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BEHAVIORAL NEUROSCIENCE

Navigation-induced ZENK expression in the olfactory system of pigeons (*Columba livia*)

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Abstract

A large body of evidence indicates that pigeons use olfactory cues to navigate over unfamiliar areas with a differential contribution of the left and right hemispheres. In particular, the right nostril/olfactory bulb (OB) and left piriform cortex (Cpi) have been demonstrated to be crucially involved in navigation. In this study we analysed behaviour-induced activation of the olfactory system, indicated by the expression of the immediate early gene ZENK, under different homing conditions. One experimental group was released from an unfamiliar site, the second group was transported to the unfamiliar site and back to the loft, and the third group was released in front of the loft. To evaluate the differential contribution of the left and/or right olfactory input, the nostrils of the pigeons were either occluded unilaterally or not. Released pigeons revealed the highest ZENK cell density in the OB and Cpi, indicating that the olfactory system is activated during navigation from an unfamiliar site. The groups with no plug showed the highest ZENK cell density, supporting the activation of the olfactory system probably being due to sensory input. Moreover, both Cpis seem to contribute differently to the navigation process. Only occlusion of the right OB resulted in a decreased ZENK cell expression in the Cpi, whereas occlusion of the left nostril had no effect. This is the first study to reveal neuronal activation patterns in the olfactory system during homing. Our data show that lateralized processing of olfactory cues is indeed involved in navigation over unfamiliar areas.

Introduction

Homing pigeons possess the extraordinary ability to return to their home loft even when displaced to an unfamiliar location up to hundreds of kilometres away. According to the 'map and compass' model of Kramer (1953), after displacement, birds determine their position with respect to the goal (map step) and orient themselves with a sun (Schmidt-Koenig, 1960) or a magnetic compass (compass step) (Keeton, 1971; Wiltschko *et al.*, 1981). When homing over previously explored areas, pigeons mostly use a map-like representation of familiar landmarks, which is processed in the hippocampal formation (HF) (Bingman *et al.*, 2006a). From unfamiliar locations, up to several hundreds of kilometres, pigeons rely on an olfactory map (Wallraff, 2005 for references). The critical role of olfaction in pigeon navigation was first discovered by Papi *et al.* (1972), who observed that anosmic pigeons were unable to home. He proposed that pigeons acquire an olfactory map by associating the odours carried by the winds at the home area with the directions from which they blow. Once at the release site, they recognize the local odours and determine the direction of displacement. Until now, the role of olfaction in pigeon navigation has been tested at a behavioural level by releasing anosmic or piriform cortex (Cpi)-lesioned birds, or by manipulating the information needed for the map learning at the home loft (Wallraff, 2005 for references). However, an investigation of the neuronal activity pattern within the olfactory system and its related structures under different homing conditions is still lacking.

Test releases on pigeons with unilateral lesion to the Cpi or with one nostril occluded have shown that both hemispheres are necessary for a successful olfactory-based navigation. However, the left and right hemispheric systems turned out to contribute differently to the initial orientation (Gagliardo *et al.*, 2005a, 2007a). In fact, occlusion of the right, but not the left, nostril/olfactory bulb (OB) leads to a disturbance of initial orientation (Gagliardo *et al.*, 2007a). This effect might be related to a perceptual asymmetry with a dominance of the right nostril/OB, as has already been shown in chicks in olfactory discrimination tasks (Vallortigara & Andrew, 1994; Burne & Rogers, 2002). As in reptiles, the OB of birds projects bilaterally onto the Cpi with a stronger input to the ipsilateral side (Reiner & Karten, 1985; Bingman *et al.*, 1994). However, contrary to what one would expect, only left but not right Cpi lesions resulted in an impairment of initial orientation in homing pigeons (Gagliardo *et al.*, 2005a).

In this study, we used the expression of the neuronal activity-dependent marker ZENK to assess whether homing from unfamiliar locations is accompanied by an activation of the olfactory system at the neuronal level and if the functional lateralization is based on asymmetrical neuronal activity patterns within the olfactory pathways. Our results provide strong evidence that the olfactory system is

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activated during navigation over unfamiliar areas in homing pigeons and shows that only the lack of olfactory input on the right olfactory mucosa produces a decreased ZENK cell density in the ipsilateral Cpi.

Materials and methods

Subjects

A total of 122 adult homing pigeons (*Columba livia*) of both sexes, born and housed in a loft at the Arnino field station (10 km SW of Pisa), were used for this study (Table 1). Of the 122 pigeons, 82 (43 female and 39 male) were used for the ZENK analysis. The sex was assessed by visual inspection of the gonads after the decapitation. Therefore, only the sex of birds that were used for the ZENK analysis could be evaluated. We used animals of both sexes, as no differences between sexes are expected on the basis of the biology of the species and consistently no homing studies have highlighted sex differences in homing abilities (Wallraff, 2005 for references). The pigeons were fed *ad libitum* and were allowed spontaneous free flights from the loft. At the time of the experiment, the pigeons were approximately 6 months old.

To map the brain activity, we used the expression of the neuronal activity-dependent marker ZENK, an immediate early gene, which was introduced by Shimizu *et al.* (2004) in homing experiments over familiar locations. In this study we compared the ZENK cell density of the following three experimental groups.

1. R: Released from an unfamiliar location to examine if the olfactory system is specifically activated during homing from an unfamiliar site.
2. TnR: Transported to the unfamiliar location and back to the loft but not released. This group was confronted with the same olfactory input as the first group but they were not required to use this information for homing.
3. RH: Released at about 200 m from the loft at the home site. This group was selected to control for arousal effects of the birds, which may have occurred during handling and release, the flying itself, and during the presumed hippocampus-based landmark orientation.

As ZENK protein expression peaks between 1 and 2 h after stimulus onset and declines thereafter (Mello & Ribeiro, 1998), pigeons had to be caught immediately after their return to the loft. For this reason, those pigeons that tended to enter the loft faster had been identified in a preliminary release at 500 m from the loft. The pigeons that entered the loft fastest were assigned to the two released groups (R and RH). The remaining pigeons were allocated to the TnR group.

TABLE 1. Pigeons used in the experiment

Group and nostril condition	No. of pigeons (released/ZENK)
R group	
No plug	14/11
Left plug	27/10
Right plug	28/12
TnR group	
No plug	8
Left plug	8
Right plug	8
RH group	
No plug	9/8
Left plug	10/9
Right plug	10/7

R group, released from an unfamiliar location; RH group, released at about 200 m from the loft at the home site; TnR group, transported to the unfamiliar location and back to the loft but not released.

The three groups were further subdivided into three subgroups: left nostril plugged, right nostril plugged and no nostril plugged. The birds had their nostril plugged on the evening before the experiment. The plugs were made out of a small amount of a paste (Xantopren®), which turns into a solid rubbery plug after inserting it into the nostril. If some pigeons lost their plugs during the night, they were replaced early in the morning before the experiment. Previous studies demonstrated that this plugging procedure is very efficient at blocking the olfactory information of one nostril completely, without causing cross-lateral contamination from the not-plugged nostril (Benvenuti & Gagliardo, 1996).

