

Olfaction based navigation in pigeons (*Columba livia*):

Examination of the neuronal substrates

and their asymmetries

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Chapter 1

General Introduction

General Introduction

Among all animals only a number of species possess the ability of true navigation. These animals are capable of returning to their home from an unfamiliar location, without using familiar landmarks, knowing the direction of displacement, or any information collected during the displacement (Griffin, 1952; Able, 2001). This extraordinary ability has been demonstrated in various types of birds, amphibians, reptiles and even in invertebrates (Rodda et al., 1992; Boles and Lohmann, 2003; Wallraff, 2005).

Although all these animals are enormously diverse in their anatomy, habitat, reproduction, foraging behaviour etc., they all have in common this extraordinary navigation skill. This begs the question how they are capable to accomplish this. Exploring this issue has fascinated scientists for a long time and is still a question of great interest. One main focus is the navigation ability of birds especially the remarkable homing capability of pigeons. Domesticated homing pigeons return back to their loft after being released up to hundreds of kilometres away from their home. Moreover, pigeons appear to possess a strong homing drive. Further, they are easily available and can be kept in man-made and controlled locations. These qualities make the homing pigeons the most and extensively studied animal model to answer the question of navigation.

1.1 History of domesticated homing pigeons

Originating from the rock dove, the domesticated pigeon has been around man for thousands of years. Evidence for domestication extends back to 4500 BC in Mesopotamia (modern Iraq) where the oldest known figurines, mosaics, and coins portraying domesticated pigeons have been found in archaeological digs. For the ancient Egyptians, pigeons have been an important food source. During the Middle Ages, nearly every manorial estate in Europe had a dovecote population specifically kept for the use as food. The use of pigeons for currier purposes is also a subject of historical record, dating back to the earliest assignment of currier pigeons at around 2500 BC, the time of King Dojoiser in Egypt. The sultan of Baghdad even established an entire pigeon mailing system in 1150 AD, and Genghis Khan used them frequently during war. The message of the conquest of Gaul to Rome was relayed by currier pigeons. Pigeons also delivered the news of

Napoleon's defeat at Waterloo to England and were commonly used for messenger service throughout Europe during the Revolution of 1848. During interruptions of the telegraphic service between Berlin and Brussels in 1849, carrier pigeons were used to keep communications up and running. In World War I and II, the military heavily relied on pigeons to convey emergency messages. The British and French governments even awarded honours to pigeons that “served” as a messenger in the time of war (Haag-Wackernagel 1998). Until 1995 the Swiss army possessed carrier pigeons for communications in the event of war.

Through millennia of selective breeding, pigeon keepers managed to raise, aside from homing pigeons, various fancy breeds, such as tumblers and pouters. Today, there are almost 800 breeds and 250 species of pigeons, all of which are suspected to have descended from the rock dove. Today, society does no longer rely on the homing abilities of pigeons to relay information from one place to another. However, this ability gave rise to the sport of pigeon racing employing a whole industrial branch (Haag-Wackernagel D., 1998).

1.2 Avian Navigation

The word navigation originates from the Latin words *navis* (“ship”) and *agere* (“to drive”). The term navigation derives from early seafaring, while developing methods of observing and while recording their position in order to plot a suitable, safe and fast course over great distances. The accumulation of these facts in journals enabled them and their successors to repeat and extend this endeavour. Every successful journey that was recorded could be retraced and integrated into a growing body of reliable information.

Humans and animals possess navigation abilities, but especially birds have been found to have an extraordinary ability for navigation. In studies conducted on birds Griffin (1952) was able to identify three types of avian navigation abilities. Type I is the simplest form of navigation on the basis of familiar landmarks. Type II is the ability to navigate in a particular direction without referring to landmarks. Type III is the most complex form of avian navigation ability. The animals can orient homeward, regardless of its direction, very likely based on mechanisms other than recognition of landmarks. This differentiation has

been widely accepted, although new findings have gradually modified the traditional view on this categorization (rev. Able 2001).

Successful Type III navigation requires information about the relative position to the destination and a mechanism providing directional information. Kramer (1953) suggested a mechanism relying on a “map” and “compass”.

“Wer als Blindekuh von seinem Heimatort entführt wurde, dem nutzt nach Abnahme der Binde vor den Augen die exakteste Bestimmung der Himmelsrichtungen gar nichts, solange er nicht weiß, wie sein neuer geographischer Ort zum Heimatort liegt. Erst wenn diese Lagebeziehung klar ist, kann mit Erfolg der Kompass oder Sonnenstand und Uhr zu Rate gezogen werden.“

„Someone, who was blindfolded and taken from his home, will not be able to find the way back after the removal of the blindfold if he solely relies on an exact determination of the points of the compass. He would also need to know about his relative geographical position to his home. Only then, measurements from a compass or the sun azimuth and time can be used successfully.“

When using the “map and compass”-mechanism, an animal first has to determine the direction of displacement (“map” step), second, the animal sets a course for the destination (‘compass’ step), and third, it finishes its journey by recognizing the goal. Therefore, all three types of navigation mechanisms described above are of great importance. The words “map” and “compass” in the context of animal navigation are used rather metaphorically than allegorically in order to describe neuronal processes during navigation. Kramer’s “map and compass”-mechanism together with Griffin’s “Type III navigation” were often depicted as *true navigation* (Able, 2001). The following paragraphs

describe the “compass and map mechanisms” in detail and further illustrate the different types of navigation.

1.2.1 Compass mechanism

The compass mechanism in the realm of animal navigation refers to the ability to orientate in a particular compass direction, without relying on landmarks. Without the knowledge of a specific target location, the animal must possess some sort of compass system in order to successfully navigate home. A variety of biological compasses have been suggested, which could be based on celestial cues or on the earth's magnetic field. In the following, the two main compass systems, the sun and the magnetic compass, used by diurnal birds, are described.

Sun compass

The sun compass was first described by Kramer in the 1950's. He observed that starlings housed in a cage showed directed migratory restlessness, using the sun to obtain directional information (Kramer, 1950). To use the sun as a compass for direction determination, an animal must know the current sun's azimuth and the corresponding time of the day. The relation between a specific point of the sun's azimuth and a specific compass direction are calibrated by the endogenous circadian rhythm. The endogenous circadian rhythm fluctuates with a periodic length of approximately 24 hours, and it is entrained against the light–dark cycle of the environment. This was first demonstrated by Hoffmann (1954) and Schmidt-Koenig (1960), who shifted the natural endogenous circadian rhythm of starlings and pigeons. The phase-shifted birds misread the sun compass and showed a $15^\circ/\text{h}$ shift (approximate sun movement per hour) in their direction orientation, according to the degree of time shift. Pigeons, which were raised under permanent 6h clock-shift conditions, could recalibrate their compass after being kept in the natural light-dark cycle (Wiltschko et al., 1976). The recalibration capability of the sun compass has led to the conclusion that the sun compass is established in a learning process and is not innate.

Magnetic compass

The use of visual cues, i.e. the sun, to help determine a compass direction, may appear quite obvious, because birds have developed a well-functioning visual system. However, what happens if the sun is not visible, for example, under an overcast sky? Even under these conditions, pigeons can navigate without problem, which indicates that pigeons must be able to use an additional compass. Indeed, clock-shifted pigeons were disorientated only during sunny days but not during overcast (Keeton, 1969). The existence of a magnetic compass was first demonstrated by Wiltschko (1968, see below 1.2). European robins were sensitive to changes in the surrounding magnetic field during migratory restlessness. In pigeons, the usage of a magnetic compass was first demonstrated by Keeton (Keeton, 1971). In his experiment, he attached a bar magnet to the beaks of pigeons, which affected their initial orientation, but only under an overcast sky, not under sunny conditions. Further studies demonstrated that the birds' magnetic compass is an 'inclination compass' (Wiltschko and Wiltschko, 1972), and in contrast to the sun compass, which is learned, it is innate and not dependent on experience and learning (Wiltschko and Gwinner, 1974). The question as to how birds process the magnetic information is still debated controversially. Recent studies provided evidence for two magneto sensory organs. The first organ is thought to consist of a radical pairs system, which is orientationally located in the eye. The second organ seems to be based on magnetite located near the beak. The radical pair mechanism was proposed to be the sensory basis for the compass mechanism (Ritz et al., 2002;2009).

Whether the sun or the magnetic compass is used, depends on several factors: prior experience of the animals, the time of day, and weather conditions. Generally, the sun compass is preferred. However, both compass systems seem to be linked to each other, in that the magnetic sense is used to calibrate the sun-compass (Able and Able, 1993; Cochran et al., 2004).

1.2.2 Map mechanism

The compass direction systems alone cannot provide the information of where in space the animal is in relation to its goal. Therefore, a second mechanism is needed, a navigational map, in order to obtain this information. Depending on the distance between

the release site and the destination, different approaches have been proposed to characterize this map mechanism. However, not all avian species require a navigation map.

Navigation without a map mechanism

Many migratory songbirds fly thousands of kilometres to their species-specific over-wintering area, and do so even alone during their first migration. They have to fly over areas they have never been before, which makes it unlikely that these birds have acquired a map-like knowledge of their migratory route, as they have not gathered any previous experience. It has been proposed that these migratory birds are born with a genetic program enabling them to find their over-wintering area (Berthold, 2003). The genetic program provides the inexperienced young birds with information about the direction and duration it should fly on its first migration. Using the compass system and the innate direction information, the birds are capable of finding their way during their first migration. After perpendicular displacement of inexperienced birds relative to the compass direction the birds continue to fly in their intended compass direction and hence arrive in an area, which is dislocated approximately in the direction and distance of their displacement. Once the migratory birds are familiar with the migratory route, they establish a navigation map and therefore can correct their flight if they are displaced and find their way to the already known over-wintering site (Perdeck, 1958). Cross-breeding between two populations of European blackcaps (*Sylvia atricapilla*) with different migratory distances resulted in an intermediate level of migratory activity (Berthold and Querner, 1981). This confirms the hypothesis that the duration of migration is genetically encoded.

Taken together, young birds on their first migratory flight successfully navigate to their species-specific over-wintering area without a map mechanism. The innate genetic program provides the animals with sufficient information about direction and duration of their flight.

Navigation over familiar areas

During training flights and exploratory free flights around the loft, pigeons gradually become familiar with their environment. It has been proposed that during these flights, pigeons establish a so called topographic map. These maps can be based on any type of sensory cues, like olfactory cues or visual landmarks, resulting in a representation of a spatial pattern that relies on individual experience. As an example for the topographic map navigation, it was demonstrated that pigeons follow highways as visual landmarks (Lipp et al., 2004). However, the limiting factor for the extent of such a topographic map is the range of the bird's experience. Two distinct mechanisms were suggested in the usage of this kind of map for landscape based navigation. First, the pigeons can navigate compass-independently by directly referring only to the pattern of visual landmarks, which is often called “piloting” or “place map navigation”. In this case, a spatial representation is formed using familiar landmarks to directly guide navigation. This mechanism is probably based on pigeons learning the spatial relationship between their loft and various other unrelated landmarks. For the second type of navigation, also known as "site-specific compass navigation" or "bearing map navigation", it was suggested that guidance between two landmarks is compass-controlled. In this case, the animals use local landmarks in the immediate vicinity of the training location only to recall a compass direction.

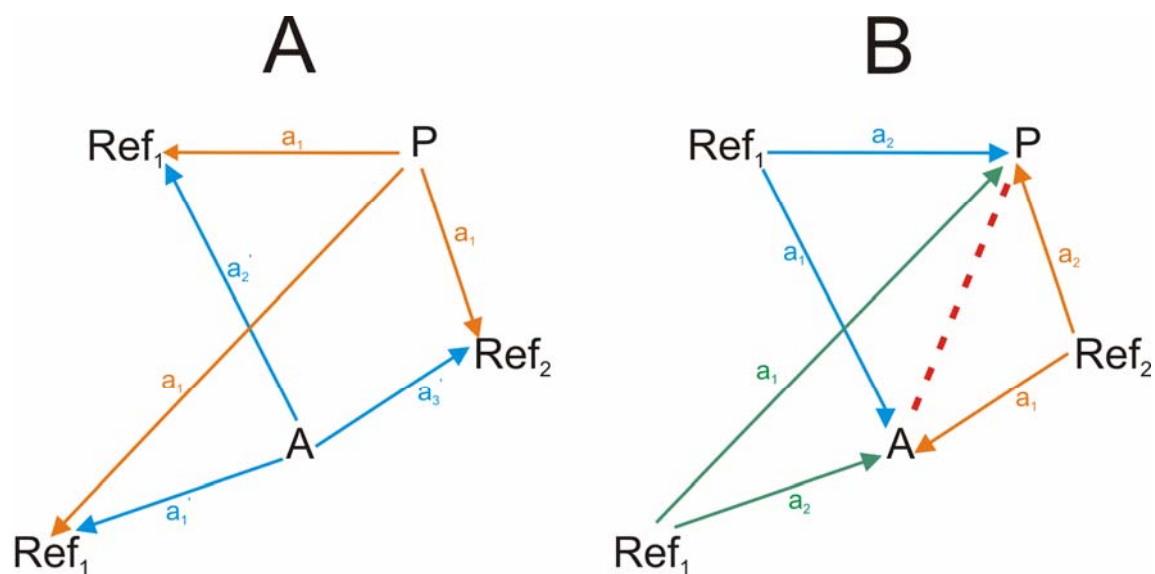


Figure 1. Topographical maps A) Place map, B) Bearing map, ‘ a_n ’ a measure of relative spatial relationship to the animal at a home loft (in A) or a compass bearing from a familiar reference site (in B) adapted from Phillips et al (2006).

The question as to which of these two mechanisms is actually used was studied in clock-shift experiments. Biro and colleagues (2004) demonstrated that with increasing familiarity, the pigeons are guided by memorized landmark cues (piloting), and are barely controlled by sun compass information. However, when the birds are less familiar with the environment the site-specific compass navigation becomes dominant, suggesting that previous experience dictates, which mechanism is used.

Navigation over unfamiliar sites

In unknown territory, pigeons do not have any more contact to familiar landmarks, reference points, or to goal animating cues. Consequently, the animals need a navigation map, which can be extrapolated from the familiar site to any unfamiliar release site. To fulfil this criterion, a gradient map was proposed by Wallraff (1974), who suggested that such a gradient map relies on at least two environmental stimuli that vary predictably in space in a gradient like fashion. In order to create a bi-coordinate grid-like system, the gradient axes of the two stimuli must intersect, though not necessarily orthogonally. To provide the animal with information about its position respective to home, the gradient map theory hypothesizes that the gradients extend monotonically beyond the familiar area. The respective physical parameters at their current position, in contrast to those remembered from the home-site, are assumed to provide the animal sufficient positional information respective to its home. For example, an animal is displaced from its home loft A with the coordinate's x^0y^0 to an unfamiliar location P x^ay^a . If it is able to at least recognize the differences Δx and Δy , a determination of its position would be possible (Fig. 2). Knowing its position it requires the direction information for successful homing. The direction information can either be attained by the use of a compass mechanism or by recognizing the direction information of the gradient. Clock-shift experiments could prove that the birds use an extra compass system, in this case the sun compass (Wallraff, 1990). The clock-shifted birds revealed a shift in the initial bearing corresponding to the angular differences between the sun's azimuth at the real local time, providing proof for Kramer's map compass theory. Hypothetically, the gradient map is unlimited in extension. Naturally occurring monotonic gradients may however limit its applicability due to spatial limitations (Wallraff, 1990; Wallraff, 2005).

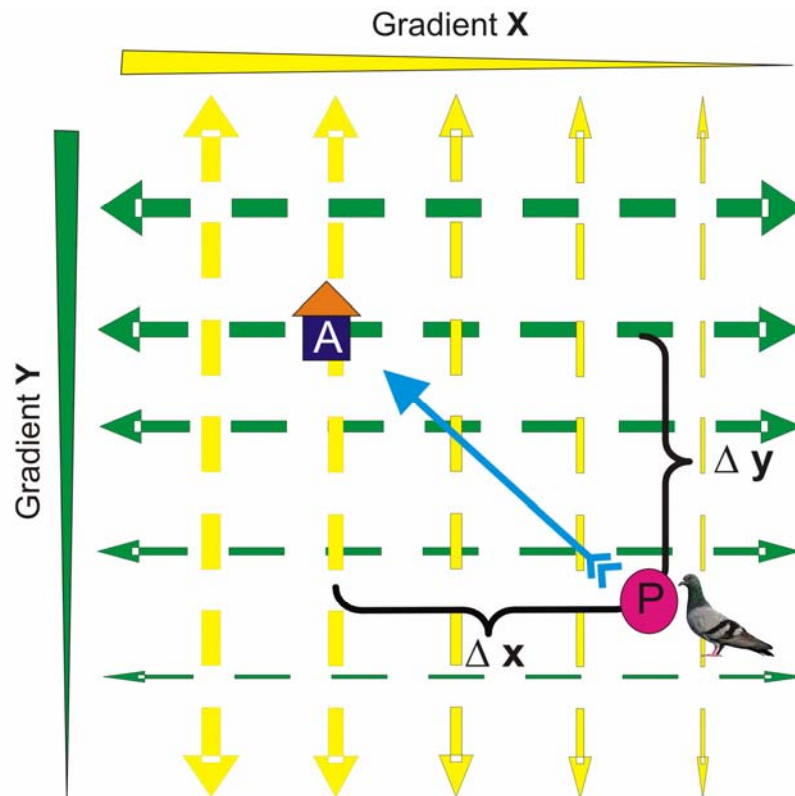


Figure2: Schematic design of Wallraff's gradient model of a navigational map. In this hypothetical example, two atmospheric odours X and Y create a bi-coordinate odour gradient grid. When transported from its home loft A (x^0y^0) to the location P ($x^a y^a$), a pigeon would measure its relative displacement by determining the difference between the local atmospheric odour intensities (Δx , Δy). Once the direction of displacement is determined, a homeward direction can be computed using a compass mechanism (adopted from Bingman et al., 2006).

Acquiring the values of the gradient cues at the home site is the first step in using this gradient map. Additionally, the animals have to know the direction towards which the respective scalar values increase or decrease. Scanning an area around the home loft that is big enough to measure differences against the home values could provide the birds with the necessary directional information. Alternatively, signals gathered at the home site may already indicate gradient directions. To establish a gradient grid, the environmental stimuli have to be stable over large areas, and at least two of them have to be available.

A number of physical variables have been discussed as possible candidates to establish a gradient map. The first two proposed candidates for the gradient map were the earth's geomagnetic field and the sun. Furthermore, possible other sources for the map were suggested, like the coriolis force caused by the earth's rotation, infrasounds

emanating from geomorphological structures, like coast or mountains, *electromagnetic sferics* originating from lightning discharge resulting in a electromagnetic pulse, *gravity inclination*, and stable *atmospheric chemicals* (Wallraff, 2005). Since the *geomagnetic field* and *atmospheric chemicals* are the most extensively studied factors, these two are described in more detail

Magnetic navigation map

The Earth's magnetic field is very similar to the dipole field of a giant bar magnet. Field lines emanate from the southern hemisphere and curve around the globe before they re-enter the planet in the northern hemisphere. The inclination angle at which the magnetic field lines intersect the Earth's field varies predictably with latitude. According to the sensory mechanism proposed by Wiltschko (1968), the magnetic compass relies on the ability of an animal to detect this field, which might enable it to determine whether its position is north or south of a particular area. Apart from the inclination angle, at least three other magnetic field elements vary across the Earth's surface (Lohmann et al., 2007). These are (1) the intensity (strength) of the total field; (2) the intensity of the horizontal field; (3) the intensity of the vertical field. Experiments with loggerhead sea turtle hatchlings (*Caretta caretta*) showed that they are able to detect at least two parameters of the Earth's magnetic field, namely magnetic inclination angle (Lohmann and Lohmann, 1994) and magnetic field intensity (Lohmann and Lohmann, 1996). Both parameters would allow them to establish a magnetic map. Furthermore, spiny lobsters (*Panulirus argus*) have been assumed to use a magnetic map as well (Boles and Lohmann, 2003). However, the question as to whether or not birds can use a magnetic map is still a controversial matter of debate. Mora et al., (2004) demonstrated that pigeons are capable of discriminating different magnetic fields. Transection of ophthalmic branch of the trigeminal nerve interrupts this discrimination ability, which is thought to transmit magnetic information from magnetite-containing structures in the beak of the bird (Fleissner, 2003; Williams and Wild, 2001). As a result of these findings, it was proposed that the section of the olfactory nerve and anaesthesia of the olfactory mucosa coincidentally interrupt the transmission of magnetic information, because of the proximity of both systems, and therefore interfere with their homing ability and is not due to olfactory deprivation. However, this was not confirmed by homing experiments. Only the section of the olfactory nerve reliably disrupted the homing ability and map acquisition. Pigeons with

a sectioned ophthalmic branch homed comparably to the controls and were able to establish a navigation map (Gagliardo et al., 2006;Gagliardo et al., 2008), contradicting the hypothesis that trigeminally mediated magnetic information is involved in the navigation map mechanism. It seems likely that birds use the geomagnetic field as a source for their magnetic compass, but unequivocal evidence for a magnetic map has yet to be presented.

Olfactory navigation map

Papi and colleagues first introduced the importance of olfactory cues during navigation in 1971. They sectioned the olfactory nerves of pigeons and could demonstrate that these birds were disorientated compared to control birds as they took off towards their home direction. Moreover, they found a poor homing performance. In a second study, the combination of unilateral nerve transection and occluding the ipsi- and/or contralateral nostril were performed to examine the question whether surgery alone would affect the navigation ability. Pigeons, which could smell with one nostril (unilateral sensory deprivation), were homeward oriented and showed a higher homing success compared to the birds, which were completely deprived of olfactory cues (bilateral sensory deprivation). Furthermore, the homing performance decreased with increasing unfamiliarity of the release site. These findings indicate that the poor navigation performance in the first experiment was truly caused by olfactory deprivation and not by the invasiveness of the surgery (Papi et al., 1972).

These experiments inspired series of olfactory deprivation studies using several techniques, for instance, inactivation of the olfactory epithelium by zinc sulphate, nasal anaesthesia using Xylocain, occlusion of nostrils or insertion of nasal tubes. All these treatments lead to disorientation at the release sites and to a poor homing performance (Wallraff, 2005). Pigeons which were not allowed to smell the air at the release site, and obtained a nasal anaesthesia before taking off were impaired in their initial orientation compared to birds which also received nasal anaesthesia but could smell the ambient air at the release site for some time. This result shows that ground level air is sufficient to determine positional information and that pigeons indeed orientate before taking off. This was supported by another study (Wallraff et al., 1992), which showed that when pigeons are exposed to the natural air of one location and then are released without further contact

with ambient odours at another unfamiliar site, pigeons behave as if departing from the site of previous olfactory exposure.

