and complementary specialisation of the two hemispheres for different aspects of song processing [87-89]. In the present experiment, the activity differences between the left and the right hemisphere in the 'own offspring odour group' were reduced compared with the 'control group'. This effect can be explained by an enhancement of the activation of the right hemisphere in birds receiving the social odour (see Table 1). This counteracted the normal, baseline, lateralization pattern, which is visible in the control group and in TnA (i.e., higher activation of the left hemisphere). A similar trend for a spontaneously higher c-Fos

expression in the left hemisphere has been reported in two previous studies on septal activation in domestic chicks exposed to visual social stimuli [90,91]. In this case, the septum of the control group showed a spontaneous left lateralisation, and an increase of activity of the right hemisphere septum equalized the activation of the left and the right septum of chicks exposed to a social stimulus. Accordingly, in a study on olfactory lateralisation in domestic chicks, the right hemisphere has been found to be important for recognising the familiar artificial imprinting objects (a red cylinder hanging in the chicks' rearing cage, containing an odorant substance) [76]. When two such visually identical objects were presented, chicks were able to use their right nostril (and so with direct olfactory input to the right hemisphere) chose the one that smelled like the imprinting object, whereas chicks with their left nostril choose at random. These findings support the general idea of a preferential involvement of the right hemisphere in social responses, social recognition and the rapid recognition of emotional stimuli [72,73,75–79,80,92–96]. Thus, the current study is in agreement with the previous literature in confirming both the presence of a task-independent, leftward lateralization of c-Fos expression in the brain of different bird species, and in implicating the right hemisphere in social odour processing. Why in the previous literature, sexual imprinting, which is learning in a social context, increases the activation of the left hippocampus, has to be determined. It might be associated with the left hemispheric dominance found in zebra finches for courtship behaviours [97-100], as testified both by behavioural and IEG-expression studies [101,102]. This has been explained based on the ability of the left hemisphere to sustain attention towards a preferred and familiar stimulus, towards which a motor response is planned [83, 101,103-104].

The idea to use fast saccadic head movements as an indicator of arousal was based on the observation that birds appear to increase the frequency of these movements in arousing situations, e.g. noise from outside the cage. As shown in this paper, this measurement is useful to determine whether an experimental animal has perceived an odour stimulus. Whether it is also useful in other perceptual contexts has to be determined. Saccades, i.e. the fast movements of the eyes or the head, or a combination of both, are the common strategy of vertebrates for changing their gaze from one fixation point to the next [105,106]. In contrast to slow scanning movements of the eye, which lead to a constantly moving, blurred image on the retina, the saccade-fixation strategy optimizes the time a certain image can be fixed without blur, and

minimizes the time for a change between consecutive targets. While the general strategy is similar between vertebrates, and neurobiological experiments indicate that there is a strong coupling of head and eye movements, there are differences between species in the proportion of head and eye movements. Birds have been shown to use mainly head movements for gaze changes, although eye movements cannot be neglected (e.g., 80-90% of gaze changes in pigeons are due to head movement, [107]). Based on the assumption that both head and eye saccades have the same motivation, we assumed that a change in head saccades in birds indicates changes of the arousal level, as suggested for humans [108,109]. This is supported by experiments with peacocks showing that head saccades are altered after presenting a predator [110]. If this interpretation is true, the arousal level of our experimental males was reduced when they were exposed to the odour of the own offspring. This gives way to a lot of speculations; further experimentation also with stimuli from other sensory domains is necessary to create a solid basis for a connection between the amount of head movement and arousal.

4.1. Conclusion

Our experiment provides the first hints of how social odour cues may be processed within the avian brain. The results indicate that the 'Social Decision Making Network' receives olfactory information, in addition to other sensory cues. While the role of TnA remains unclear, the hippocampus is obviously involved in social odour processing in a lateralised way. It is tempting to speculate that odours are also included into the information about events stored in episodic memory. As ever, our results cause more questions than they solve. The spectrum of odours eliciting brain activation has to be broadened to see whether there are differences in responses due to familiarity and specific social relations (e.g. kin vs. non-kin). It is also urgent to broaden the number of brain areas examined for responses to odours, within and outside the 'Social Decision Making Network'. Our study has shown that the zebra finch is an ideal subject for such studies, and that our experimental setup is well suited to answer the questions raised above.