Release and circular statistic procedures

The experimental release took place on three consecutive days under sunny conditions with no or only light wind. The pigeons of the R and TnR group were transported to one of the unfamiliar release sites [release sites: 1, Fornacette (23 km, home direction 271°) and 2, La Costanza (18 km, home direction 190°)]. The distance of both release sites was similar with respect to the approximate time that the pigeons needed for homing, which should not exceed 120 min to ensure optimal ZENK visualization (Mello & Ribeiro, 1998). During transportation, the pigeons had the possibility of smelling the surrounding air through the open windows of the car. Prior to release, the position of the plug was controlled again. A tape was applied around a leg of each bird and the time of release was recorded to enable the experimenter waiting for the pigeons at the home loft to kill only the subjects homed within 120 min of the toss. The homing time was recorded for each bird and pigeons arriving together were excluded from the experiment. The birds were released singly, alternating among the three nostril conditions. The initial orientation was recorded by an observer who was blind to the nostril conditions. Each bird was followed with the aid of 10×40 binoculars until it disappeared from the observer's view and the azimuth of the vanishing bearing was recorded with a compass. For each group, we calculated the mean vector and the homeward component relative to the initial orientation distribution of either all released pigeons or only those pigeons for which the ZENK expression was measured. The initial orientation distribution was tested for randomness by employing both the Rayleigh and the V-test (Batschlet, 1981). The RH group was released in front of the loft, at about 200 m from it. The pigeons belonging to the R and RH groups were caught as soon as they entered the loft. Pigeons caught before 60 min after release were kept in cages and killed at least 60 min, but not later than 120 min, after they had been released. The drive to enter the loft was much higher in the R pigeons than in the RH birds. The RH birds first performed a flight over the loft and then usually stayed for a while on the roof of the loft before entering it. TnR pigeons stayed for approximately 60 min at the release site and were then transported back to the loft, which took approximately 15–20 min. They were then killed at least 60 min, but not later than 120 min, later than their journey back from the release site started. Pigeons that lost their plug during the flight were not used for histology. The study was performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the animal ethics committee of the Landesamt für Natur, Umwelt und Verbraucherschuta NRW, Germany.

Fixation

Animals were killed by decapitation. The brains were removed and fixed for 3 h in 5% Acrolein in 0.12 M phosphate-buffered saline

(PBS) (pH 7.4). They were then rinsed briefly in PBS, washed twice for 30 min in PBS and cryoprotected in 30% sucrose in PBS. To avoid loss of the OB during immunohistochemistry, we embedded the brain in 15% Galatine in 30% sucrose in PBS. The embedded brains were cryosectioned in the frontal plane (40 μm). The left or right brain side was marked by a hole stuck with a small needle. Slices were collected in five parallel series for the OB and 10 parallel series for the rest of the brain and stored in 0.12 M PBS containing 0.1% sodium azide at 4°C until they were subjected to immunohistochemistry.

Immunohistochemistry

The immunohistochemical detection of ZENK was performed with free-floating slices according to the standards of the immuno-ABC technique (Hellmann & Güntürkün, 2001). After each incubation step, the slices were washed three times for 5 min with PBS. Slices of one series were incubated in 0.1% $NaBH_4$ in PBS for 15 min. Endogenous peroxidases were blocked with 0.3% H_2O_2 in deionized water for 30 min. Slices were incubated with 10% normal goat serum in 0.12 M PBS+0.3% Triton X-100 for 1 h to block non-specific binding sites in the tissue. The slices were then incubated with primary antibody solution (1/5000 + 1% normal goat serum; rabbit erg-1, sc-189, Santa Cruz) (Shimizu *et al.*, 2004) for 72 h at 4°C. The secondary antibody reaction was carried out with biotinylated goat anti-rabbit IgG (1/200 in 0.12 M PBS+0.3% Triton X-100; Vectastain Elite kit; Vector, Burlingame, CA, USA) for 1 h at room temperature (22°C). Afterwards, the tissue was incubated in an avidin-biotin-peroxidase solution (1/100 in PBST; Vectastain ABC-Elite kit). The peroxidase activity was detected using a heavy metal-intensified 3′3-diaminobenzidine (Sigma) reaction, modified by the use of 1% β-D-glucose/glucose oxidase (Sigma-Aldrich, Munich, Germany) (Hellmann & Güntürkün, 2001). The slices were mounted on gelatinized slides, dehydrated and coverslipped with Permount (Fisher Scientific, NJ, USA). One corresponding serial set was stained with cresyl violet to visualize neuronal structures.

Quantification and data analysis

The quantification of ZENK expression was conducted blindly to the experimental conditions and hemisphere. The density of ZENK-positive cells was analysed bilaterally in the OB, Cpi and hippocampus for each pigeon. Pictures of a representative region of 800 × 800 or 1300 × 1030 pixels (136.64 × 136.64 or 225.29 × 178.49 μm^2, magnification 40 × 2.5) were captured with a camera-equipped microscope (Olympus BH-2, Axio Vision 3.4). The pictures were converted to eight-bit grey-scale images by Adobe Photoshop (CS2). ZENK-positive cells were counted automatically using the ImageJ program (Rasband, 1997–2008). Both strong and faintly stained cells were included in the cell counting, thus avoiding a bias based on differences in staining intensity (Shimizu *et al.*, 2004). The threshold was set manually according to Shimizu *et al.* (2004). Our quantification aimed to test possible differences between the three different groups as well as both hemispheres. Thus, a reconstruction of the complete number of labelled neurons within an anatomically defined area was not intended.

For OB analysis, only slices with a U-shape of the granular cell layer including the ventricle were examined (Fig. 1A). In these slices, the OB was subdivided into three regions of interest: medial, ventral and lateral. Two regions of interest were defined in OB slices: medial and lateral, in case these slices were too small to be divided into three regions. In a pre-analysis of five brains, we concluded that the number of five randomly chosen regions (Mat lab, The Mathworks Inc. Biopsychology Toolbox, Rose *et al.* 2008) of interest in the OB was sufficient to obtain reliable results for the ascertainment of ZENK cell density. For the Cpi analysis, one picture of each slice with a visible Cpi was taken (Fig. 1B). For Cpi and OB, a picture size of 136.64 × 136.64 μm^2 was used due to the narrow size of these two areas. The hippocampus was analysed at A 5.75 (anterior coordinate according to the Atlas of Karen and Hodos, 1967) (Karten & Hodos, 1967) in the dorsolateral (DL), dorsomedial and triangular part, according to Atoji & Wild (2004) (Fig. 1B) in a representative area of 225.29 × 178.49 μm^2. The sampling window in all analysed brain areas was taken from the middle of the area of interest. We made use of parallel cresyl violet-stained slices to determine the region of interest.

