Odours stimulate neuronal activity in the dorsolateral area of the hippocampal formation during path integration

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The dorsolateral area of the hippocampal formation of birds is commonly assumed to play a central role in processing information needed for geographical positioning and homing. Previous work has interpreted odour-induced activity in this region as evidence for an ‘olfactory map’. Here, we show, using c-Fos expression as a marker, that neuronal activation in the dorsolateral area of the hippocampal formation of pigeons is primarily a response to odour novelty, not to the spatial distribution of odour sources that would be necessary for an olfactory map. Pigeons exposed to odours had significantly more neurons activated in this area of the brain than pigeons exposed to filtered air with odours removed. This increased activity was observed only in response to unfamiliar odours. No change in activity was observed when pigeons were exposed to home odours. These findings are consistent with non-home odours activating non-olfactory components of the pigeon’s navigation system. The pattern of neuronal activation in the triangular and dorsomedial areas of the hippocampal formation was, by contrast, consistent with the possibility that odours play a role in providing spatial information.

1. Introduction

Homing pigeons are well known for their ability to return home even from unfamiliar areas hundreds of kilometres from their home lofts. The navigational ability of young, inexperienced pigeons is based primarily on path integration [1–3], also referred to as route-based navigation [1]. Older, experienced pigeons rely on map-based navigation that requires birds to learn the spatial distribution of one or more geophysical factors around the loft (the ‘map’). While the navigational map is being established, path integration allows young pigeons to perform exploratory flights and learn the distribution of local environmental gradients without the risk of getting lost [1–3].

Olfactory cues have been proposed to provide one source of route-based directional information [4]. Olfactory information has also been proposed to be the basis of a gradient map [4,5] used by experienced birds to determine geographical position [6]. However, findings from behavioural experiments have challenged the idea that odours provide navigational map information, suggesting instead that unfamiliar odours activate brain areas involved in processing non-olfactory cues [7,8] (i.e. the dorsolateral area of the hippocampal formation). If so, exposure to unfamiliar odours, both natural (potentially providing a source of map information) and artificial (shown in earlier behavioural studies not to provide map information [7]), should activate dorsolateral hippocampal neurons [9], while there should be little, if any, activity produced by exposure to familiar natural odours from the vicinity of the home loft [10], or to filtered air without odours either at the home loft [10] or during displacement to a release site [5].

In behavioural experiments, we confirmed previous findings from other laboratories (for a review, see [5]) showing that homing orientation was disrupted when pigeons were deprived of olfactory cues either during displacement to
or after arrival at [8], a release site. However, our behavioural experiments also showed that while pigeons exhibited homing orientation when they had access to natural odours, they also exhibited homing orientation when exposed to a fixed sequence of artificial odours that did not provide path integration or site-based map information [7,8]. The possibility that one or more of the artificial odours present in the natural environment provided map information cannot explain the homing orientation, because pigeons exposed to the same sequence of artificial odours were able to orient and return home from release sites located in opposite directions from the home loft. Therefore, the fixed sequence of artificial odours used in the behavioural experiments did not provide information about spatial position (‘map’ information). In the present experiments, the same sequence of artificial odours was used to distinguish activational effects of odours from the use of olfactory navigation cues.

To investigate the role of olfactory cues in the early phase of the development of the pigeon’s navigational system, we undertook a c-Fos functional mapping analysis of neuronal activation in several brain regions involved in processing homing information [10–13]. Young pigeons, with ages ranging between seven and eight weeks, were exposed to natural odours, artificial odours or filtered air without odours during displacements to a release site prior to being released for the flight home, or during a simulated displacement with no homing flight during which pigeons remained in the home loft (see figure 1a; see also Material and methods). At the end of the olfactory manipulation (i.e. at the end of the displacement or simulated displacement), pigeons were made anosmic by anaesthesia of the olfactory mucosa and, 8 min later, released for the flight home or inside the loft. All the pigeons were perfused 1–2 h after the end of the olfactory manipulation, and their brains were dissected, cryoprotected and sectioned in the coronal plane (figure 1b). The free-floating sections were then stained for the neuronal activation marker c-Fos (see Material and methods).

