

COGNITIVE NEUROSCIENCE

The motion of a living conspecific activates septal and preoptic areas in naive domestic chicks (*Gallus gallus*)

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Keywords: avian, early social behaviour, immediate early genes, preoptic area, septum

Edited by John Foxe

Received 3 October 2016, revised 15 November 2016, accepted 15 November 2016

Abstract

Predispositions to attend to animate objects are ubiquitous in newborn vertebrates, but little is known about their neural bases. In this study, we wanted to know if exposure to the motion of a living, behaving conspecific will selectively activate septal, preoptic and amygdaloid areas in visually naive domestic chicks. For this purpose, newly hatched chicks were exposed to a live conspecific, whose natural motion presents of course several features typical of animate motion to which chicks are known to be sensitive. In the control group, chicks were exposed to a rotating stuffed chick that showed rigid non-biological motion. The two stimuli were visually matched with regard to their static features. We measured brain activity by visualizing the immediate early gene product c-Fos with a standard immunohistochemical procedure. Notably, dorsal right septum and left preoptic area showed higher activation in experimental subjects compared to the control animals. This is, to the best of our knowledge, the first demonstration of septal and preoptic areas involvement in response to the animate motion of a social partner, as opposed to rigid motion of a similarly looking stimulus. Moreover, these results indicate that previous visual experience and specific learning events are not necessary to establish the septal and preoptic areas function, which is present shortly after birth.

Introduction

Early social behaviours are believed to play a crucial role in the ontogenesis of social cognition, in both human and non-human primates (Johnson, 2005; Sugita, 2008; Simion *et al.*, 2011) and in avian species (Mascalzoni *et al.*, 2010; Rosa-Salva *et al.*, 2010, 2011, 2012, 2015, 2016). Newborn vertebrates of these distant species show similar predispositions to preferentially approach or look at socially relevant stimuli, which can be detected by the presence of some general configuration of static features (e.g. the typical face-like configuration in the head region) and dynamic cues distinguishing animate motion from that of inanimate objects (for a review see Rosa Salva *et al.*, 2015). These social predispositions are active since birth and, at least in animal models, emerge in the absence of any specific learning experience. One of their adaptive functions might be to guide the action of learning mechanisms, such as imprinting, towards appropriate social objects, which are important for shaping the normal development of social function and the related neural circuitry (Johnson, 2005; Rosa Salva *et al.*, 2015; Di Giorgio *et al.*, 2016a,b; Di Giorgio *et al.*, in press). Domestic chicks are an ideal model to investigate early social responses. Being the precocial offspring of a social species, chicks can be tested for social behaviours soon after hatching, with strict control of pre- and post-hatching experiences. Before any learning occurs, chicks are

attracted by animate stimuli, which will ensure subsequent imprinting towards appropriate social objects (Bateson, 1966; Rosa Salva *et al.*, 2015).

The detection of social stimuli based on motion cues that are typical of animate creatures is of particular importance for this study. Research in naïve chicks and human newborns revealed spontaneous preferences toward biological motion displays characterized by semi-rigidity, compared to rigid or random motion (Vallortigara *et al.*, 2005; Vallortigara & Regolin, 2006; Simion *et al.*, 2008). Animate motion can also be recognized because of its self-propulsion, which reveals the presence of an internal energy source to the moving object, as opposed to the motion of inanimate creatures caused only by external forces. Cues of self-propulsion, such as speed changes or starts from rests (autonomous initiation of movement from a resting state), increase the perception of animacy in human observers (Tremoulet & Feldman, 2000) and are preferred by infants (Frankenhuis *et al.*, 2013) and newborn babies (Di Giorgio *et al.*, 2016a,b). Also visually naïve newly hatched chicks show preferential imprintability toward objects that perform starts from rest (Mascalzoni *et al.*, 2010) and spontaneously prefer a simple object that autonomously changes its speed (accelerating and then decelerating back to the initial speed) to an identical one that moves at constant velocity (Rosa-Salva *et al.*, 2016).

The neural basis of early social predispositions is mostly unknown. However, social behaviour in mammals and birds often

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involves brain areas that are nodes of the social behaviour network (Newman, 1999; Goodson, 2005; O'Connell & Hofmann, 2011). These nodes, by definition, are in control of multiple forms of adult social behaviour, they contain sex steroids receptors and are reciprocally interconnected (Newman, 1999). As a first step to investigate the role of this network also in early social behaviours of newborn animals, we have demonstrated that a brief exposure of visually naïve chicks to a live, behaving conspecific selectively activated septal and amygdaloid areas [arcopallium and nucleus taeniae (TnA)], revealing their involvement in responses to social companions directly after birth (Mayer *et al.*, 2017). The septal nuclei are an evolutionarily well-conserved part of the limbic system, present in all vertebrate groups (Northcutt, 1981). Similar to the mammalian septum, its avian homologue is located medial to the lateral ventricles and it also shares the connectivity profile of the mammalian septum, with massive inputs from the hippocampus and projections to the hypothalamus and mid-brain (Krayniak & Siegel, 1978; Atoji & Wild, 2004; Montagnese *et al.*, 2004, 2008). It contains dense dopaminergic fibres (Bailhache & Balthazart, 1993) and shows subdivisional organization that resembles that of the mammalian septum (Goodson *et al.*, 2004a). Research on the social functions of the avian septum have also uncovered similarities to mammalian species (Goodson, 2005; Goodson & Kingsbury, 2013). In adult birds, septal nuclei participate in territorial behaviour, aggression, dominance hierarchies, individual discrimination, social communication, pair-bonding, gregariousness, appetitive and consummatory sexual behaviours (Ramirez *et al.*, 1988; Goodson, 1998a,b, 2005; Goodson & Adkins-Regan, 1999; Goodson *et al.*, 1999, 2004a,b; Goodson *et al.*, 2005a,b; Tomaszycski *et al.*, 2006; Goodson *et al.*, 2009; Montagnese *et al.*, 2014; Nishizawa *et al.*, 2011; Klatt & Goodson, 2013; Kelly & Goodson, 2014; Taziaux *et al.*, 2006, 2008; Nagaranjan *et al.*, 2014).