Statistical analysis was carried out using the program Statistica (StatSoft, Tulsa, USA). The mean density of ZENK-positive cells per group in the OB and Cpi was subjected to a mixed 3 × 3 × 2 analysis of variance with 'releasing condition' [released (R), transported to the released site but not released (TnR), released in front of the loft (RH)] and the 'nostril condition' (no plug, left plugged, right plugged) as between-subject factors and with 'hemisphere' (left, right) as repeated measure. For statistical analysis of the hippocampus, we used the same procedure as above but, as the hippocampus was subdivided into three areas, a second factor of repeated measures, the 'area condition' (DL, dorsomedial, triangular part), was added. As the number of animals varied among the groups from 7 to 12, we used the honestly

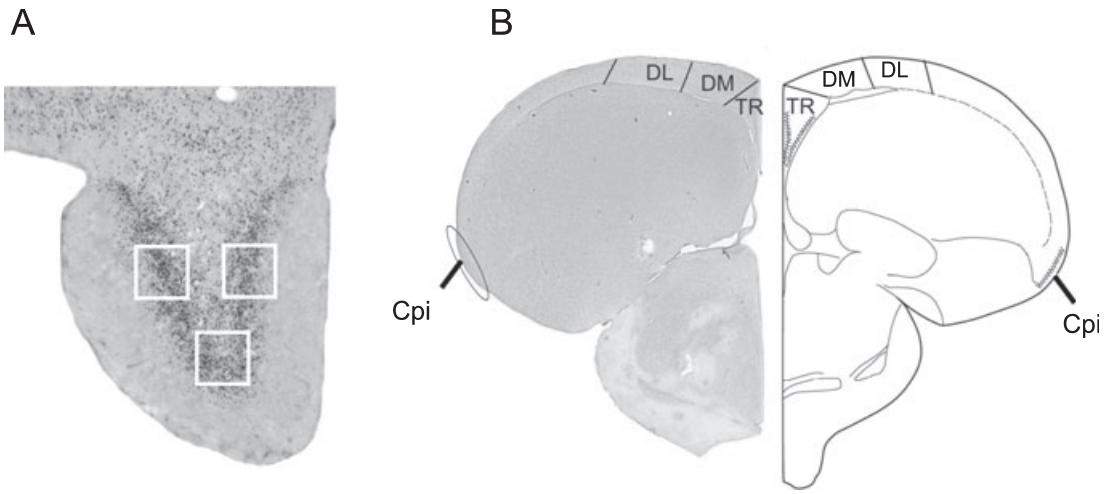

FIG. 1. Overview of the analysed areas. (A) OB. Squares indicate the sampling windows (lateral, ventral and medial) chosen for analysis. (B) Cpi and HF. The HF was subdivided into three subareas [DL, dorsomedial (DM) and triangular part (TR)] for analysis.

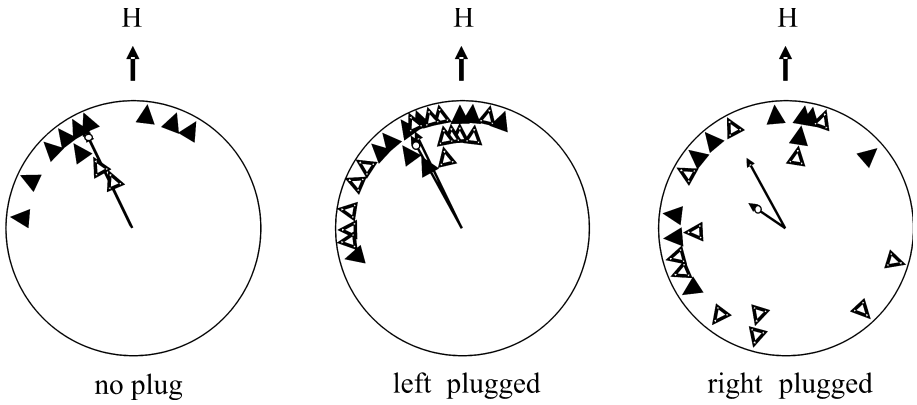

FIG. 2. Pooled initial orientation of pigeons with no plug, pigeons with the right nostril plugged and pigeons with the left nostril plugged. Each symbol represents the bearing of a single pigeon. Filled triangles and open triangles represent the birds used in the ZENK experiment and those excluded, respectively. The mean vector relative to the distribution of all pigeons is represented by the inner white spotted arrow; the mean vector relative to the distribution of the pigeons used in the ZENK experiments is represented by the solid inner arrow. The outer arrow represents the home (H) direction. See text for further explanations.

significant difference (HSD) test for post-hoc analysis with unequal sample sizes. The *post-hoc* Fisher least significant difference (LDS) test for equal sample was used to analyse the differences between the three subareas of the hippocampus.

Results

Initial orientation

The pooled (home direction set to 360°) initial orientation distributions of the pigeons released from two sites are presented in Fig. 2 and Table 2. The initial orientation displayed by the three experimental groups is consistent with previous results (Gagliardo *et al.*, 2007a) if all released pigeons are considered. In fact, both not-plugged and left nostril-plugged pigeon groups displayed initial orientation distributions significantly different from random, whereas the distribution of the right nostril-plugged birds turned out to be randomly scattered (see Table 2 for the Rayleigh and V-test results).

When selecting the bearings of the pigeons included in the ZENK experiment, the three experimental groups were all significantly oriented (see Fig. 2 and Table 2). This was due to the fact that for the analysis of the ZENK expression we had to select only the birds homing within 2 h, which were more likely to be those birds displaying an initial orientation closer to the home direction.

TABLE 2. Summary of behavioral results

Group	*N*	*n*	α	*r*	HC
All pigeons					
No plug	14	12	334°	0.86***	+0.77***
Left plug	27	23	331°	0.83***	+0.72***
Right plug	28	22	304°	0.35	+0.20
Pigeons used in the ZENK experiment					
No plug	12	10	334°	0.83***	+0.74*
Left plug	12	9	334°	0.84*	+0.75***
Right plug	12	10	331°	0.63*	+0.55**

Group, control pigeon with no plug, pigeons with the left nostril plugged and pigeons with the right nostril plugged; *N*, birds released; *n*, birds for which the initial orientation was recorded; α, mean vector direction; r, mean vector length; HC, homeward component. The asterisks in the *r* and HC columns indicate the results of the Rayleigh and V-test, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Olfactory bulb

The multivariate analysis revealed significant main effects of 'releasing' ($F_{2,71} = 14.18$, $P < 0.001$) and 'nostril condition' ($F_{2,71} = 16.39$, $P < 0.0001$). No main effect of 'hemisphere' was found ($F_{1,71} = 0.14$ $P = 0.71$).

Post-hoc analysis showed that the R group ($5845 \pm 2127/mm^2$) had a higher ZENK cell density than the TnR ($4289 \pm 1835/mm^2$; $P < 0.001$) and RH ($3487 \pm 2232/mm^2$; $P < 0.01$) groups, indicating that orientation in an unfamiliar environment increases the expression of the neuronal activity marker ZENK.

Birds with no plug ($6153 \pm 1612/mm^2$) displayed the highest ZENK cell density compared with the left- ($3789 \pm 2315/mm^2$) and right- ($4163 \pm 2189/mm^2$) plugged ($P < 0.001$) groups (Figs 3B and 4).

The significant interaction between 'hemisphere' and 'nostril condition' ($F_{2,71} = 48.08$, $P < 0.0001$) (Fig. 3C) suggested that the hemisphere-specific activation depended on the 'nostril condition'. Moreover, the significant triple interaction of 'hemisphere', 'nostril' and 'releasing condition' revealed that the hemisphere-specific activation was not only modulated by the 'nostril condition' alone but also by its combination with the 'release condition' ($F_{4,71} = 7.79$, $P < 0.001$). Although no differences between the hemispheres could be detected in the TnR and RH condition in all three nostril conditions, R pigeons showed a decreased ZENK cell density in the ipsilateral OB in both plugged 'nostril conditions' (left plugged, $P < 0.0001$; right plugged, $P < 0.001$) (Figs 3D and 4). No further significant interactions were observed.