Olfaction navigation hypothesis

The results of olfactory deprivation studies lead to the conclusion that pigeons are able to rely on an olfactory navigation map. According to the original hypothesis, it was proposed that pigeons associate different odour information with winds from different directions, and thereby they establish an olfactory mosaic map of the surrounding environment (Papi et al., 1972; Papi, 1990). Different compass directions are associated with areas of different atmospheric odour qualities. This means that pigeons determine the direction of displacement in an unfamiliar location by using the ambient odour profile and comparing it to the wind direction associated with that odour profile experienced at the loft. Upon using their sun or magnetic compass, they fly off in a direction opposite to the associated wind direction. Winds carry the odour profile from an unfamiliar release site to the home loft, enabling the animals to recognize the odour profile of the new site as "familiar". According to this hypothesis, birds experience odour profiles as familiar landmarks that can be detected remotely (Bingman et al., 2006). However, a mosaic map would not contain enough information because of its relatively short operational radius of about 50 to 100km to the home loft. This radius is limited by the physical properties of wind-born odours to be transported reliably to the site of the pigeon loft. The fact that successful navigation can occur over hundreds of kilometres, well beyond the reach of wind-born odours, contradicts the theory of the olfactory mosaic map. Therefore, Wallraff proposed that pigeons make use of an odour gradient-like map for navigating over long distances based on atmospheric tracer gas gradients that are stable over a large area (Wallraff, 1990). In conclusion, pigeons may learn two types of dissociable olfactory navigational maps. The neuronal basis for this will be discussed below.

According to the olfactory navigation hypothesis, a permanent shielding of winds at the home site during the first three months post-fledging prevents the ability to acquire an olfactory map (Gagliardo et al., 2001a). During this time, the manipulation of wind direction should lead to misleading information about the wind direction at the loft and therefore might result in the acquisition of an inaccurate olfactory map. Deflector loft

experiments have been commonly used to manipulate the wind directions. Three main types of wind manipulations have generally been employed: (1) blocking one or more directions of the natural winds (2) deflection of the wind so that it appears to come from another direction, and (3) reversing the winds direction by the means of fans (Papi, 1990; Wallraff, 2005). Pigeons reared according to the first condition were unable to home, indicating that the olfactory information from one direction is not enough to establish an olfactory map. Pigeons could acquire an olfactory map when they were exposed to at least two wind directions. Shifts of the wind (condition 2) in either a clockwise or a counter-clockwise direction resulted in the deflection of the homing direction according to the degrees of the deflectors. Reversal of one wind direction causes the pigeons to take off in the opposite direction to the home when released from the direction of the loft axis. In another experiment, testing the olfactory navigation hypothesis, artificially created winds carried false odours and elicited similar behavioural results in pigeons (Ioalè et al., 1990)

1.3 The olfactory system of birds

In order to better understand the mechanisms underlying the olfactory maps in navigating birds, it is important to know its olfactory system. For a long time, the olfactory sense for smell in birds was believed to be not important or not even functional. This was amplified by observations that birds do not sniff at one another or at objects like mammals do. The nostrils high up on the surface of the beak are not appropriately located for the investigation of localized olfactory sources. Moreover, the small size of the OBs in relation to the rest of the brain was interpreted as a hint for a rather underdeveloped sensory system (Roper, 1999). Nevertheless, comparative anatomical studies revealed a high degree of similarity between the avian olfactory system and those of amphibians, reptiles, and mammals both on the macroscopic and microscopic level (Roper, 1999). Most birds possess paired external nostrils on the posterior dorsal surface of the beak and a series of nasal chambers where the most caudal chamber is lined with the olfactory epithelium containing the olfactory receptor cells (Roper, 1999). The olfactory receptors constitute the cellular basis of the sense of smell among vertebrates. The total number and proportion of functional olfactory receptors is generally positively correlated with olfactory acuity (Rouquier et al., 2000; Gilad et al., 2004). Recently, it was demonstrated that the majority of avian olfactory receptor genes are potentially functional (Steiger et al., 2008) and that they are not, as previously believed, non-functional pseudogenes. All these findings

provide evidence that birds actually have very well developed olfactory system. The olfactory receptor cells are connected via paired olfactory nerves with the olfactory bulbs (OBs), which is located at the rostral end of the brain (Rieke and Wenzel, 1978). The avian OB is composed of a concentric seven-layer structure, just as the mammalian one, but the avian OB lacks an accessory olfactory system (Rieke and Wenzel, 1978). The OBs project and receive projections from various brain areas. The piriform cortex is the main projection area, receiving bilateral input from the OBs (Reiner and Karten, 1985; Bingman et al., 1994). Beside these anatomical similarities electrophysiological studies performed on the olfactory epithelium, olfactory nerves, and OB could also reveal a fully functional olfactory system (Tucker, 1965; Sieck and Wenzel, 1969; McKeegan et al., 2002). Most birds tested on an odour discrimination task in which odour stimulus was paired with an aversive stimulus have shown olfactory capabilities comparable to mammals (Mason and Clark, 2000). Taken together, the behavioural data of the olfactory based homing ability in pigeons strongly support the notion that the olfactory sense of bird is well developed and comparable to the mammalian one.

Neuronal basis for olfaction based navigation

The piriform cortex (Cpi) is the main projection area of the OB (Reiner Karten 1985; Bingman et al., 1994). Bilateral ablations of the Cpi disrupt the homing ability of pigeons from unfamiliar sites but not from familiar site. As a consequence the determination of displacement in respect to the goal using olfactory cues is disturbed. Accordingly, lesions of the Cpi during the sensitive period of olfactory map acquisition prevent the establishment of the olfactory map even when the pigeons are allowed to perform spontaneous training flight around the loft (Gagliardo et al., 1997). Another important neural region underlying navigation is the hippocampal formation (HF, Bingman et al., 2006). Lesions of the HF disrupt learning of olfactory maps, but only if the pigeons are not allowed to fly around the loft freely (Bingman et al., 1990). Pigeons that have the opportunity of free flight are not impaired. These findings are consistent with the fact that pigeons may acquire two different kinds of olfactory map. For the acquisition of an olfactory mosaic map, the pigeons require a fully functional HF to associate different odour information to different wind directions. The olfactory gradient map is acquired during training flights around the loft, while the birds can scan the field around home and thereby learn the differences against the home values. This complex process requires a

fully functional Cpi (Bingman et al., 2006). The HF was indeed found to be important for a landmark-based, i.e., map-like, representation of space (Shimizu et al., 2004). Taken together, the Cpi is important for the processing of olfactory cues of both olfactory maps and for the acquisition of the olfactory gradient map. This lends support for the important role of the Cpi, which is not only a relay station for processing olfactory cues but also plays a crucial role in olfactory-guided map navigation.

Apart from the Cpi, lesion studies indicated the role of further telencephalic brain areas to be involved in the navigation process. Ablation of the of nidopallium caudolaterale (NCL), the analogue of the mammalian prefrontal cortex, disturbs homing from unfamiliar site, but not from familiar locations (Gagliardo and Divac, 1993). This suggests that the NCL is important for map-based navigation. However, further studies are needed to examine the degree of involvement of the NCL in olfaction-based navigation. Ablation of the visual wulst did not disrupt the homing ability neither from the familiar nor from the unfamiliar site. However, evidence suggests that the wulst is important for the acquisition of familiar landmark-based navigation (Bingman et al., 1984).

Functional lateralization of the navigation system

The functional and structural lateralization of the left and right hemisphere is not only a feature of the human brain but is common in the entire animal kingdom from the fruit fly to mammals (Vallortigara et al., 1999; Vallortigara and Rogers, 2005). One of the most extensively studied lateralized systems is the visual system of birds, revealing functional as well as anatomical lateralization (Manns and Güntürkün, 2009). Having a lateralized brain is considered to enhance the efficiency and neuronal capacity of the brain, by reducing the interhemispheric conflict. Pigeons with a higher degree of visual lateralization can better discriminate grit from grains binocularly than less lateralized birds (Güntürkün et al., 2000). Another advantage of a lateralized brain was shown in chicks. In a task simultaneously engaging both hemispheres, visually lateralized chicks performed better than non-lateralized ones (Rogers et al., 2004; Dharmaretnam and Rogers, 2005). This finding suggests that a lateralized brain enables parallel and separate processing of tasks that engage opposite hemispheres simultaneously.

In addition to the extensively studied lateralisation of the avian visual system (Manns and Güntürkün, 2009), pigeons were found to have a functionally lateralized olfactory system, too. Unilateral lesions of the Cpi lead to an impairment of the homing ability. However, pigeons with an intact left Cpi revealed an initial orientation compared to the control birds, which had received a sham surgery. Pigeons with an intact right Cpi showed a randomly scattered, thus impaired, initial orientation, arguing for a functional dominance of the left Cpi in processing olfactory cues for the determination of the direction of displacement (Gagliardo et al., 2005). Unilateral occlusions of the nostrils demonstrated the same pattern of functional lateralization for the nostril/OB, but with reversed dominance. Here, the right nostril/OB is of great importance for initial orientation (Gagliardo et al., 2007). Such a lateralized olfactory perception was previously shown in chicks with a dominance of the right nostril/OB (Vallortigara and Andrew, 1994; Burne and Rogers, 2002). A structural lateralization of the olfactory system in pigeons yet remains to be proven.

As mentioned above, HF lesions can prevent the acquisition of the olfactory map if pigeons are not allowed for free flights around home. However, this is only true when the left HF is lesioned (Gagliardo et al., 2001b). Pigeons reared with an intact right HF do not manage to establish an olfactory map.

1.4 Concluding remarks

Pigeons possess different navigation map mechanisms, which they use on demand at the release site (Figure 3). During exercise flights pigeons establish a topographic map. When familiar with the environment, pigeons can use the relationship between familial landmarks and the loft, to navigate (piloting). With decreasing familiarity, pigeons use their "bearing map". Here pigeons use local landmarks in the immediate vicinity of the training location only to recall a compass direction. At an unfamiliar site, which is in the range of wind borne odours pigeons determined the direction of displacement to the home loft using the "olfactory mosaic map". Out of the range of familiarity pigeons make use of their "gradient map", which is most likely based on atmospherically stable tracer gases. Depending on the navigation mechanism different brain areas are of importance. Landmark based navigation requires the HF, whereas olfactory-guide navigation requires the Cpi.

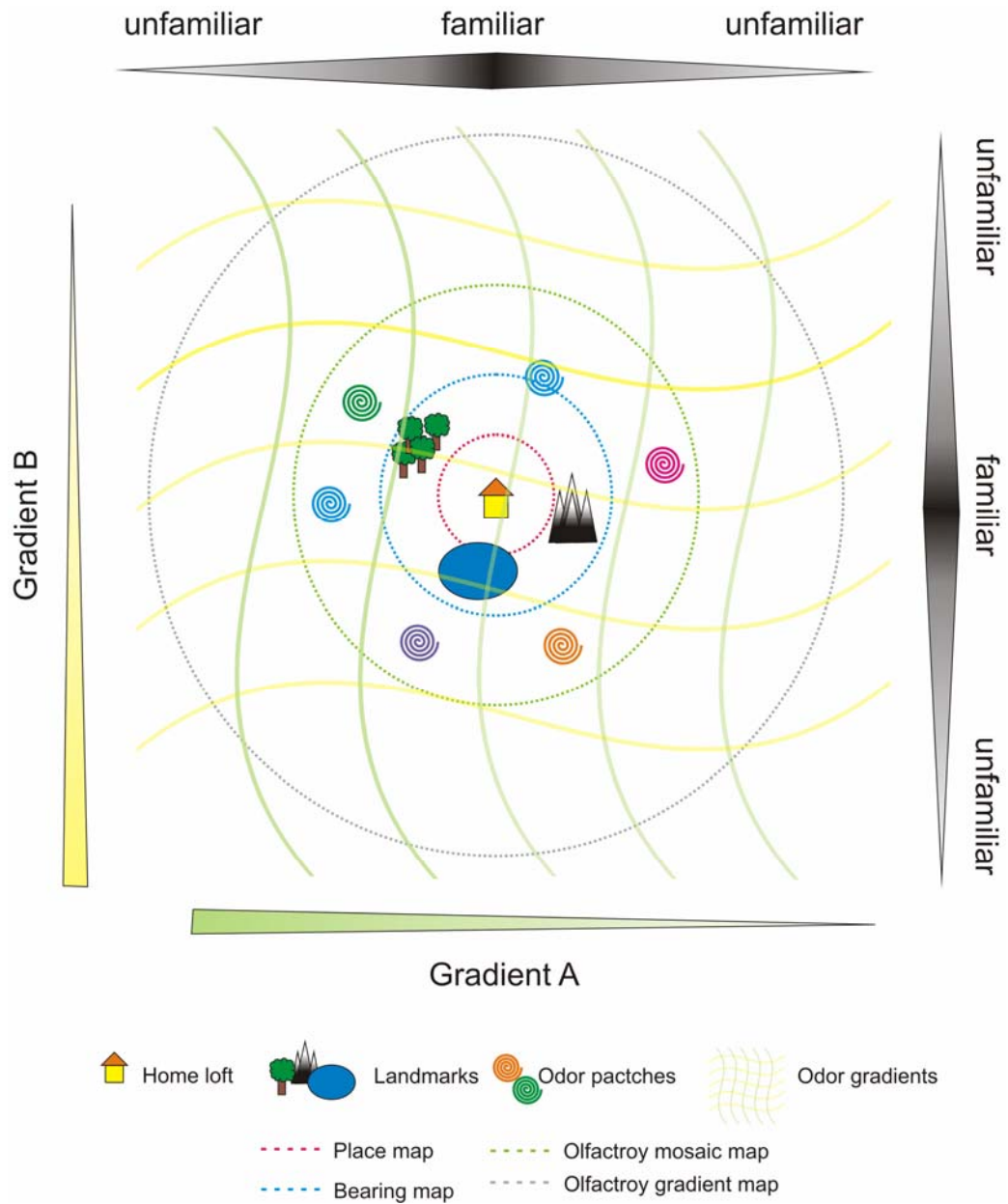


Figure 3: Depiction of the principal map mechanisms used by pigeons: Concentric rings indicate the borders of a map mechanism. The lines of the rings are dashed to indicate that the borders are not fixed and can be extended by the experience of the pigeon. Hypothetically there is no limit for the gradient map. In familiar environment, pigeons use the relationship between familial landmarks and the loft to navigate (piloting). With decreasing familiarity, pigeons use their compass-based "bearing map navigation". At an unfamiliar site, within range of wind borne odours pigeons use the "olfactory mosaic map" for homing. Out of the range of familiarity pigeons make use of their "gradient map".

1.5. Aims of the present thesis

Behavioural studies have demonstrated that the olfactory system is a key feature of the navigation ability in pigeons. However, up to now the neuronal processes underlying this navigation performance are still largely unknown. In my thesis, I investigated the olfactory neuronal processing mechanisms in pigeons, proposed by the olfactory navigation hypothesis. Following this approach, three experimental studies were conducted:

First Study: Organization of the olfactory system in homing pigeons (Columba livia).

Previous behavioural experiments with unilateral manipulations of the olfactory system indicated that the piriform cortex (Cpi, secondary olfactory area) is necessary for an intact homing performance. The left Cpi was found to play a crucial role in processing olfactory cues that are needed for determining the direction of displacement. This function seems to be triggered by the right olfactory system, as demonstrated in earlier experiments by plugging the left or right nostril of homing pigeons. This strongly argues for an asymmetric projection pattern with a stronger projection from the right OB to the left Cpi than vice versa. Such large bilateral innervations of the dominant brain structure are also known from the visual pathway in birds. However, nothing is known about quantitative differences in projection. To address this question, I used a retrograde tracing technique. Cholera toxin subunit B (CtB) was injected unilaterally into either the left or right Cpi of adult pigeons. After immunohistochemical detection of CtB-positive cells, the number of ipsi- and contralaterally projecting neurons located in the OB was estimated, and the asymmetry index was calculated. In order to better understand the olfactory information processing in pigeons, my goal was to clarify the projection pattern from the OB to the telencephalon, as different tract tracing studies have revealed inconsistent results. To reliably identify these projections, I performed unilateral injections of the anterograde tracer BDA either in the left or right OB.

Second Study: Navigation induced ZENK expression in the olfactory system of homing pigeons (Columba livia)

A large number of behavioural studies have been conducted to prove that the olfactory system is involved during navigation. Nevertheless, unequivocal evidence for a navigation-dependent neuronal activation is still lacking. Delivering this kind of evidence is certainly a great technical challenge, as neuronal recording during flight is very difficult, and the technique is still in development. Though, electrophysiological recordings can already be conducted in free moving animals in a skinner box. An elegant tool to measure the activation of a brain area during homing was introduced by Shimizu et al., (2004). They used ZENK, an immediate early gene (IEG), which detects protein expression, peaking at a plateau around 1-2 hours after the stimulus onset. Hence, this can be elegantly used to monitor the activity of a certain brain area while performing a task.

In this study, I used the IEG technique to visualize the neuronal activation of the olfactory system (OB and Cpi) during navigation performance in pigeons. The experimental procedure included releasing one experimental group from an unfamiliar site, while one control group was transported to the unfamiliar site but not released and the other was released in front of the loft. To analyse the differential contribution of the left and right olfactory input at the neuronal level, the nostrils of the pigeons were either unilaterally plugged or not. I found the behavioural data to point towards a higher demand of the olfactory system during navigation from an unfamiliar area. I also expected a higher ZENK expression in the olfactory system of the released birds, as they had to use their olfactory map mechanism actively in contrast to the control groups. This study was conducted in collaboration with Anna Gagliardo from the University of Pisa, Italy. The behavioural experiments for this study took place in Pisa. The neuroanatomical analysis was carried out at the Ruhr-University Bochum, Germany.

Third Study: Adult neurogenesis in the olfactory bulb of homing pigeons (Columba livia)

The question, whether or not there is any neurogenesis in the OB, was the focus of my third study. In the mammalian brain, the OB is - apart from the hippocampus - the only region of lifelong creation of new nerve cells, revealing a plastic mechanism, which

contributes to the perceptual and memory functions performed by the bulb. Elevated perceptual and memory demands associated with olfactory-guided homing behaviour suggests neurogenesis in the avian OB. However, this has not been verified yet. To address this question, I conducted Bromodeoxyuridine (BrdU) injections in one-year old pigeons. BrdU is a thymidine analogue that becomes incorporated into the DNA of dividing cells during the S-phase of the cell cycle. Therefore, it can be used to estimate the neurogenesis rate. Immunohistochemical double labelling of BrdU and neuronal marker proteins were used to characterise newly generated bulbar neuron types. Moreover, I examined the question if there are any asymmetries in the neurogenesis rate between the right and left OB, because the right OB has previously been shown to play a crucial role in olfactory-guided navigation.

Chapter 2

Study I: Organization of the olfactory system in homing pigeons (*Columba livia*).

Organization of the olfactory system in homing pigeons (*Columba livia*).

Introduction

The olfactory sense is phylogenetically the oldest sensory system, and it is used to establish contact with the environment. The olfactory sensory system seems to be pronounced differently in the animal kingdom, and birds were believed for a long time to be microsmatic (Roper, 1999). However, a number of previous studies have demonstrated that the olfactory system of birds strikingly resembles that of amphibians, reptiles and mammals at the anatomical level (Roper, 1999). The olfactory system of birds consists of paired external nostrils, a series of nasal chambers where the most caudal chamber is lined up with the olfactory epithelium, which contains the olfactory receptor cell. Axons of this cell build up one pair of olfactory nerve fibres terminating in the olfactory bulbs (OBs). The OBs are located at the rostral end of the brain (Rieke and Wenzel, 1978). They project to various brain areas where the piriform cortex (Cpi) has been identified as the main projection area, receiving bilateral input (Reiner and Karten 1985, Bingman et al., 1994). Apart from these anatomical similarities, electrophysiological studies could also reveal a fully functional olfactory system (Tucker, 1965; Sieck and Wenzel, 1969; McKeegan, 2001). Moreover, it was recently shown that the majority of avian olfactory receptor genes are potentially functional (Steiger et al., 2008) and not, as it was previously believed, non-functional pseudogenes.

In avian species, like pigeons, which display extraordinary navigation abilities, olfaction plays a critical role (Wallraff, 2005). Manipulation of the olfactory system, for instance, plugging the nostrils (Gagliardo et al., 2007), anaesthesia of olfactory mucosa (Wallraff, 1988), transection of the olfactory nerve (Papi 1971), or ablation of the piriform cortex (Cpi, Papi and Casini 1990) generate remarkable disruption of initial orientation and homing performance in pigeons (for rev. see Wallraff 2005). However, behavioural studies have indicated that the left and right hemispheric systems differentially contribute to olfactory-dependent navigation. Pigeons with a defective right Cpi behaved comparable to the controls and orientated significantly towards their home direction, whereas pigeons with a defective left Cpi were heavily impaired in their initial orientation skills (Gagliardo et al., 2005). This argues for a dominance of the left Cpi in processing olfactory cues

during the displacement in relation to the goal. Nevertheless, both groups were significantly impaired in their homing performance compared to the control group, indicating that both hemispheres are needed for an optimal navigation performance. On the other hand, unilateral nostril occlusions revealed that the right nostril/OB is more important in the step of initial orientation than the left nostril/OB (Gagliardo et al., 2007). Here again, the homing performance of occluded birds was significantly reduced than in the control pigeons. Since the OB projects bilaterally to the Cpi (Reiner and Karten, 1985; Bingman et al., 1994) it is conceivable that the contralateral projection from the OB to Cpi might be asymmetrically organized, with a stronger projection from the right OB to the left Cpi. A larger bilateral innervation of the dominant brain structure is also known from visual pathways in birds (Güntürkün et al., 1998; Rogers and Deng, 1999). Such a more pronounced bilateral visual input to the left hemispheric system may be related to dominant processing (Manns and Güntürkün, 2009). Alternatively, asymmetrically organized side-projections may be involved in the mediation of asymmetric processing. However, findings regarding the general projection pattern of the olfactory bulb are inconsistent. Therefore, we reanalyzed OB projections using biotinylated dextran amine (BDA). Quantitative differences in the projection pattern between OB and Cpi were analyzed by injections Cholera toxin subunit B (CtB) unilaterally into the Cpi.

