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Single-Neuron Correlates of Social Identity in Freely Interacting Female Rats

Durham University, Department of Psychology

Francesca Pibiri

31/05/2017



Submitted for admission to the degree of DOCTOR OF PHILOSOPHY

Declaration

I, Francesca Pibiri, confirm that the work presented in this thesis is my own. The only exception is in chapter 11 of the present thesis where a collaborator (Ricardo Bindi, University of São Paulo) contributed in the behavioural scoring. His contribution has been explicitly referenced in the text. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Francesca Pibiri

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Abstract

The neuronal bases of social interaction are poorly understood, especially in terms of social motivation and social cognition. The present study used single-unit recording to investigate neuronal firing patterns in two inter-connected regions: the amygdala and the piriform cortex, focusing on the latter region. Olfactory signals strongly shape rodents' social interaction: previous anatomical and physiological studies implicate the piriform cortex in olfactory pattern separation and pattern completion functions which could support memory for the odour profile of familiar conspecifics. As a first step in characterising the rodent social amygdala and piriform cortex, I paired Lister Hooded rats in an apparatus where they are fully free to engage in a variety of positive social interactions including anogenital sniffing, face to face contacts, and body contact. The apparatus was a 40x40 cm wooden square box with a wall height of 50 cm. I performed extracellular electrophysiological recordings from ensembles of single neurons tested in various social and non-social conditions (e.g. familiar rat in box vs empty box, or familiar rat vs novel rats). In addition, I simultaneously recorded behaviour with images time-stamped in synchronization with the electrophysiological recordings.

The present thesis shows that there are pyramidal neurons in the rodent amygdala and piriform cortex which respond strongly to social interaction. The main finding was that about 20% of cells in the piriform cortex showed firing patterns specific to either familiar or novel conspecifics, and a similar proportion showed firing patterns specific for one of two familiar sisters.

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List of abbreviations

µm	microns
AB	accessory basal nucleus (amygdala)
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AOB	accessories olfactory bulb
AOT	accessory olfactory tract
AP	anterior-posterior
AVP	arginine vasopressin
B	basal nuvleus (amygdala)
Base	baseline
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CA	<i>Cornu Ammonis</i> areas (CA1,CA2,CA3)
CeA	central nuclei (amygdala)
CeI	intermediate subdivision
CeL	lateral subdivision
CeM	medial subdivision
CoA	cortical nucleus (amygdala)
CREB	cyclic adenosine monophosphate responsive-element-binding protein
CRH	corticotropin releasing hormone
DV	dorsal-ventral
EEG	electroencephalography
Fam Bed	familiar bedding
Fam Obj	familiar object
Fam	familiar
GABA	gamma-aminobutyric acid
ICV	intracerebroventricular
KO	knock out
LA	lateral nucleus (amygdala)
LEDs	infrared light-emitting diodes
LFP	local field potential
LS	lateral septum
MeA	medial Amygdala
ML	medial-lateral
Mm	millimetre
MOB	main olfactory bulb
ms	milliseconds
N	number
NAc	nucleus acumbens
NMDA	n-methyl-D-aspartic acid
Nov	novel
OVX	ovariectomized
OXT	oxytocin
OXT-R	oxytocin receptor

OXYKO	oxytocin knock out
PAC	peryamygdaloid cortex
PIL	personal licence
Pir	piriform cortex
s.e.m.	standard error of mean
sec	seconds
TRH	thyrotropin releasing hormone
V1aR	V1a receptors
V1Br	V1b receptors

Chapter 1: Introductory Overview

In this opening section, I try to offer a concise overview of the rationale and overall approach for the whole project, presenting the thesis' aims and methods, and a general idea of the key results. Subsequent sections of the Introduction set out the different elements of the background material to this thesis in more detail, with full citations. Here, the emphasis is on presenting the main arguments in a simple fashion, and so references are kept to a minimum.

1.1 The need to understand coding of social identity in rodents

The neurobiological bases of non-aggressive social interaction in typically developing rodents, are not well understood, especially in terms of both social motivation and social cognition.. Exciting advances have come from the literature on the 'social neuropeptides' oxytocin and vasopressin (Donaldson and Young, 2008). Many studies have shown that these social neuropeptides augment social bonding, including adult pair bonding in voles (Donaldson and Young, 2008). However, there is clearly a gap in our understanding of the neurobiological bases of social cognition, particularly the coding of social identity, at the 'cell assembly' level. The present thesis attempts to address these gaps in our understanding. How should one begin to address these gaps?