Piriform cortex

Significant main effects of 'releasing' ($F_{2,73} = 71.69$, $P < 0.001$) and 'nostril condition' ($F_{2,73} = 20.22$, $P < 0.001$) were found. No main effect of 'hemisphere' was observed ($F_{1,73} = 0.78$, $P = 0.38$).

As expected, R pigeons ($1349 \pm 448/mm^2$) revealed a higher ZENK cell density compared with the TnR ($736 \pm 351/mm^2$, $P < 0.001$) and RH ($417 \pm 333/mm^2$, $P < 0.001$) groups. In contrast to the OB, the TnR and RH groups also differed in ZENK cell density with more ZENK-positive cells in the TnR birds ($P < 0.001$) (Figs 5A and 6).

The groups with no plug revealed the highest ZENK cell density ($1193 \pm 532/mm^2$, $P < 0.001$) compared with the left- ($758 \pm 545/mm^2$) and right- ($711 \pm 469/mm^2$) plugged groups ($P = 0.84$) (Fig. 5B).

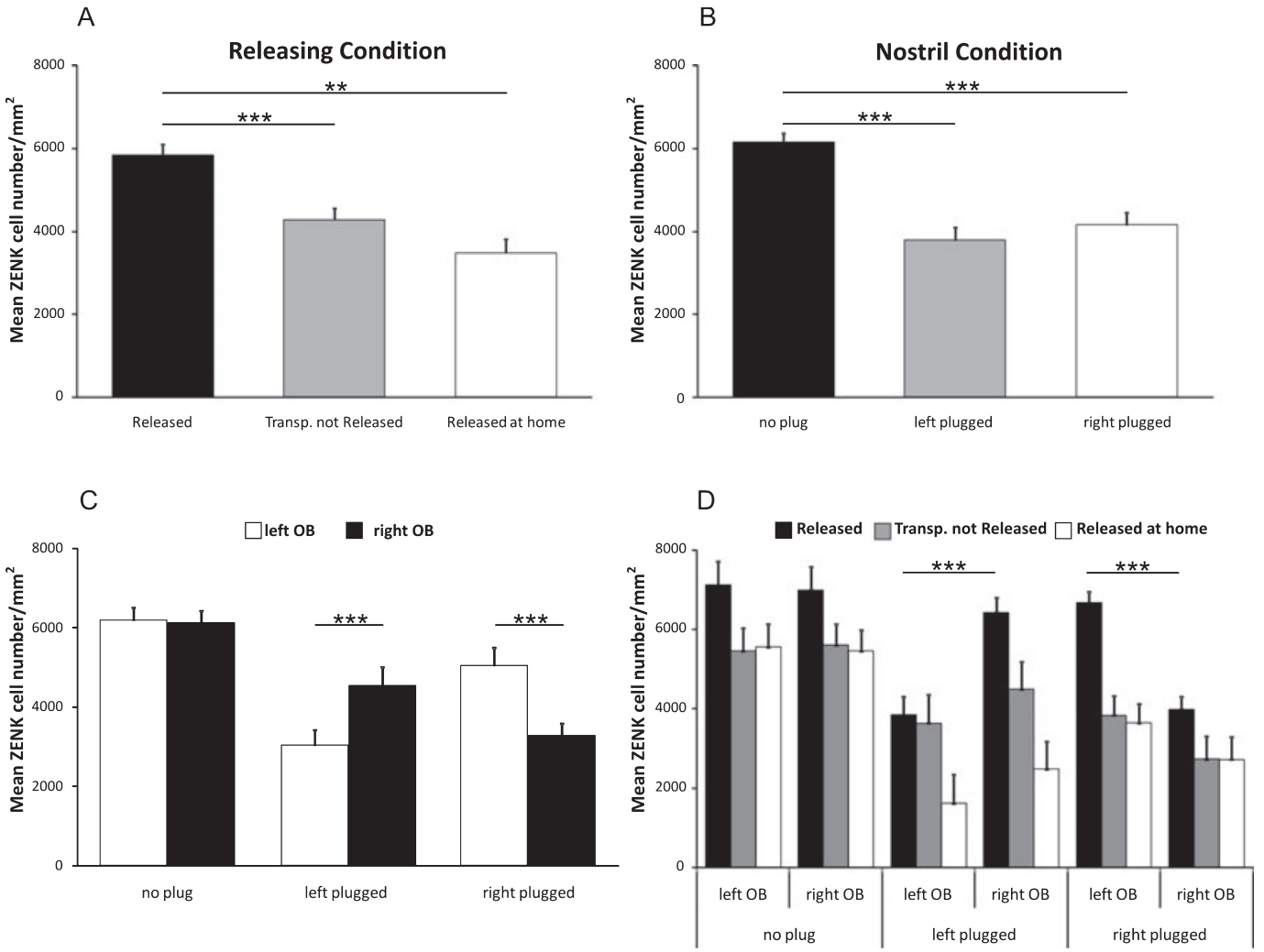

FIG. 3. (A) Mean density of ZENK-labelled cells in the OB of pigeons from the three releasing conditions: R, TnR and RH. (B) Mean density of ZENK-labelled cells of the three nostril conditions: not plugged, left plugged and right plugged. (C) Mean density of ZENK-labelled cells of pigeons from the three releasing conditions plotted against hemisphere: left and right. (D) Mean density of ZENK-labelled cells of pigeons from the three releasing conditions plotted against nostril and hemisphere condition. $^{**}P < 0.01$, $^{***}P < 0.001$.

As in the OB, the significant interaction between 'hemisphere' and 'nostril condition' ($F_{2,73} = 8.14$, $P < 0.001$) (Fig. 5C) suggested that the hemisphere-specific activation was modulated by the nostril condition. Moreover and similar to the OB, a significant triple interaction indicated that the differences in hemisphere-specific ZENK expression between the 'nostril conditions' depended on the 'releasing conditions' ($F_{4,73} = 3.1376$, $P < 0.05$). However, contrary to the OB data, only the released pigeons with a right-plugged nostril showed significantly reduced ZENK expression in the ipsilateral Cpi (right Cpi, 974 ± 309/mm^2; left Cpi, 1251 ± 293/mm^2, $P < 0.05$) (Figs 5D and 6). Moreover, after the occlusion of the right nostril only the right Cpi revealed a significantly reduced ZENK expression compared with the right Cpi of the not-plugged condition ($P < 0.0001$). However, no differences in ZENK expression were found between the left Cpi of the not-plugged condition and the left Cpi after occlusion of the left nostril ($P = 0.79$). For further verification of the lateralized ZENK expression in the Cpi, we calculated the asymmetry index (AI = cell number$_{left}$ – cell number$_{right}$/cell number$_{left}$ + cell number$_{right}$), which expresses the degree of asymmetry as a score between – 1 and 1. Only the AI of the right-plugged Cpi (AI = 0.11) revealed a significant difference to the not-plugged condition (AI = −0.08, $P < 0.01$). No differences were found between the left-plugged (AI = −0.07) and not-plugged ($P < 0.97$) condition, underlining the fact that only the occlusion of the right nostril, and not occlusion of the left nostril, had a significant effect on the ZENK expression of the ipsilateral Cpi. No further significant interactions were observed.

