Introduction

1. Advanced mammals live in social groups

Living in social groups contributes to the evolutionary success of advanced mammals (Moore et al., 1997; Pedersen, 2004). Social interactions like mate selection, pair bond formation, inter-male aggression, pup recognition, maternal care including feeding familiar and related pups, territoriality, and formation of dominance hierarchies require that the animal is able to identify and remember the identity, sex and social status of conspecifics. For this purpose and for proper communication between groups, animals use different types of signals including vocalisation, visual, tactile and olfactory cues, pheromones (see section 2.2) and even distinctive patterns of movement.

Olfactory signals regulate social behaviours in a large variety of animals. Rodents rely on olfaction for identification of individuals, social interaction, and reproduction (Schaefer et al., 2001; Schaefer et al., 2002; Restrepo et al., 2004). They have a remarkable ability to detect genetic differences between individuals. For example, male mice prefer to mate with females with a dissimilar phenotype of the major histocompatibility complex (MHC). MHC-derived proteins function as specialised display devices, binding peptides that are generated by intracellular proteolytic degradation (Boehm and Zufall, 2006), shuttling them to the cell surface, and presenting them to T-type lymphocytes. Non-self peptides are recognised by the receptors of cytotoxic T lymphocytes, and the corresponding cells are destroyed. In this process, the peptide/MHC complex plays a well-established role in the immune system.

In addition, however, MHC molecules fulfil a completely different function. They display the genetic make-up of an individual at the cell surface. Subsequently, MHC molecules are cleaved by so far unknown mechanisms, and MHC-derived peptides are released into the extracellular fluid (Pearse-Pratt et al., 1998). Finally, they appear in the urine and other bodily secretions (Schaefer et al., 2002), acting as sensory stimuli for a subset of vomeronasal receptors (Beauchamp and Yamazaki, 2003; Leinders-Zufall et al., 2004). This role of MHC peptides as individuality signals is now widely accepted (Boehm and Zufall, 2006).

Individual recognition is perhaps the earliest stage in the process of developing social relations. Consequently, individual recognition is crucial for reproduction and species

survival (Winslow and Insel, 2004). Individual recognition can be defined as the ability of an animal to recognise and differentiate between two and/or more familiar or unfamiliar conspecifics. This ability is essential for many aspects of social behaviour.

2. Two separate systems detect olfactory signals

The sense of smell and its capacity to detect small extraneous molecules are of critical importance to most animals. Detection of odorous cues significantly contributes to the identification and evaluation of food, predators, territories, and reproductive partners. Animals use two distinct systems to detect and convey olfactory signals: the main and the accessory olfactory system. In fact, the olfactory systems use highly specialised chemodetectors capable of recognising highly or less volatile odorants at very low concentrations. They are able to distinguish myriads of chemical compounds, and may sense most of the volatile chemicals, which the animal encounters.

2.1 The main olfactory system preferentially detects highly volatile odorants

In the main olfactory system, receptor neurons in the olfactory region of the nasal cavity are activated by small, volatile molecules either passively during respiration or actively by sniffing. In rodents the number of olfactory receptor genes is close to 1,200 (Zhang and Firestein, 2002). Each olfactory receptor neuron expresses one or a few of these genes (Ngai et al., 1993). All receptor neurons in the olfactory epithelium, which express the same receptor, project to a few, typically two, glomerula (Mombaerts, 1999) in the main olfactory bulb (MOB). In mammals, the MOB contains about 1,000 to 3,000 glomerula (Royet, C. et al., 1988). About 3,000 axons enter one single glomerulum (Meisami, 1989) with no branching before entry (Belluscio et al., 1999).

In the MOB glomerula, incoming olfactory nerve fibres synapse with apical dendrites of mitral and tufted cells. Each mitral cell extends its apical dendrites to one single glomerulum only. On the other hand, one glomerulum may receive dendrites from more than twenty mitral cells (Meisami, 1989). Mitral and tufted cells provide the output from the MOB, sending their axons through the medial or the lateral olfactory tract to their target areas. Some of the fibres pass via the anterior commissure to the olfactory tract of the opposite side. A portion of axons of the mitral cells forms the medial olfactory tract, which terminates in the medial septal nucleus and the olfactory tubercle (Zeman and Innes, 1963).