1.2 Female rats as subjects: neural representation of individual conspecifics?

In terms of subjects, given limited resources, the present thesis investigated responses in female-implanted rats only, since females are thought to have superior social motivation and cognition to males. The idea was then to focus upon female-female interactions, since this affords better opportunities for recording non-aggressive social interaction.

How specific are representations of social identity in rodents? It seems clear that social identity discrimination can extend to such discriminations as kin vs non-kin, male vs female, and mate vs non-mate, but it is not fully clear whether this extends to individual conspecifics. Arguably, the widespread use in behavioural testing of adult males as subjects with juvenile rats as social stimuli may not best capture the potential for coding of individual conspecifics. Aggression is lessened but so too is social motivation. Rather, ethologically oriented observations of diverse behaviors in rodents such as compliance with social hierarchies, the existence of partner preference (Donaldson and Young, 2008), and more recently reciprocal altruism (Zentall, 2015), all suggest that coding for individual conspecifics is at least highly plausible. Indeed, there is already a small literature strongly indicating representation of individual conspecifics in hamsters (Johnston and Jernigan, 1994; Petrulis, 2009), and some suggestion of this in rats (Gheusi et al, 1997). Accordingly, a key aim of the present thesis was thus to offer novel insights into the neural coding of individual conspecifics in rats. In particular, the use of females and an inhouse-breeding program permitted testing neural representations of sisters of the implanted rats.

The classic behavioural paradigm used to test social memory in mouse models of autism, builds upon the Novel-Object recognition paradigm, and compares approach behaviour to familiar and novel conspecifics, often using the Crawley three-chamber apparatus (Silverman et al 2010). The standard result is that the novel conspecific is explored more, revealing memory for

the familiar conspecific. Accordingly, a key method in this thesis involved comparing neural responses to a familiar sister to responses to novel female conspecifics. The aim was to provide knowledge of cellular responses underlying discrimination of novel and familiar conspecifics. Indeed, the present thesis does provide encouraging results in this respect, revealing neurons that fire more to novel conspecifics than to the familiar conspecific, and neurons that fire more to the familiar conspecific than the novel conspecifics. These neurons, particularly the latter category (familiar-higher), could reflect a specific memory for the social identity of the familiar conspecific. Recording neural responses allows us to test this idea more directly, by comparing neural responses to two familiar sisters.

There is no Crawley-type behaviourally equivalent task for this, since there is no expectation that behavioural responses to familiar conspecifics will be different. Constructing robust separate representations of two sisters, especially with in-bred lines like Lister Hooded rats, involves relatively difficult discrimination; two sisters have the same sex, and are genetically rather similar. Accordingly, if sister-specific representations (i.e. one cell assembly for sister A, and a different cell assembly for sister B) are seen in these data, this could suggest that coding for individual conspecifics is robust in rodents. The present thesis does indeed provide encouraging results for this idea, revealing neurons that fire more to one sister than another. Where in the brain might one find such neurons, and what would the best way to go about finding them? The next sections address these questions.

1.3 The Piriform Cortex and Amygdala

The present work focuses upon the piriform cortex and amygdala. Amygdalar dysfunction needs no special additional justification here, since it has been repeatedly linked to autistic spectrum disorders, a neurodevelopmental syndrome which among other abnormalities is characterised by deficits in reciprocal social interaction. The focus on the piriform cortex perhaps requires further explanation. Firstly, while the piriform cortex receives multimodal inputs, the dominant input is olfactory, and it is well established that olfaction contributes to social motivation and social recognition in mammals (including humans where it supports kin recognition (e.g. Porter et al, 1986), partner recognition and partner preference). Secondly, the piriform cortex is strongly bidirectionally connected with the amygdala (Majak et al, 2004), and orbitofrontal cortex (Illig et al, 2005; Chen et al, 2014), and the amygdala and orbitofrontal cortex are well known to be important in social cognition. Thirdly, the piriform cortex shows a very high density of receptors for social neuropeptides oxytocin and vasopressin (Smith et al 2016; Mitre et al, 2016). As mentioned above, the present study was built upon the potentially superior social motivation and cognition in females. It makes sense to sample a brain region where it is known that females exhibit a higher density of receptors for the social neuropeptides. Definitively establishing such regions has been hampered by a lack of reliable OXT-R antibodies. To address this problem, a recent study (Mitre et al, 2016) created four antibodies for the mouse, and selected the best one (called OXTR-2) to analyse OXT-R density across the brain. They surveyed 39 brain regions, and found that the only region where oxytocin receptor density was higher in females than males was the piriform cortex. This strongly suggests that the piriform cortex is a good candidate for examining neural responses in social interaction. Finally, it seems likely that social identity coding for an individual conspecific relies heavily on circuitry supporting pattern separation and pattern completion, and the extensive