The homology of the amygdala in birds is less clear. However, although controversial, most literature agrees that at least part of the arcopallium in birds is homologous to the pallial part of the amygdala in mammals (Martínez-García *et al.*, 2007; Butler *et al.*, 2011), whereas the TnA within the posterior and medial arcopallium, is homologue to the subpallial, medial amygdala of the mammals (Cheng *et al.*, 1999; Reiner *et al.*, 2004; Jarvis *et al.*, 2005; Yamamoto & Reiner, 2005; Yamamoto *et al.*, 2005). TnA receives direct inputs from olfactory bulb (Reiner & Karten, 1985; Swanson & Petrovich, 1998) and sends hippocampal and hypothalamic output (Casini *et al.*, 1986; Canteras *et al.*, 1997; Cheng *et al.*, 1999). Like its mammalian counterpart, this area shows an enrichment in androgen and estrogen receptors (Martínez-Vargas *et al.*, 1976; Balthazart *et al.*, 1992, 1998b; Bernard *et al.*, 1999) and is in control of different social functions, such as sexual behaviours and social interactions (Thompson *et al.*, 1998; Cheng *et al.*, 1999; Absil *et al.*, 2002; Ikebuchi *et al.*, 2009). In an altricial species, the zebra finch, TnA can already be delineated at post-hatching day one (Ikebuchi *et al.*, 2013), which supports the idea that the early development of TnA is necessary for social control already at the time of hatching.

The avian preoptic area (POA) of the hypothalamus represents another important node of the social behaviour network (Newman, 1999). This area is rich in sex steroid receptors (Balthazart *et al.*, 1998b; Gahr, 2001) and plays a conserved role in aggression, parental care, male sexual behaviour as well as in sexual behaviour (Akerman *et al.*, 1960; Balthazart & Surlemont, 1990; Slawski & Buntin, 1995; Balthazart *et al.*, 1998a; Ritters *et al.*, 1998; Ruscio & Adkins-Regan, 2004; Bharati & Goodson, 2006; Taziaux *et al.*, 2006, 2008). The avian POA is similar to the mammalian POA in neurochemistry, hodology, development and topography; it contains

dopamine- and neuropeptide-producing cells (Viglietti-Panzica, 1986; Bailhache & Balthazart, 1993) and is highly interconnected with the amygdaloid complex (Berk & Butler, 1981).

In a recent study (Mayer *et al.*, 2017), we observed higher neuronal activity in the septal and amygdaloid nuclei of visually naïve chicks in the presence of a conspecific, compared to baseline chicks that were exposed to the same visual and acoustical environment without seeing the conspecific. However, it remains unclear which visual features of the alive conspecific elicited the effect (e.g., its overall appearance, its face region or its animate motion). In this study, we were thus interested to know if motion cues, that are typically associated with the presence of animate creatures, are sufficient to activate nuclei of the social behaviour network. We compared neuronal activity in the septum, arcopallium/TnA and preoptic area of visually naïve experimental chicks, which were exposed to a living conspecific, to that of control chicks exposed to a stuffed chick constantly rotating on its axis. The two stimuli were visually matched in their static features (overall appearance, presence of a face configuration, colour, size etc.), but differed considerably with regard to their motion. While the live conspecific represents, in the most natural way, a summary of all the features of 'animate motion' that attract chicks' attention, the control stuffed chick was showing only rigid motion, that is considered to be inanimate (Vallortigara *et al.*, 2005). We measured neuronal activity by labelling the immediate early gene product c-Fos with a standard immunohistochemical procedure. The expressions of these genes are reliably induced by behaviourally relevant neuronal activity and their products have often been used to map brain activities in different species (Cole *et al.*, 1989; Sheng & Greenberg, 1990; Lanahan & Worley, 1998; Jones *et al.*, 2001; Guzowski, 2002; Mayer & Bischof, 2012; Mayer *et al.*, 2016a).

Material and methods

Subjects

Thirty-three laboratory-hatched, domestic chicks (*Gallus gallus domesticus*), of the 'Hybro strain' (a local variety derived from the white leghorn breed), were used. Fertilized eggs were obtained from a local commercial hatchery (Agricola Berica, Montegalda (VI), Italy) and were hatched in groups inside dark incubators (Marans P140TU-P210TU). Hatching took place at a temperature of 37.7 °C, with 60% humidity. During the first day after hatching chicks were moved to individual compartments (cardboard box 11 cm × 11 cm × 25 cm) and reared at constant temperature of 33 °C. All procedures were performed in complete darkness, so that the chicks remained visually unexperienced. The experiment was conducted on the second day after hatching.

Ethical statement

The experiments reported here comply with the current Italian and European Community laws for the ethical treatment of animals and the experimental procedures were licensed by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanità Pubblica Veterinaria (permit number 20269/A).

Testing apparatus

The testing apparatus (Fig. 1) consisted of a rectangular arena with an inner space of 30 cm × 45 cm × 23.5 cm (W × L × H) that was divided by a wall in the middle into two identical corridors

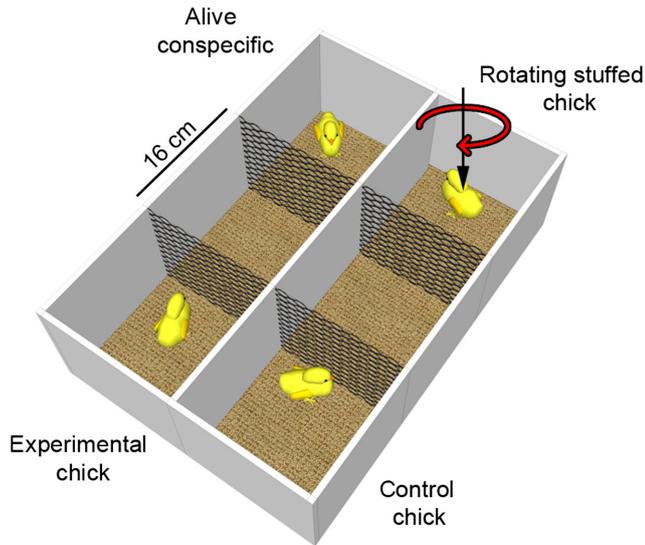


FIG. 1. Experimental setup: the experimental chick was observing the live conspecific on the other end of the corridor, visible through the grids, whereas the baseline chick was facing a stuffed chick rotating at 5 rpm. [Colour figure can be viewed at wileyonlinelibrary.com]