The exposure of birds to different olfactory treatments was restricted to the period of the displacement or simulated displacement (see above and Material and methods). This allowed the change in neuronal activity caused by odours (natural or synthetic) during the olfactory manipulation to be compared with that of birds exposed to filtered air (i.e. air deprived of odours) during the same period of time as controls. Because previous behavioural experiments have shown that the sequence of artificial odours used in the current experiments did not provide the birds with navigational information [7,8] (see earlier discussion), any effect of these artificial odours on neuronal activity in the dorsolateral area of the hippocampal formation is unlikely to be the result of their involvement in providing map information by means of path integration or map-based cues. At the same time, exposing the birds to comparable olfactory treatments during displacement and simulated displacement (i.e. pigeons that stayed in the home loft) helps to distinguish effects of olfactory exposure from non-olfactory effects of the displacement and homing flight. Finally, comparison of the effects of exposure to natural and artificial odours both during the simulated displacement at the home loft and during the actual displacement to the release site helps to discriminate between home and non-home effects of odour exposure.
2. Material and methods

(a) Subjects, behavioural procedures and stimulus conditions

Experiments were carried out in our loft at Marinhais (39°03' N; 8°43' W), Portugal. Young pigeons at the age of three to four weeks were placed in the experimental loft and were allowed spontaneous daily flights from the loft. A commercial multigrain food mixture was provided once a day, in the evening, and water and grit were provided ad libitum.

Forty-eight young (seven to eight weeks old) homing pigeons were used in this experiment; of those four got lost during the return flight and 10 had their brains poorly perfused. The fly/natural odours group \((n = 9)\) was exposed to natural odours during the outward journey to the release site; the fly/purified air group \((n = 8)\) was exposed to filtered air (filters remove 99.9\% of the atmospheric odours); and the fly/artificial odours group \((n = 9)\) was exposed to filtered air with a fixed sequence of artificial odours (in each chamber of the odour release device, 100\(\mu\)l of a single commercially available odour were placed). The artificial odours were administered by manually opening valves on either side of a small chamber containing a cotton swab to which the odour had been applied. Opening the valves allowed air to pass over the cotton swab and carry the odour into the transportation box in which the pigeons were housed. To switch odours, the valves on either side of a chamber containing a different odour were opened, after which the valves to the chamber containing the previous odour were closed. Odours were presented in 5 min intervals in the following order: lavender, camellia, eucalyptus, rose and jasmine. The last odours remained until the end of the outward journey. For additional information, see [7].

The release site was located at a distance of 9.8 km ESE from the loft and the transport to the release site took 23–25 min. Immediately after arriving at the release site, all the groups were placed in purified air for 30 min. Eight minutes prior to releasing the birds, the nostrils of all pigeons were anaesthetized with a Xylocaine spray (for further details, see [8]). Then all pigeons were released in a single flock composed of one to two individuals from each group treatment plus four additional individuals with no treatment. This procedure minimizes pigeon loss and assures that most arrive home in an appropriate time window to be perfused.

The home/natural odours group \((n = 3)\) was exposed inside the home loft to ambient air; the home/artificial odours group \((n = 5)\) was exposed inside the home loft to a sequence of artificial odours equivalent to that experienced by the fly/artificial odours group (see above; figure 1a). In order to keep conditions as similar as possible between the home groups and the fly groups, the boxes of home groups were placed on top of a wheelchair that was moved around inside the loft to simulate the turns of the outward journey and the turbulence associated with the car movement. During the simulated journey, the car was placed next to the boxes with the engine on ensuring that the distance between the engine and the boxes in the trunk (i.e. in the case of the fly groups) and the engine and the boxes in the loft (i.e. in the case of the home groups) were approximately the same. Moreover, similarly to the boxes inside the car trunk, pigeons in the home groups could see part of the sky through the loft window. Like the fly groups, the home groups were exposed to odour and vestibular stimulation for 25 min followed by a 30 min period where pigeons were kept inside the stationary boxes in purified air. Eight minutes before the end of this 30 min period, all the pigeons had their nostrils anaesthetized with a Xylocaine spray. At the end of the 30 min period, pigeons were released inside the loft where they stayed until perfused.