Method

A total of 26 adult homing pigeons (*Columba livia*) of both sexes from local breeding stocks were used in this study. For the qualitative determination of the projection from the OB to the Cpi, 16 birds successfully received unilateral injections of the retrograde tracer Cholera toxin subunit B (CtB; 1% in deionised water; Sigma, Deisenhofen, Germany) either to the left or to the right Cpi. For anterograde pathway tracing, a successful BDA (10,000 MW; 10% in 2% DMSO; Molecular Probes, Leiden, The Netherlands) injection into the OB of four pigeons (left n=2; right n=2) was performed. The study was carried out according to the specifications of the German law for the prevention of cruelty to animals. All efforts were made to minimize the number of animals used and to decrease their suffering as much as possible.

Prior to surgery, pigeons were anesthetized with equithesin (0.33 ml per 100 g body weight) and secured in a standard stereotaxic apparatus (Karten and Hodos, 1967). For Cpi

injections, a modified device was used that allowed lateral rotation of the head along the longitudinal axis over 100° to the left or right (Hellmann and Güntürkün, 1999). After opening the skull with a dental drill, the tracers were injected via a glass micropipette (outer tip diameter 15–20 µm for CtB and 25–30µm for BDA) mounted to a mechanic pressure device (WPI Nanoliterinjector; World Precision Instruments, Berlin, Germany). The micropipette was inserted either into the Cpi or the OB. To estimate potential asymmetries in the projection pattern, we had to ensure a complete filling of the Cpi. Therefore, we made several injections along the complete antero-posterior elongation of the Cpi between level A 7.5, and 4.5 according to stereotaxic coordinates of the pigeon brain atlas by Karten and Hodos (1967), with an injection depth of 0.15mm. Placing of OB injections were visually controlled, since the OB is a clearly delimited area on which six injections with two depths (0.2mm and 0.4mm) were performed. At each depth about 60/90nl, CtB/BDA were applied.

After two (for CtB injection) or fourteen days (for BDA injection) of survival time, animals received an injection of 2000 units heparin and were then deeply anaesthetized with equithesin (0.45 ml per 100 g body weight). The pigeons were perfused through the left ventricle with 0.9% saline (40°C), followed by 4% paraformaldehyde in 0.12M PBS (4°C, pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde + 30% sucrose for 2h at 4°C, cryoprotected in 0.12M PBS +30% sucrose at 4°C for 48h. The brains were cut in frontal plane at 40µm on a freezing microtome. The left or the right brain side was marked by a hole stuck with a small needle. Sections were collected in five parallel series for the OB and ten parallel series for the rest of the brain and stored in 0.12M PBS containing 0.1% sodium azide at 4°C until they were subjected to immunohistochemistry.

Brain slices were treated free-floating according to the immuno- ABC-technique (Hellmann and Güntürkün, 2001). The slices of one serial set were incubated in 0.3% hydrogen peroxide in distilled water for 30 min to reduce endogenous peroxidase activity. For CtB immunostaining, the slices were incubated in 10% normal goat serum for one hour in order to block unspecific binding sites. Sections were incubated overnight at 4 °C in the primary antibody solution (rabbit anti-CtB; Sigma; 1/10,000 in 0.12 M phosphate-buffered saline 0.3% Triton X-100 (PBST). After being rinsed, the sections were incubated for 60 min at room temperature in the biotinylated secondary antibody solution (goat anti-rabbit; Vectastain, Vector, Camon (Wiesbaden, Germany); 1/250 in PBST). After additional

rinsing, the slices were incubated for 60 min in avidin–biotin–peroxidase solution (Vectastain ABC-Elite kit, Vector, Camon; 1/100 in PBST). For the BDA staining, one serial of slices was incubated for 60 min. in avidin–biotin–peroxidase solution. The peroxidase-activity was detected using a heavy metal intensified 3'3-diaminobenzidine (DAB; Sigma) reaction, modified by the use of β D-glucose/glucose-oxidase (Sigma; Hellmann and Güntürkün, 2001). The slices were mounted on gelatin-coated slides, dehydrated, and coverslipped with Permount (Fisher Scientific, Hampton, NH, USA).

The number of ipsi- and contralaterally CtB labelled cells within the OB and the contralateral Cpi were counted with 40x1.6 magnification at a Leica DML microscope (Leica Microsystems, Wetzlar, Germany) in one series of slices. The asymmetry index (AI) was calculated according to Güntürkün et al. (1998):

$$AI = \frac{\text{cell number ipsy} - \text{cell number contra}}{\text{cell number ipsy} + \text{cell number contra}}$$

We did not compare absolute cell numbers, since they depend on the amount of the applied tracer. However, it was not possible to estimate the injection volume due to the damages caused by the injection needles (Fig. 2).

Statistical analysis was performed with the statistic program Statistica (StatSoft, Tulsa, OK, USA). Photographic documentation was carried out using a digital camera-system (Zeiss Axiocam; Zeiss, Jena, Germany) attached to the microscope. Images were processed with Zeiss Axiovision 3.0. Colour balance, contrast, and brightness were adjusted with Photoshop CS2 software (Adobe, Germany).

Results

Anterograde tracing of bulbar efferents

In all four cases, BDA injections were successfully placed in the OB. No spread into adjacent brain areas was observed. BDA-labelled fibres could be detected in several telencephalic areas. A great number of BDA positive fibres was found in the entire ipsilateral OB mostly confined to the mitral cell layer (Fig. 1). Some fibres were observed in the contralateral OB (Fig. 1). Fibre terminals were massively present bilaterally in the prepiriform cortex (Cpp), but with more fibres on the ipsilateral site (Fig. 1). A continuum of a few fibres extended dorsally from the OB to the ventral and then to the lateral wall of the ipsilateral telencephalon. Few fibres were scattered in the medial septum (Fig. 1). A large compact fibre bundle was traced, running from the OB along the ventral telencephalic wall to the Cpi, where a great number of terminating fibres were seen on both hemispheres. Few fibres were detected to terminate somewhat above the ipsilateral Cpi in the dorsolateral corticoid area (CDL, A 6.00). Some fibre terminals were also observed bilaterally in the TnA (Fig. 1). A large fibre bundle entered the diencephalon via the stria medularis bridge in the habenula commissure and ascended to the contralateral telencephalon (Fig. 1). Thus, our tracing experiment verified largely the observations of Reiner and Karten (1985). However, there was one exception. In contrast to their findings, we could not identify any fibres within the olfactory tubercle (TuO).

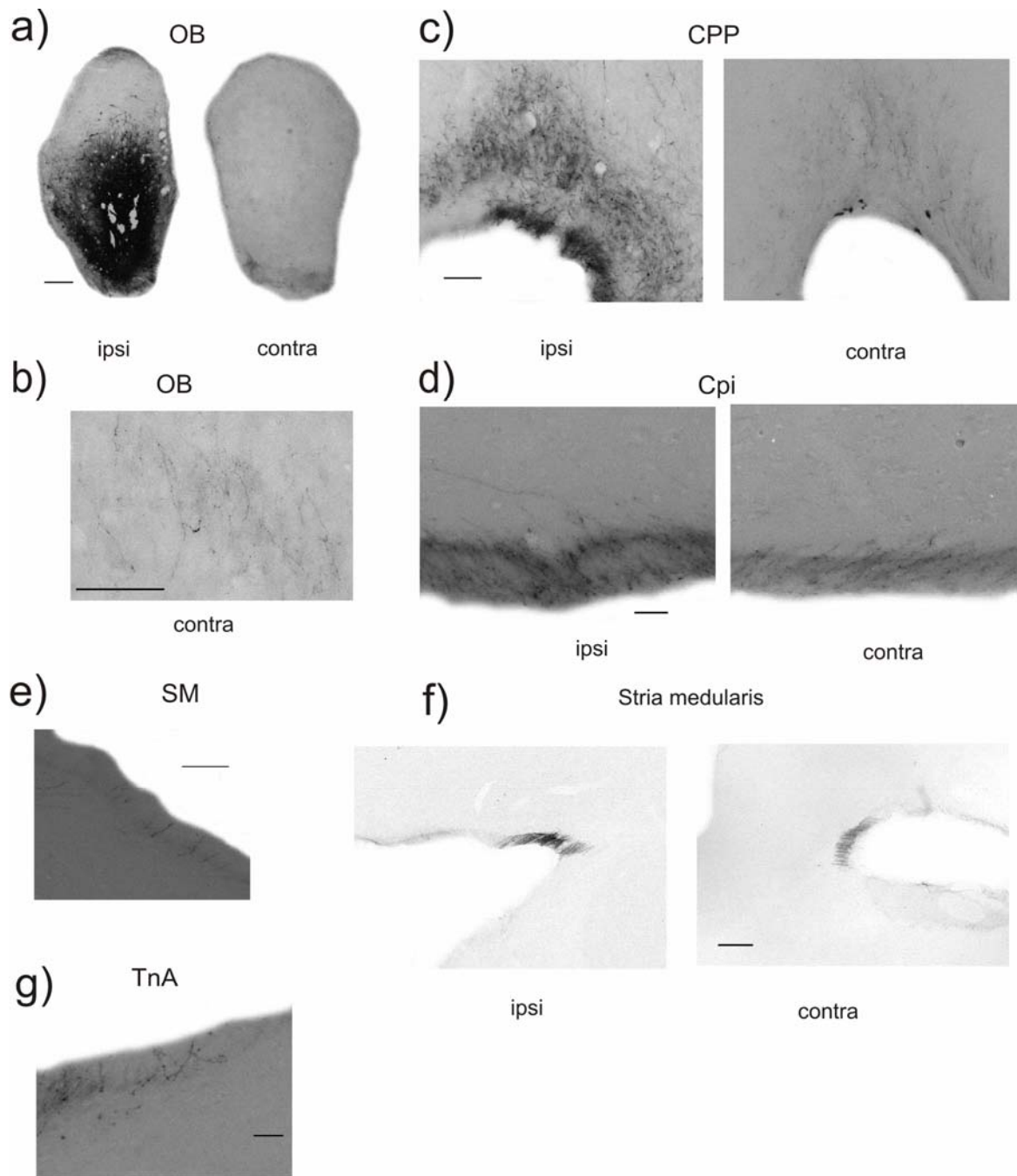


Figure 1: Anterograde labelling with BDA of OB projection targets. (a) Injection site of the OB and the corresponding contralateral OB. The OB projects in various areas: ipsilaterally into the SM (e), contralaterally into the OB (c), bilaterally into the Cpp (b), Cpi (d), TnA(g). The projection mediates the contralateral site via the stria medularis bridging in the habenula commissure. Scale bar a,f = 200 μ m, b=100, e,c=50 μ m, g=20 μ m

Retrograde tracing of afferents to the Cpi

All injections were successfully placed into the Cpi, spanning along the complete dorsoventral dimensions of this area. But since the Cpi is a thin structure on the surface of the telencephalon, it was inevitable that some traces spread out to the nearby acropallium and amygdaloid nuclei. Nevertheless, in four cases the injection site was mostly restricted to the Cpi. These cases were used to describe the qualitative projection pattern of the Cpi.

In principal, our CtB injection into the Cpi confirmed the results of an earlier study by Bingman et al. (1994). Retrogradely labelled cells were bilateral found in the entire OB (A 14.50-13.50), exclusively in the mitral cell layer, which constitutes the bulbar output layer (Fig. 2). A large number of retrogradely labelled cells were also detected within the prepiriform cortex (CPP, A 14.50-13.50; Fig. 2), the hyperpallium densocellulare (HD A 14.25- 9.00; Fig. 2) and frontolateral nidopallium (NFL A 14.00-11.00). Few ipsilateral labelled cells were detected near the vallicula (Va A 14.00), the olfactory tubercle TuO (A 12.00), the nucleus of the diagonal band (NDB A 9.00; Fig. 2) and very few cells in the nucleus accumbens. Ipsilateral to the injection site, labelled neurons, which formed a continuum, were found throughout the ventrolateral and then lateral wall of the telencephalon and throughout the entire hippocampal formation in the dorsomedial hippocampus (DM; Fig. 2). Few cells were observed in the contralateral caudoventral wall of the telencephalon. Bingmann et al., (1994) classified them as belonging to the Cpi (Fig. 2), but according to the new classification of the amygdaloid nuclei (Atoji et al. 2006), it is more likely that most of them are part of the basal division of the nucleus posterioris amygdalopalli (PoAb). Only some were located within the contralateral Cpi. However, it is arguable whether the PoAb is really projecting to the contralateral Cpi or if it is due to the reciprocal connections between the amygdaloid substructures, since we cannot exclude tracer spread into the compact division of PoA (PoAc). PoAc has already been shown to project to the contralateral PoAb (Atoji et al. 2006). The dorsal acropallium (AD) revealed CtB labelled cell on the ipsilateral side. Ipsilateral CtB labelled cells were also seen in the dorsolateral Corticoid Area (CDL), the temporo-parieto-occipital area (TPO), the caudal nidopallium (NC), PoAc and PoAb. However, it is not clear if they are really projecting to the Cpi or are merely spread from the injection site. We did not identify projections to the TnA and septum, as observed by Bingman et al (1994).

In the diencephalon, labelled neurons were found ventrally to the nucleus dorsomedialis anterior thalami (DMA) and nucleus dorsomedialis posterior thalami

(DMP). Sparsely distributed cells were observed in the lateral hyperthamalic nuclei (LHy) and lateral mammillary nucleus (ML).

CtB- positive fibres showing terminal-like labelling were found in the bed nucleus of the stria terminalis (BNST) and in the area subpallial amydala (SpA). Among labelled cells, the TuO and the LHy showed also fibre terminals (Fig. 2).

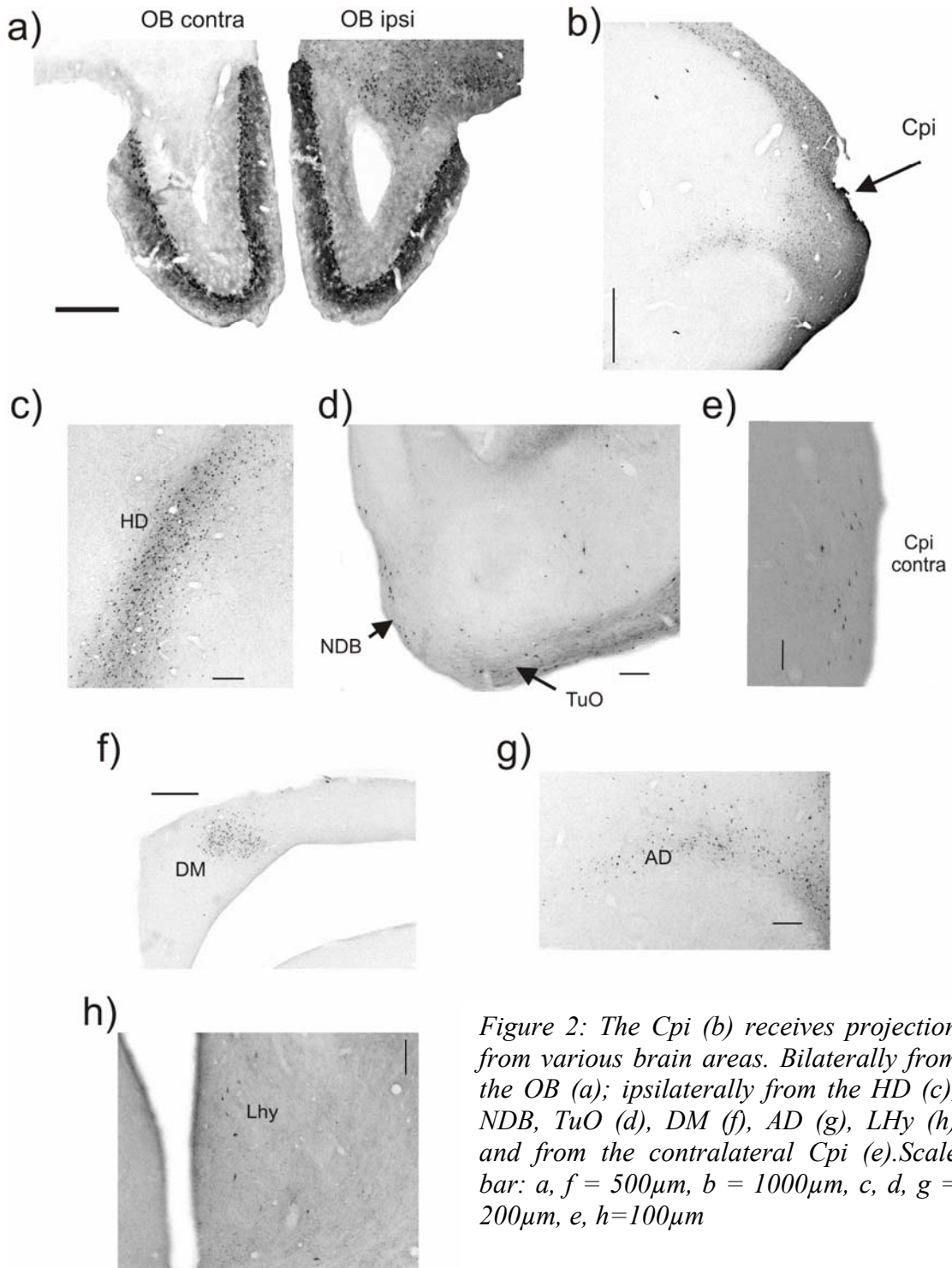
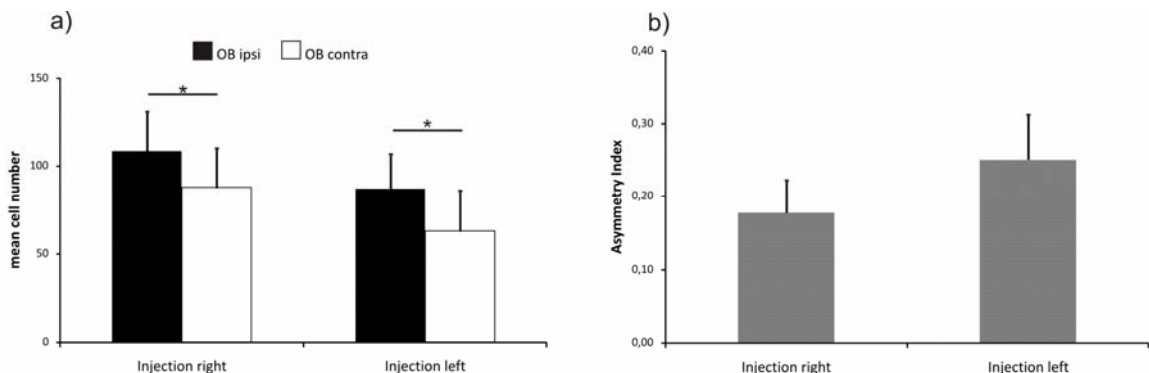


Figure 2: The Cpi (b) receives projection from various brain areas. Bilaterally from the OB (a); ipsilaterally from the HD (c), NDB, TuO (d), DM (f), AD (g), LHy (h) and from the contralateral Cpi (e). Scale bar: a, f = 500 μ m, b = 1000 μ m, c, d, g = 200 μ m, e, h=100 μ m

Quantitative analysis of the OB Cpi Projection:

The number of CtB-positive cells in the OB ipsilateral to the injection site was significantly higher than in the contralateral OB (right Cpi injection ipsi: 108.68 ± 63.06 , contra: 87.79 ± 63.50 ; Wilcoxon-Test $Z= 2.52$ $p < .05$; left Cpi injection ipsi: 87.21 ± 55.69 , contra: 62.83 ± 65.66 Wilcoxon-Test $Z= 2.24$ $p < .05$; Fig. 3a). The asymmetry index of CtB positive cells in the OB revealed no significant differences (right injection AI: 1.18 ± 0.13 ; left injection AI: 0.25 ± 0.17 ; Mann-Whitney U-Test $Z=-0.74$ $p=0.46$; Fig. 2b), indicating that the contralateral projection from the OB to the Cpi is not asymmetrically organized.

To determine a relationship in the projection pattern from the OB to the contralateral Cpi, a Pearson correlation was performed. While after left-sided injections, the number of labelled cells in the contralateral Cpi showed a strong positive correlation in relation to cell numbers in the OB ($r=0.77$, $p < 0.05$), no corresponding correlation could be detected after right-sided injections ($r=0.17$, $p=0.684$).



*Figure 3: The Cpi receives projection from both OBs but with a higher innervation from the ipsilateral OB (a). The asymmetry indexes did not differ, indicating that the contralateral projection from the OB to the Cpi is not asymmetrically organized. * $p < .05$,*

Discussion

Although behavioural data indicates an asymmetrical projection pattern from the OB to Cpi with a stronger input from the right OB to the left Cpi (Gagliardo et al. 2005, 2007), the present tracing experiment could not identify any asymmetry in the neural projection from the OB to Cpi. Thus, we propose that the functional lateralization is not based on an asymmetrical projection pattern on the neuronal level.

Fibre connection of the OB

Two earlier studies on the OB projection revealed different results with respect to the projection pattern. To track-trace the projection they used the anterograde degeneration technique and the autoradiograph technique. However, these two techniques do not always provide reliable results. Therefore, we used BDA as an anterograde tracer to reverify the OB projection.

In accordance with the results of Reiner and Karten (1985), olfactory information of both nostrils reaches the left as well as the right hemisphere. Ascending olfactory input from the olfactory mucosa passes along the ipsilateral olfactory nerve entering into the OB. The olfactory bulbs in turn send the information mostly bilaterally into telencephalic brain areas. Only the projection to the medial septum (SM) is confined to the ipsilateral hemisphere. The main target of bulbar efferents is the Cpi. The extent of this OB projection corresponds approximately to the previously described Cpi in the atlas of Karten and Hodos (1967). The projection to the contralateral hemisphere runs via the habenular commissure (Reiner and Karten 1985) and not via the anterior commissure as described by Rieke and Wenzel (1978). Only the TnA, a part of the avian amygdale, receives olfactory input (Reiner and Karten 1985). OB projections were not detected in any other amygdaloidal area. In contrast to the tracing study of Reiner and Karten (1985), no projection to the TuO could be observed. As the authors pointed out, they had difficulties to limit the injection site to the OB due to its small size. Thus, their detected staining of the TuO could result from tracer spread into adjacent brain areas. This would mean, however, that the TuO does not receive olfactory input and may not be an olfactory brain area. Using the anterograde degeneration technique, Rieke and Wenzel (1978) reported that the OB of pigeons projects to the ipsilateral mesopallium, medial striatum and nucleus acumbens, as well as to the contralateral globus pallidus. Such projections were not found in this study.

Instead, our study revealed projections to the TnA, Cpi, and the septum. This indicates that the detected differences by our study and the study of Reiner and Karten (1985) are based on the technique used by Rieke and Wenzel (1978).