2.2 The accessory olfactory system detects and processes low-volatile odorants

Many animals need to detect low-volatile odorants to properly communicate with conspecifics. Evoked behaviours include the nursing and recognition of the young in sheep (Kendrick et al., 1997), the pregnancy block (Bruce effect) in female mice caused by exposure to unfamiliar males (Brennan and Keverne, 1997), the arousal of sexually satiated male rodents by a novel female (Bunnell et al., 1977; Dewsbury, 1981), or the recognition of a familiar winner in male golden hamsters (Lai et al., 2004). Low-volatile odorants, which can provoke such behaviours, are called pheromones.

Pheromones convey species-specific information about gender, reproduction status, and social hierarchies (Halpern, 1987; Luo et al., 2003; Luo and Katz, 2004). Either released to the outside world by bodily fluids such as urine, or produced and kept on the emitter's body in, for example, sweat or saliva, they provide the conspecific recipient with information about the emitter (Rodriguez, 2004). Pheromones may even cause neuroendocrine changes in the receiver such as an advanced onset of puberty or oestrous cycling (Halpern, 1987; Doving and Trotier, 1998; Keverne, 1999).

2.2.1 The receptive area of the accessory olfactory system is the vomeronasal organ

The vomeronasal organ (VNO) is a more or less straight tube, bilaterally located in the rostral portion of the nasal septum. Its cavity is filled with fluid produced by the vomeronasal glands. Its medial side is lined with receptor neurons, which are able to detect pheromones. In some species (goats and horses), the access of pheromones to VNO can be facilitated by active dilation and constriction (pumping) of the VNO vessels.

The VNO neuroepithelium is subdivided into two morphologically and molecularly distinct compartments: the luminal (or apical) and the basal layer. Pheromone receptors in the apical layer belong to the V1 receptor family, which uses Gαi2 as transducers. Receptors of the V2R family are located in the basal layer and express Gαo proteins (Dulac and Axel, 1995; Matsunami and Buck, 1997).

2.2.2 Main and accessory olfactory bulbs show similar cytoarchitectures

The axons of the VNO receptor neurons bypass the MOB and proceed to the accessory olfactory bulb (AOB). In mammals, the AOB is located at the dorsocaudal edge of the MOB (Fig. 1 A). In principle, the cytoarchitecture of the AOB resembles that of the MOB.

The incoming fibres, bundles of unmyelinated axons from vomeronasal receptor neurons (Halpern, 1987; Takami and Graziadei, 1990), enter the glomerular layer (GL). Terminating in the glomerula, they make synaptic contacts with the apical dendrites of mitral cells. Vomeronasal nerve fibres remain segregated according to their origin from the apical or basal layers of the VNO (Fig. 1 C and D). Fibres from apical layer receptor cells (Gαi2-positive) target the anterior portion of the AOB and fibres from basal layer receptor cells (Gαo-positive) terminate in the posterior half of the AOB (Imamura et al., 1985; Halpern et al., 1995; Jia and Halpern, 1996; Sugai et al., 1997; Sugai et al., 2000).

The dichotomous nature of the VN system extends to the secondary neurons, the mitral cells. Anterior mitral cells extend their apical dendrites exclusively to glomerula in the anterior AOB. Conversely, posterior mitral cells send their primary dendrites exclusively to glomerula in the posterior AOB (Jia and Halpern, 1997). The basolateral dendrites of these mitral cells, however, cross the anterior/posterior boundary, providing an avenue for “cross-talk” between the two subdivisions.

Mitral cells are the AOB output cells. There are about 5,000 mitral cells in the rat AOB (Guillamon and Segovia, 1997), with glutamate as their transmitter (Fuller and Price, 1988; Trombley and Westbrook, 1990; Hayashi et al., 1993). In contrast to the main olfactory bulb, every AOB mitral cell has up to six apical dendrites, each entering a different glomerulum (Takami and Graziadei, 1992). The granule cells are GABAergic and inhibitory (Takami and Graziadei, 1992). Their deep processes receive input from the amygdala and their superficial processes reciprocally interact with the basal dendrites of the mitral cells.