(45 cm × 14.5 cm × 23.5 cm). All inner surfaces of the two corridors were covered with white non-reflective material. The two ends of each corridor were delimited by black metal grids (1 cm × 1 cm), providing compartments for individual chicks (14.5 cm × 14.5 cm large, 23.5 cm). The section of the corridor between the two grids (or central compartment) was 16 cm and served to prevent a direct tactile interaction of the experimental chicks. The apparatus was positioned on foots 15 cm above the ground. A portion of the floor of the stimuli compartments was removable (12 cm × 12 cm), and could be substituted by a construction including an electrical motor for the rotation of the control stimulus (taxidermised chick). This way, on the side of the apparatus in which the control stimulus needed to be presented, the simple floor was removed and substituted with the altered floor including the rotation mechanism on which the taxidermised chick could be located. A layer of sawdust covered the floor to prevent chicks from seeing and pecking their own feces. The stimulus side of the experimental setup was uniformly illuminated from above (60 cm) with diffused warm light (60 W). A digital camera was suspended centrally above the arena to record the behaviour of the chicks.

Test session for *c-Fos* labelling

Animals were tested on the second day after hatching. For this purpose, the 33 chicks were divided into three groups of 11. Chicks from one group served as 'demonstrators', while the other two groups were the 'experimental' group and the 'control' group. They were placed in the three compartments of the experimental apparatus (Fig. 1). During the exposure, each experimental chick was able to see a live chick (demonstrator), whereas the control group was facing a stuffed chick that was rotating at constant speed of 5 rpm. Temperature in the experimental room was maintained constant at 25 °C. Each subject was extracted from the individual compartments in the incubator in complete darkness, placed singly into a closed transportation box and carried to the experimental room. During the transportation, special care was taken to avoid unwanted stimulation prior to the test. For each test, the demonstrator was moved first,

followed by the experimental and control chicks that were carried simultaneously in two different compartments of the same box. In order to counterbalance all variables between the experimental and the control chicks, the order of entrance and removal of the animals and their left-right position in the experimental corridor were counterbalanced between test pairs. All test sessions were video recorded. After 10 min of exposure, both the experimental and control chicks were transported back to their individual home compartments inside the dark incubator, where they remained until perfusion. The demonstrators were not used for further investigation; they were caged in groups with food and water *ad libitum* in the animal house until they were donated to local farmers.

Immunohistochemistry

Seventy minutes after the start of the test session, subjects used for brain activity measurements were overdosed with an intramuscular injection of 0.05 mL Ketamine/Xylazine Solution (1 : 1 Ketamine 10 mg/mL + Xylazine 2 mg/mL) per 10 g of body weight. After 5 min, when the animals became unresponsive (tested by gently pulling the feet and wings), they were immobilized on a plate, the thorax was opened and the heart was exposed. The chicks were perfused transcardially via the left ventricle with cold phosphate buffered saline (PBS; 0.1 mol, pH = 7.4, 0.9% sodium chloride, 4 °C) for 5 min and then fixed with 4% paraformaldehyde (PFA) in PBS for 7 min. The head was then severed from the body, the skin and the eyes were removed, and the skull was transferred to 4% PFA where it was post-fixed for 7 days. In following, the skull was secured in a stereotaxic head holder (Stoelting, using a Kopf Instruments pigeon head holder). A coronal-plane cut was made through the skull and the brain with a scalpel blade attached to a micromanipulator, at an orientation of 45° to ensure that the subsequent brain sections had the same orientation as in the chick brain atlas of Kuenzel & Masson (1988). The brain was then removed from the skull, post-fixed for approximately 48 h in 4%PFA/PBS-containing 20% sucrose at 4 °C, and then transferred to 30% Sucrose/0.4% PFA/PBS for 48–72 h until it sunk. The left and the right hemispheres were separated and processed independently. Each hemisphere was embedded in gelatin containing egg yellow (7%), post-fixed for approximately 48 h in 4% PFA/PBS containing 20% sucrose at 4 °C, and then transferred to 30% sucrose in 0.4% PFA/PBS where they were stored for multiple days (at least 48 h) until processing. The brain hemispheres were frozen at –80 °C in plastic molds covered with O.C.T (Tissue-Tek freezing medium). For free-floating staining, four series of 40 µm coronal sections were cut on a Cryostat (Leica CM1850 UV) at –20 °C. The sections were collected only from the regions of interest (septum, POA and A/TnA). The sections of the first series were used for processing and labelling. The sections of the other series were kept in PBS at 4 °C as backup or for testing antibody specificity (processing without the primary antibody). Endogenous peroxidase activity was depleted by incubation in 0.3% H₂O₂ in PBS for 20 min. After washing in PBS (3 × 5 min), the sections were treated with 3% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min. The sections were then transferred to the first antibody solution (*c-Fos* antibody made in rabbit, 1 : 2000; K-25, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated 48 h at 4 °C on a rotator. After several washes in PBS, the secondary antibody reaction was carried out using a biotinylated anti-rabbit solution (1 : 200, BA-1000; Vector Laboratories) in PBS for 60 min at room temperature. The ABC method was used for signal amplification (Vectastain Elite ABC Kit, PK 6100; Vector Laboratories). The

c-Fos-immunoreactive (c-Fos-ir) neurons were visualized with the VIP substrate kit for peroxidase (SK-4600; Vector Laboratories). This produced a purple reaction product confined to the cell nuclei of activated neurons. Sections were then transferred to distilled water and serially mounted on gelatin-coated slides. They were dried at 50 °C on a heating plate and counterstained with methyl green (H-3402; Vector Laboratories). After gradual dehydration in ethanol (70%, 80%, 90% and 99% EtOH for 3 min each, and then placed in Xylene) the mounted sections were cover slipped with Eukitt (FLUKA).