Fibre connection to the Cpi

The projections to the Cpi observed in this study largely reflect the findings by Bingman et al., (1994). The main telencephalic areas projecting to the Cpi are the OB, the dorsomedial hippocampus (DM) and the hyperpallium densocellularis (HD).

The hippocampal formation (HF) as well as the Cpi have been found to play a crucial role in spatial memory formation and navigation ability of birds (Bingman et al., 1990; Papi and Casini, 1990). Ablations of either the HF or the Cpi can interrupt the acquisition of the olfactory map. This projection pattern reflects the functional cooperation of the hippocampal formation with the Cpi for olfactory-guided navigation. Moreover, the HF and Cpi are reciprocally connected to the HD (Bingman et al., 1994). The HD was identified to be a polymodal processing area that receives afferents from the visual (Karten, 1979), somatosensory (Delius and Benetto, 1972), and the olfactory system (Bingman et al., 1994). HD is also connected to other hyperpallial areas (Shimizu et al., 1994). Recent studies revealed that it sends efferents to parts of the amygdala (Atoji et al., 2006). A lesion study has found the HD to be important for reversal learning (Shimizu and Hodos, 1989). In how far the HD may be further involved in navigation processes still needs to be determined. Additional evidence that the HD receives olfactory information comes from electrophysiological studies, according to which electrical stimulation of the olfactory bulb elicits a neural response (Rieke and Wenzel, 1978).

Unlike the observations of Bingman et al (1994), no projection from the TnA or from the septum to the Cpi could be observed in the current study. This is consistent with track tracing analysis of the TnA in ringdoves where a projection from the TnA to the Cpi could not be detected (Cheng et al., 1999). Atoji and Wild (2004) reported projections from the medial septum to the PoA, which might reach the Cpi. Since the PoA and the Cpi are adjacent areas, the observed projection by Bingman et al., (1994) may be due to a spread of tracer into the PoA.

Another important aspect is that tract tracing studies of the PoAc revealed nearly the same projection pattern as the Cpi. Aside from the OB, afferents from HD, NFL, HF and contralateral Cpi to the PoAc have been described (Atoij et al., 2006). PoAc and the Cpi are neighbouring structures. Therefore, it is not clear if they share the same projection pattern or if the common projections are due to a spread out of the tracer. Nevertheless, only the injection into the Cpi revealed afferents from the OB. Furthermore, anterograde track-tracing studies demonstrated that the Cpi as well as the PoA receive input from the HF, which at least in part must be due to the fact that these areas share the same projection pattern.

Olfactory projections and functional lateralization

One of the primary aims of this study was to examine if the functional lateralization of the olfactory system is based on an asymmetrical projection pattern between OB and Cpi. The behavioural results revealed a functional dominance of the left Cpi, which appears to be triggered by the right nostril/OB, as demonstrated by plugging the left or right nostril of homing pigeons (Gagliardo et al., 2005; 2007). Following this line of thought, the contralateral connection of the olfactory bulb could be based on an asymmetric projection with a stronger innervation from the right OB to the left Cpi. However, our tracing experiment did not reveal any left right differences in the relation to ipsi- and contralateral projections arising from the left and right OB

As a consequence of the anatomical data, we have to deduce that the asymmetrical bottom-up effect shown in behavioural data is not directly linked to left-right differences in the amount of ascending OB projections. Thus, functional asymmetries presumably originate from asymmetries at a different neural level. The number of afferent cells from the contralateral Cpi and the OB were significantly correlated after injection into the left Cpi, but not vice versa. This indicates a more closely connected circuitry in the left Cpi compared to the right one and leads to the assumption that the left Cpi is stronger modulated through the right Cpi than the right Cpi through the left Cpi. Such finer tuned projections may provide evidence for different ways of information processing in the left and right Cpi. A lateralized modulation through the interhemispheric connection that is not based on asymmetric organisation is also known from the visual system (Manns and

Güntürkün, 2009). However, this kind of Cpi-Cpi modulation has not been verified yet and requires further analysis.

In conclusion, our results demonstrate that the functional lateralization of the olfactory system is not based on a stronger bilateral input, but probably is a result of a stronger interhemispheric modulation through the contralateral hemisphere.

Chapter 3

**Study II: Navigation induced ZENK expression in
the olfactory system of pigeons (*Columba livia*)**

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

Introduction

Homing pigeons possess the extraordinary ability to return to their home loft when displaced to an unfamiliar location up to hundreds of kilometres away. After determining the direction of displacement with respect to their home loft, they use the sun (Schmidt-Koenig, 1960) or the earth's magnetic field (Keeton, 1971; Wiltschko et al., 1981) to orientate homewards ("map and compass" model of Kramer, 1953). Numerous studies indicate that pigeons use olfactory cues when starting to determine the direction of displacement for successful homing (Wallraff, 2005). According to the olfactory navigational map hypothesis, pigeons acquire an olfactory navigational map by associating different odours carried with winds from different directions (Papi et al., 1971; 1990; Wallraff, 1990), so that, any impairment of the olfactory system may disturb the homing performance (rev. see Wallraff, 2005). However, the neuronal activation of the olfactory system during homing has not been verified yet.

Behavioural studies have demonstrated that the contributions of the left and right hemispheric system differ in the olfactory-based navigation. Impairment of the olfactory input, induced by plugging the right nostril/olfactory bulb (OB), leads to a disturbance of initial orientation but not after plugging the left one (Gagliardo et al., 2007). Such a lateralised olfactory perception was previously shown in chicks with a dominance of the right nostril/OB (Vallortigara and Andrew, 1994; Burne and Rogers, 2002;). The piriform cortex (Cpi) receives bilateral projections from the OB with a stronger input from the ipsilateral side (Reiner and Karten, 1985; Bingman et al., 1994). However, contrary to what one would predict, only the lesion of the left Cpi resulted in an impairment of initial orientation but not after a lesion of the right one (Gagliardo et al., 2005). Nevertheless, both hemispheres appear to be necessary for successful navigation, since the impairment of either the left or right hemispheres of both olfactory areas leads to a reduced homing performance (Gagliardo et al., 2005, 2007)

Apart from the olfactory system, the hippocampal formation (HF) has been identified to be important for homing in familiar locations, processing a map-like representation of familiar landmarks (Bingman et al., 1988).

In this study, we followed several goals: first, we wanted to find out whether navigation performance over unfamiliar locations leads to an activation of the olfactory system at the neuronal level. Secondly, we aimed to clarify if the functional lateralization may be based on asymmetrical activation processing of the olfactory information. To address these questions, we used the expression of ZENK, an immediate early gene (IEG), which was introduced by Shimizu et al., (2004) in homing experiments in familiar locations. In order to achieve this, we released one experimental group from an unfamiliar site. The first control group was transported to the unfamiliar site and back without being released, while the second control group was released in front of the loft. The nostrils of the pigeons were either unilaterally plugged on the left or on the right side or not plugged at all. Thus, we were able to investigate a differential contribution of the left and right olfactory input on the neuronal level.

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 (10km SW from Pisa) were used for this study (Tab.1). The pigeons were fed *ad libitum* and were continually allowed spontaneous free flights from the loft. At the time of the experiment, the pigeons were approximately 6 months old. To investigate homing-dependent ZENK activation, we compared one experimental group with two control groups. Thus, the pigeons were divided into three groups:

- 1) Released from an unfamiliar location (R), to examine if the olfactory system is activated during homing from an unfamiliar site.
- 2) Transported to the unfamiliar location but not released (TnR). This group was chosen to examine the influence of the new environment, since it has been demonstrated that pigeons already orientate before taking off (Gagliardo et al., 2001c); however, active navigation was not demanded.

- 3) Released at about 200 meters from the loft at home site (RH). The third 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.

Since ZENK protein expression peaks between 1 and 2 hours after stimulus onset and declines thereafter (Mello and Ribeiro, 1998), pigeons were caught immediately after their return to the loft. The fastest pigeons to enter the loft were assigned to the two released groups (R and RH). The remaining pigeons were allotted to the TnR group. The fastest-enterers among the pigeons had been identified in a preliminary release where all pigeons were released at 500 meters from the loft.

The three groups were further subdivided into three sub-groups: left plugged, right plugged and unplugged. 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 during the night some pigeons lost their plugs, they were replaced early in the morning before the experiment.

Table 1: Experimental pigeons

Experimental group	Nostril Condition	Number of pigeons (released/analysed)
Released	No plug	14/11
	Left plug	27/10
	Right plug	28/12
Transported not released	No plug	8
	Left plug	8
	Right plug	8
Released at home	No plug	9/8
	Left plug	10/9
	Right plug	10/7

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 (1. releasing site: Fornacette [23 km, home direction 271°], 2. releasing site: La Costanza [18 km, home direction 190°]). The distance of both release sites was comparable, considering the time the pigeons approximately needed for homing, which should not exceed 120 min to ensure optimal ZENK visualization (Mello and Ribeiro, 1998). During transportation, the pigeons had the possibility to smell the surrounding air through open windows of the car. Prior to release, the position of the plug was controlled again. The birds were released singly, alternating the three nostril conditions. The flight was documented by an observer blind to the nostril conditions. Each bird was followed with the aid of 10 x 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 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). At the home loft, another observer waited for the birds documenting the time of arrival to obtain the homing time. Pigeons, which arrived together, were excluded from the experiment.

The RH group was released at about 200m in visual distance of the loft. After the pigeons of the R and RH group entered the loft, they were caught and committed to the analyzing procedure. The TnR pigeons stayed for approximately 60 min at the release site and were then transported back to the loft and directly committed to the procedure described in the following.

Fixation

Animals were sacrificed via rapid decapitation directly after arrival at the loft between 60 and 120 min after release. Pigeons, which lost their plug during the flight, were excluded from the analysis. The removed brains were fixed for 3h in 5% Acrolein in 0.12M phosphate buffer saline (PBS, pH of 7.4), rinsed briefly in PBS, washed two times for 30 minutes in PBS and cryoprotected in 30% sucrose in PBS. To avoid loss of the OB

during immunohistochemistry, we embedded the brain in 15% Gelatine in 30% sucrose in PBS. The embedded brains were cryosectioned in frontal plane (40 μ m). The left or the right brain side was marked by a hole stuck with a small needle. Slices were collected in five parallel series for the OB and ten parallel series to the rest of the brain and stored in 0.12M PBS containing 0.1% sodium azide at 4°C until they were subjected to immunohistochemistry.

Immunohistochemistry

The immunohistochemical detection of ZENK (rabbit erg-1, sc-189, Santa Cruz) was performed with free-floating slices according to the standards of the immuno-ABC-technique (Hellmann and Güntürkün, 2001). After each incubation step, the slices were washed 3 times for 5 min with PBS. Slices of one series were incubated in 0.1% NaBH₄ in PBS for 15 min. Endogenous peroxidases were blocked with 0.3% H₂O₂ in deionised water for 30 min. Slices were incubated with 10% normal goat serum in 0.12M PBS+0.3% Triton X-100 (PBST) for 1h to block non-specific binding-sites in the tissue. Then the slices were incubated with primary antibody solution (1/5000+ 1% normal goat serum) for 72h at 4°C. The secondary antibody reaction was carried out with biotinylated goat anti-rabbit IgG (1/200 in PBST; Vectastain Elite kit, Vector, Burlingame, CA), for 1h at room temperature. Afterwards, the tissue was incubated in an avidin-biotin-peroxidase solution (1/100 in PBS-X; Vectastain ABC-Elite kit). The peroxidase-activity was detected using a heavy metal intensified 3´3-diaminobenzidine (DAB, Sigma) reaction, modified by the use of 1% β D-glucose/glucose-oxidase (Sigma) (Hellmann and Güntürkün, 2001). The slices were mounted on gelatinised slides, dehydrated and coverslipped with Permount (Fisher Scientific, New Jersey, USA). For visualising neuronal structures one corresponding serial set was stained with cresyl violet.

Quantification and Data analysis

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, the Cpi, and the hippocampus. Pictures of a representative region of 800 pixels x 800 pixels or 1300 pixels x 1030 pixels (136.64 μ m² x 136.64 μ m² or 225.29 μ m² x

178.49 μm^2 , magnification 40 x 2.5) were captured with a camera-equipped microscope (Olympus BH-2, Axio Viosin 3.4). The pictures were converted to 8-bit gray scale images by Adobe Photoshop (CS2). ZENK positive cells were counted automatically using the ImageJ program (Rasband, 2003). Both strong and faintly stained cells were included into the cell counting, thus avoiding bias based on differences in staining intensity (Shimizu et al., 2004). The threshold was set manually according to Shimizu et al. (2004).

For OB analysis, only slices with a U-shape of the granular cell layer including the ventricle were examined (Fig. 1). In these slices, the OB was subdivided into three or two regions of interest; medial, ventral, and lateral. Two regions of interests were defined in OB slices, medial and lateral, when these slices were too small to be divided in three regions. In a pre-analysis of five brains, we defined that the number of five randomly chosen regions of interest (Mat lab 2006b) was sufficient to obtain reliable results for the ascertainment of ZENK cell density. For the Cpi analysis, a picture of each slice with a visible Cpi was taken (Fig. 1). For the Cpi and the OB, a picture size of 136.64 μm^2 x 136.64 μm^2 was used due to the narrow size of these two areas. The hippocampus was analysed at A 5.75 (Karten and Hodos, 1967) in the dorsolateral (DL), dorsomedial (DM) and triangular part (TR), according to Atoji and Wild (2004; Fig. 1) in a representative area of 225.29 μm^2 x 178.49 μm^2 . The sampling window in all analysed brain areas was taken from the middle of the area of interest. To determine the region of interest, we made use of parallel stained cresyl violet slices.

Statistical analysis was carried out using the program Statistica (StatSoft, Tulsa, USA). Density of ZENK positive cells in the OB and Cpi was subjected to a mixed 3x3x2 analysis of variance (MAVONA) with “hemisphere” (left, right) as repeated measure and “releasing condition” (released (R), transported to the released site but not released (TnR), released in front of the loft (RH)) and the “nostril condition” (unplugged, left plugged, right plugged) as between-subject factors. For statistical analysis of the hippocampus, we used the same procedure as above but since the hippocampus was subdivided in three areas, a second factor of repeated measures of area (DL, DM and TR) was added. Since the number of animals varied between the groups from 7 to 12, we used the HSD test for post hoc analysis with unequal sample sizes.

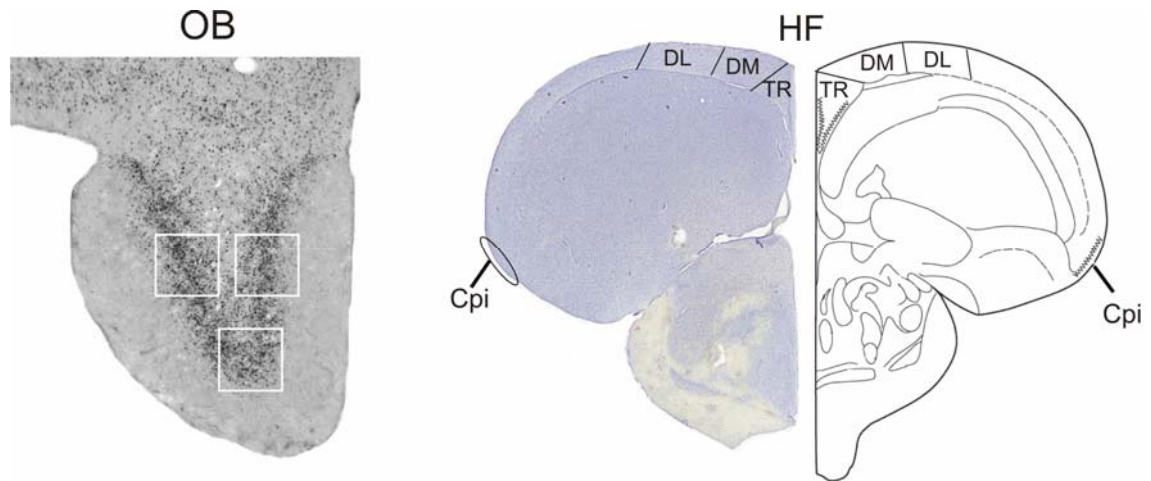


Figure 1: Overview of the analysed areas. Left OB: squares indicate sampling windows lateral, ventral and medial chosen for analysis. Right Cpi and HF: the HF was subdivided in three subareas: DL, DM and TR for analysis.

Results

Initial Orientation

The pooled (home direction set to 360°) initial orientation distributions of the pigeons released from two sites is presented in Figure 2 and Table 2. The initial orientation displayed by the three experimental groups is consistent with previous results (Gagliardo et al., 2007), if we consider all the pigeons that were released. In fact, both unplugged and right plugged pigeons displayed initial orientation distributions significantly different from random, while the left plugged bird's distribution turned out to be randomly scattered (see Tab. 2 for the Rayleigh and the 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, Tab. 2). This was due to the fact that for the analysis of the ZENK expression we had to select only the birds that homed within two hours, which were more likely to be those displaying an initial orientation closer to the home direction.

Table 2: Group: intact control pigeon with no plug; pigeons with the right nostril plugged; pigeons with left 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 indicates the results of the Rayleigh and V test respectively. ***, $p < .001$, **, $p < .01$, *, $p < .05$

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

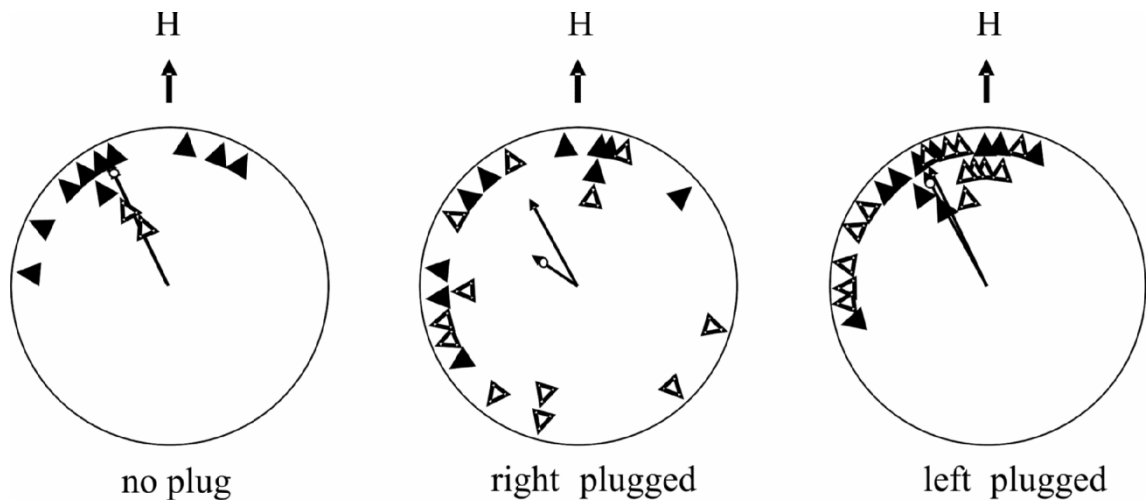


Figure 2: Pooled initial orientation of pigeons with no plug, pigeons with right nostril plugged and with the left nostril plugged. Each symbol represents the bearing of a single pigeon. Filled triangles and open triangles represent the birds used in the ZENK experiment and those excluded, respectively. 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 filled inner arrow. The outer arrow represents the home direction. See text for further explanations.

Olfactory Bulb (OB)

The multivariate analysis revealed significant main effects of “releasing” ($F_{(2,71)} = 14.18, p < .001$) and “nostril condition” ($F_{(2,71)} = 16.39, p < .0001$). No main effect of “hemisphere” was found ($F_{(1,71)} = 0.14, p = .71$). Post hoc analysis showed that the R group ($5844.79/\text{mm}^2 \pm 2127.42/\text{mm}^2$) had a higher ZENK activation than the TnR ($4289.62/\text{mm}^2 \pm 1834.54/\text{mm}^2; p < .001$) and RH group ($3487.31/\text{mm}^2 \pm 2232.35/\text{mm}^2; p < .01$), indicating that orientation in an unfamiliar environment increases ZENK expression. The TnR and the RH group did not differ in their ZENK expression ($p = .18$ Fig. 3a; Fig. 4). The birds with no plug ($6153.09/\text{mm}^2 \pm 1612.23/\text{mm}^2$) displayed the highest ZENK expression ($p < .001$). No differences were found between left ($3789.29/\text{mm}^2 \pm 2314.85/\text{mm}^2$) and right ($4163.45/\text{mm}^2 \pm 2188.72/\text{mm}^2$) plugged groups ($p = .66$, Fig. 3b; Fig. 4). However, the significant interaction between “hemisphere” and “nostril condition” ($F_{(2,71)} = 48.08, p < .0001$) suggests that the hemisphere-specific activation depended on the “nostril condition”. While no hemispheric differences were found in the unplugged “nostril condition” (left OB = $6184.87/\text{mm}^2 \pm 1672.19/\text{mm}^2$, right OB = $6122.53/\text{mm}^2 \pm 1583.63/\text{mm}^2, p = .99$), a decreased ZENK expression was detected for the left (left OB = $3038.43/\text{mm}^2 \pm 1998.56/\text{mm}^2$, right OB = $4540.16/\text{mm}^2 \pm 2399.56/\text{mm}^2, p < .0001$) as well as for the right plugged nostril condition (left OB = $5043.85/\text{mm}^2 \pm 2390.86/\text{mm}^2$, right OB = $3283.05/\text{mm}^2 \pm 1561.78/\text{mm}^2; p < .0001$, Fig. 3c) in the ipsilateral OB to the plugged nostril, suggesting that olfactory stimulation induces ZENK expression. Moreover, the significant triple interaction of “hemisphere”, “nostril” and “releasing condition” reveals that the hemisphere-specific activation is not only modulated by the “nostril condition” alone but also by its combination with the “release condition” ($F_{(4,71)} = 7.79, p < .001$). While 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 expression in the ipsilateral OB in both plugged “nostril conditions” (left plugged: left OB = $3844.74/\text{mm}^2 \pm 1839.96/\text{mm}^2$, right OB = $6429.40/\text{mm}^2 \pm 1703.34/\text{mm}^2, p < .0001$; right plugged: left OB = $6672.37/\text{mm}^2 \pm 1561.79/\text{mm}^2$, right OB = $3980.87/\text{mm}^2 \pm 930.51/\text{mm}^2, p < .001$, Fig. 3d, Fig. 4). No significant interactions of “releasing” and “nostril condition” ($F_{(4,71)} = 1.17, p = .33$) as well as “hemisphere” and “releasing condition” ($F_{(2,71)} = 0.02, p = .98$) could be observed.