Hippocampal formation

Statistical analysis revealed a significant main effect of 'releasing' ($F_{2,73} = 125.88$, $P < 0.001$) and 'area condition' ($F_{2.146} = 71.58$, $P < 0.001$) (Figs 7B and 8). No main effect of 'hemisphere' was found ($F_{1,73} = 1.68$, $P = 0.19$). In contrast to the results of the OB and Cpi, no main effect of 'nostril condition' ($F_{2,73} = 0.52$, $P = 0.59$) was observed in the hippocampus.

Post-hoc analysis showed that the R group (974 ± 583/mm^2) displayed higher ZENK cell density than the TnR (150 ± 167/mm^2, $P < 0.001$) and RH (391 ± 323/mm^2, $P < 0.001$) groups. Moreover, ZENK cell density was higher in the RH group compared with the TnR birds ($P < 0.001$) (Figs 7A and 8).

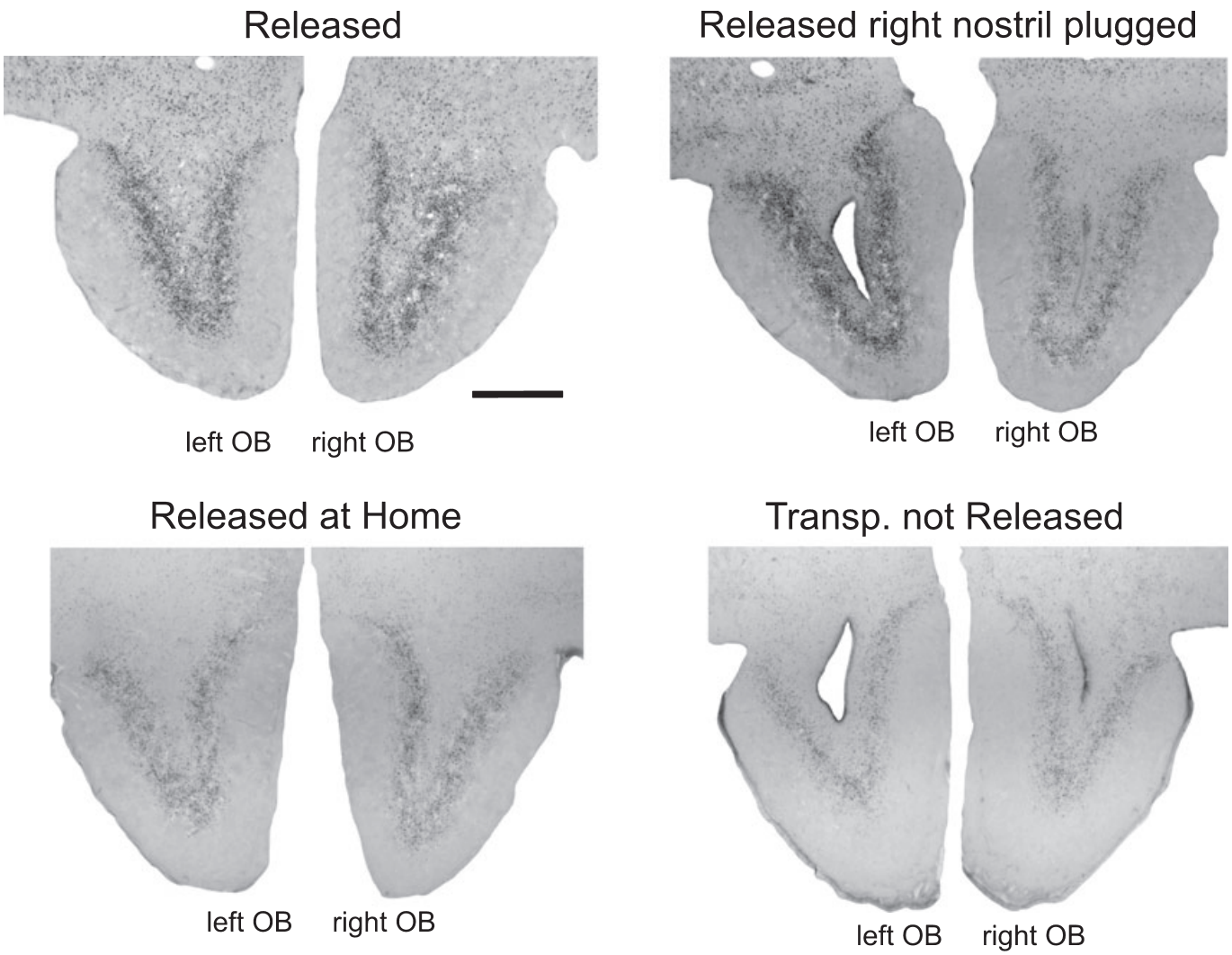

FIG. 4. ZENK cell staining in the right and left OB of the three experimental conditions (R, TnR and RH) of pigeons with no plug and in the OB of released pigeons with the right nostril plugged. Scale bar, 500 μm.

A significant interaction of 'area' and 'releasing condition' ($F_{4,146} = 5.17$, $P < 0.001$) indicated that the ZENK expression in the three hippocampal subareas depended on the 'releasing condition' (Figs 7C and 8).

The significant three-way interaction of 'hemisphere', 'area' and 'nostril condition' demonstrated that the hemisphere-specific activation of the three hippocampal subareas is modulated by the 'nostril condition' ($F_{4,71} = 7.79$, $P < 0.001$). Only in the not-plugged 'nostril condition' did the DL hippocampus show a higher ZENK expression in the left hemisphere compared with the right hemisphere (DL left, $1048 \pm 848/\text{mm}^2$; DL right, $805 \pm 649/\text{mm}^2$, $P < 0.001$) (Figs 7D and 8). No further significant interactions were observed.

In principle, it is conceivable that the ZENK expression is directly proportional to the homing time as pigeons might smell with an increased intensity during flight. To exclude this possibility, we calculated a Pearson correlation of ZENK expression and flight time. As no main effect of 'hemisphere' was found, we pooled the data from both hemispheres. No significant correlation of homing time and ZENK expression could be observed for all three analysed areas (OB, $r = -0.11$, n.s.; Cpi, $r = 0.16$, n.s.; hippocampus: DL, $r = 0.25$, n.s., dorsomedial, $r = 0.23$, n.s., triangular part, $r = 0.23$, n.s.).

Discussion

The present study demonstrates that navigation during homing in pigeons results in higher ZENK cell density in the olfactory system and HF. The highest ZENK expression was observed after homing from unfamiliar terrain, particularly in the olfactory system. These results strongly support the hypothesis that olfactory cues are used to navigate from unfamiliar sites (Papi *et al.*, 1972; Wallraff, 2005 for references).