hippocampal-CA3-like recurrent circuitry in the piriform cortex appears ideal for pattern completion (Haberly, 2001; Barnes and Wilson, 2008; Franks et al, 2011). Haberly (2001) noted the substantial similarity between the piriform cortex and hippocampus, both of which are allocortical. Hasselmo's computational model of cholinergic neuromodulation of pattern separation and pattern completion in the hippocampus (Hasselmo et al, 1986), which has proved highly predictive (Douchamps et al, 2013; Siegle and Wilson, 2014), was first worked out in the piriform cortex (Hasselmo et al, 1991; Hasselmo and Bower, 1993). In summary, the piriform cortex is an appropriate region to start in exploring neural codes for individual identity.

1.4 Neuro-ethological approach: naturalistic social interaction

The next important issue to address was what would be the best way to go about finding the neurons representing social identity. As a first step towards characterising cellular responses during social behaviour, the approach adopted here might be described as a neuro-ethological approach, sacrificing controlled cue delivery in favour of relatively unconstrained, naturalistic, social interaction. The only obvious constraints were the use of a small arena for social interaction, and trials of fixed duration (three minutes long).

Some studies present social-relevant stimuli (e.g. Petruslis et al, 2005; Parsana et al, 2012), and report detailed cellular responses which are precisely time-locked to stimulus delivery, which is certainly informative, but almost all lack genuine social interaction. Those few cell-recording studies which do involve social interaction typically limit the kind of behaviours the rodents can engage in. For instance, one interesting and careful study which specifically looked for coding of individual conspecifics in the rodent hippocampus produced entirely negative results, despite techniques designed to reveal social responses and an ample yield of sampled cells (von

Heimendahl et al, 2012). These negative results could be due to several factors, such as the region sampled (i.e. dorsal hippocampus, though including CA2 neurons). Arguably, an important candidate explanation is that the social interaction paradigm was too restrictive, and did not for instance permit anogenital sniffing, despite the importance of this behaviour for rodent (and much mammalian) social behaviour. In the (von Heimendahl et al, 2012) study, the apparatus set up permitted only whisker and snout contact, when both rats extended their heads into the gap between the platforms of the implanted and stimulus rat.

In contrast to most cell recording paradigms, the approach adopted in this thesis, leveraging good recording stability derived from spatial cell recording techniques, permitted relatively unconstrained interaction. The approach afforded non-aggressive engagement of all sensory modalities including the somatosensory, olfactory, gustatory, auditory and visual, all of which likely play a significant role in shaping social cognition. Importantly, for instance, our freely-behaving paradigm affords both anogenital and face-to-face contact. This open-ended approach means that a relatively narrow interpretation of the effective stimulus cannot be given to cellular responses. Such an approach, however, is seen here as the necessary first step in revealing an important phenomenon, with future work needing to limit the parameter space of variables potentially explaining the phenomenon. This ethological initially open-ended approach was successful in O'Keefe's discovery of hippocampal place cells (e.g. O'Keefe, 1976).

Constraining interpretation is aided in the present work by behavioural scoring, which was done blind by collaborators. Moreover, in some cases in the present work, the standard rat-present trials were supplemented by probe trials to attempt to narrow down interpretation. Probe trial results varied, but a brief summary is that that volatile olfactory cues were sometimes sufficient, and sometimes insufficient, to re-activate the individual-conspecific

patterns of firing observed in rat-present trials. These results are certainly informative, but cannot indicate by themselves whether such re-activation is purely sensory or involves a pattern completion type memory response. Future work should be able to explore if coding of individual conspecifics is highly associative.