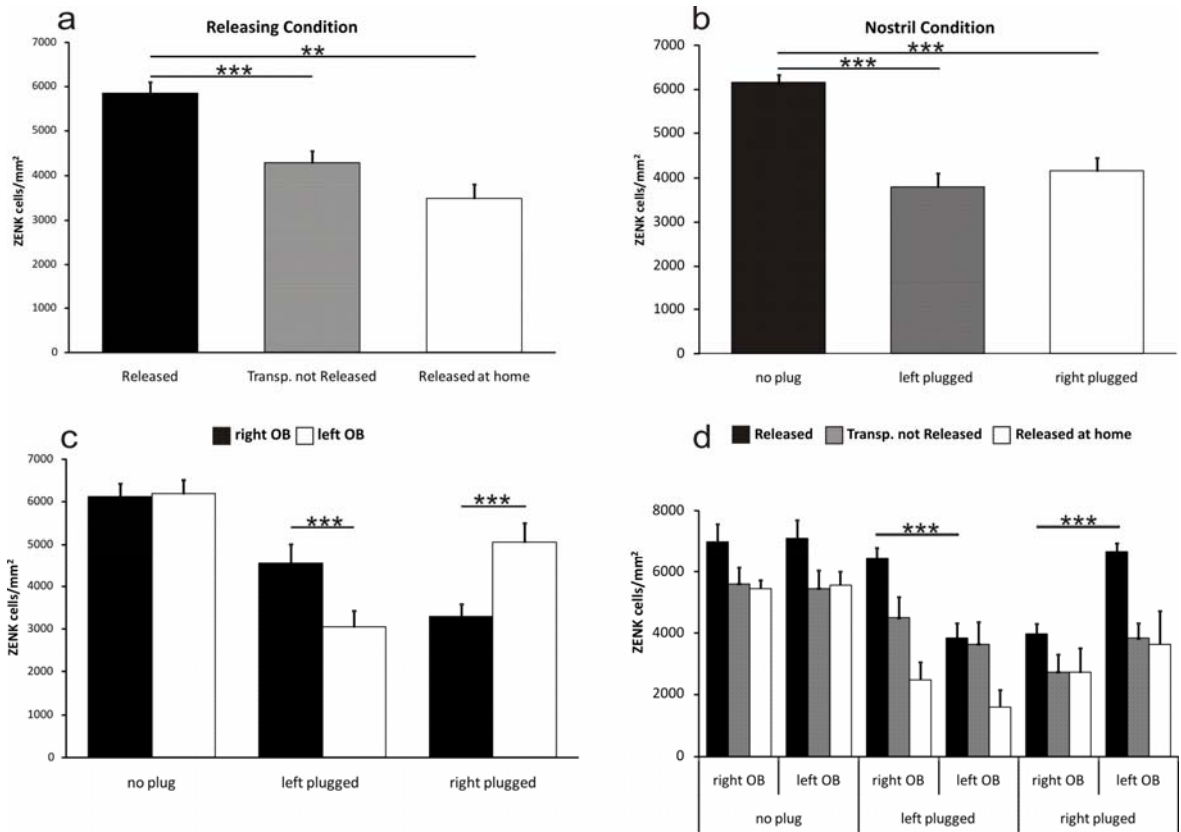


Figure 3: Effects on the ZENK expression in the OB. (a) ZENK cell density of the three releasing conditions. (b) ZENK cell density of the three nostril conditions. (c) ZENK cell density plotted against hemisphere. (d) ZENK cell density plotted against nostril and hemisphere condition. ** $p < .01$, *** $p < .001$.

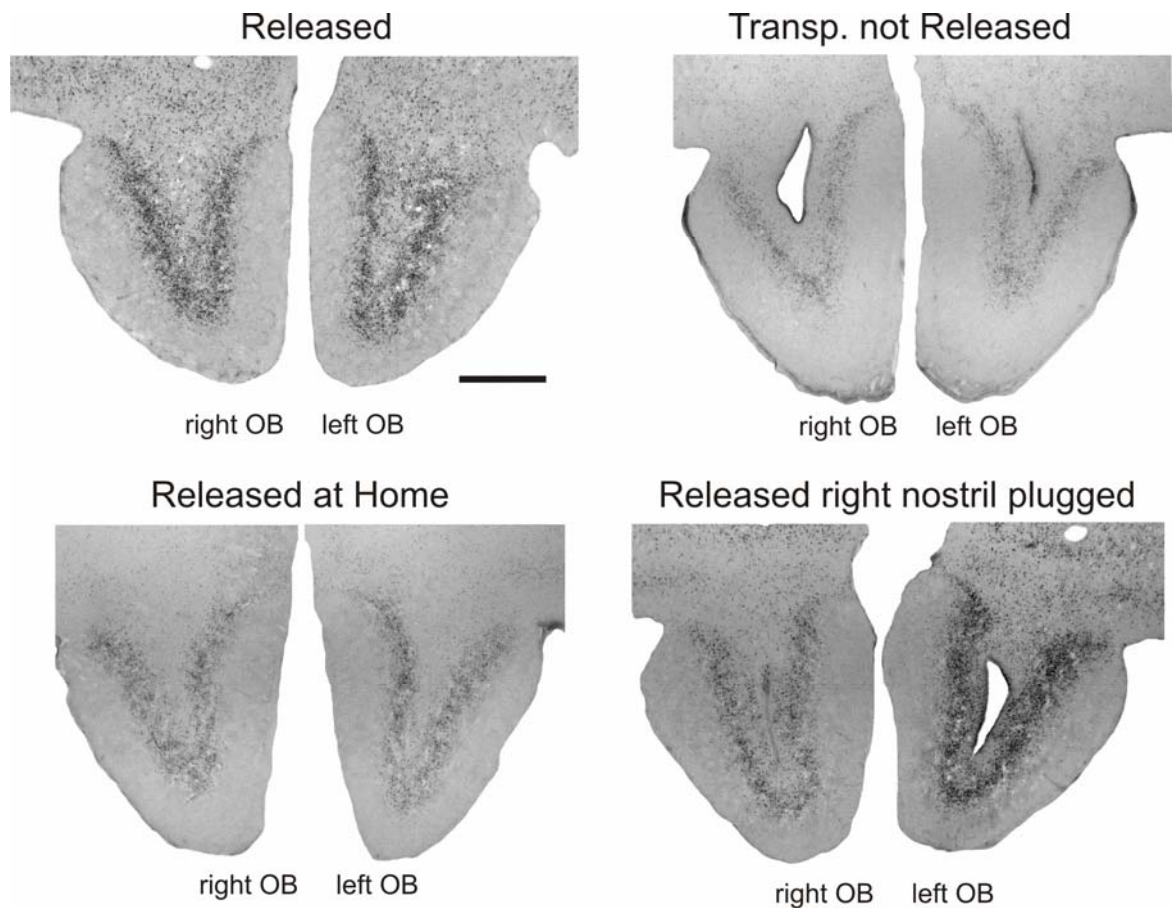


Figure 4: ZENK cell staining of the right and left OB of the three experimental conditions of pigeons with no plug and of the OB of released pigeons with the right nostril plugged. Scale bar=500 μ m

Piriform Cortex (Cpi)

Significant main effects of “releasing” ($F_{(2,73)} = 71.69$, $p < .001$) and “nostril condition” ($F_{(2,73)} = 20.22$, $p < .001$) were found. No main effect of “hemisphere” was observed ($F_{(1,73)} = 0.78$, $p = .38$). As expected, R pigeons ($1349.02/\text{mm}^2 \pm 448.81/\text{mm}^2$) revealed a higher ZENK expression compared to the TnR ($735.98/\text{mm}^2 \pm 350.71/\text{mm}^2$; $p < 0.001$) and RH group ($416.53/\text{mm}^2 \pm 333.23/\text{mm}^2$; $p < .001$). In contrast to the OB, the TnR and RH also differed in ZENK expression with higher values in the TnR birds ($p < .001$, Fig. 4a; Fig 5).

The groups with no plug ($1193.24/\text{mm}^2 \pm 532.29/\text{mm}^2$) revealed the highest ZENK expression ($p < .001$), no differences were found between right ($711.45/\text{mm}^2 \pm 469.09/\text{mm}^2$ ranging from $52.03/\text{mm}^2$ to $1838.26/\text{mm}^2$) and left ($757.63/\text{mm}^2 \pm 545.16/\text{mm}^2$ ranging

from 0.00/mm² to 2021.59/mm²) plugged groups ($p=.84$ Fig. 4b; Fig. 5). As in the OB, the significant interaction between “hemisphere” and “nostril condition” ($F_{(2,73)} = 8.14$, $p < .001$) suggested that the hemisphere-specific activation was modulated by the nostril condition. However, other than in the OB, ZENK expression was decreased only in the right Cpi (right Cpi = 636.858/mm² ± 416.11/mm²; left Cpi = 786.046/mm² ± 513.39/mm²) after plugging the right nostril ($p < 0.018$). Plugging the left nostril did not reduce ZENK activity in the left Cpi (right Cpi = 816.47/mm² ± 586.26/mm²; left Cpi = 698.802/mm² ± 504.90/mm²) ($p < .05$, Fig. 4c; Fig. 5).

Moreover and comparable to the OB, a significant triple interaction indicated that the differences in hemisphere-specific ZENK activation between the “nostril conditions” depended on the “releasing conditions” ($F_{(4,73)} = 3.1376$, $p < .05$), whereas only the released pigeons with a right plugged nostril showed a reduced ZENK expression in the ipsilateral Cpi (right Cpi = 974.44 ± 308.69/mm², left Cpi = 1250.69 ± 293.62/mm²; $p < .05$, Fig. 4d; Fig. 5). No significant interactions, between “releasing” and “nostril condition” ($F_{(4,73)} = 0.43$, $p = .79$) or of “hemisphere” and “releasing condition” ($F_{(2,73)} = 0.16$, $p = .86$) was observed.

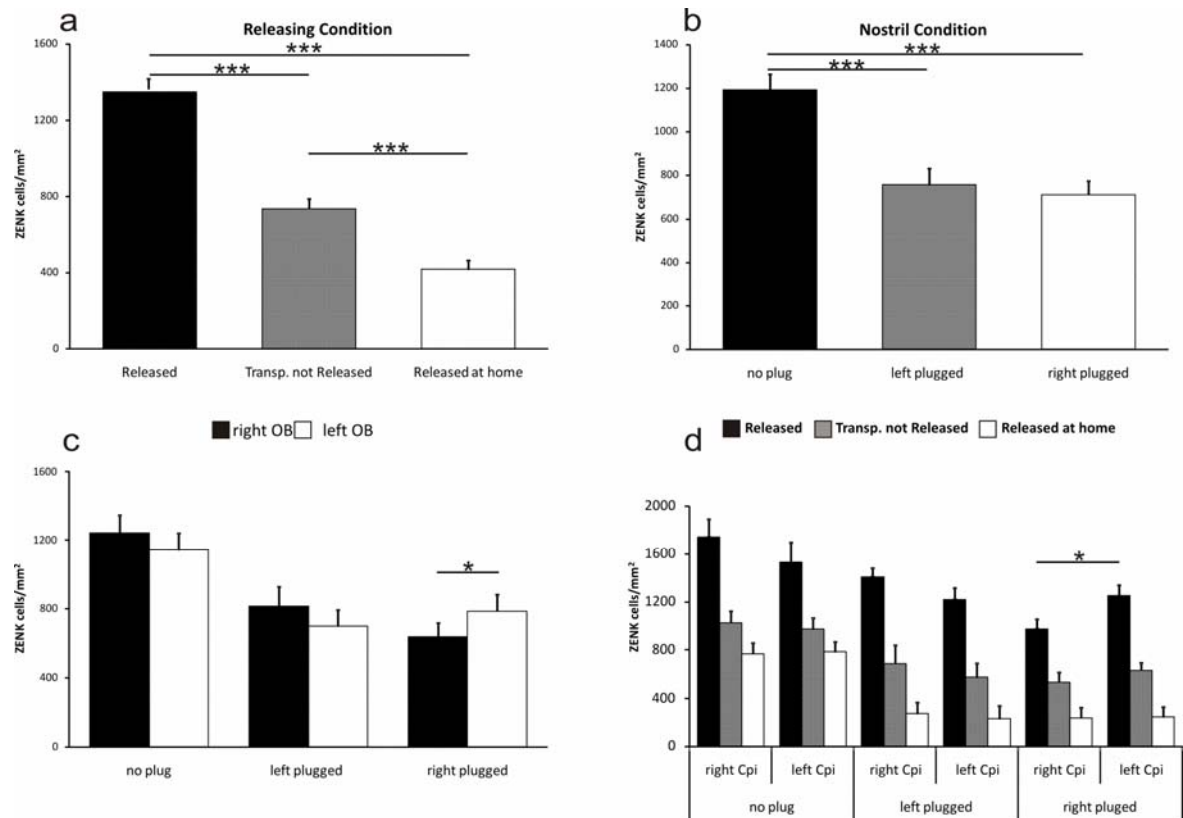


Figure 5: Effects on the ZENK expression in the Cpi. (a) ZENK cell density of the three releasing conditions. (b) ZENK cell density of the three nostril conditions. (c) ZENK cell density plotted against hemisphere. (d) ZENK cell density plotted against nostril and hemisphere condition. * $p < .05$, *** $p < .001$.

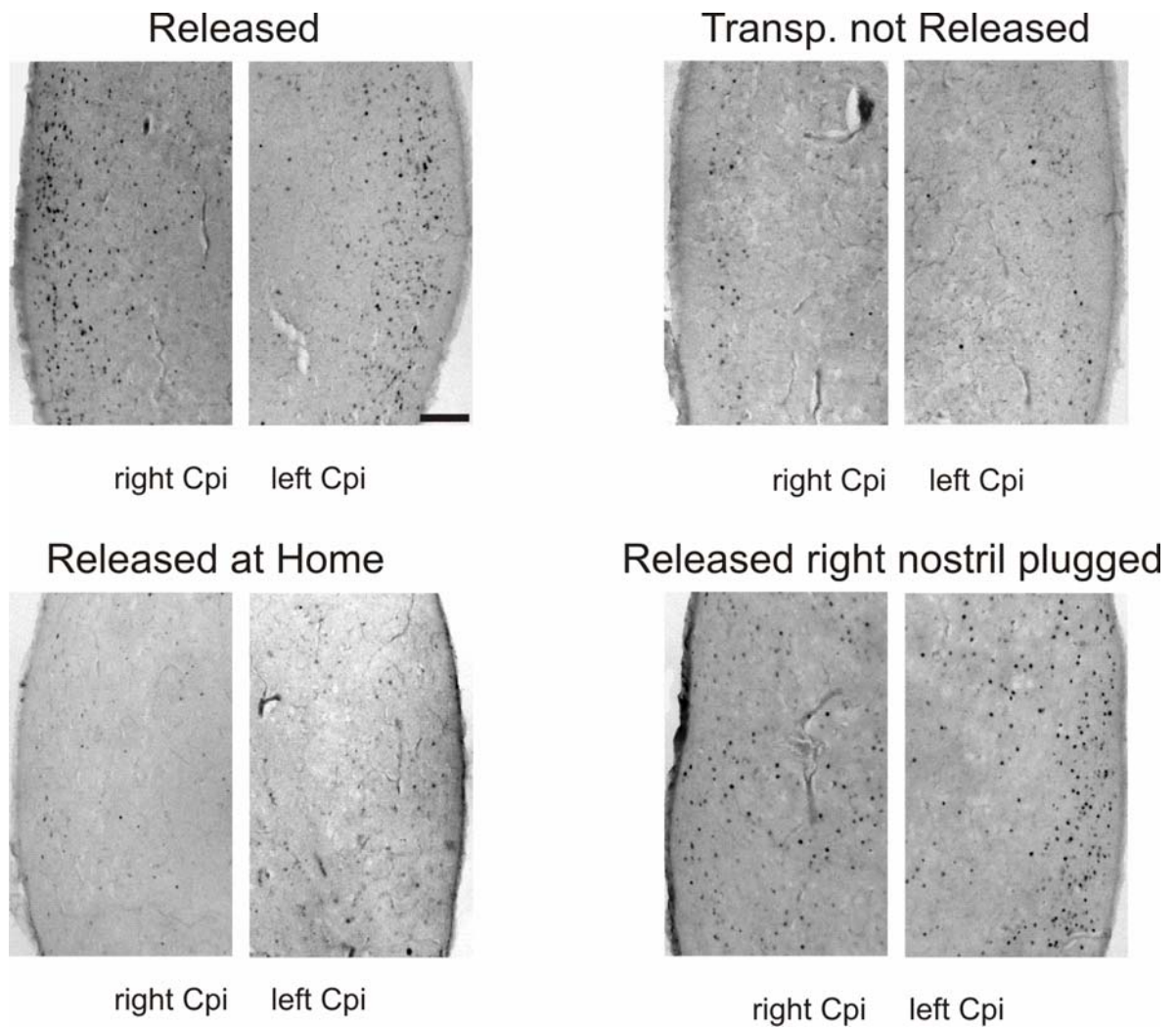


Figure 6: ZENK cell staining of the right and left Cpi of the three experimental conditions of pigeons with no plug and of the Cpi of released pigeons with the right nostril plugged. Scale bar = 200 μ m

Hippocampal formation

Statistical analysis revealed a significant main effect of “releasing” ($F_{(2,73)} = 125.88$, $p < .001$), and “area condition” ($F_{(2,146)} = 71.58$, $p < .001$). Post hoc analysis showed that the R group ($973.70/\text{mm}^2 \pm 582.83/\text{mm}^2$) had a higher ZENK activation than the TnR ($149.89/\text{mm}^2 \pm 167.01/\text{mm}^2$; $p < .001$) and RH ($391.07/\text{mm}^2 \pm 323.05/\text{mm}^2$; $p < .001$), while higher ZENK expression of the RH group compared to that of the TnR birds ($p < .001$ Fig. 6a; Fig 7) was detected.

The DL ($836.83/\text{mm}^2 \pm 666.97/\text{mm}^2$; Post hoc Fisher LDS Test for equal sample $p < .001$) and DM ($531.61/\text{mm}^2 \pm 495.84/\text{mm}^2$; Post hoc Fisher LDS Test for equal sample $p < .001$) showed a higher ZENK expression than the TR ($296.43/\text{mm}^2 \pm 273.18/\text{mm}^2$; Post

hoc Fisher LDS Test $p < .001$), whereas the DL had a higher ZENK expression than the DM (Post hoc Fisher LDS Test for equal sample $p < .001$). A significant interaction of “area” and “releasing condition” ($F_{(4,146)} = 5.17$, $p < .001$) indicated that the area-specific ZENK activation depended on the “releasing condition” with a higher ZENK expression in the DL ($1429.85/\text{mm}^2 \pm 584.4565/\text{mm}^2$; $p < .001$) and DM ($973.955/\text{mm}^2 \pm 441.1952/\text{mm}^2$; $p < .001$) than in the TR ($517.308/\text{mm}^2 \pm 265.5141/\text{mm}^2$ ranging from $49.73/\text{mm}^2$ to $1367.68/\text{mm}^2$) and a higher ZENK expression in the DL than in the DM in the R condition. In the RH condition, only a difference between the DL ($577.41/\text{mm}^2 \pm 388.3784/\text{mm}^2$; $p < .001$) and TR ($227.284/\text{mm}^2 \pm 166.0930/\text{mm}^2$) was observed. The TnR group showed no differences between the three hippocampal subareas (DL vs. DM $p = .17$, DL vs. Tr $p = .99$, DM vs. Tr $p = .19$, Fig 3c). No main effect of “hemisphere” was found ($F_{(1,73)} = 1.68$, $p = .19$). In contrast to the results of the OB and Cpi, no main effect of “nostril condition” ($F_{(2,73)} = 0.52$, $p < .59$) was observed in the hippocampus.

The significant interaction of “hemisphere” and “nostril condition” ($F_{(2,73)} = 3.54$, $p < .035$) indicated that the hemisphere-specific activation is modulated by the “nostril condition”. However, further post hoc analysis showed no significant effects. The significant three-way interaction of “hemisphere”, “area” and “nostril condition”, demonstrated that the hemisphere-specific activation is not only modulated by the joined influence of the “nostril” and the “area condition” ($F_{(4,71)} = 7.79$, $p < .0001$). However, only in the unplugged “nostril condition”, the DL showed a higher ZENK expression in the left hemisphere (DL right = $804.952/\text{mm}^2 \pm 649.41/\text{mm}^2$, left $1048.09/\text{mm}^2 \pm 848.19/\text{mm}^2$ $p < .001$, Fig. 6d; Fig. 7) compared to the right one.

No interaction of “releasing” and “nostril condition” ($F_{(4,73)} = 0.79$, $p = .53$), “area” and “nostrils condition” ($F_{(4,146)} = 1.16$, $p < .36$), “area” and “hemisphere condition” ($F_{(2,146)} = 1.81$, $p = .37$) and “hemisphere” and “releasing condition” ($F_{(2,73)} = 1.59$, $p = .33$) were observed. Furthermore, no triple interaction of “hemisphere” “releasing” and “nostril condition” ($F_{(4,73)} = 0.73$, $p = .17$) and “area”, releasing” and “nostril condition” ($F_{(8,146)} = 0.65$, $p = .29$) were found and no quadruple interaction of “area”, hemisphere”, releasing” and “nostril condition” either ($F_{(8,146)} = 1.71$, $p = .10$).

To evaluate any possible association between ZENK expression of the released pigeons and homing time, we calculated a Pearson correlation. Since 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 $r = -0.11$ ns., Cpi $r = 0.16$ ns., Hippocampus: DL $r = 0.25$ ns., DM $r = 0.23$ ns., TR $r = 0.23$ ns.).