2.3 The two olfactory systems mainly project to different targets

The main olfactory bulb transmits information mainly to the piriform cortex, the entorhinal cortex, the amygdala-cortical transitional area, the anterior cortical amygdala, the posterolateral cortical amygdaloid area, and the nucleus of the lateral olfactory tract. Less intense projections target the bed nucleus of the accessory olfactory tract, the anteroventral medial amygdaloid nucleus, and the ventral anterior amygdaloid area (Meredith, 1998). Secondary connections end in the hippocampus and the thalamus. Finally, olfactory information gain access to neocortical processing either through projections to the neocortex at dorsal to the rhinal fissure (Scalia and Winans, 1975) or

through projections via the mediodorsal thalamus to the orbitofrontal and the insular cortices.

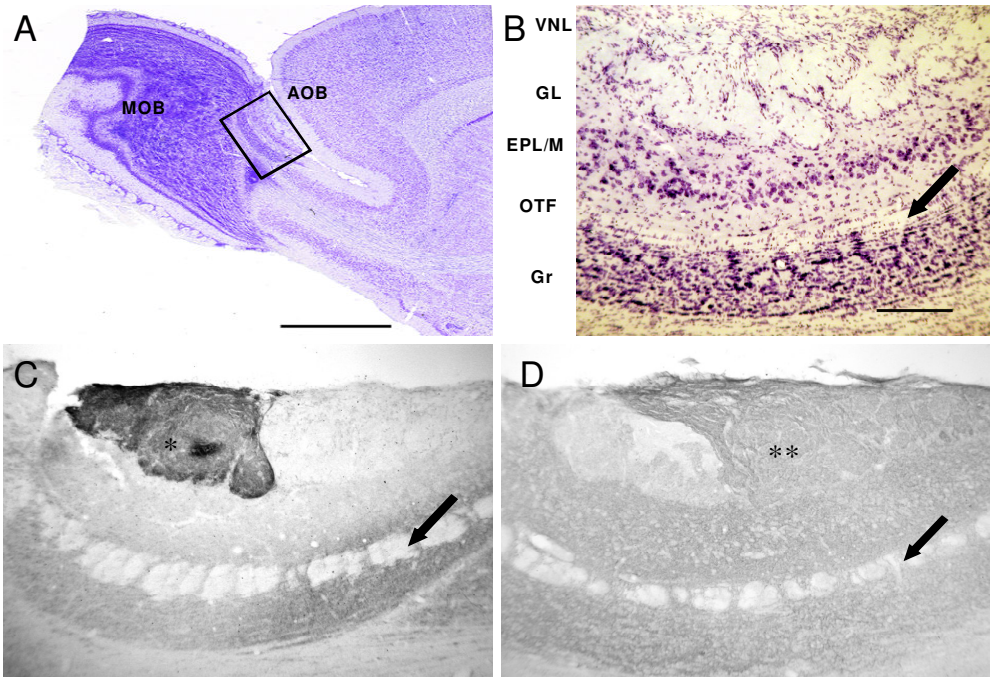

Fig. 1. Cytoarchitecture of the rat accessory olfactory bulb.

Localization and cytoarchitecture the accessory olfactory bulb are easily recognized in sagittal sections after staining with cresyl violet (A, B). The external plexiform and the mitral cell layers (EPL/M) are merged and cannot be easily separated from each other (B). Note that the efferent fibers from the MOB forming the lateral olfactory tract traverse the AOB thereby separating the mitral cell and the granule cell layers (B to D, arrows). Vomeronasal nerve fibers enter and terminate in the glomerular layer, where their axons synapse on to dendrites of mitral cells. Note that the anterior and posterior parts of the AOB may be identified by its strong Gαi2 immunoreactivity (C, asterisk) or its moderate staining for Gαo (D, double asterisks), respectively. Abbreviations: AOB: accessory olfactory bulb; EPL: external plexiform layer, M: mitral cell layer; GL: glomerular layer; Gr: granule cell layer; MOB: main olfactory bulb OTF: olfactory tract fibers; VNL: vomeronasal nerve layer. Bar represents 2 mm in A and 200 µm in B, C, and D.