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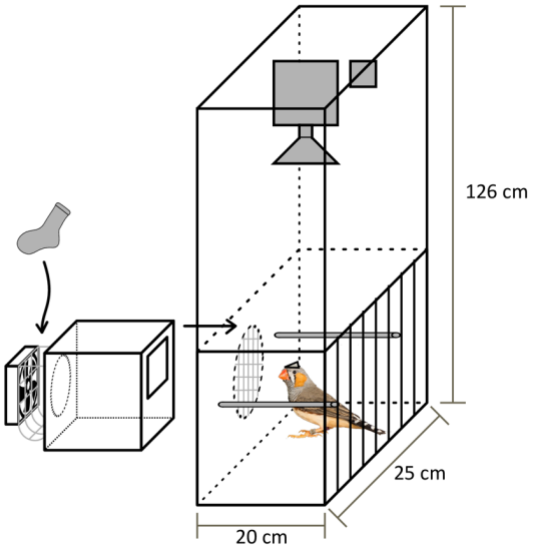

Figure 1: Schematic representation of the experimental set up. On the left, a nest-box connected to a ventilator was attached to the experimental cage (a grid prevented the finch from entering the box). The odour stimulus (the sock) was placed between the box and the ventilator. On the right, a zebra finch male is shown inside the experimental cage, which contained two perches. One of the long walls of the experimental cage consisted of a grid that enabled the zebra finch male to see the room, as the animals were used to in their holding cages. Above the experimental cage, an additional compartment contained the infra-red camera and an infra-red light source, for recording the subject's behaviour. On the finch head a triangular tracking marker, made

of infra-red reflecting tape, has been attached, allowing automated counting of the head saccades.

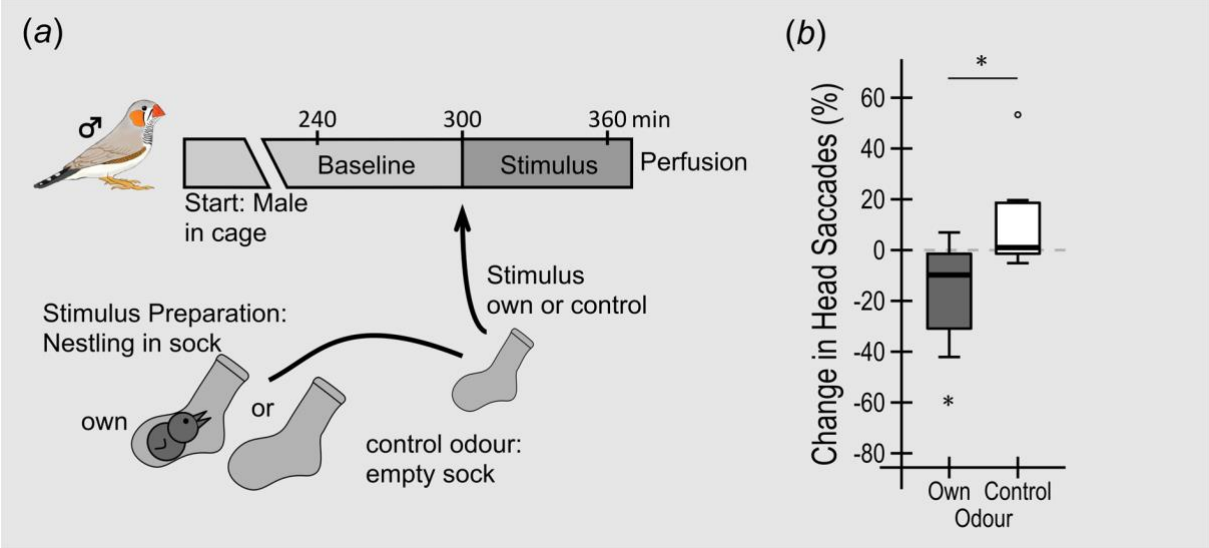

Figure 2: Experimental procedure (a) and results of the behavioural experiments (b). Male zebra finches received either the odour of the own nestling (n=8), or a control, neutral odour (empty sock, n=7). In the results, the y-axis represents the change in the rate of head saccades (in %) between the baseline measurement and after stimulus presentation. Positive values indicate an increase in the rate of head saccades after the stimulus, negative values represent a decrease. Data is given as boxplots with median and quartiles. The circle represents an outlier, whereas an asterisk represents a significance difference between the two groups or departure from the no-change level (0). Significance level was set up to with $p<0.05$.