Brain anatomy

Brain sections were examined with a Zeiss microscope at a magnification of 200× (eyepiece: 10×, objective: 20× with a numerical aperture of 0.5) and a digital camera (Zeiss AxioCam MRc5). The ZEN imaging software (Zeiss) was used for the manual counting of c-Fos-ir neurons on a computer screen. Counting was performed blind to the experimental conditions. For counting, a rectangular 'enclosure', 150 × 250 μm, was positioned over spots of highest number of c-Fos-ir neurons within brain areas of interest (Fig. 2). Contrast and exposure time of the camera were adjusted so that the image on the screen matched the view under the microscope. Successful immunostaining produces dark purple-black-stained nuclei and minimal background staining. Thus, the nuclei of c-Fos-ir neurons were easily discerned from background and non-activated neurons, which were stained light green (Fig. 3). Every activated c-Fos-ir neuron within the sample areas was marked on the screen with the 'event marker' of the ZEN software, which automatically computed the total number of c-Fos-ir neurons.

Several brain regions within the collected telencephalic parts contained c-Fos-ir neurons, however, we measured the density of labelled neurons only in the brain areas of interest: septum, preoptic area, arcopallium and TnA. To measure cell density within the septum three to eight sections of both hemispheres were selected by the shape and anatomical landmarks that would correspond to the A(terior) 8.8 to A7.6 (Kuenzel & Masson, 1988). It is important to note that the anterior-posterior coordinates that are provided here for orientation within the atlas do not represent the real coordinates in 1-day-old chicks, because the Kuenzel & Masson (1988) atlas was based on brains from 2-week-old chicks that are substantially bigger in size. The septum of each section was parsed into three subdivisions: dorsal septum, ventrolateral and ventromedial septum (Fig. 2a). The border between the ventrolateral and medial septum was based on anatomical landmarks that are visible after methyl-green counterstain (similar to giemsa staining). The dorsal part was defined as the dorsal 1/2 of the septum starting from the ventral border of the lateral ventricle (Fig. 2a). Labelled cells within the preoptic area were counted from one to two sections of each hemisphere selected from a region where the anterior commissure was apparent A8.2 (Kuenzel & Masson, 1988). The counting area was positioned beneath the anterior commissure (Fig. 2a). Labelled cells within the arcopallium and TnA were counted from four to six sections of each hemisphere, selected from the region extending from A8.2 to A6.4 (Kuenzel & Masson, 1988). Arcopallium is delimited in its upper boundary by the *lamina arcopallialis dorsalis*, whereas TnA is a region beneath the arcopallium which can be visually distinguished on the basis of different cell densities. Arcopallium was parsed into dorsal and ventral regions (Fig. 2b). After completing the cell counts, for each animal mean values from the different sections were calculated for each brain region in each hemisphere and cell