Olfactory bulb

The OB of the released group revealed the highest ZENK cell density compared with the two control groups. Sensory input triggers ZENK expression in the OB, which was reduced by occluding the ipsilateral nostril. However, this stimulation-induced ZENK expression was only observed in the pigeons released from unfamiliar locations and not in the TnR and RH groups, where no significant effect of nostril occlusion on the ZENK cell density could be observed. Nonetheless, these two groups had a functioning olfactory input via at least one of their nostrils. They either smelled the local odours at the release site

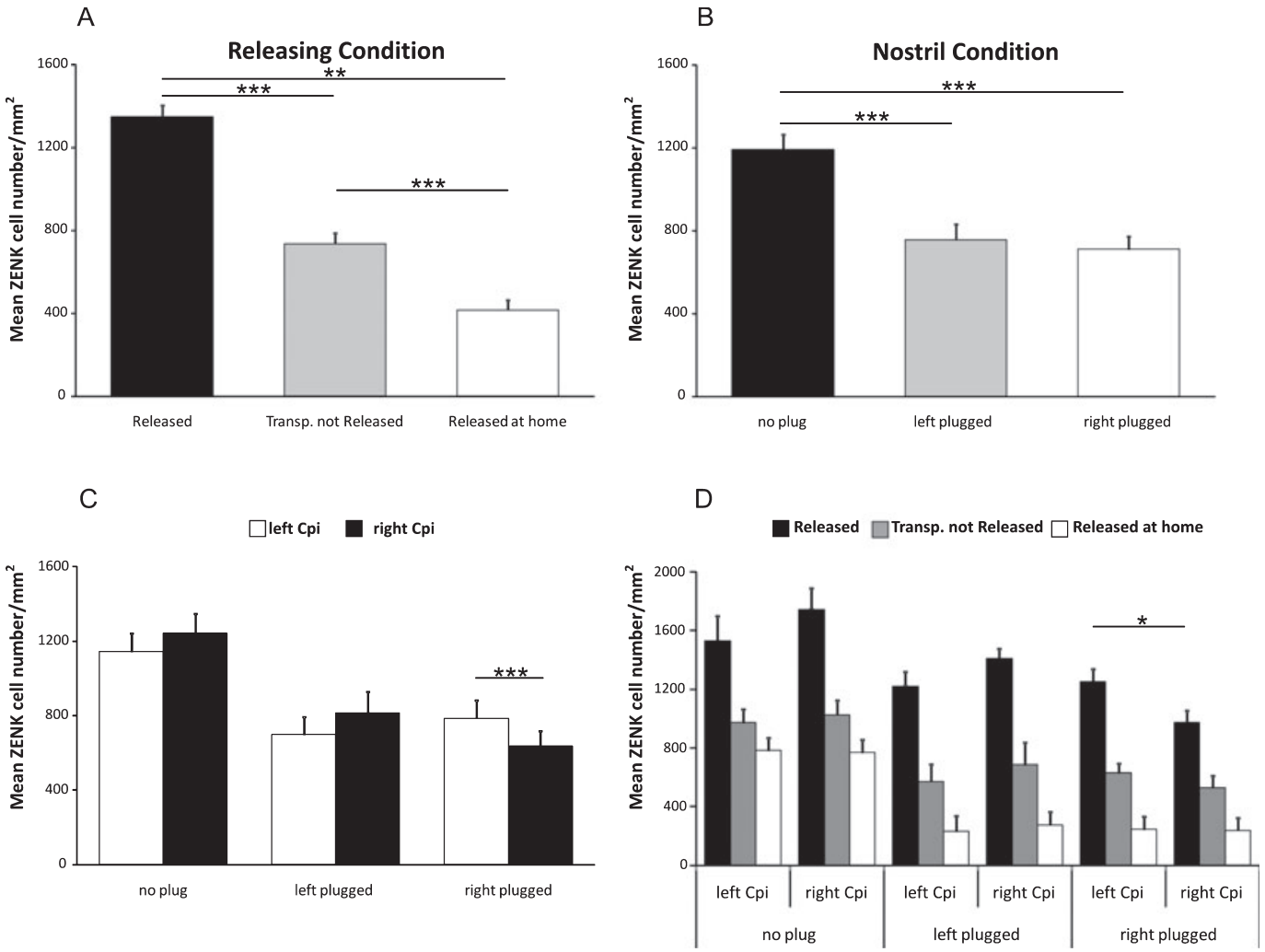

FIG. 5. (A) Mean density of ZENK-labelled cells in the Cpi of pigeons from the three releasing conditions: R, TnR and RH. (B) Mean density of ZENK-labelled cells of the three nostril conditions: not plugged, left plugged and right plugged. (C) Mean density of ZENK-labelled cells of pigeons from the three releasing conditions plotted against hemisphere: left and right. (D) Mean density of ZENK-labelled cells of pigeons from the three releasing conditions plotted against nostril and hemisphere condition. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(TnR) or at the familiar area around the loft (RH). The fact that these stimulations resulted in a lower OB ZENK cell density compared with the R birds favours the assumption that the olfactory system is more strongly activated when pigeons have to navigate actively over unfamiliar areas.

The OB as a primary sensory target is presumably not directly involved in navigation processing. Therefore, the highest ZENK cell density in the R birds implies that OB activity is modulated by a top-down input depending on behavioural context.

In contrast to behavioural experiments, which suggest that the right nostril/OB is functionally dominant (Gagliardo *et al.*, 2007a), no hemispheric differences in ZENK cell density could be observed. This finding indicates that the functional lateralization either cannot be visualized by using the ZENK method or must be triggered through other, perhaps higher, processes within the Cpi or beyond.

Piriform cortex

The Cpi is the main projection area of the OB (Reiner & Karten, 1985; Bingman *et al.*, 1994). Lesion studies demonstrated that the Cpi is crucial for olfactory-based navigation from unfamiliar sites (Papi & Casini, 1990). Moreover, the Cpi receives diverse input from other brain areas yielding evidence that its role is not limited to olfactory stimuli but might be regarded as an associative area (Bingman *et al.*, 1994) involved in olfactory map processing. Therefore, very similar to the OB, a higher ZENK cell density would also be expected in the Cpi of released pigeons, compared with the two control groups. We successfully confirmed this assumption in our study. As predicted, the highest ZENK expression was detected in the Cpi of released birds, again demonstrating that processing of olfactory cues is a key feature of navigation from unfamiliar locations. Unlike in the OB, the data from the Cpi revealed a higher ZENK cell density in TnR pigeons compared with the RH pigeons that were released in front of the loft. It is possible that the olfactory environment of the release site near the loft stimulates associative areas of the olfactory system to a smaller degree than in pigeons that have to actively find their way back. Furthermore, this result is consistent with several behavioural findings according to which pigeons orient at the release site before taking off (Chelazzi & Pardi, 1972; Mazzotto *et al.*, 1999; Gagliardo *et al.*, 2001c). The higher ZENK cell density in the Cpi could be a result of using olfactory cues before and during take-off. The lowest ZENK cell density in the olfactory system was found in RH birds. This

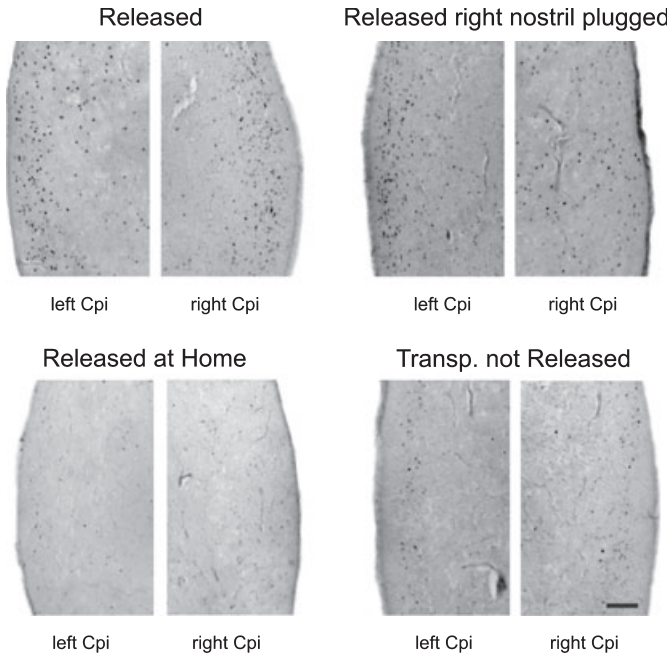

FIG. 6. ZENK cell staining in the right and left Cpi of the three experimental conditions (R, TnR and RH) of pigeons with no plug and in the Cpi of released pigeons with the right nostril plugged. Scale bar, 100 μm.