1.5 Summary

The present work recorded from neurons in the piriform cortex and amygdala during naturalistic, female-to-female rat social interaction within a small arena. The most interesting findings are the discovery of piriform neurons whose firing rates discriminate familiar from novel conspecifics, and piriform neurons whose firing rates discriminate familiar sisters of the implanted rat. To my knowledge, these are entirely original findings. Future work should narrow down interpretations of these neuronal correlates, but they are compatible with the hypothesis that the piriform cortex contributes to social identity coding of individual conspecifics.

Chapter 2: Anatomy of the Amygdaloid Complex

2.1 Nomenclature

The amygdala is an almond-shaped structure located deeply and medially in the temporal lobe of the mammalian cerebral hemisphere. Discovered first in the early 19th century by Karl Friedrich Burdach as the ‘amygdalar nucleus’, corresponding to the now known basolateral complex, the recent model of the amygdala counts a large number of structures identified as amygdaloid complex.

Because there is a persisting controversy related to the number, the extent of the nuclei’ borders and the classification of the amygdala subdivisions, it is important to establish that this thesis adopts the nomenclature introduced by Price et. al (1987) and Sah et al (2003).

The amygdala nuclei are divided into three main groups on the base of the cytoarchitectonics, histochemistry and connections with other regions:

- 1) The *deep nuclei* include the lateral nucleus, basal nucleus and accessory basal nucleus
- 2) The *supercifical nuclei* include the cortical nuclei and the nucleus of the lateral olfactory tract
- 3) The *centromedial nuclei* include the medial and the central nuclei
- 4) The *remaining nuclei* include the intercalated cell masses and the amygdalohippocampal area

The location of the different amygdaloid regions is shown in **figure 2.1**.

As mentioned previously the amygdaloid complex is a heterogeneous structure of 13 nuclei divided into four main groups.