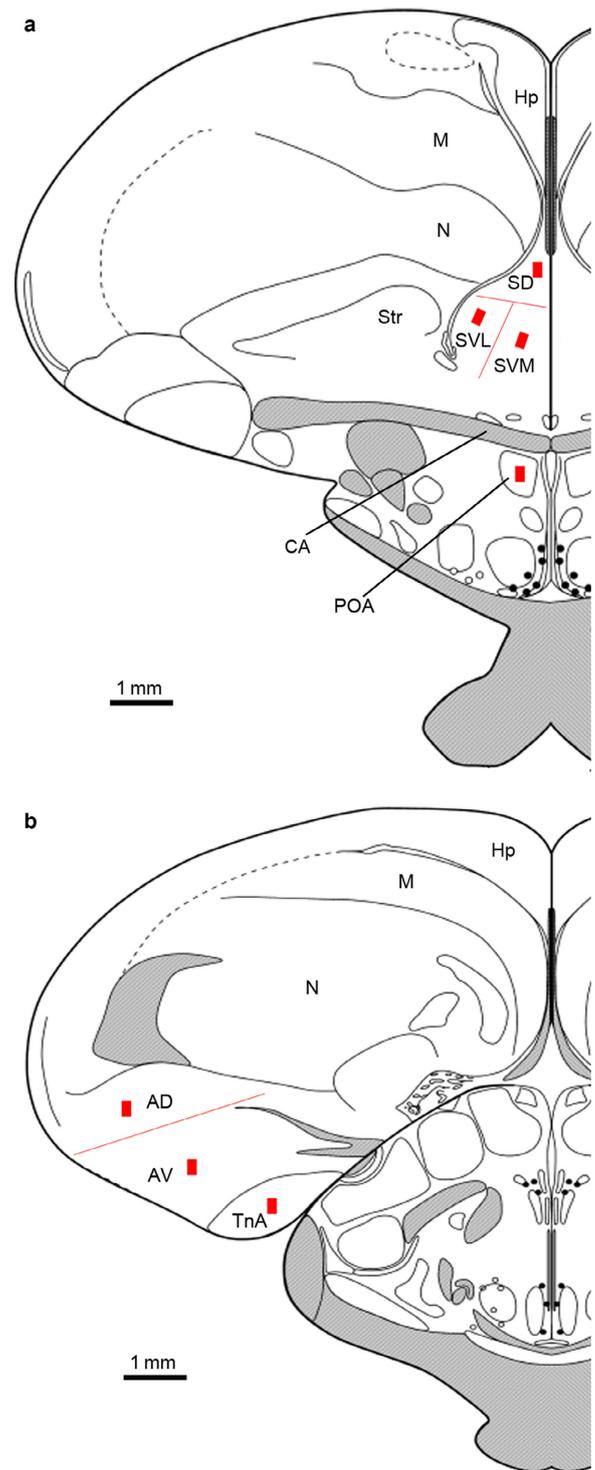


FIG. 2. Typical placement of cell count zones (red rectangles). (a) Schematic view of a coronal section showing a typical placement of the cell count zone within the septum with its porportioning into dorsal, ventrolateral and ventromedial subdivisions (red lines) and within the preoptic area. (b) Schematic view of a coronal section showing a typical placement of the cell count zone within arcopallium and nucleus taeniae. The arcopallium was subdivided into dorsal and ventral parts (red line). Drawings were adapted from the atlas of Kuenzel & Masson (1988). AD, dorsal arcopallium; AV, ventral arcopallium; CA, anterior commissure; Hp, hippocampus; M, mesopallium; N, nidopallium; POA, preoptic area; SD, dorsal septum; Str, striatum; SVL, ventrolateral septum; SVM, ventromedial septum; TnA, nucleus taeniae of the amygdala. [Colour figure can be viewed at wileyonlinelibrary.com]

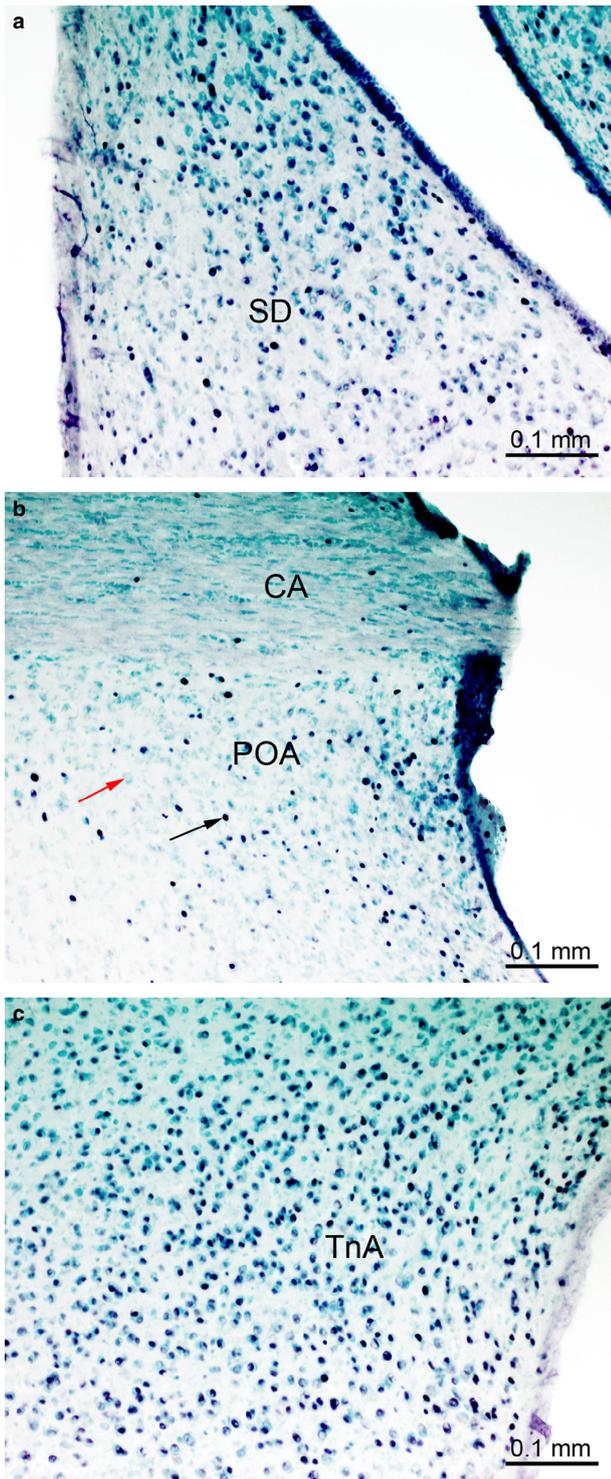


FIG. 3. Labelled neuronal nuclei in the sampled areas of an experimental chick. Immunoreactive neurons are stained black (black arrow) and can easily be distinguished from the c-Fos-negative, green-stained neuronal nuclei (red arrow). (a) A high number of labelled c-Fos-ir nuclei within the dorsal septum. (b) Labelled nuclei within the preoptic area. (c) Labelled nuclei within the nucleus taeniae. SD, dorsal septum; TnA, nucleus taeniae; POA, preoptic area. [Colour figure can be viewed at wileyonlinelibrary.com]

densities were standardized to 1 mm^2 . Furthermore, the cell counts pooled from the two subdivisions of ventral septum were averaged to estimated overall activity in ventral septum. Also the cell counts

pooled from the two subdivisions in arcopallium and the counting in TnA were averaged to estimate overall activity in this area. The resulting individual bird means were considered overall indicators for the number of c-Fos-ir neurons and were employed for further statistical analysis.

Statistical analysis

In this study, chicks of both groups were handled pairwise at all steps of the procedure. In particular, chicks were transported in pairs (simultaneously in two compartment of the same transporting box) to and from the experimental room and were simultaneously exposed to the same acoustical environment. Also the subsequent processing of the brains, as well as the immunohistochemical reactions were carried out pairwise. Thus, measurements obtained from the experimental and control chicks of each pair could not be treated as independent. Following this logic, data from the two groups were analysed as pairwise interdependent data points: the presence of difference in the density of c-Fos-ir neurons was tested by a repeated measures ANOVA with brain area, hemisphere and group as repeated measures. For *post hoc* analyses, paired *t*-tests (two tailed) were carried out for each area. The standard errors of the means and the standard deviations presented in the results part and in the graphs have been corrected as recommended by Loftus & Masson (1994) for pairwise comparisons. The correction represents analogous confidence intervals that reflects the real variability in the data used in within-subject designs, without affecting the mean values (see also Field, 2014). Please note that of course all the statistical analyses were run on the original uncorrected data. All statistical analyses were performed with the software IBM spss Statistic for Windows (Version 22.0).