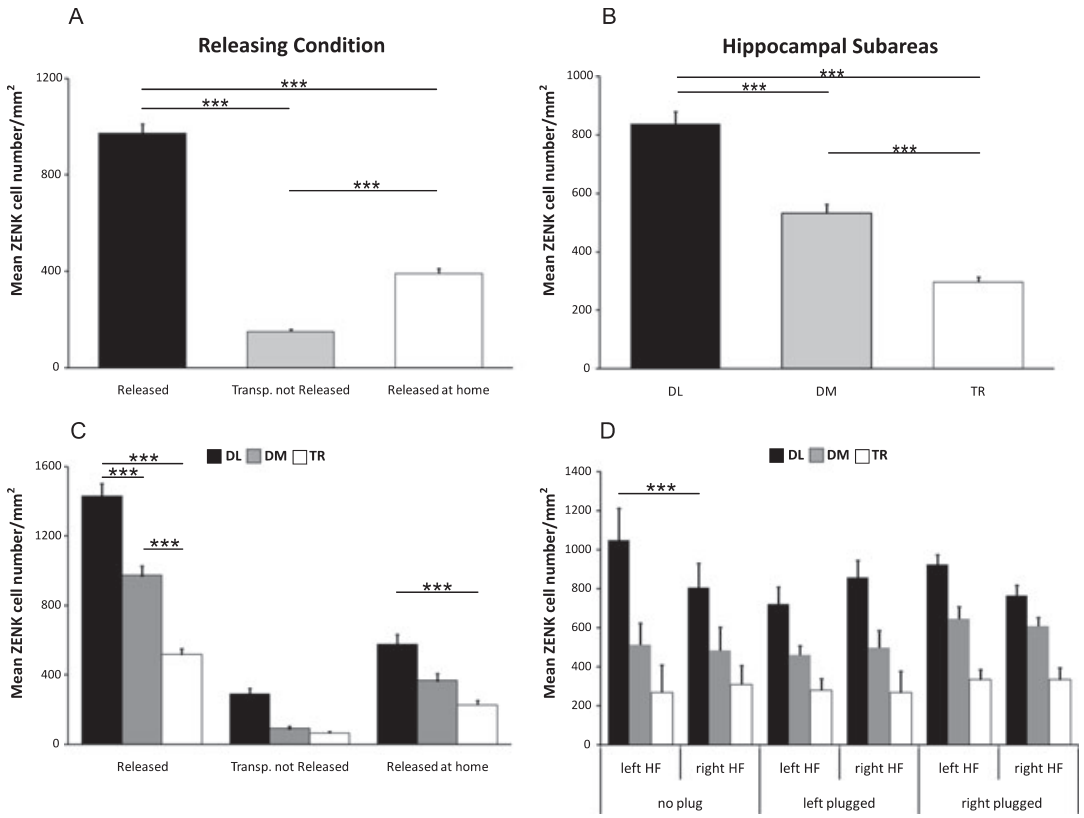

FIG. 7. (A) Mean density of ZENK-labelled cells in the HF of pigeons from the three releasing conditions: R, TnR and RH. (B) Mean density of ZENK-labelled cells in the three hippocampal subareas: DL, dorsomedial (DM) and triangular part (TR). (C) Mean density of ZENK-labelled cells of the three hippocampal subareas plotted against releasing condition and subarea. (D) Mean density of ZENK-labelled cells of the three hippocampal subareas plotted against nostril condition, hemisphere condition and subareas. ***$P < 0.001$.

corresponds to behavioural data that show that pigeons do not have to use their olfactory system to home over familiar areas (Papi & Casini, 1990). Moreover, pigeons use other navigational mechanisms like the hippocampus-based visual landmark orientation [see Hippocampal formation section below] within a familiar area (Shimizu *et al.*, 2004; Gagliardo *et al.*, 2009).