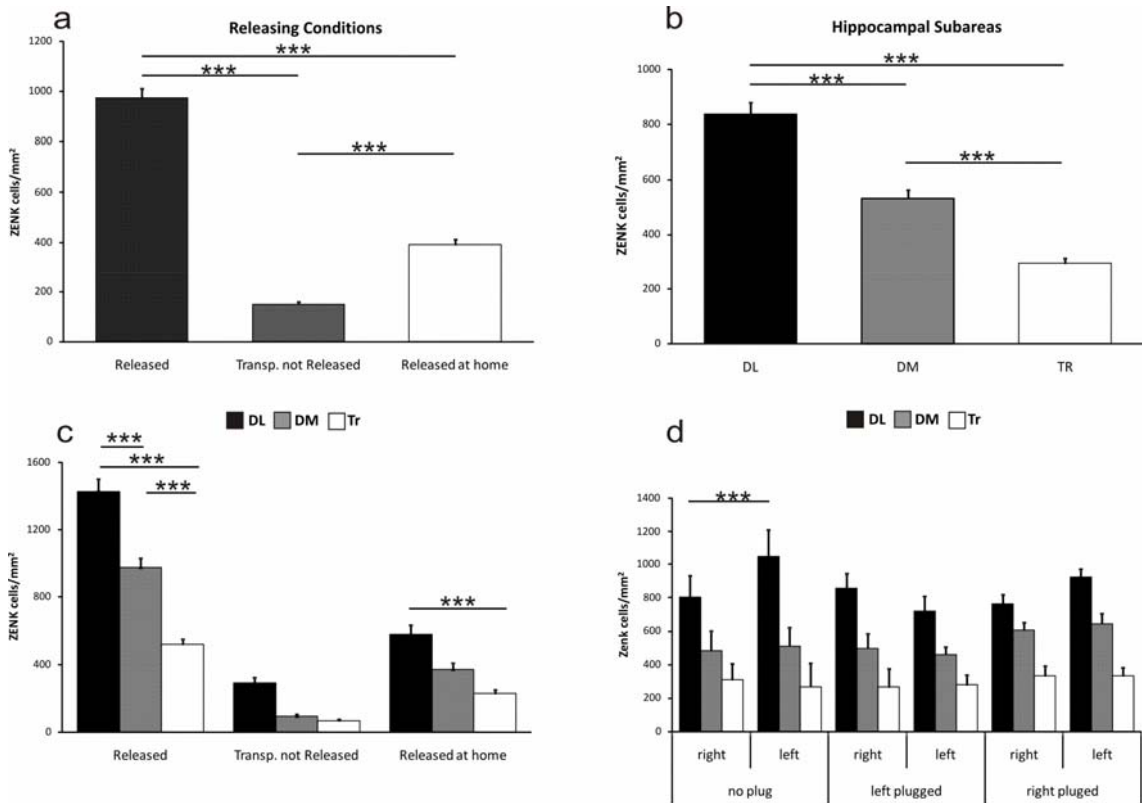


Figure 7: Effects on the ZENK expression in the HF. (a) ZENK cell density of the three releasing conditions. (b) ZENK cell density in the tree subareas. (c). ZENK cell density plotted against releasing condition and subarea. (d) ZENK cell density plotted against nostril condition, hemisphere condition and subarea. *** $p < .001$.

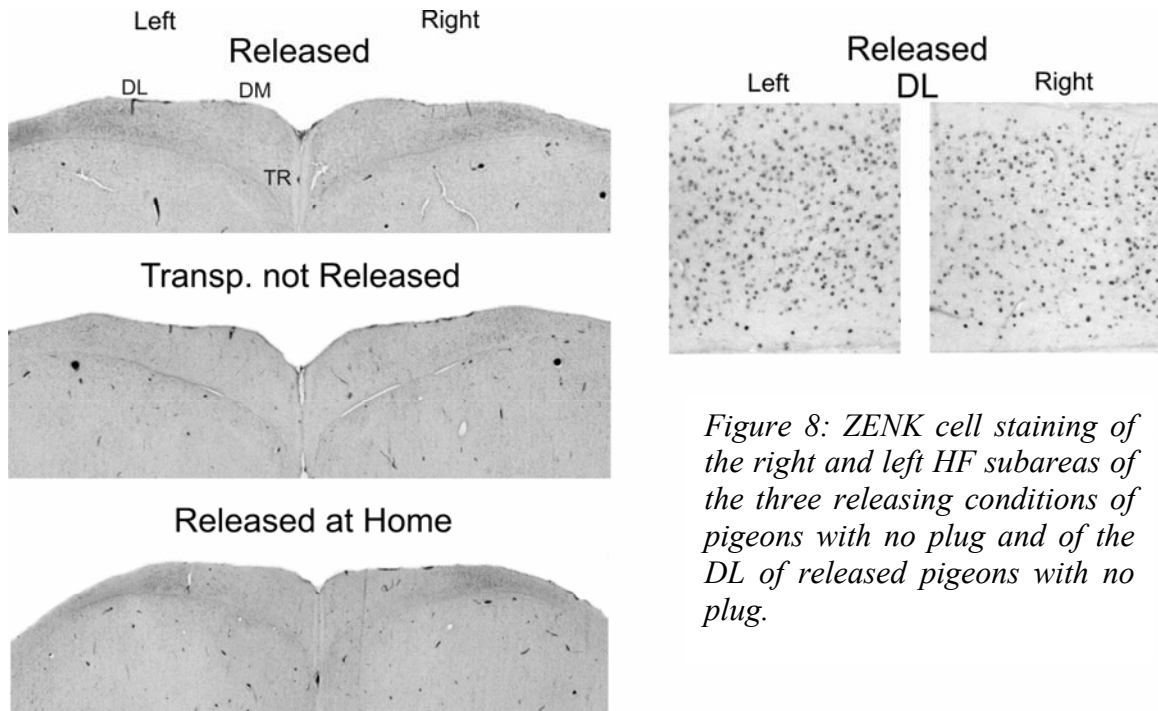


Figure 8: ZENK cell staining of the right and left HF subareas of the three releasing conditions of pigeons with no plug and of the DL of released pigeons with no plug.

Discussion

This study demonstrated that the olfactory system as well as the hippocampal formation elicits navigation induced ZENK expression. In particular, only the homing from unfamiliar terrain leads to an activation of the olfactory system. These results are consistent with numerous behavioural data on the manipulation of the olfactory information (Wallraff, 2005), showing that the processing of olfactory cues is necessary for navigation from unfamiliar sites and thus in turn leads to the activation of the respective brain areas.

Olfactory bulb (OB)

The olfactory bulb of the released group revealed the highest ZENK expression compared to the two control groups. Sensory input triggers the ZENK expression in the olfactory system, which was impaired by plugging the ipsilateral nostril. However, this is only true for the released pigeons and not for the TnR or RH group, where no effect of nostrils occlusion on the ZENK expression could be observed. Nonetheless, the two control groups had a different olfactory experience of either smelling the familiar air around the loft, or they had the same experience of new odours at the release site, showing

no differences in the activation of the OB. This favours the assumption that the olfactory system is only activated on demand when pigeons have to navigate actively over unfamiliar areas.

In contrast to behavioural experiments, which suggest that the right nostril/OB is functionally dominant (Gagliardo et al., 2007), no hemispheric differences in ZENK expression could be observed. This finding indicates that the functional lateralization must be triggered through other, maybe higher processes (see below Cpi).

Piriform Cortex (Cpi)

The Cpi is the main projection area of the olfactory bulb (Reiner and Karten, 1985, Bingman 1994). Lesion studies demonstrated that the Cpi is crucial for olfactory-based navigation from unfamiliar sites (Papi et al., 1990). Therefore, very similar to the OB, a higher activation would also be expected in the Cpi of released pigeons, whereas the two control groups should show a lower activation. We successfully confirmed this assumption in our study. As predicted, the highest sensory-triggered ZENK expression was detected in the Cpi of released birds, again demonstrating that the 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 expression in TnR pigeons compared to pigeons that were released in front of the loft. It can be assumed that a new olfactory environment does stimulate the olfactory system but less pronounced than in pigeons that have to actively find their way back. Furthermore, this result is consistent with several behavioural findings in that pigeons already orientate at the release site before taking off (Gagliardo et al., 2001c). For this orientation process to be successful, the birds have to use their olfactory system, which would lead to a higher ZENK expression in the Cpi. The lowest ZENK expression in the olfactory system in the RL birds corresponds to the behavioural data, which showed that pigeons do not have to use their olfactory system to home over familiar areas. Moreover, as verified by Shimizu et al., (2004), pigeons use other navigational mechanisms like the hippocampus based visual landmark orientation (see below HF).

In contrast to the OB, only the right Cpi of the released birds reacted vulnerably to the ipsilateral sensory deprivation. This higher sensory sensitivity of the right Cpi may be responsible for the impairment of the initial orientation after plugging the right nostril (Gagliardo et al., 2007). This would indicate that the assumed dominance of the right

nostril/OB is due to the dominance of the right Cpi and is not regulated by a bottom-up process. Rather, the OB appears to be a relay station for olfactory information processing likely with less regulative modulation. However, this finding is in contrast to the results of unilateral Cpi lesion studies (Gagliardo 2005) where a dominance of the left Cpi was observed. One explanation could be that the left Cpi is maybe stronger modulated through the right Cpi since both are reciprocally connected (Bingman et al., 1994). A strong additional input to the left Cpi can stabilize its activity and would thus counteract the down-regulation of ZENK expression after left nostril occlusion. In addition, this could also explain the finding that the occlusion of the right nostril leads to disorientation in the initial orientation because of the insufficient input from the right Cpi. Such a lateralised interhemispheric modulation has been well documented in the visual processing in pigeons (Manns and Güntürkün, 2009). The functional lateralization does not appear to be based on stronger bottom-up bilateral input of the dominating brain area (Study I), but rather on an interhemispheric modulation through the contralateral Cpi.

Further studies are needed to clarify the inconsistent behavioural and neuronal observations. Nonetheless, the ZENK expression pattern emphasizes the crucial role of the Cpi during navigation over unfamiliar areas with a striking asymmetrical involvement of both Cpi's in processing olfactory cues.

Hippocampal formation (HF)

The HF is involved in landmark-based homing. Hippocampal lesions disrupt the homing ability over familiar areas (Bingman and Mench, 1990;Gagliardo et al., 1999), whereas initial orientation when released from an unfamiliar site remains unaffected (Bingmann et al., 1988). This leads to the assumption that at the last phase of homing, pigeons rely on navigation guided by familiar landmarks rather than on olfactory cues. Especially the parahippocampal area (APH) has been shown to be activated during homing over familiar terrain (Shimuzu et al., 2004), which confirms our observations, where released pigeons revealed the highest ZENK expression in the dorsolateral hippocampus (DL, corresponding with the APH) compared to other subareas. In contrast to olfactory brain areas, the ZENK expression was not triggered through olfactory input, arguing for a landmark based navigation system that is not relying on olfactory cues. In addition, the pigeons, which were released at home, had a higher activation of the HF compared to the

TnR birds. Their olfactory system revealed the lowest ZENK expression rate, which is in sharp 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 the environmental necessity. But as demonstrated by the ZENK activation in the released birds, both mechanisms can also be used simultaneously.

Furthermore, the DL exhibits an asymmetric ZENK expression with more ZENK positive cells in the left DL compared to the right DL in the birds with no plugs, independent of the releasing condition. The HF is functionally lateralised. Although the left and the right HF are considered to be important for landmark-guided navigation, they appear to have different foci (Gagliardo et al., 2001c). The right HF is assumed to be important for representation of landmarks in a map-like fashion, whereas the left is more important for recognition and guidance by landmarks (piloting, Gagliardo et al., 1999). 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). However, our findings can be viewed in a more general way, since the asymmetrical activation is independent of the experimental condition. Nonetheless, numerous studies demonstrated a superiority of the right eye (left hemisphere) in various discrimination tasks (Manns and Güntürkün, 2009), which could account for the higher ZENK expression in the left DL.

In conclusion, our findings provide further evidence for the olfactory navigational hypothesis. The olfactory system seems to provide the neuronal substrate for navigation over an unfamiliar location in a lateralised pattern. Even though we could not replicate the functional lateralization found in behavioural studies at the neuronal level, we were able to provide evidence that the left and right hemispheres contribute differently to the navigation process. Moreover, we showed that the navigation over familiar and non-familiar locations is processed at least in part by two different neuronal systems.

Chapter 4

Study III: Adult neurogenesis in the olfactory system of pigeons (*Columba livia*)

Adult neurogenesis in the olfactory system of pigeons (*Columba livia*)

Introduction

Adult neurogenesis is a widely spread phenomenon common from reptiles to humans (Gould, 2007; Kaslin et al., 2008). In mammals, the generation of new neurons is restricted to the subgranular zone of the hippocampal dentate gyrus and the subventricular zone (SVZ). From the SVZ, neuroblasts migrate along the rostral migratory stream to the olfactory bulb (OB), where they differentiate into two types of interneurons, either granule or periglomerular cells (rev. Gould, 2007). The newly generated OB neurons reveal a plastic mechanism contributing to the perceptual and memory functions performed by the bulb. Accordingly, new granule cells preferentially respond to new odours (Magavi et al., 2005), and olfactory enrichment increases the survival of new cells in the OB leading to improvements of olfactory memory (Rochefort et al., 2002), whereas impaired olfactory discrimination is proposed to be linked to low neurogenesis rates (Gheusi et al., 2000; Enwere et al., 2004).

In birds, adult newborn neurons are scattered throughout the entire telencephalon, where the ventral and dorsal edges of the lateral wall display the highest density of proliferating cells (Gahr et al., 2002). However, there are areas of enhanced proliferation activity, which are related to species-specific behaviour. The hippocampus of adult food storing birds shows seasonal neurogenesis corresponding to storing behaviour (Lee et al., 1998). Adult songbirds show neurogenesis in brain nuclei involved in seasonal singing (Gahr et al., 2002). Both networks are crucial for memory formation. Thus, it is assumed that the newborn cells are a key feature of new memory formation.

However, since the olfactory system of birds has traditionally been considered a sense of minor importance (Roper, 1999), the existence of adult neurogenesis in the olfactory bulb of birds had been completely neglected (Kaslin et al., 2007). The pioneer works of Papi and colleagues on the navigation of homing pigeons (1971, 1972, 1990) demonstrated the important role of olfaction in pigeons. Manipulation to the olfactory system, like ablation of the olfactory nerve (Papi et al., 1971), anaesthesia of the olfactory

mucosa (Ioalé P., 1983), lesion of the piriform cortex (Papi and Casini, 1990), and plugging the nostrils (Gagliaro et al., 2007), resulted in a severe impairment of the initial orientation and homing performance (for rev., see Wallraff 2005). This has led to the assumption that the olfactory sense must be strongly involved in the navigation performance of pigeons. In addition, the olfactory bulbs (OBs) of homing pigeons are enlarged compared to non-homing breeds, possibly representing a functional adaptation to olfactory-guided homing behaviour (Rehkamper et al., 1988;2008). Such an increased demand on perception and memory associated with olfactory-guided homing behaviour indicates that neurogenesis also takes place in the OBs of pigeons. Moreover, olfactory deprivation experiments suggest a differential role of the left and right OB in olfactory-guided behaviour with a dominance of the right OB (Gagliardo et al., 2007), which is probably supported by different capabilities of brain plasticity in the two hemispheres. A differential number of newborn cell types may reflect neuronal mechanisms mediating differential olfactory processing. In the present study, we investigated whether there is any neurogenesis in the OB of adult pigeons, which cell types are newly generated, and if there are left-right differences in the number of newborn cells.

Methods

Animals

Twenty-four one-year old pigeons received interperitoneal injections of Bromdeoxyuridine (BrdU, Sigma, dissolved in saline solution 0.9; 100 mg x 3 days / 1kg bodyweight). BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells during the S-phase of the cell cycle. Immunohistochemical detection of BrdU can be used to estimate the rate of newborn cells. The complex process of neurogenesis includes proliferation, differentiation and survival or cell death of newborn cells. Therefore, eight pigeons were perfused either after 14, 28 or 56 days, which ensures an overview of the entire neurogenesis processes in the OB of adult pigeons.

Fixation

The animals were first injected with 1.000 IU heparin. Fifteen minutes later, they were deeply anaesthetised with equithesin (0.45ml/100g bodyweight) and perfused through the left ventricle with 0.9% saline (40°C), followed by 4% paraformaldehyde in 0.12M

PBS (4°C, pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde + 30% sucrose for 2h at 4°C and cryoprotected in 0.12M PBS +30% sucrose at 4°C for 24h. The brains were embedded in 30% Gelatine in 4% paraformaldehyde + 30% sucrose over night at 4°C and stored in 0.12M PBS + 30% sucrose. The embedded brains were cryosectioned in the frontal plane (40µm). The left or the right brain side was marked by a hole stuck with a small needle. Slices were collected in five parallel series for the OB and ten parallel series for the rest of the brain and stored in 0.12M PBS, which contained 0.1% sodium azide at 4°C until they were subjected to immunohistochemistry. The experiments were carried out according to the specifications of the German law for the prevention of cruelty to animals.

Immunohistochemistry

The immunohistochemical detections were performed with the following antibodies: monoclonal mouse anti-BrdU (Roche, Germany; 1/100), polyclonal goat anti-doublecortin (DCX, Santa Cruz Biotechnology; 1/100) as a marker for proliferating cells, polyclonal rabbit anti-Calbindin (Swant, Switzerland; 1/1000) as a marker for mature OB granule cells, anti-tyrosin hydroxylase (TH, Chemicon, Germany; 1/1000) as a marker for matured periglomerular cells in the OB.

Free-floating sections were processed according to the ABC-technique (Hellmann and Güntürkün, 2001). All steps were performed on a shaker table at room temperature unless specified otherwise. After each incubation step, the slices were washed three times for 10 min with PBS. Endogenous peroxidases were blocked with 0.3% H₂O₂ in deionised water for 30 min. In case of the BrdU staining, the slices were incubated with 2M HCl at 37 °C for 60 minutes, following a two times 5 minutes washing step in 0.1M Borate buffer (pH 8.4). Slices were incubated with 10% normal serum (Vectastain Elite kit, Vector, Burlingame, CA) from the host of the secondary antibody in 0.12M PBS+0.3% Triton X-100 (PBS-X) for 1h to block non-specific binding-sites in the tissue. Then the slices were incubated with primary antibody solution for 48h at 4°C. The secondary antibody reaction was performed for 1h at room temperature (1/200 in PBS-X; Vectastain Elite kit, Vector, Burlingame, CA). Afterwards, the sections were incubated in an avidin-biotin-peroxidase solution (Vectastain ABC-Elite kit, 1/100 in PBS-X). Peroxidase-activity was detected using a heavy metal intensified 3'3-diaminobenzidine (DAB, Sigma) reaction, modified by

the use of 1% β D-glucose/glucose-oxidase (Sigma; Hellmann and Güntürkün, 2001). The sections were mounted on gelatinized slides, then dehydrated and coverslipped with Permount (Fisher Scientific, New Jersey, USA). Corresponding serial sets were stained with cresyl violet.

Double-labelling was performed by using three representative sections from one serial of the OB. BrdU-immunopositive cells were identified by the primary rat anti-BrdU (1/100, Abcam) detected by rabbit anti-rat Fluorescein-conjugated secondary antibody (1/100, Molecular Probes). NeuN and Calbindin were detected by a biotinylated anti-mouse or anti-rabbit secondary antibody (1/100, Vector) detected by Alexa 594-Streptavidin (1/1000, Invitrogen).

Cell counting and data analysis

The number of BrdU positive (BrdU⁺) cells was counted in the ipsi- and contralateral olfactory bulbs (OB) in every fifth section with 20 x 1.6 magnification at a Leica DML microscope (Leica Microsystems, Wetzlar, Germany). The quotient of counted cells divided by the number of analyzed sections was used as a measure for cell quantity in each preparation. Moreover, we estimated the area of the OBs with the image analyzing system analySIS 3.0 (SIS) and calculated the volume and density of labelled cells.

Statistical analysis was performed with the statistic program Statistica (StatSoft, Tulsa, OK, USA). For the photographic documentation a digital camera-system (Zeiss Axiocam; Zeiss, Jena, Germany) was used that was attached to the microscope. Images were processed with Zeiss Axiovision 3.0. Colour balance, contrast, and brightness levels were adjusted with Photoshop 5.5 software (Adobe, Germany).

Results

As in mammals, the olfactory bulb of pigeons is a seven layer structure consisting of the following layers from the outside moving in: (1) the olfactory nerve layer (ONL), (2) glomerular layer (GL), (3) external plexiform layer (EPL), (4) mitral cell layer (MCL), (5) internal plexiform layer (IPL), (6) granule cell layer (GCL), and (7) periventricular layer

(PVL), which bends around the lateral ventricle. In pigeons, the lateral ventricles extend into the OBs (Kosaka and Kosaka, 2009; Rieke and Wenzel, 1978) (Fig. 1).

An immunohistochemical characterization of bulbar cell types demonstrated a lamination that closely resembles the mammalian one. From studies with rodents, it is known that the inhibitory interneurons in the granular cell layer and the periglomerular neurons in the glomerular layer are the only two cell types in the OB, which are newly generated at a permanent rate (Lledo et al., 2006). Immunohistochemical staining of Calbindin and TH verified that these two cell types are also present in the OB of pigeons (Fig. 1).

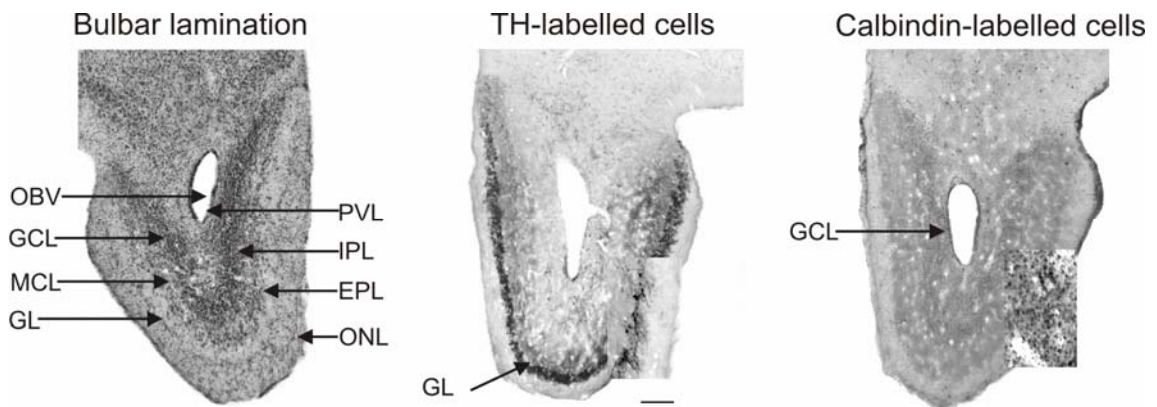


Figure 1: Lamination of the OB of pigeons: the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer, mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL), and periventricular layer (PVL), which bends around the lateral ventricle (OBV). Immunohistochemical staining of Calbindin cells in the GCL and TH cell in the GL. scale bar = 200µm

BrdU⁺ cells in the OB

Two weeks after the BrdU-injection, BrdU⁺ cells were mainly restricted to the PVL (Fig. 2). Only a very small number of BrdU⁺ cells could also be detected in the GCL (Fig. 2). This pattern was consistent with the Doublecortin (DCX)-immunolabelling. DCX labelled cells were found in the PVL and GCL; however, the whole OB was permeated with DCX-positive fibres. After four weeks, more BrdU⁺ cells were seen in the PVL and a substantial number of BrdU⁺ cells were present in the GCL. Only after eight weeks of BrdU-injection, BrdU⁺ cells could also be observed in the GL. Moreover the number of newly born cells increased mainly outside the PVL.