Results

Results of immunohistochemical procedure

We processed the brains of all 11 experimental and 11 control group chicks. In all of these brains, the nuclei of c-Fos-ir were stained black after the immunohistochemical procedure and thus easily distinguishable from other neurons that were counterstained with methyl green (Fig. 3). The distribution of c-Fos-ir neurons showed individual variations. In some birds, the number of c-Fos-ir neurons in septum was very low and also isolated neurons were found. In some other birds labelled neurons appeared in high densities and more or less homogeneously distributed over the entire septum. Also the distribution along the rosto-caudal axis was variable. Some brains showed c-Fos-ir neurons only in the rostral parts of septum, others caudally or in both parts. The c-Fos activity was almost always present in the dorsal septum. In fact, very often, c-Fos expression was observable only in the dorsal and dorsomedial parts of septum, whereas the ventrolateral septum remained empty. However, in some other cases also the ventrolateral septum contained high densities of c-Fos-ir neurons. The activity pattern was very often also visibly asymmetrical between the two hemispheres.

Similar to what was observed in the septum, also the activity pattern within arcopallium and TnA showed high individual variations. Also in these areas, the activity pattern was often rather clustered than homogeneously distributed. Very often the activity pattern was not limited by the anatomical subdivisions (e.g. if labelled cells were present in the TnA, the activity pattern continued in the surrounding ventral arcopallium). In contrast to what was observed in the septum

or arcopallium, the individual variations within the preoptic areas were less visible.

Results of the quantitative analysis

For the repeated measures ANOVA, Mauchly's test indicated that the assumption of sphericity had been violated, $\chi^2(5) = 14.079$, $P = 0.02$, therefore Greenhouse–Geisser corrected tests are reported ($\epsilon = 0.54$). The following main effects were significant: area ($F_{2,152,21,507} = 4.069$; $P = 0.029$) and hemisphere ($F_{1,000,10,000} = 45.695$; $P < 0.001$). Moreover, a significant interaction of area*group*hemisphere ($F_{1,623,16,233} = 3.932$; $P = 0.048$) revealed significant differences in densities of c-Fos-ir neurons between the experimental and control group in a brain region and hemisphere-dependent fashion. The post hoc comparisons (two tailed, paired *t*-tests) revealed significant differences between the two groups in the right dorsal septum ($t_{10} = 2477$; $P = 0.03$) and in the left preoptic area ($t_{10} = 3237$; $P = 0.009$). The density of c-Fos-ir cells within the right dorsal septum (Fig. 4a) of the experimental birds (mean \pm SEM: 969.5 ± 120.4 cells/mm²) was nearly twice as high ($\sim 48\%$) as in the septum of control birds (506.2 ± 73.1 cells/mm²). However, the density of c-Fos-ir cells in the left preoptic area (Fig. 4c) was $\sim 27\%$ higher in the experimental group (994.1 ± 110.1 cells/mm²) compared to the control group chicks (723.6 ± 79.9 cells/mm²). Such differences were not present in the left

dorsal septum (experimental group: 1033 ± 131.3 cells/mm²; control group: 1065.1 ± 135.3 cells/mm²; $t_{10} = -0.143$; $P = 0.889$) or the right preoptic area (experimental group: 779.8 ± 115.2 cells/mm²; control group: 762.4 ± 83.8 cells/mm²; $t_{10} = 0.132$; $P = 0.898$).

Differences between the groups were not present in the left ventral septum (experimental group: 1327.9 ± 127.3 cells/mm²; control group: 1262.3 ± 139.6 cells/mm²; $t_{10} = 0.346$; $P = 0.736$) or in the right ventral septum (experimental group: 1027 ± 136.8 cells/mm²; control group: 857.8 ± 78.1 cells/mm²; $t_{10} = 0.916$; $P = 0.381$).

No differences between the groups were present in the left arcopallium (experimental group: 1012.1 ± 122.1 cells/mm²; control group: 918.4 ± 78.1 cells/mm²; $t_{10} = 0.682$; $P = 0.511$) or in the right arcopallium (experimental group: 670.8 ± 113.6 cells/mm²; control group: 727.2 ± 108.2 cells/mm²; $t_{10} = -0.313$; $P = 0.761$).

Discussion

The results of this study demonstrate that previous visual experience is not required to activate septal and preoptic areas in response to the animate motion of a living, behaving conspecific. We found higher neuronal activity in the right dorsal septum and left preoptic area of visually naïve chicks after the first exposure to a conspecific, compared to control chicks exposed to the same visual and acoustical environment, containing a rigidly rotating stuffed chick. Such