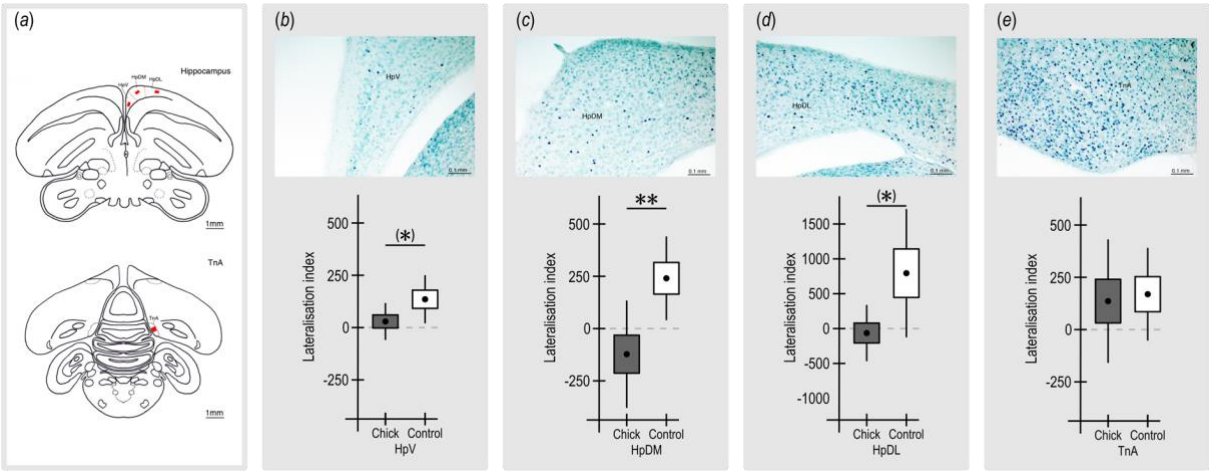

Figure 3: (a) Typical placements of the cell count zone (red) in coronal sections for the three subdivision of hippocampus (HpV, HpDM, HpDL) and TnA. (b) - (e) Upper panel: Photomicrographs showing typical examples of c-Fos-ir cell distribution patterns within the three subdivision of hippocampus and TnA of the own odour treatment. c-Fos-ir cells are stained black after the immunohistochemical procedure. The non-activated cells are counterstained in green. Lower panel: Lateralisation of c-Fos-ir densities after presentation of an own nestling odour (dark grey box) or a neutral control stimulus (white box). Lateralisation index represents the difference in number of c-Fos-ir cells between left and right hemisphere (see methods). Positive values of the lateralisation index indicate a stronger response of the left, negative values correspondingly of the right hemisphere. Dots represent the mean, boxes the standard error and vertical lines give the range of the standard deviation. Two asterisks represent significant differences (p=0.01) and one asterisk in brackets indicates a trend ($0.05 < p < 0.07$). HpV - ventral hippocampus; HpDM - dorsomedial hippocampus; HpD - dorsolateral hippocampus; TnA - nucleus taeniae of the amygdala.

Table 1: Measured densities of c-Fos-ir cells/mm^2 (mean ± s.e.m) within the three hippocampal subdivisions and nucleus taeniae of the amygdala (TnA)

	Offspring odour (n=8)		Control (n=7)	
	Left Hemisphere	Right Hemisphere	Left Hemisphere	Right Hemisphere
HpDL	1395.8 ± 333	1460 ± 326	1780 ± 389	985.7 ± 242
HpDM	705.8 ± 126	828.3 ± 210	720 ± 135	479.7 ± 133
HpV	395 ± 100	365.8 ± 101	361.3 ± 61	226 ± 43
TnA	956.7 ± 217	820.7 ± 244	1122.3 ± 177	953 ± 161