(b) Immunohistochemistry

Sixty to 120 minutes after the end of stimulation [10,13], pigeons were deeply anaesthetized with an intra-peritoneal injection of sodium pentobarbital (0.5 ml per pigeon) and transcardially perfused with phosphate buffered saline (0.09\% NaCl in 0.1 M phosphate buffer, pH 7.4) followed by fixative (4\% paraformaldehyde in phosphate buffer—PB, pH 7.4). Brains were dissected and postfixed for an additional 24 h in the same fixative. For sectioning, the brains were first cryoprotected in sucrose buffer (30\% sucrose in PB) and then embedded in sucrose–gelatin (30\% sucrose, 10\% gelatin in distilled water). The embedded brains were sectioned on a freezing microtome in the coronal plane at a thickness of 60\µm. Free-floating sections were stored in PB containing 0.001\% of sodium azide at 4°C until they were stained.

The immunohistochemical detection of c-Fos was performed with free-floating sections according to a previously published protocol [14,15]. Staining was performed in two distinct batches (Batch 1: five fly/art. odours, four fly/pure air, four fly/nat. odours; Batch 2: four fly/art. odours, four fly/pure air, five fly/nat. odours, five home/art. odours, three home/nat. odours). Briefly, sections were incubated in 3\% hydrogen peroxide to reduce endogenous peroxidase activity, rinsed and incubated in 3\% normal goat serum to block non-specific binding sites in the tissue. Avidin- and biotin-binding sites were blocked with the Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories). The primary antibody was detected using a biotinylated anti-rabbit goat serum (1: 200, Vector Laboratories) and an avidin–biotin–peroxidase complex (Vectastain Elite ABC-Kit, Vector Laboratories). The peroxidase reaction was developed in a solution containing 0.05\% 3,3’–diaminobenzidine tetrahydrochloride hydrate, 0.01\% ammonium nickel(II) sulfate hexahydrate, 0.0125\% cobalt chloride and 0.02\% hydrogen peroxide. Sections were then rinsed and stored in a PB solution containing sodium azide at 4°C until they were mounted. The sections were mounted on gelatin-coated slides and coverslipped with DPX (Fluka). Every sixth section was counterstained with cresyl violet and used for general orientation following the pigeon atlas of Karten & Hodos [16].

(c) Analyses

Three tissue sections from each subject (sections selected according to [16]) were photographed for the olfactory bulbs (BO; atlas sections A6.75, A6.50 and A6.25). In these sections, c-Fos-IR nuclei were counted in a total of 54 representative counting frames (i.e. 27 frames for each brain hemisphere; frame area 0.018714 mm²). One tissue section from each subject (sections selected according to [10–12]) was used for the dorsal thalamic nucleus (NDT; atlas section A5.25), the vestibular nucleus (NVe; atlas section P1.75) and the three subdivisions of the hippocampal formation (TR, DM and DL; atlas section A5.75). Here, the number of c-Fos-IR nuclei was quantified in a total of 18 counting frames (i.e. 9 frames for each brain hemisphere; frame area 0.040221 mm²).

Photographs were taken at equal light intensity for all sections by a technician blind to the experimental conditions. Then they were converted to 8-bit greyscale photographs and the number of c-Fos-immunopositive cells was quantified using the ImageJ program, by two technicians blind to the experimental conditions (i.e. each section was counted twice, once by each technician, and averaged). A threshold was defined manually based on background staining (here we used 150), and we counted the number of cell nuclei that had higher optical density than the threshold.

Statistical comparisons were made with a general linear model repeated-measures analysis of variance (STATISTICA). Factors included the olfactory stimulus (natural air, artificial air and...
3. Results and discussion