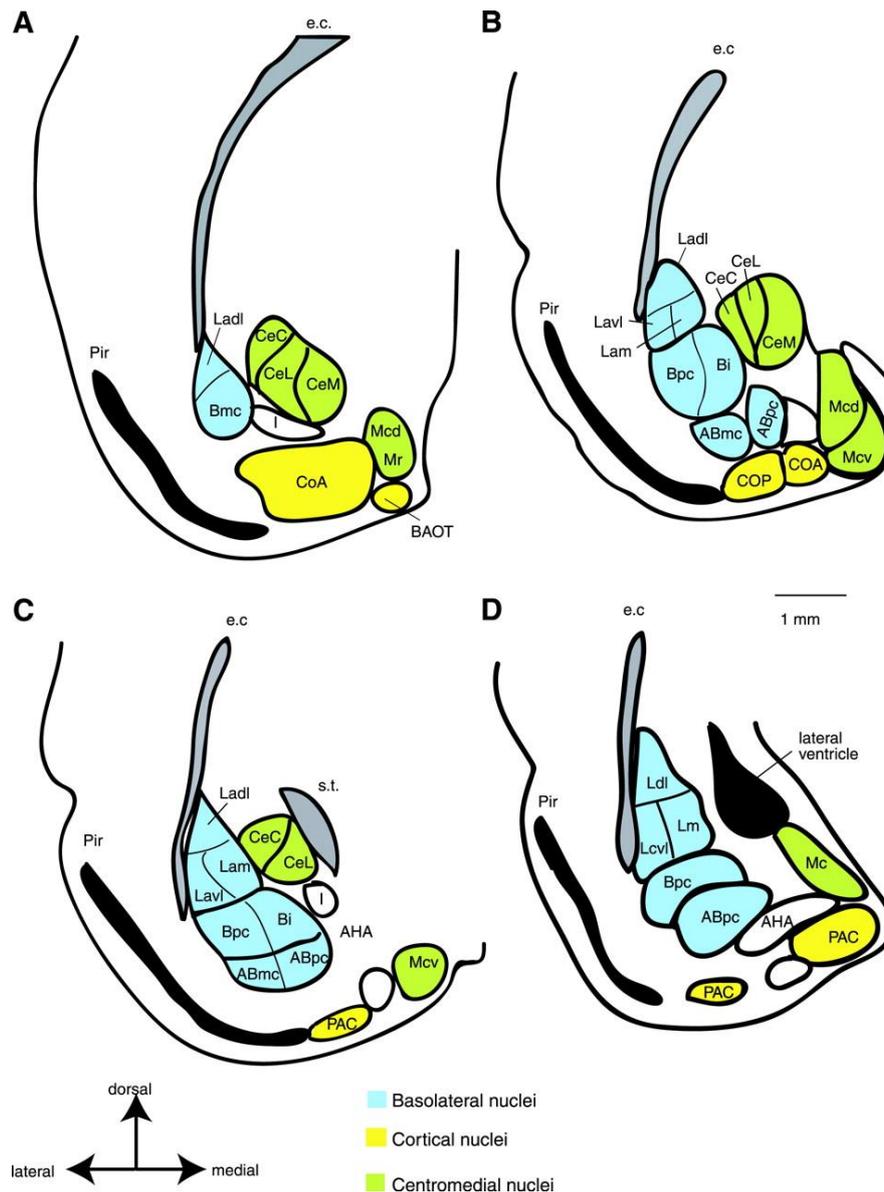


Figure 2.1. Representation of the rat amygdaloid nuclei. Coronal sections are drawn from rostral (A) to caudal (D). In blue the deep nuclei, in yellow the superficial nuclei, and in green the centromedial group. ABmc, accessorybasal magnocellular subdivision; ABpc, ac-cessory basal parvocellular subdivision; Bpc,basal nucleus magnocellular subdivision;e.c., external capsule; Ladi, lateral amygdalamedial subdivision; Lam, lateral amygdalamedial subdivision; Lavi, lateral amygdalaventrolateral subdivision; Mcd, medialamygdala dorsal subdivision; Mcv, medialamygdala ventral subdivision; Mr, medialamygdala rostral subdivision; Pir, piriformcortex; s.t., stria terminalis. Reprinted from Sah et al (2003)

2.2 Gross morphology

The deep nuclei also known as basolateral complex include the lateral nucleus (LA), the basal nucleus (B), often referred together as basolateral nucleus, and the accessory basal nucleus (AB). The lateral nucleus lies dorsally in the amygdala bordered ventrally by the basal nucleus, laterally by the external capsule and medially by the central nucleus. LA has three subdivisions: dorsolateral, ventrolateral and medial. The basal nucleus, located ventrally to the LA, has three subdivisions: rostral, caudal-intermediate and parvicellular. The basal nucleus is located close to the amygdalo-hippocampal area and it has three subdivisions: magnocellular, intermediate and parvicellular. The accessory basal nucleus lies adjacent to the amygdalo-hippocampal area, ventral to the basal nucleus.

The superficial nuclei have cortical features with a layered structure. This group comprises the anterior cortical nucleus (CoA), bed nucleus of the accessory olfactory tract (AOT), nucleus of the lateral olfactory tract, periamygdaloid cortex (PAC) and posterior cortical nucleus.

The nomenclature described by Price et al. (1987) include the medial (M), central nuclei (CeA) and the amygdaloid part of the bed nucleus of the stria terminalis (BNST) into the superficial nuclei group. However, Sah and colleagues (2003) and McDonald (1998) introduced a forth group ‘the *centromedial nuclei*’ based on the histochemical and developmental characteristics that distinguish these nuclei from the rest of the superficial nuclei. These areas are located in the dorsomedial portion of the amygdala. Th CeA has four divisions: the capsular subdivision (CeC), lateral subdivision (CeL), intermediate subdivision (CeI) and medial subdivision (CeM). The medial nucleus has 4 subdivisions: rostral, central dorsal, central ventral and caudal.

The remaining areas are included in the other amygdaloid nuclei. This group comprises the anterior amygdaloid area, the amygdalo-hippocampal area and the intercalated nuclei (I). The anterior amygdaloid area is not a very defined region close to the rostral pole of the amygdala. The intercalated nuclei are cluster of small neurons associated with the fibre bundles that separate the amygdaloid nuclei. The amygdalo-hippocampal area is located between the hippocampal formation and the caudomedial corner of the amygdala.

2.3 Morphology and Physiology

2.3.1 Deep Nuclei: Morphology

The Basolateral complex comprises different type of cells categorised on the base of axon and dendritic pattern; among those: extended neurons, cone cells, chandelier cells and neurogliaform cells. However, the main population of neurons is constituted by 2 types of cells. The first main group has been described as pyramidal or cortical-like cells and constitutes the 70% of the total neurons. These cells present some characteristics that recall the cortical pyramidal neurons; in fact, many of them have a pyramidal-like soma with three to seven dendrites. One of the dendrites of these cells is usually more prominent and the secondary and the tertiary dendrites are spiny. However, in many ways these cells differ from the cortical neuron. These Basolateral cells do not have a rigid orientation but they appear to be randomly organised in particular in proximity of the nuclear border. The primary dendrite of the apical and basal dendrites is equivalent in length and the distal dendrites do not have an elaborate terminal ramification. Their axon can originate from the soma or from the proximal portion of

the primary dendrite. B and LA have a very similar morphology, but neurons in the B have a larger soma diameter (15-20 μm) compared to the LA neurons (10-15 μm).