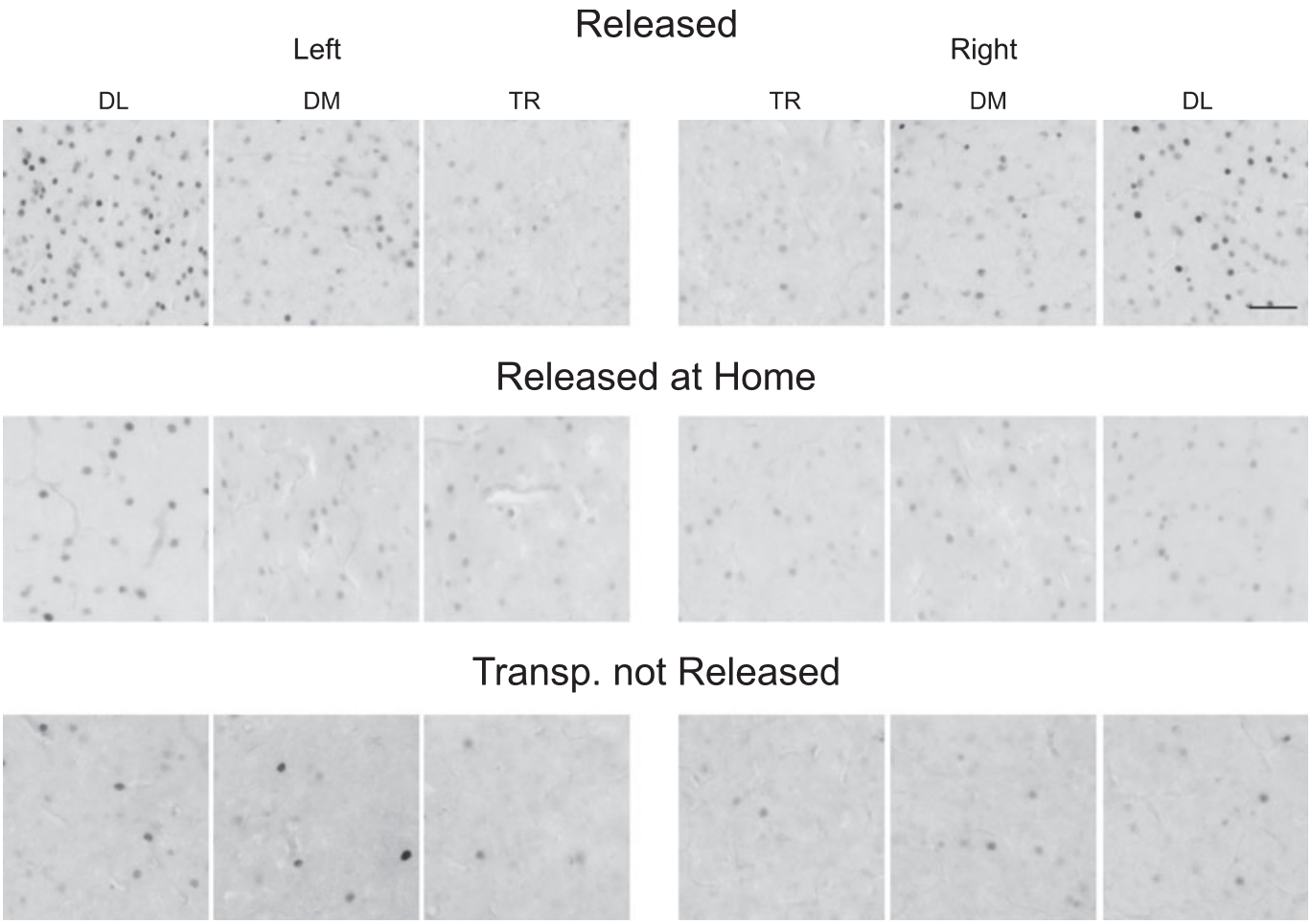

FIG. 8. ZENK cell staining in the right and left hippocampal subareas [DL, dorsomedial (DM) and triangular part (TR)] of the three releasing conditions (R, TnR and RH) of pigeons with no plug. Scale bar, 50 μm.

Behavioural experiments demonstrated that the left and right Cpis are important for navigation over unfamiliar locations, with a predominant role of the left Cpi (Gagliardo *et al.*, 2005a). As in the OB, no hemispheric differences were observed in the not-plugged nostril condition in the Cpi. However, in contrast to the OB, only the right Cpi, but not the left Cpi, of the released birds showed reduced ZENK cell density after ipsilateral sensory deprivation. This higher dependence on olfactory input of the right Cpi could mediate the impaired initial orientation after right nostril plugging (Gagliardo *et al.*, 2007a). Thus, the assumed right nostril/OB dominance could result from a higher sensory sensitivity of the right Cpi and not from an asymmetry of bulbar function as such. This still does not explain the predominant role of the left Cpi during navigation. However, it might be that the critical role of the left Cpi is based on neuronal mechanisms, which are not visualized by ZENK. Although further neuroanatomical studies are necessary to clarify the structural basis, the ZENK expression pattern emphasizes the crucial role of the Cpi during navigation over unfamiliar areas with a striking asymmetrical involvement of both Cpis in processing olfactory cues.

It has recently been proposed that olfactory cues do not provide any navigational information to the pigeons but are needed for activating a non-olfactory, presumably magnetic, navigational system (Jorge *et al.*, 2009, 2010). According to this hypothesis the navigational impairment observed in anosmic pigeons would be due to a lack of activation rather than to a lack of olfactory information. Nevertheless, the activation hypothesis is contradicted by a large body of evidence (Benvenuti *et al.*, 1973, 1977; Fiaschi *et al.*, 1981; Gagliardo *et al.*, 2001a; Ioalè, 1980; Ioalè *et al.*, 1990; Papi *et al.*, 1974; Wallraff, 2005 for references) coming from experiments in which birds with an intact olfactory system were exposed to manipulated environmental stimuli. The present results further support a navigational utilization of olfactory cues. If olfactory information is not used for navigation itself, we would have observed the same ZENK activation pattern of the olfactory system in both transported groups (R and TnR). This, however, was not the case. Instead, we could determine significant differences in the activation pattern between the groups, making it likely that active navigation increases the processing of olfactory information.

Hippocampal formation

Hippocampal lesions do not affect orientation from unfamiliar locations, indicating that the hippocampus is not involved in the operation of the olfactory map learned before the lesions (Bingman *et al.*, 1987). By contrast, a large body of evidence has demonstrated the involvement of the HF in landmark-based navigation (Bingman *et al.*, 2005; Gagliardo *et al.*, 2009). It has also been shown that during the final step of the homing process, when localizing the loft

within the home area, pigeons rely on familiar landmarks (Gagliardo *et al.*, 2007b) and that hippocampal lesions produce an impairment in birds performing this task (Bingman & Mench, 1990). This is consistent with our findings that pigeons released in the vicinity of the loft had a higher activation of the HF compared with the TnR birds. The parahippocampal area in particular has been shown to be activated during navigation over familiar terrain (Shimizu *et al.*, 2004). Our results show that released pigeons revealed the highest ZENK expression in the DL hippocampus (corresponding to the parahippocampal area) compared with other subareas. The activation of this hippocampal substructure is consistent with an involvement of the DL hippocampus in learning the spatial array of visual landmarks during the homing flights (Gagliardo *et al.*, 1999). In contrast to olfactory brain areas, the ZENK expression was not triggered through olfactory input, arguing for a landmark-based navigation system that does not rely on olfactory cues. The olfactory system of RH birds revealed the lowest ZENK expression rate, which is in remarkable contrast to the released birds. Again this supports the assumption that the olfactory and visual landscape-based navigation mechanisms may be used independently according to environmental necessities. The ZENK expression in the HF and olfactory areas of the R birds argues, albeit indirectly, that both mechanisms can be used simultaneously.

Furthermore, the DL hippocampus exhibits an asymmetric ZENK expression with more ZENK-positive cells in the left DL hippocampus compared with the right DL hippocampus in the birds with no plugs, independent of the releasing condition. Several studies demonstrate a functional lateralization of the avian hippocampus. The left HF is assumed to be important for navigational processes, whereas the right HF is more important for representing the locations of events (Bingman *et al.*, 2006b). Studies on the firing pattern of the hippocampal cells in pigeons indicated a critical involvement of the left HF in the navigational processes, whereas the right HF seemed to be more important for representing the locations of events (Siegel *et al.*, 2006). The electrophysiological data are consistent with behavioural studies indicating the specific role of the left hippocampus in processing the geometric properties of the environment (Nardi & Bingman, 2007) and the sun compass-mediated spatial learning (Gagliardo *et al.*, 2005b). The latter would explain the observed impairment of the left hippocampal-ablated pigeons in developing the olfactory navigational map (Gagliardo *et al.*, 2001b). In releasing experiments from familiar locations where one eye of the pigeons was occluded, a superiority of the right eye/left hemisphere was shown (Ulrich *et al.*, 1999). Moreover, the right eye/left hemisphere of migratory birds was indicated to be predominant for magneto-reception in compass orientation (Wiltschko *et al.*, 2002). These various facts could account for the higher activation of the left DL hippocampus.

In conclusion, our findings provide strong evidence for the olfactory navigational hypothesis. The olfactory system seems to supply the neuronal substrate for navigation over an unfamiliar location where the left and right olfactory systems contribute differently to the navigation process. In addition to the olfactory system, an activation of the HF, which is involved in visual landmark orientation, demonstrates that the navigation over non-familiar locations is processed by the olfactory system where navigation over familiar location is processed, at least in part, by the HF.

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Abbreviations

AI, asymmetry index; Cpi, piriform cortex; DL, dorsolateral; HF, hippocampal formation; OB, olfactory bulb; PBS, phosphate-buffered saline; R group, released from an unfamiliar location; RH group, released at about 200 m from the loft at the home site; TnR group, transported to the unfamiliar location and back to the loft but not released.

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