We quantified c-Fos immunoreactive (IR) neurons in regions of the brain previously documented to be involved in aspects of homing [13,18] and/or in processing different types of sensory [11,12,19] and/or spatial information [13,18,20]: (i) olfactory bulbs; (ii) the piriform cortex; (iii–v) three subdivisions of the hippocampal formation—the triangular area, dorsomedial area and dorsolateral area; (vi) the ventricular nucleus; and (vii) the vestibular nucleus (figures 1c–g and 2). Overall, the experimental manipulation(s) affected the neuronal activity in the targeted regions (main effect: ‘odour’, p < 0.05; electronic supplementary material, table S1). Olfactory treatment differentially affected neuronal activity in the olfactory bulbs, and especially in all areas of the hippocampal formation (figures 3 and 4), which is proposed to function as a hub for the processing of navigational information in other vertebrates [26], including mammals [27–29]. Pigeons that homed after exposure to filtered air without odours exhibited lower neuronal activity in this region of the hippocampal formation relative to the olfactory-stimulated groups. Moreover, the interaction of treatments (‘odour × hemisphere’ effect; electronic supplementary material, table S4) provides further evidence for the specialization of the left dorsolateral and dorsomedial areas of the hippocampal formation in processing navigational-related information [13,18,20,25].

As mentioned previously, exposure to odours (i.e. natural or artificial) during displacement to the release site increased neuronal activity in the dorsolateral area of the hippocampal formation (figures 3 and 4), which is proposed to function as a hub for the processing of navigational information in other vertebrates [26], including mammals [27–29]. Pigeons that homed after exposure to filtered air without odours exhibited lower neuronal activity in this region of the hippocampal formation relative to the olfactory-stimulated groups. Moreover, the increased neuronal activity in the dorsolateral area was virtually indistinguishable in birds exposed to artificial odours at the home loft and in those exposed during displacement to the release site. These findings suggest that olfactory input, rather than non-specific effects of the...
displacement and homeward flight (i.e. neuronal activity driven by stress, arousal or interaction with conspecifics during the group release), was responsible for the increased neuronal activity (figures 2 and 4).

Interestingly, the pattern of neuronal activation in the triangular and dorsomedial areas of the hippocampal formation are consistent with the possibility that odours play a role in providing spatial information (i.e. effects of artificial odours and pure air were indistinguishable, while those of natural odours perceived during displacement to the release site were elevated in the triangular and dorsomedial areas; figure 4c,d). However, lesion studies indicate that neither of these areas is essential for homing (for a review, see [5]).

Importantly, natural odours from the home loft did not increase neuronal activity in the dorsolateral area of the hippocampal formation relative to that of birds exposed to filtered air (figure 3f). By contrast, both in birds that had to fly home after exposure to natural or artificial odours during displacement to the release site (figures 3f and 4c).
and in birds at the home loft exposed to artificial odours during simulated displacements (figure 3f), there was an increase in neuronal activity relative to controls. Therefore, the characteristic of olfactory stimuli that consistently resulted in increased neuronal activity in the dorsolateral area of the hippocampal formation was not ‘natural’ as

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**Figure 3.** Neuronal activation for different brain regions and stimulus conditions. (a–f) Mean numbers of c-Fos-IR neurons (symbols) in (a) the olfactory bulb, (b) the piriform cortex, (c) the dorsal thalamic nucleus and (d–f) the hippocampal formation (HF): (d) the triangular area, (e) the dorsomedial area (DM) and (f) the dorsolateral area (DL). The stimulus ‘fly’ compares neuronal activity in birds that had to fly home (squares) with birds that stayed in the home loft (circles). The stimulus ‘odour’ compares neuronal activity resultant from exposures to natural odours (black symbols), artificial odours (white symbols) or no odours (grey symbols). The error bars represent ± s.e.m. Significance is given by the repeated-measure ANOVA. Significance level according to: *p < 0.05; **p < 0.01; ***p < 0.001.

**Figure 4.** Neuronal activation in pigeons that had to fly home. Activity was analysed at each brain hemisphere (right/left). Symbols represent the mean number of c-Fos-IR neurons observed in (a) the olfactory bulb, (b) the piriform cortex and (c–e) the hippocampal formation: (c) the triangular area, (d) the dorsomedial area and (e) the dorsolateral area. Additional legends as in figure 3.
opposed to ‘artificial’, and was not ‘odours carrying potential navigational information’ as opposed to ‘odours without navigational information’, but was ‘novel’ as opposed to ‘familiar’. These findings indicate that activity in this region of the brain is primarily triggered by unfamiliar odours that under natural conditions would signal that the pigeon has been displaced from its home loft (i.e. the characteristics expected of olfactory cues that ‘activate’ the navigational systems necessary to return home).

Animal husbandry and all experimental procedures complied with EU and Portuguese law for animal welfare.

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