The second type of cell is GABAergic and constitutes the local circuit interneurons. Contrary to the pyramidal-like cells, interneurons have two to six primary dendrites lacking spines and form a spherical dendritic field. On the base of their dendritic trees, these cells have been subdivided into multipolar, bitufted and bipolar cells. The axons originate from the soma or from the initial portion of the primary dendrite. Some of the interneurons form a distinct array called cartridge around the soma of the pyramidal-like cells which allow a tight inhibitor control on these cells. There are different class of interneurons in the basolateral complex; some of them express parvalbumin, others calbindin and/or calretin with a significant overlap. The soma is slightly smaller than pyramidal-like cells (10-15 μm). Sah et al (2003).

2.3.2 Deep Nuclei: Physiological properties

The pyramidal-like neurons and the local circuit interneurons in the BLA show different electrophysiological properties. The 95% of the cells classified as pyramidal, show broad action potentials with half-width 1.2 ± 0.1 ms (Mahanty and Sah, 1998). Following a prolonged current injection, the pyramidal cell response varied from a full spikes frequency adaptation with cells most firing only two to three spikes, to no frequency adaptation with cells firing repetitively. In between were cells that fired several times but showing clear spike frequency accommodation.

In contrast to what has been observed in the cortex, this variety of firing properties of pyramidal neurons in the BLA does not match clear morphological differences. Faber and co-worker (2001) concluded that these different responses in firing are determined by the differential

distribution of voltage-gated and calcium-activated potassium channels and not by mechanisms that rely on cell morphology. A large slow after-hyperpolarizing potential always followed trains of action potentials, and it has been found larger in cells with spike frequency adaptation (Mahanty and Sah, 1998; Faber et al, 2001, Donald et al, 1992; Sah et al, 2003).

The pyramidal neurons in the basolateral complex are glutamatergic and they represent the output cells of these nuclei (Smith and Paré, 1994). These cells receive cortical and thalamic glutamatergic inputs which form asymmetrical synapses. Immunocytochemical results revealed the localization of AMPA and NMDA in the basal nuclei, NMDA receptors are most prevalent within spines while AMPA receptors are more prevalent within dendritic shafts (Faber et al, 1995). Kainate receptors, present at some glutamatergic inputs to the pyramidal cells, have been proposed to be involved in basal synaptic transmission (Li and Rogawski, 1998). Finally, the activation of metabotropic receptors has both pre-synaptically and post-synaptically action.

The second population was classified as interneurons. These cells have fast-spiking phenotype with half-width 0.76 ± 0.04 ms. No slow after-hyperpolarizing potentials were observed in these cells. In response to prolonged depolarizing current, interneurons show very little frequency adaptation (Mahanty and Sah, 1998). In vivo recording in cats shows that interneurons, unlike pyramidal-like cells, fire spontaneously at high frequency (10-15 Hz) and show discharge patterns ranging from tonic to phasic (Paré and Hélène Gaudreau, 1996).

As most of the interneurons in the central nervous system, these cells are GABAergic and constitute local circuit interneurons (Paré and Smith, 1993). They receive local, cortical and thalamic excitatory inputs and inhibitory input from the synaptic connections with each other. The activation of these cells generates inhibitory synaptic potentials that have a fast component

mediated by GABA_A receptors and a slow component mediated by GABA_B receptors (Sugita et al 1992).

As described above, several classes of interneurons have been identified based on their content of calcium binding proteins. Physiological studies do not report any different firing properties between different cells. However, it seems likely that different electrophysiological properties are attributed to interneurons involved in different local circuits and with distinct afferent/efferent connections (Sah et al, 2003).