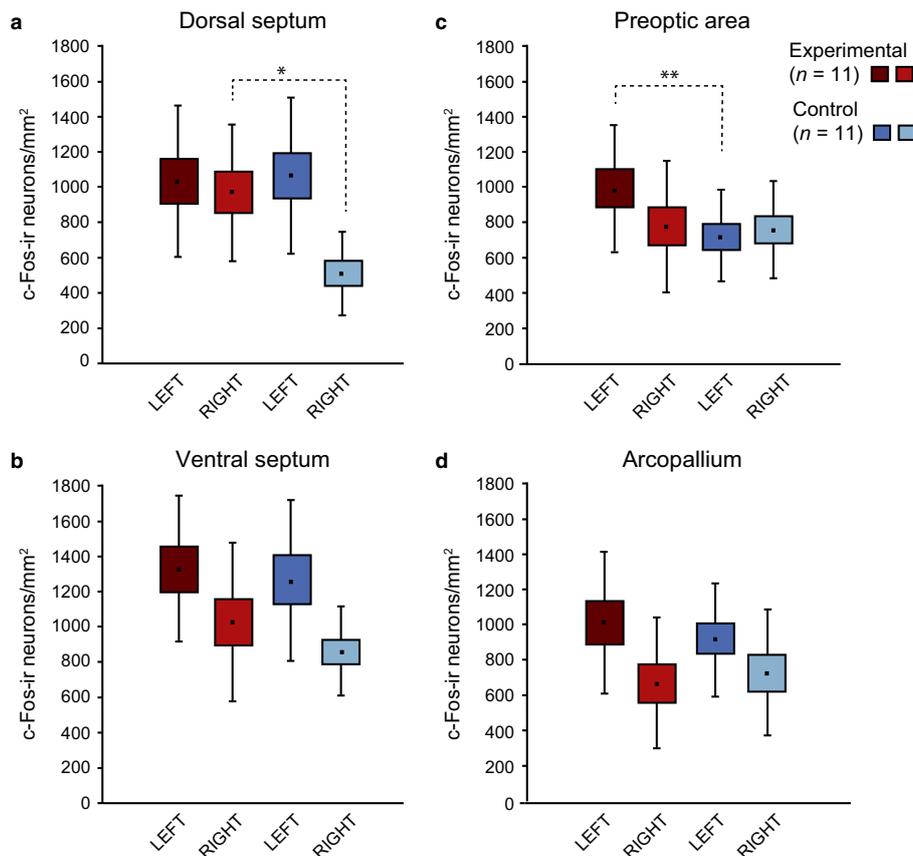


FIG. 4. Measured c-Fos-ir densities in two groups of chicks ($n = 11$ in each condition). (a) Significantly higher number of c-Fos-ir neurons is present in the right dorsal septum of experimental chicks compared to controls. (b) A high number of c-Fos-ir neurons is present in the ventral septum of both groups. (c) Significantly higher number of c-Fos-ir neurons in the left preoptic area of experimental chicks compared to controls. (d) A high number of c-Fos-ir neurons is present in the arcopallium of both groups. Graph-plot: mean (black square), SEM (box) and SD (whisker) ($*P < 0.05$; $**P < 0.01$). Densities of c-Fos-ir neurons per mm² are represented on the Y-axis. [Colour figure can be viewed at wileyonlinelibrary.com]

differences between the two groups were not present in the arcopallium, showing that the observed differences were region-specific and not due to the overall activity of the brains. This is, to the best of our knowledge, the first demonstration of septal and preoptic areas involvement in response to animate motion of a social partner, as opposed to rigid motion of a similarly looking stimulus.

The differential activation of septal and preoptic areas found between the two groups can be explained only by the different visual stimulation provided by the different types of motion of the two presented stimuli. For both groups, the visual scenarios were matched in complexity, luminosity and contrasts. In fact, both groups of chicks were exposed to the same visual environment in which both received patterned visual stimulation (e.g. provided by the black grid separating the different compartments, the visual texture of the sawdust, white walls, etc.). Also both groups of chicks saw visually salient and very similar looking objects (a live or stuffed chicks), only differing in their type of motion (animate motion of a social partner, as opposed to the unnatural motion of the stuffed chick). Moreover, the experimental chick could not receive any tactile stimulation from the 'demonstrator', because they were divided by the empty central compartment of the corridor. Also olfactory cues would be equally available to both groups, due to the open structure of the apparatus and to the intermixing of the sawdust and due to the interchange in the compartment used for the two groups, after each pair. Finally, also the acoustical stimulation received by each pair of experimental and control chicks was matched (the two chicks composing each pair were tested simultaneously side by side and the apparatus was completely open from above).

At the anatomical level it is of particular interest to understand how visual information could modulate the neuronal activity in septum and preoptic areas. In fact, so far there is no evidence that septum or preoptic areas are directly devoted to visual processing. The thalamofugal pathway that terminates in the visual Wulst is one of the major visual projections to telencephalon of birds (Bischof & Watanabe, 1997). However, no direct projections from the visual Wulst have been found to terminate in the septum of chicks (Montagnese *et al.*, 2004, 2008), see also (Shimizu *et al.*, 1995; Atoji & Wild, 2004) for pigeons. Connections could be observed only between the rostral SL and the rostral HA (the upper layer of the Wulst) of chicks (Montagnese *et al.*, 2004, 2008). This part of Wulst is probably somatosensory rather than visual (Miceli *et al.*, 1980). Also the septopallio-mesencephalic tract, which runs through the fronto-medial septum and is thought to be the major outflow from the visual regions of the Wulst (Karten *et al.*, 1973; Bagnoli *et al.*, 1980; Miceli *et al.*, 1987), has never been shown to terminate in septum and thus probably does not contribute to the septal activation. More likely, visually modulated information could arrive to septum through the hippocampus. The area parahippocampalis receives direct connections from the visual Wulst (Atoji *et al.*, 2002) and in turn it projects to the hippocampus, where visually responsive cells have been recently observed (Scarf *et al.*, 2016). Septum receives direct input from the dorsal parts of the hippocampus (Atoji & Wild, 2004) and thus might undergo at least some visually dependent modulation of its activity through this route. In addition, it is important to consider that subtelencephalic regions might play important role in innate complex stimulus recognition in chicks (Zachar *et al.*, 2008; see also Rosa Salva *et al.*, 2015) and some of them, such as the pretectal nucleus, sends projections to the septum (Montagnese *et al.*, 2008). Thus, it is also possible to hypothesize a contribution of subtelencephalic regions to the septal activation found in this study.