Fibres from the accessory olfactory bulb principally terminate in the medial nucleus and the posteromedial cortical nucleus of the amygdala (Kevetter and Winans 1981). The AOB also projects to the bed nucleus of the accessory olfactory tract, the anterior cortical amygdaloid nucleus, the posterolateral cortical amygdaloid area, and the nucleus of the lateral olfactory tract. In addition, the AOB projects to the cortex-

amygdala transitional zone and the ventral anterior amygdaloid area (Mohedano-Moriano et al., 2007).

2.4 The amygdaloid complex is the common target of the two olfactory systems

The amygdala is a highly complex structure, consisting of more than ten individual nuclei. Each of them may further be divided into subregions (De Olmos and Beltramino et al., 2004). Correspondingly, the amygdala is divided functionally into several subsystems, namely the frontotemporal, olfactory, vomeronasal, and autonomic subsystem (Petrovich and Canteras et al., 2001).

The olfactory amygdala includes the anterior cortical amygdaloid nucleus (ACo) and the posterolateral cortical amygdaloid nucleus (PLCo). In contrast, the vomeronasal amygdala consists of the medial amygdaloid nucleus (Me), the posteromedial cortical amygdaloid nucleus (PMCo) and the amygdalohippocampal transitional area (AHi). However, none of the two olfactory subdivisions of the amygdala is limited to afferents from a single system. Rather, they receive projections from both main and accessory olfactory bulbs (Pro-Sistiaga et al., 2007). Each of the amygdalar subsystems has a wide range of connections, especially with the hypothalamus. These pathways provide the anatomical basis for pheromonal effects on animal behaviour (Scalia and Winans, 1975; Dudley and Moss, 1999).

3. There are two experimental approaches to investigate social recognition in rodents

It might be assumed that social interaction can be investigated properly in humans only. But animals also interact socially to a considerable extent. Sexual reproduction requires animals to come in close contact with each other in order to mate. In addition, animals that show a degree of parental care form minimal social units consisting of one or more parents and their offspring. Social behaviours necessitate the ability of individuals to interact with each other; therefore, social recognition, the primary step in social interaction, can well be recognised in non-human and even non-primate species. Consequently, several methods have been developed to analyse the processes of social recognition in detail.

3.1. The individual recognition paradigm

The “individual recognition paradigm” is used to investigate experimentally whether an animal recognises familiar conspecifics (Thor and Holloway, 1982). The method is based on the olfactory discriminative capabilities and on the innate desire of an animal to investigate conspecifics (Engelmann et al., 1995). With this paradigm, a male juvenile rat (20-30 days of age) is introduced to an adult male rat for a 5-minute trial, and the duration of social investigation (sniffing, following, or general inspection) is measured (Thor and Holloway, 1982). Thirty minutes later, the same juvenile is reintroduced to the resident, and the duration of his investigatory behaviour is measured again. Usually the second investigation is much shorter than the first one. The ratio of the investigation times in the two subsequent encounters is taken as an index of how well the resident recognises the juvenile.

The individual recognition paradigm is one of the most powerful techniques to study social recognition. Nevertheless, the technique has two important limitations. The paradigm cannot assess whether an attenuated investigation during the second exposure really reflects juvenile-related memory or whether it is simply due to fatigue or other secondary mechanisms. Furthermore, sexually naive animals fail to reduce the duration of their investigatory behaviour towards the previously presented juvenile after an interval of 30 minutes between the exposures. Therefore, sexual experience is required in the animal to detect individual recognition.