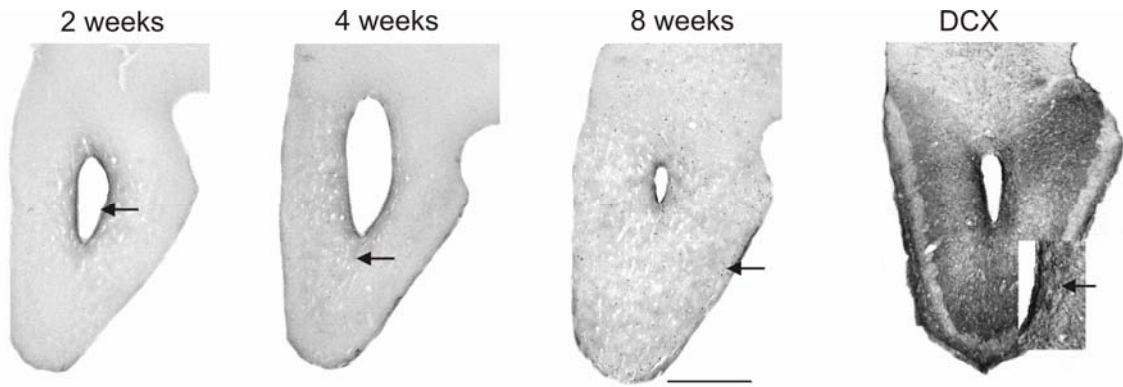


Figure 2: BrdU staining of the OB after two, four and eight weeks. DCX of the OB. Scale bar = 500 μ m

The number of BrdU⁺ cells doubled between the second (BrdU⁺ cells: 19.50 \pm 6.67) and fourth week (BrdU⁺ cells: 41.00 \pm 23.26) after BrdU injection. After eight weeks of survival time, a dramatic increase of nearly 20 times of BrdU⁺ cells was observed (BrdU⁺ cells: 769.69 \pm 530.03) in the OB. The difference in the number of newly born cells did not reach significance between the right and left OB; however there was a strong trend towards more generated cells in the right OB compared to the left OB after 4 weeks of survival time (2 weeks BrdU⁺ cells: right 20.50 \pm 7.25, left 18.50 \pm 6.35, Wilcoxon Matched Pairs Test $z = 0.93$ $p = 0.35$; 4 weeks BrdU⁺ cells: right 43.87 \pm 23.50, left 38.12 \pm 24.26, Wilcoxon Matched Pairs Test $z = 1.94$ $p = 0.052$; 8 weeks BrdU⁺ cells: right 816.72 \pm 601.78, left 722.66 \pm 484.57, Wilcoxon Matched Pairs Test $z = 0.70$ $p = 0.48$; Fig. 3)

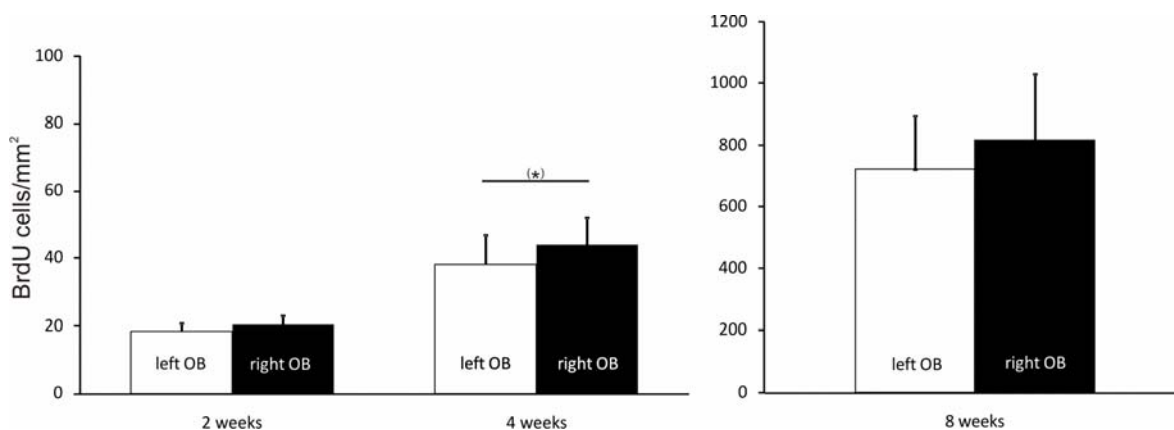


Figure 3: Density of BrdU labelled cells in the left and right OB after two, four and eight weeks, (*) $p=0.052$.

Double staining of BrdU⁺ cells

Since BrdU does not only accumulate in newly born neurons but also in newly generated glia cells, the cell type of the newborn neurons was verified by neuronal markers. No double-labelling with neuronal markers could be observed after 2 weeks in the OB, revealing that at this stage, the neurons are still not differentiated to functional granular cells. The BrdU-Calbindin positive cells were first detected in the GL after 4 weeks and could also be observed after 8 weeks of survival time (Fig. 4). BrdU-NeuN double-labelled neurons were found after 8 weeks in the GL and GCL, revealing that newborn periglomerular cells have a prolonged differentiation period compared to the granular cells (Fig. 4).

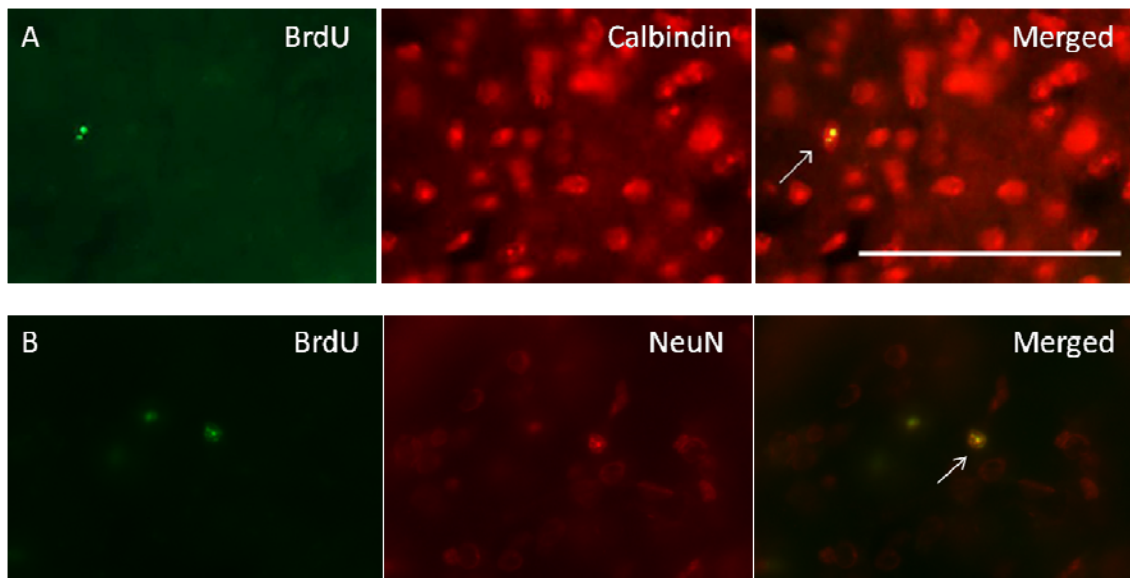


Figure 4: (A) Double staining showing a BrdU– Calbindin colabelled granular cell; (B) Double staining showing a BrdU– NeuN colabelled periglomerular cell; Scale bar A= 50 μ m

BrdU⁺ cells in the telencephalon

Emanating from the ventricle of the OB, a continuum of BrdU⁺ cells was detected to be spread out into the hyperpallium. Furthermore, BrdU⁺ cells were observed along the lateral wall of the lateral ventricle. Two main spots of newborn cells were found in the dorsolateral and ventral end of the lateral ventricle (Fig. 5). From the dorsolateral edge of the ventricle, a band of BrdU⁺ cells spread out along the dorsolateral telencephalon. This band was also visible in the DCX - immunolabelling.

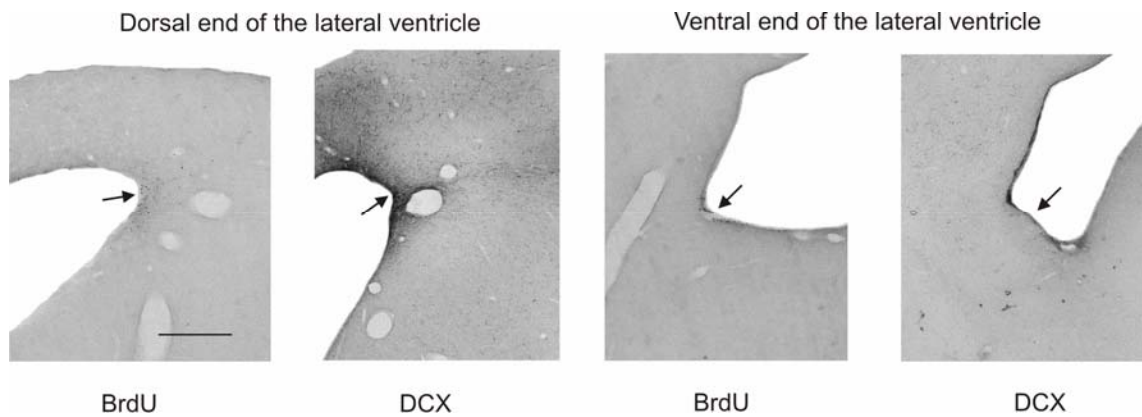


Figure 5: *BrdU* and the corresponding *DCX* staining of the dorsal and ventral end of the lateral ventricle. Scale bar = 500 μ m

DCX-positive cells were also found in the lateral V-shaped layer, which is thought to be the analogue of the dentate gyrus of the hippocampus of mammals (Fig. 5, Atoji and Wild 2004). However, only very few newborn cells were present in the entire hippocampus. *BrdU*⁺ cells were scattered throughout the whole telencephalon without any clustering, including the, hyperpallium (H), mesopallium (M), nipodallium (ND) and striatum (St), with a slightly higher cell density in the H and ND.

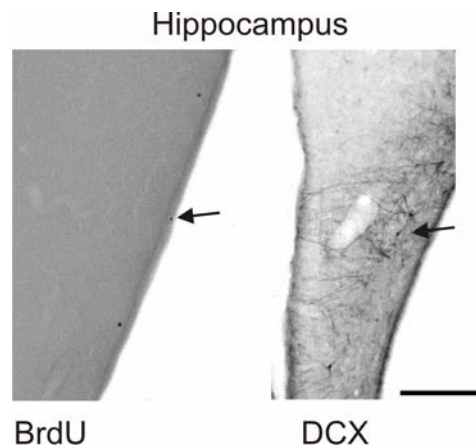


Figure 5: *BrdU* and the corresponding *DCX* staining of the Hippocampus. Scale bar = 200 μ m

Discussion

This study, verified for the first time adult neurogenesis to occur in the olfactory bulb of adult pigeons, a process, which might be related to olfactory-guided navigation. The presence of newborn neurons in the GL and GCL very strikingly resemble those cell types found in the mammalian OB.

Adult neurogenesis in the OB of pigeons

As the immunohistochemical studies verified, the OB of pigeons have a similar neuroanatomical organization to the one in mammals. Immunohistochemical staining revealed, in principal, the same layers and layer-specific cell types with calbindin positive granular cells in the GCL and TH-positive periglomerular cells in the GL. However, unlike in mammals, the lateral ventricle in pigeons projects into the OB. Therefore, newborn cells do not appear to migrate from the SVZ along the RMS into the OB. It rather seems that they are directly generated in the area of the ventricle reaching into the OB (OBV), from where the cells directly migrate radially into the respective OB layers. This line of thought finds support by the presence of BrdU⁺ and DCX immunolabelled cells around the OBV. The presence of BrdU⁺ cells within the GCL and GL of the OB may be due to the generation of the same cell types as found in the mammalian OB. This was verified with double labelling of cell-specific neuronal markers in this study. Double labelling of BrdU and Calretinin in the GCL provide evidence for the generation of granular cells, whereas double labelling of BrdU and NeuN displayed newly build periglomerular cells. However, the maturation process of both cell types seems to be prolonged compared to that in mammals. Rodent studies showed that granular cells become fully functional morphologically within 2 weeks after their birth (Petreanu and Alvarez-Buylla, 2002), whereas the development of periglomerular cells takes 4 weeks (Belluzzi et al., 2003). In the present study, the first newly generated granular cells were seen after 4 weeks, and periglomerular cells could be observed after 8 weeks after injection. Interestingly, 8 weeks of survival time was also the period after which the 20fold increase of newly born cells occurred. In summary, the OB of pigeons revealed the same pattern of neurogenesis as the mammalian one with some prolonged differentiation time compared to the mammalian neurogenesis pattern.

In general, mitral cells mediate the transmission of olfactory information to higher brain areas. These cells simultaneously activate inhibitory granular cells that in turn inhibit

the mitral cells via dendro-dendritic connections. This lateral inhibition by the granular cells synchronises the firing of the mitral cells and is therefore thought to play a key role in olfactory coding. Newly generated granular cells in the OB are very sensitive to new odours (Magavi et al., 2005). This greater responsiveness was identified to be stimulus and experience dependent. Furthermore it was proposed that newborn neurons optimize the functionality of the OB, enabling it to process sensory information of new and complex stimuli, and they may contribute to perceptual and memory functions of the OB (Gheusi and Lledo, 2007). Therefore, it would not be surprising that animals, like homing pigeons, which use their olfactory system to navigate, indeed revealed neurogenesis in the OB. The number of newborn cells differed slightly between the left and right OB. This difference may cause the differential role of the left and right OB in navigational processing. The greatest difference between the right and left OB was detected after 4 weeks of survival time. This was also the point in time when the granular cells matured. Such asymmetrical generation of newborn cells indicates that neurogenesis in the pigeon's olfactory system is probably involved in neuronal processes mediating olfactory-guided navigation presumably in a lateralized manner.

One restricting factor of this study was that pigeons were kept in single cages in a closed housing without any olfactory stimulation. It would be interesting to examine if another pattern in differentiating time, number and asymmetry of newborn neurons could be observed if the pigeons were kept in an open aviary where they would be allowed to fly around freely.

Adult neurogenesis in the telencephalon of pigeons

Similar to mammals, two hot spots of cell proliferation were found at the dorsal and ventral edges of the lateral ventricle. The DXC immunolabelling verified that these two areas are potential hot spots for the generation of new neurons. A higher density of newborn cells in these areas was previously approved in ring doves (Ling et al., 1997). In contrast to the OB, only very few BrdU⁺ cells were observed in the hippocampus. Again, this supports the notion that the new cells in the OB may be specifically involved in processes mediating olfactory-guided navigation. Unlike in songbirds, where increased neurogenesis is pronounced in some nuclei involved in vocal control, vocal learning, and singing (Gahr et al., 2002), no clustering of newborn neurons was found in the present study. But the hyperpallium and the caudal nidopallium were observed to have a higher

density of newborn neurons. This finding is consistent with the results of ring dove studies (Ling et al., 1997), which claim that the increased neurogenesis rate in these brain areas may be due to the development of environmental control and reproductive function.

In conclusion, the present study not only revealed the known areas of adult neurogenesis in birds, but also indicated a neurogenesis pattern strikingly similar to that of mammals in the OB of homing pigeons. The tendency that more newly generated cells were found in the right OB, might be due to the greater sensory demand of the right side, which are probably specifically required by the complexity of olfactory-guided navigation performance in birds.

Chapter 5

General Discussion

General Discussion

The aim of this thesis was to investigate the neuronal substrate possibly underlying the behavioural data on olfactory-guided navigation in homing pigeons. In the following, the main results of the studies are briefly summarized and the implications of the findings are discussed.

5.1 Summary of the results:

The first study was conducted to visualize any functional lateralization of the olfactory system with a predicted dominance of the right OB and left Cpi, which could be based on an asymmetrical projection. The results indicate that this is not the case, but the left olfactory system seems to possess a more closely connected circuitry compared to the right one.

With the help of the immediate early gene technique in the second study, it was possible for the first time to show that the olfactory system is actively involved in the process of olfactory-goal navigation. Pigeons, which had to actively navigate from a non-familiar location, revealed the highest ZENK expression in the olfactory system compared to the control groups, although one of the control groups had the same olfactory experience at the release site. The second control group was released in visible distance of the loft and hence did not have to use their olfactory map to find their way back, but had the experience of flying and handling procedures during release, as the experimental group did. This study lends strong support for the olfactory navigation map hypothesis.

The intention of the last study was to visualise neurogenesis in the OB of pigeons. With the BrdU-technique, it was possible to reveal that the OB of pigeons is similar to the mammalian one with respect to its organization. The study further showed neurogenesis to occur in the same cell types as in mammalian OB, thus providing an important tool for olfactory learning.

In summary, pigeons actively use their olfactory system to navigate from an unfamiliar release site to their home loft. The functional lateralization of the olfactory system with a dominance of the right OB and left Cpi was not found to be based on an asymmetrical projection pattern and did not reveal asymmetrical activation during homing. However, the functional lateralization may still be modulated through interhemispheric

Cpi-Cpi connection. Also, the asymmetrical generation of new cells in the OB strongly argues for an asymmetrical processing of the olfactory information.

5.2 Involvement of the olfactory system during navigation

The important role of the olfactory system in pigeons during homing was examined with different types of experiments, from nasal anaesthesia to ablation of olfactory brain areas. It is widely accepted that olfactory cues play a key role in homing over non-familiar areas in pigeons (Abel, 2001; Wallraff 2005). The use of olfactory cues is not limited to pigeons. Other species have also been shown to use this sense for navigation, although the exact conditions yet need to be identified. Swifts (*Apus apus*) and European starlings (*Stutnus vulgaris*) have been identified to use olfactory cues to find their way back to their nesting sites.

In a study (Fiaschi et al., 1974), swifts were displaced from their nesting colony 47-66km away. Before the release, these swifts received unilateral olfactory nerve sections, and the nostrils, either ipsilateral or contralateral to the nerve section, were plugged. Only three out of 23 birds, which were bilaterally deprived of olfactory input, returned back to their colony, whereas 15 out of 20 birds, which could still smell with one nostril, homed successfully. European starlings showed reduced homing ability after bilateral olfactory nerve section when released more than 100km away from their nests (Wallraff et al., 1995). Moreover procellariiform seabirds (e.g., petrels, albatrosses and shearwaters) return back to their breeding islands, thereby travelling great distances often at nights, over foggy and featureless oceans. Nocturnal petrels use olfactory cues to home to their burrows (Bonadonna et al., 2001; Bonadonna and Bretagnolle, 2002). However, it is still unclear whether they use olfactory cues to navigate during their foraging flights.

In addition to birds, fish were also found to use olfactory cues to navigate home. Many species of salmon (*Oncorhynchus spp.*) use odours to travel over hundreds of kilometres to their natal river. For example, Pacific salmon born in fresh water move to the ocean during adulthood. Before dying, they return to the stream where they were born in order to breed. The mechanism that enables them to find their way back to their specific home river is still unknown, but olfaction has been shown to play an essential role in freshwater homing (DeBose and Nevitt, 2008).

Nevertheless, a recent study claims that olfactory sensation may only be important for the activation of the non-olfactory path integration system (Jorge et al., 2009). In the study, young inexperienced pigeons were transported to the release site under three different conditions. The first group of pigeons could smell the natural environmental air, the second group could smell synthetic air, which contained no natural odour, and the third group was exposed to novel odours. When released from a distance of 8km away from the loft, pigeons, which were exposed to novel odours, behaved similar to the controls in their initial orientation. In contrast, birds, which were odour deprived during the transportation, were randomly scattered in their initial orientation. The authors concluded that a new odour environment activates the acquisition of non-olfactory directional information in pigeons during displacement. Hence the olfactory system itself does not provide any navigational information (Jorge et al., 2009). However, this effect disappeared when experienced pigeons were released from a distance of 24km. Following the authors' explanation, pigeons acquire a site-based map during training flights and thus do not have to rely on route integration. Consequently, exposure to different odours would not influence their initial orientation.

In the second experiment, we analyzed the activation of the olfactory system during homing. We used inexperienced birds, which have never homed before. In accordance to Jorge et al., (2009), we observed activation of the olfactory system, which can be attributed to path integration during the displacement. However, this should also be the case in birds, which were transported to the release site but not released. Since both groups were transported together, they should reveal the same activation pattern of the olfactory system, given that it is not used for the navigation step itself. However, only the released pigeons showed a significant activation of the olfactory system, implying that the olfactory system is not used for activation of path integration per se. The increased activation of the olfactory system in all birds is probably not due to the handling during release, because pigeons released at home, experienced the same handling experience and revealed no significant activation of the olfactory system. This further lends support to the notion that the activation of the olfactory system of released birds must be due to active navigation by using olfactory information. However, pigeons, which were transported to the release site and were not released, revealed significantly higher activation of the Cpi compared to the group, which was released at home. This can be explained by the observation that pigeons can already orientate on the ground before taking off, a process, which requires the processing of olfactory information (Gagliardo et al., 2001c). In this experiment they

examined the directional choice of anosmic and control pigeons leaving a circular arena. Anosmic pigeons left the arena randomly, while control pigeons chose their approximate home direction. This study demonstrated that pigeons use olfactory information to determine the direction of displacement before taking off.

Moreover, rearing experiments demonstrated that pigeons, which were kept in a wind shielded loft during their first three months of life, showed poor homing performance, presumably because they were prevented from acquiring an olfactory navigation map (Gagliardo et al., 2001a). Such a dramatic effect of their navigation ability would not be expected if pigeons would only use a path integration system that would be activated by new odours during displacement. In conclusion, pigeons and maybe other animals, very likely use their olfactory system to attain navigational information.

5.3 Neuronal basis of olfactory map

The neuronal system for landmark-based navigation has been extensively investigated in a variety of studies. In rodent studies, the hippocampus was discovered to be the neuronal substrate of spatial cognition, especially in the representation of familiar landmarks, forming a so-called cognitive map (O'Keefe and Nadel, 1978; Best et al., 2001). The avian homologue structure, the hippocampal formation (HF), was found to fulfil comparable functions (Colombo and Broadbent, 2000). Lesions of the hippocampus prevent young pigeons from learning to navigate in the direct vicinity of the loft (Strasser et al., 1998), indicating that the pigeons cannot acquire a landmark-based representation important for piloting navigation behaviour.