In this study, a significant difference was present only in the dorsal part of the right septum. Topologically, the dorsal septum is very close to the ventral hippocampus in avian species. Especially here the density of myelinated fibers connecting hippocampus and septum is visibly high (Karten *et al.*, 2013). Thus, the differential activity in the dorsal septum in our study might probably reflect the interconnectivity with the hippocampus that is of thalamofugal origin, and could thus provide the source of visual input to septum. This interpretation is also supported by the observed lateralization of the effect, which was limited to the right side of the dorsal septum. In a recent study it has been shown that chicks preferentially use their left eye during observation of a biological motion display (Rugani *et al.*, 2015). Because of a total decussation of the optic fibers in avian species, the visual information from the left eye would stimulate mainly the activity in the right hemisphere. The thalamofugal visual pathway is also known to be lateralized in chicks. In chicks hatched from eggs that were exposed to light this pathway shows more prominent projections to the left hemisphere (Rogers & Sink, 1988; Rogers & Bolden, 1991; Rogers & Deng, 1999; Deng & Rogers, 2000, 2002a; Andrew *et al.*, 2004; Rogers, 2008). Chicks used in the present experiment were not hatched from eggs exposed to light during incubation. However, behavioural lateralization has been found also in dark incubated chicks in some tasks, notably a right hemisphere dominance in the recognition of social companions (Deng & Rogers, 2002b; and see Vallortigara & Andrew, 1991, 1994). Similar to that, even at the brain activity level right hemisphere dominance for early processing of social stimuli can be found in dark-incubated chicks (see Mayer *et al.*, 2016b for a c-Fos expression study). Moreover, also the presence of a lateralized glutamate NMDA type receptor binding has been reported in the intermediate medial mesopallium (IMM) of dark incubated and hatched chicks (Johnston *et al.*, 1995).

Overall these results indicate that at least the dorsal part of right septum is involved in processing motion patterns of a living, conspecific. However, our results may not be sufficient to determine without ambiguity whether visual information caused an upregulation in the presence of animate motion in the right dorsal septum of experimental group or a downregulation in the absence of animate motion in the control group. A puzzling finding of this study concerns the absence of significant differences between the two groups in the ventral parts of the septum. Considering that immediate early genes studies with other avian species showed that the ventrolateral zones of the caudal lateral septum are the most responsive to social stimuli (Goodson *et al.*, 2005a,b; Taziaux *et al.*, 2006) we expected to find the strongest differences especially in the ventral sub-regions of septum. However, it is important to keep in mind that in this study both groups were exposed to social stimuli, that were matched with regards to their static features. Thus, social response might have been present in both groups, which would explain the lack of difference between the groups. This would point to a specific role of dorsal septum in responses elicited by the motion cues provided by social partners, a subdivisional aspect that should be investigated in more detail in future studies.

Interestingly, differences between the two groups were absent in the arcopallium. Even this is somewhat surprising because in a previous study with visually naïve chicks arcopallium activation was found in the presence of a social companion, compared to baseline chicks that were exposed to the same environment without a conspecific (Mayer *et al.*, 2017). The results of this study suggest, that dynamic features alone are not sufficient to elicit this effect in the arcopallium. Future studies should be designed to study arcopallium

activation with particular attention to static stimuli such as features of the head region and face-like configurations, that are known to be preferred by visually naïve chicks (Rosa Salva *et al.*, 2015). Arcopallium contains high proportions of visually selective cells for certain colours and shapes (Scarf *et al.*, 2016). It also receives intratelencephalic projections from the entopallium (Benowitz & Karten, 1976), which is a telencephalic station of the tectofugal visual pathway in birds involved in object, shape and motion recognition (Watanabe, 1996; Bischof & Watanabe, 1997; Watanabe *et al.*, 2008; Shimizu *et al.*, 2010; Cook *et al.*, 2013). The presence of visual stimulus selective activity in arcopallium (Scarf *et al.*, 2016) and its direct connection back to the optic tectum (Zeier & Karten, 1971; Bischof & Watanabe, 1997) are of particular interest in the study of early social behaviours. At least in humans, amygdala and superior colliculus (homologue of the optic tectum) are believed to be involved in early social orienting responses, especially detection of face stimuli, to which in our study of both groups were exposed (Johnson, 2005).

Another important finding of our study is the significantly higher activity in the left preoptic area of the chicks that were exposed to a living conspecific compared to controls. Preoptic area is a key brain area for the control of sexual behaviour in another galliform species, namely the male Japanese quail (Panzica *et al.*, 1996; Balthazart *et al.*, 1998a; Taziaux *et al.*, 2006, 2008). At least in adult quails, the medial preoptic area involvement in the sexual behaviour is mediated by testosterone (Riters *et al.*, 1998). The role of the preoptic area for early social behaviours has never been investigated so far. The studies with quails would suggest that activation of this area could be mediated by hormones, in particular by testosterone, rather than by direct visual information. However, being part of the social behaviour network in which all nodes are reciprocally interconnected (Newman, 1999), the activity in the preoptic area can also be mediated by the activity in other areas. An interesting fact to this regard is the contrasting pattern of lateralization between the septum and the preoptic area: the difference between the experimental and the control chicks was limited to the right side for the septum, but to the left side for the preoptic area. This suggests a possible inhibitory interaction of septal and preoptic area activities, another interesting aspect for future studies.

Author contributions

Conceived designed the experiments: UM, ORS, GV. Performed the experiments: FM and ORS. Analysed the data: FM, UM and ORS. Contributed reagents/materials: GV. Wrote the manuscript: UM, ORS, FM and GV.

Conflict of interests

The authors declare that they have no conflict of interest.

Data accessibility

The article's supporting data and materials can be accessed upon request to the corresponding author. Moreover the data files were uploaded on the journal website.

Acknowledgements

Giorgio Vallortigara was supported by a grant from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007–2013)/Advanced Grant ERC PREMESA G.A. no 295517.

Abbreviations

A, arcopallium; AD, dorsal arcopallium; AV, ventral arcopallium; CA, anterior commissure; Hp, hippocampus; IMM, intermediate medial mesopallium; ir, immunoreactive; M, mesopallium; NMDA, *N*-methyl-D-aspartate receptor; N, nidopallium; PBS, phosphate buffered saline; PFA, paraformaldehyde; POA, preoptic area; SD, dorsal septum; SL, lateral septum; SM, medial septum; S, Septum; SVL, ventrolateral septum; SVM, ventromedial septum; TnA, nucleus taeniae.

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