3.2. The individual discrimination paradigm

To overcome these limitations, a novel procedure has been developed, called the “individual discrimination paradigm” (Engelmann et al., 1995). In this method, the first trial is the same as in the individual recognition paradigm. But in the second trial, another unfamiliar juvenile rat is simultaneously introduced to the adult male in addition to the previous one. The times that the resident spends to investigate each of the juveniles (familiar and unfamiliar) are recorded. The time the resident spends with the familiar juvenile during the second exposure is divided by the time spent to investigate the unfamiliar one, and the ratio is taken as a measure of social recognition.

4. Neuronal stimulation activates different mechanisms in the cell

To understand fully the mechanisms which underlie social recognition, a more complete knowledge of the corresponding circuits is necessary. The neuronal cell groups involved must be identified and their sequential activation needs to be described.

Neurons become activated in response to a wide variety of stimuli from the molecular (neurotransmitters, neuropeptides, and growth factors) to the systemic (seizures, ischemia, brain injury, and sensory stimulation) level. During these responses, neurons alter their proteomics either by modification of available proteins or by expressing new ones. Posttranslational modifications like phosphorylation, sulfatation, or glycosylation with N-acetyl glucosamine result in altered protein activities, internalisation of receptors and channels, or regulation of gene expression. For example, protein kinase A can phosphorylate, thereby activating transcription factors like CREB (cAMP response element binding) protein. Active transcription factors enter the nucleus and bind to target sites on the promoter regions of genes. Thus, phosphorylated CREB can bind to a DNA segment called CRE (cAMP response element), which is upstream of genes (Davis et al., 2003), such as c-fos (cellular Finkel-Biskis-Reilly murine osteosarcoma (Finkel et al., 1966)), egr.1 (early growth response factor 1), and arc, which encodes the arc protein [activity- regulated cytoskeletal-associated protein (Chowdhury et al., 2006)].

Genes like c-fos, egr.1, and arc are called immediate early genes (IEGs). The expression of their mRNAs occurs within minutes, while the synthesis of their corresponding proteins may last from minutes to hours. Such proteins like c-fos, egr.1 (also known as Krox 24, NGFI-A, TZS8 and Zenk), and arc are widely used as neuronal activity markers (Chiasson and Dennison et al., 1995; Dudley and Moss, 1999; Tischmeyer and Grimm, 1999; Polston and Heitz et al., 2001). Homer (Inoue and Udo et al., 2007) and Narp (neuronal activity regulated pentraxin (Reti and Miskimon et al., 2008)) proteins may be used in a similar way. Other proteins which can be used to detect activated neurons include phosphorylated DARPP-32 (dopamine and cAMP-regulated phosphoprotein-32 kDa).

5. Aims of the project

To understand the process of social recognition with regard to cellular and molecular aspects, the present study aims to explore which brain areas are sequentially involved

in the corresponding biological processes. For this purpose, several questions have to be addressed:

1) Which neural system is primarily involved in the formation of social memory?

To answer this question, the brain areas which are activated during the social recognition process need to be identified. Comparing neuronal activities after social stimulation with neuronal activities after pure olfactory stimulation or non-stimulated controls will help to determine which areas are specifically involved.

2) Are both or predominantly one of the two distinct compartments of the vomeronasal system responsible for social recognition?

This question requires the cytochemical identification of the two subdivisions of the AOB. Subsequently, the activation of neurons in both of these areas may be followed.

3) Which type of neurons, glutamatergic projection neurons or GABAergic interneurons, is predominantly activated during juvenile stimulation in specific areas?

Studying the co-expression of c-Fos with mRNAs encoding the two forms of glutamic acid decarboxylase (GAD65 or GAD67) and vesicular glutamate transporters (vGLUT1 and vGLUT 2) will allow comprehending the GABAergic or glutamatergic nature of activated neurons.

4) Are socially active neuropeptides like vasopressin or oxytocin involved in social recognition?

Studying the expression of c-fos in vasopressinergic or oxytocinergic neurons will show their presence or absence in the areas of interest, and their activation during the experiment.

Answering the above-mentioned questions will help understand some mechanisms that underlie social interaction and social memory. Furthermore, a better knowledge of these mechanisms will improve our understanding of disorders characterised by marked dysregulation of social behaviour such as autism, schizophrenia, and social phobia.