However, the landmark-based site-specific compass navigation appears to remain unaffected as tested in clock shift experiments (Gagliardo et al., 1999). The HF seems to play a key role in landmark-based navigation only when the landmarks are presented in a map-like way (Bingman et al 2006). It is therefore not surprising that pigeons homing over a familiar area exhibit an activation of the HF similar to that previously verified by Shimizu et al., (2004). This is also consistent with the findings of our second study where both released groups showed HF activation comparable to the group that was not allowed to fly. These results further support the assumption that pigeons rely on the HF-based landmark map to find their way home in the immediate vicinity of their loft.

However, less is known about the neuronal system of the olfactory navigation map. Lesions of the hippocampus do not interrupt the initial orientation at an unfamiliar release site (Bingman et al., 1989), indicating that the hippocampus is not essential for the olfactory navigation map mechanism. One possible candidate for processing olfactory information used for olfaction-based navigation is the Cpi. The Cpi receives bilateral input from the olfactory bulb and projects into various brain areas (Bingman et al., 1994, Study I). Accordingly, ablations of the Cpi severely decrease the initial orientation as well as homing performance when the pigeons have to home from an unfamiliar site. In contrast, homing from a familiar location remained unaffected (Papi and Casini, 1990). The results of our second study confirmed that the activation of the Cpi was only found when the pigeons had to home from a non-familiar location, but not if they were released in visible distance to the loft using the piloting navigation. The Cpi is also critical for the olfactory map acquisition. Pigeons reared with an ablated Cpi did not learn an olfactory navigation map (Gagliardo et al., 1997). These results suggest that the pigeons' Cpi plays a crucial role in formation and retrieval of the olfactory navigation map.

This is further supported by studies in humans and rodents, where it has been proposed that the Cpi not only serves as a sensory relay station but is also involved in learning and memory. This leads to assume an association of odour stimuli with the formation of memory traces of previously experienced events (Royet and Plailly, 2004). This has been verified by a variety of studies. For example, odour learning induced an increase in L1 expression at an early training stage in the rat piriform cortex. L1 is a cell adhesion molecule involved in the formation of neural circuits and synaptic plasticity. Hence, it can be used as a marker of memory formation (Knafo et al., 2005). Activity dependent plasticity in the Cpi was found in several studies. Long-term potentiation in the rat Cpi *in vitro* (Jung et al., 1990; Jung and Larson, 1994) and *in vivo* ((Roman et al., 1993; Litaudon et al., 1997) was also demonstrated to occur after olfactory training. Similar results were obtained in human PET studies. Only after the recognition of odours, an activation of the Cpi was observed, although not during encoding (Dade et al., 2002). These results provide further evidence that the Cpi probably encodes the olfactory map in pigeons.

5.4 Functional and structural lateralization of the olfactory system

Like the visual system, the olfactory system of pigeons seems to be lateralized. Pigeons released with the right nostril occluded were severely impaired in their initial orientation when released from an unfamiliar area (Gagliardo et al. 2005). Such a dominance of the right olfactory system was also found in bees, chicks and to some extent in humans. Bees are able to form olfactory memories of the scents of flowers from which they gained their nectar. To obtain food, bees have to extend their proboscis. They can learn to associate a scent with a sugar reward, and upon learning bees extend their proboscis in anticipation of a food reward after receiving a scent stimuli. Bees are better in scent associative learning when using their right antenna, but obtain poor results when they learn the same task with the left antenna (Letzkus et al., 2006; Rogers and Vallortigara, 2008). Similar results were obtained in chicks. Chicks were reared in the presence of a small cylinder in their cage containing the odour of clove oil. At three days of age, either the left nostril or the right nostril was occluded, and the chicks were placed in a runway with two cylinders at each end, similar to that of their home cage. One of the cylinders contained the familiar clove oil scent the other was odourfree. Chicks, which could only use their right nostril, chose the cylinder that smelled like their rearing object. Chicks, which could smell with the left nostril, chose the cylinders randomly without any preference. This leads to the assumption that chicks learn and prefer the odour of their rearing objects, but only if they receive the scent through the right nostril (Vallortigara and Adrew 1994). In another experiment, it was tested whether one-day-old chicks exhibited a lateralized olfactory response to the scents eugenol and iso-amylacetate. Chicks smelling with the right nostril responded stronger to 100% eugenol than with the left nostril. However, no lateralization of the olfactory response to 100% iso-amylacetate was found. These results are consistent with the observations of Vallortigara and Andrew (1994), who found that the perception of olfactory cues is lateralized, although not to all odours. Interestingly and unlike the lateralization of the visual system, the olfactory response lateralization appears to be independent of the epigenetic light triggering (Rogers et al., 1998).

In human studies, the results with respect to the dominance of the right nostril are not consistent. Some studies demonstrated a clear advantage of the right nostril over the left nostril in odour discrimination tasks (Zatorre and Jones-Gotman, 1990) and in the sensitivity of odour detection (Thuerauf et al., 2008). Another study demonstrated that the

odour perception itself is not lateralized but dependent on the airflow rate of each nostril (Sobel et al., 1999).

Taken together, a large body of experiments provide evidence that odour information collected by the right nostril plays an important role in odour perception and discrimination. The question arises whether this superiority of the right nostril is based on processes within the olfactory chamber, or if it is due to the lateralized neuronal processing of the olfactory information, for example in the OB or Cpi. In the second experiment, we could not reveal any left-right differences in OB activation. Further, the sensitivity to olfactory deprivation resulted in a similar down regulation of the OB activity. These results indicate that it is not probable for the functional dominance of the right nostril to be caused by the processing of olfactory information in the OB; instead it may be a result of asymmetrical processes, either in the olfactory epithelium or in higher olfactory brain areas, like the Cpi. Analysis of the olfactory receptor cells (ORC) in the antennae of bees revealed more ORC in the dominant right antenna than in the left one (Letzkus et al., 2006). These findings suggest that the sensitivity of olfactory perception is may be proportional to the number of ORC, and hence would lead to a dominance of the right antenna. In conclusion, the functional dominance of the right nostril during the initial orientation in pigeons might be based on a higher sensitivity of the right olfactory epithelium due to more ORC, although this remains to be clarified.

In addition to the olfactory bulb, the Cpi also shows a functional dominance in which the left Cpi has been verified to play a crucial role in the initial orientation step during navigation (Gagliardo et al., 2005). In the first study, we examined if this functional lateralization of the Cpi could be based on stronger bilateral innervation from the OB. However, this track tracing study did not reveal any asymmetrical innervations of the Cpi from the OB, although the dominance of the left Cpi might be a result of interhemispheric modulation by the contralateral Cpi, since both are reciprocally connected (Bingman et al., 1994). This is supported by the finding according to which the number of afferent cells from the contralateral Cpi and the OB were significantly correlated after tracer injection into the left Cpi, but not after injection into the right Cpi. Such a strongly connected circuitry of the left Cpi compared to the right one, lead us to conclude that the left Cpi is stronger modulated through the right Cpi than vice versa. This is also nicely paralleled by the results from the second study. Only the released pigeons with the right nostril occluded revealed a down regulation in Cpi activity, but this down regulation was not detected after

occluding the left nostril. This is probably due to a strong additional input from the right Cpi, which stabilizes the activity of the left Cpi and therefore counteracts the down-regulation of ZENK expression after left nostril occlusion.

Such an interhemispheric modulation is well known from the visual system (Manns and Güntürkün, 2009). The optic tecta are reciprocally connected by the inhibitory intertectal commissure. This connection shows a functional lateralization with a stronger inhibitory modulation of the right tectum by the left one (Keysers et al., 2000). Hence, the activation of the left hemisphere results in a stronger inhibition of the right hemisphere than vice versa. Transection of the intertectal commissure results in the reversal of behavioural asymmetries (Güntürkün and Böhringer, 1987).

Nevertheless, not much is known about the interhemispheric modulation of the Cpi-Cpi connection. One study examined the influence of the transection of the anterior commissure, which mediates the interhemispheric Cpi-Cpi connection, and focused on the acquisition of the olfactory map. When pigeons are reared in a cage where the natural wind directions are deflected either clockwise (CW) or counter-clockwise (CCW), they revealed a corresponding deflection in the initial orientation, indicating that they had learned a false olfactory map corresponding to the deflected wind directions. Foa et al. (1986) sectioned the anterior commissure to prevent interhemispheric transfer. Afterwards, the birds were kept alternately with either the right nostril occluded in a CW cage or with the left nostril plugged in a CCW cage. Every third day, the experimental conditions were changed over the duration of 69 days. Twenty-three pigeons out of 28 revealed CW deflections when released with the right nostril occluded and CCW deflections when released with the left nostril occluded. These results demonstrate that pigeons can learn two different olfactory maps, one with each hemisphere. Unfortunately, the authors did not test pigeons without any nostril occlusion. It would be interesting to know what happens in such a conflict situation. Presumably, one hemisphere might take over the dominance. In the control condition, pigeons were reared in a normal loft with either a transected anterior commissure (AC⁻) or an intact one (AC⁺). The pigeons were released with one nostril plugged. The AC⁺ pigeons were not affected in the initial orientation and homing performance. Interestingly the experiment with the AC⁻ birds was performed twice with two different sets of pigeons. In the first set, the left and right AC⁻ pigeons revealed different initial orientation, bearing and slower homing than the AC⁺ pigeons. The authors concluded that this is probably due to an asymmetry of lesions produced by the surgery.

Therefore, they performed a slightly different transection of the commissure in a second set of pigeons, which did not show any differences in navigation performance compared to the AC⁺ birds. Nevertheless, the difference in the initial orientation in the first set of AC⁺ pigeons could be a result of different processing of the olfactory information in the two hemispheres, although this still does not explain the results of the second set of AC⁺ pigeons. What should also not be forgotten at this point is that both Cpis, regardless of the occluded nostril, receive olfactory input from the contralateral OB.

In summary, we conclude that the left Cpi is functionally dominant. However, this is probably not based on a stronger bilateral innervation from the OB, but may rather be a result of an interhemispheric Cpi-Cpi interaction.

As a side note, the Cpi lesion data should be considered with caution. The higher level of disorientation after left Cpi ablation may also be caused by the damage of amygdaloidal nuclei in the vicinity, which would not be surprising, since the Cpi comprises a thin structure on the lateral surface of the telencephalon. The amygdaloidal nuclei are the origin of the occipitomesencephalic tract (OM), and left OM transections were found to induce a visuomotoric deficit (Güntürkün and Hoferichter, 1985). However, no visual deficits were found after right OM transection. The left Cpi lesion induced disorientation, which could primarily be caused by the damage of the OM projection origins and not by the Cpi itself. The visuomotoric deficit could also account for the significantly longer vanishing time of the left Cpi lesioned birds (Gagliardo et al., 2005). Taken together, all these findings still cannot explain why birds with lesions of their right Cpi, showed no impairment in their initial orientation.

5.5 Implication of neurogenesis in the olfactory bulb of homing pigeons

The continuous generation of newborn neurons in the olfactory bulb of mammals is widely accepted (Lledo et al., 2006). However, due to the assumption that the olfactory sense is less important, neurogenesis in the OB of birds has never been analyzed. In the third study, we showed for the first time that the OB of pigeons reveals neurogenesis comparable to that in mammals. Newly generated periglomerular and granular interneurons could be observed in the OB of pigeons, the same neuron types, which are also newly generated in the mammalian OB.

The OB is the first relay station of the olfactory pathway. The OB features a high level of plasticity in response to diverse olfactory experience, such as short-term exposure, enrichment and associative learning (Mandairon and Linster, 2009). Twenty minutes of exposure to a new odour can already modify the odour response pattern of the mitral cells, also defined as the output neurons of the OB (Buonviso and Chaput, 2000). Even more strikingly, the exposure towards an odour for less than one minute of an anaesthetized rat can already fine-tune the receptive field of these output neurons (Fletcher and Wilson, 2003)). Associative learning was also shown to modulate the dynamics of olfactory bulb odour response. For example, mother sheep develop a selective recognition memory of their newborn lamb, by increasing the number of mitral cells in the OB that respond to the specific lamb odour (Kendrick et al., 1992). The activity of the mitral cells in turn is modulated by lateral inhibition through granular cells. These cells are continuously replaced by newborn granular cells, leading to the assumption that the gradual integration of new neurons is important for plasticity of olfactory circuits. However, the function of the lifelong renewal of granular cells is still not fully understood. Do newborn cells in the adult brain constitute a specific population of neurons that replace each other and hence, play a critical role in olfactory learning and memory formation? Or do newborn neurons fulfil a more general role by just replacing older neurons?

In an attempt to answer the first question, it was hypothesized that the survival of newborn neurons in the OB is activity-dependent, given the assumption that newborn neurons can adjust to the tuning of odour-specific spatiotemporal pattern of bulbar activity to improve odour-discrimination (Cecchi et al., 2001). Consistent with this hypothesis, it was demonstrated that a low neurogenesis rate in the OB is accompanied by impairment of odour-discrimination (Gheusi et al., 2000; Enwere et al., 2004), and in the same vein, an

olfactory deprivation decreases the survival rate of newborn cells (Mandairon et al., 2006). On the other hand, an enriched environment enhances the survival of newborn cells (Rocheffort et al., 2002). Newborn neurons were also shown to preferably respond to new odours (Magavi et al., 2005).

According to a recent study, neurogenesis in the OB may only serve a critical role in tissue maintenance, similar to its role in other tissue like gut and skin. In this study, Imayoshi et al. (2008) suppressed neurogenesis and observed shrinkage of the granular cell layer, although the size of the hippocampus remained unaffected. Apparently, most old neurons of the deep regions are replaced by new neurons, whereas only about half of the population in the superficial regions of the OB is replaced. Hence, not all granule cells are replaced, and a subset of cells even persists throughout life, lending support to the assumption that these cells may regulate the olfactory long-term memory for smell. Following this line of argumentation, adult neurogenesis would not be necessary for the acquisition and long-term perpetuation of odour-associated memory. In their study, Imayoshi et al. (2008) showed that the spontaneous discrimination and innate olfactory preference between two different odours were not affected in mice when neurogenesis was suppressed. Furthermore, the trained mice learned to associate one of two odours with a sugar reward. Even after 6 months, the trained mice spent substantially more time near those odours that were associated with sugar rewards (Imayoshi et al., 2008). Nevertheless, the authors also pointed out that may not be the case for all kinds of odour memory formation, and, for instance, more difficult tasks about odour-associated memory could depend on neurogenesis.

A further question arises as to what role neurogenesis plays in the OB of pigeons. Does it simply supply cells for tissue replacement or does the neurogenesis enable pigeons to better respond to changes in the environment, which would make it an important tool for olfactory-guided navigation? The OB is the first station in the processing of olfactory information and therefore acts like a gateway for higher olfactory information processing. This means that all olfactory information first has to pass the OB, which represents a bottleneck of information processing of smell. As more newly generated cells were observed in the right OB, possibly due to a greater demand of the right nostril/OB in olfactory-guided navigation, neurogenesis probably does more than just ensuring tissue supply. Very likely, the asymmetrical generation of newborn cells supports the notion that neurogenesis in the pigeons' olfactory system indeed is involved in neuronal processes

mediating olfactory-guided navigation, presumably in a lateralized manner. This would mean that, aside from the function of tissue replacement, neurogenesis in the OB of pigeons is a key factor in comprehending the mechanism underlying olfactory navigation. Yet, more research is required to further investigate the role of neurogenesis in the OB of pigeon.

5.6 Summary and outlook on further research

The three studies conducted in the realm of this thesis examined the important role of the olfactory system in homing pigeons at the neuronal level, with the focus on olfactory-based navigation. I was able to demonstrate that the olfactory system of pigeons is activated on the neuronal level during navigation from an unfamiliar site. These results provide for the first time evidence on the neuronal level for the olfactory navigation hypothesis, which proposes that pigeons use olfactory cues to determine their direction of displacement at a remote non-familiar release location. I could also show that at the level of the Cpi the olfactory system reveals different sensitivity to olfactory cues. With these findings, together with the result of the track tracing study, where I verified the projection pattern of the olfactory system, I could further provide evidence that asymmetrical bottom-up effect shown in behavioural data is not directly linked to left-right differences in the amount of ascending OB projections. However, it is probably based on an interhemispheric modulation by the contralateral Cpi. Further, I was also able to show that the OB of pigeons exhibits the same neurogenesis pattern in pigeons as in mammals. Finally, I demonstrated that neurogenesis differs between the right and left OB, probably reflecting the higher demand of the right OB. Taken together, these studies strongly support the assumption that pigeons use the olfactory system for homing over unfamiliar areas. Nevertheless, some open questions remain to be answered, which will be discussed below.

Study I: Track tracing studies provide insight into the connectivity pattern. However, they cannot reveal any information about the kind of connection itself. Even though we did not demonstrate that the functional lateralization is not based on asymmetrical bottom-up connectivity, the modulation can still be asymmetrical. Therefore,

immunohistochemical characterisation as well as electrophysiological recordings of the Cpi could provide further insight in the Cpi-Cpi modulation.

Study II: Employing the ZENK technique to visualise the activation of the olfactory system during homing is accompanied by one big limitation of this technique, namely that it offers only a limited temporal resolution. Right nostril/OB and left Cpi are of great importance in the very first step of navigation, although no differences were detected in homing performance between the right or left site treated groups. In this case, the ZENK technique is probably good enough to visualise the activation of the olfactory system per se, but it cannot appropriately visualize interhemispheric differences. In order to test this assumption, a future study would need to investigate whether a stronger inhibition of the right tectum is accompanied by a difference in ZENK expression. This would verify whether the ZENK technique actually provides an optimal tool to conduct such kinds of analyses. Future electrophysiological experiments conducted during homing could provide a deeper understanding of the involvement of the olfactory system during navigation. Transection experiments of the interhemispheric connection in combination with behaviour release experiments could also help to understand the Cpi-Cpi modulation.

Study III: The third experiment was conducted with pigeons kept in single cages under laboratory conditions. One great advantage of this kind of housing is that it provides internal validity and controlled experimental conditions. However such a housing system has the limitation that it does not provide species-specific and hence deprived environment conditions. More natural housing conditions, for instance, an open aviary, where the pigeons are allowed free flights around it and thereby using their navigation skills, would provide further – externally valid - insight into the role of neurogenesis in the OB of pigeons. It is possible that with an increasing degree of natural housing conditions other patterns in differentiating time, number and asymmetry of newborn neurons could be observed.

Future studies aiming to analyse the role of neurogenesis in the olfactory bulb could, for example, relocate pigeons and thereby expose them to a new odour environment. Some studies demonstrated that pigeons can acquire a new map if they are kept in another loft for some time. Follow-up experiments might be able to investigate: (1) the neurogenesis rate

in the relocated birds compared to birds, which were not relocated, (2) by using the neurogenesis suppression technique the impact of neurogenesis in odour acquisition process could be verified.

As the odour environment is permanently changing with the seasons, pigeons are forced to react to these permanent changes, probably by means of neurogenesis. It would be interesting to examine if a suppression of neurogenesis could affect the olfactory navigation mechanism, possibly depending on differential preexposure to these kinds of environmental odour changes.

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List of abbreviations

°	Degrees
µl	microlitres
µm	micrometres
AC	Anterior Commissure
AC ⁻	transected Anterior Commissure
AC ⁺	intact Anterior Commissure
AD	dorsal Acropallium
AD	Anno Domini
AI	Asymmetry Index
BC	Before Christ
BDA	Biotinylated Dextran Amine
BNST	Bed nucleus of the Stria Terminalis
BrdU	Bromodeoxyuridine
C	Celsius
CCW	counter-clock-wise
CDL	Dorsolateral Corticoid Area
Cpi	Piriform Cortex
Cpp	Prepiriform Cortex
CtB	Choleratoxin subunit B
CW	clock-wise
DAB	3'3-diaminobenzidine
DCX	Doublecortin
DL	Dorsolateral part of the Hippocampus
DM	Dorsomedial part of the Hippocampus
DMA	nucleus of the Dorsomedial Anterior thalami
DMP	nucleus of the Dorsomedial Posterior thalami
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EPL	External Plexiform Layer
g	gramm
GCL	Granule Cell Layer

GL	Glomerular Layer
h	hour/hours
H	Hyperpallium
H ₂ O ₂	Hydrogen Peroxide
HCl	Hydrochloric Acid
HD	Hyperpallium Densocellulare
HF	Hippocampal Formation
IEG	Immediate Early Gene
IPL	Internal Plexiform Layer
LHy	Lateral Hyperthalamic nuclei
M	Molar
M	Mesopallium
MCL	Mitral Cell Layer
ml	millilitres
ML	Lateral mammillary nucleus
MW	Molecular Weight
NaBH ₄	Sodium Tetrahydridoborate
NC	Caudal Nidopallium
NCL	Caudolateral Nidopallium
ND	Nidopallium
NDB	Nucleus of the Diagonal Band
NFL	Frontal Nidopallium
nl	nanolitres
OB	Olfactory Bulb
OM	Occipitomesencephalic tract
ONL	Olfactory Nerve Layer
ORC	Olfactory Receptor Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Triton
pH	potentia Hydrogenii
PoA	Posterior nucleus of the Amygdalopalli
PoAb	basal division of the Posterior nucleus of the Amygdalopalli
PoAc	compact division of the Posterior nucleus of the Amygdalopalli
PVL	Periventricular Layer

R	Released
RH	Released at Home
RMS	Rostral Migratory Stream
SM	Medial Septum
SpA	Subpallial Amygdala
S-Phase	synthesis phase
St	Striatum
SVZ	Subventricular Zone
TH	Thyrosine Hydroxylase
TnA	Nucleus Taeniae of the Amygdala
TnR	Transported and not Released
TPO	Temporo-parieto-occipital area
TR	Triangular part of the hippocampus
TuO	olfactory tubercle
U	Units
Va	Vallecula

Curriculum Vitae

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Publikationen

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Erklärung

Die hier vorgelegte Dissertation wurde von mir selbst und ohne unerlaubte fremde Hilfe angefertigt. Außer den in den Anmerkungen im Text und im Literaturverzeichnis genannten Hilfsmitteln wurden keine weiteren benutzt. Ich habe diese Dissertation weder in dieser noch in irgendeiner anderen Fassung bereits einer anderen Fakultät vorgelegt. Ich habe darüberhinaus bislang auch keine andere Dissertation vorgelegt.

Bochum den,

Nina Patzke