2.3.3 Centromedial nuclei: Morphology

In contrast to their neighbouring pyramidal-like neurons in the BLA, central amygdala neurons have a “striatal-like” cytoarchitecture and they are predominantly GABAergic, like the majority of cells in the striatum. Using Golgi preparation, McDonald (1982) recognised several cells types in each of the 4 subdivisions on the base of different dendritic morphology. However, there is a general agreement that the central amygdala mainly comprises a cell type with a moderate number of dendritic spines (McDonald, 1982; Schless et al, 1999; Sah et al 2003). This type of neuron has an ovoid and fusiform soma with three to five aspiny primary dendrites, and secondary and tertiary dendrites with a medium number of spines. A second type of neuron found in this area has a larger soma and a thicker aspiny primary dendrite and spiny secondary dendrites. At last, a third type of cell has been described as totally aspiny, but it represents a very small portion of the CeA population.

The medial nucleus and the bed nucleus of the stria terminalis are characterised by a cell type similar to the main neurons observed in the central amygdala. The neurons have a small to medium size body with multipolar spiny dendrites (Sah et al, 2003).

2.3.4 Centromedial nuclei: Physiological properties

In accordance with morphological studies, electrophysiological recordings revealed the presence of abundant local GABAergic connections within the central nucleus. Using intracellular current-clamp recordings from rat brain slices, Schiess et al (1993) identified two types of neurons on the base of their accommodation characteristics and the nature of their after-hyperpolarisations. In response to a prolonged current injection, type A shows a lack of accommodation and medium after-hyperpolarisation while type B showed a clear spike frequency adaptation and exhibited both a medium and slow after-hyperpolarisations. Type A represents 75% of the population, type B 26% of the population.

Neurons in the central nuclei receive glutamatergic input from the lateral and the basal nuclei activating receptors AMPA and NMDA. Excitatory inputs to the lateral subdivision also express metabotropic glutamate receptors which activation leads to depression of the synaptic input. Two different types of ionotropic GABA receptors have been identified in the CeL localized to different GABAergic inputs: GABA_A and GABA_c. Both receptors types are co-expressed onto dendrites which form synapses with inputs from the intercalated nucleus. Instead, somatic synapses onto CeL neurons with inputs from a dorsomedial source express only GABA_A. Thus, the different location of these two types of GABA receptors suggests that they play a different role in the local circuit of the central amygdala (from Sah et al, 2003).

2.3.5 Superficial nuclei and remaining nuclei: Morphology

Most of the studies have examined morphology and properties of deep and centromedial nuclei, while very little is known regarding the cortical nuclei and the remaining nuclei.

The anterior amygdaloid area contains cells similar to the second class of neurons observed in the CeM with ovoid cell bodies, three to four primary dendrites and secondary dendrites with few spines (Hall, 1972).

The nucleus of the lateral olfactory tract, the amygdalo-hippocampal area and the cortical nuclei contain cortical-like cells similar to the main cells observed in the basolateral amygdala while spiny stellate cells and neurogliaform cells represent a smaller population. Conversely to pyramidal-like cells in the basal nuclei, neurons in the olfactory areas have an organization more cortical-like with apical dendrites oriented parallel to each other.

The intercalated cells are very similar to the neurons observed in the striatum. These cells are GABAergic and act as inhibitors for the neurons in the CeA to which follows stimulation in the BLA. The neuronal population accounts two main types of cells. The first type has a ovoid soma (10-15 μm) with spiny and large bipolar dendritic trees and axons that send collateral to BLA and CeA. The second type has a large body (50 μm) with thick spiny or aspiny dendrites that travel in parallel to the borders of BLA and CeA (From Sah et al, 2003).

2.4 Afferent and Efferent connections

Amygdala connections with other brain areas and vice versa have been studied using anterograde and retrograde tracers injected in various amygdaloid nuclei. From a general point of view, on the base of the information contents, three are the main connectional networks of the amygdala with other brain areas. Sensory information to the amygdala is supplied by a large part of the forebrain including: olfactory cortex, visceral pathways, posterior thalamus and sensory association cortical areas. Visceral information to emotional stimuli are modulated by

connections between amygdala and the part of the brain stem included between hypothalamus and medulla. Emotional behaviour and mood are regulated by amygdaloid connections with various forebrain areas: ventromedial frontal, rostral insular, rostral temporal cortex, medial thalamus and ventromedial basal ganglia (Price, 2003). This section will describe the main afferent and efferent connections of the amygdaloid complex in rodents. **Figure 2.2** shows the main afferent connections to the amygdaloid nuclei and **figure 2.3** showed the main efferent connections from the amygdaloid nuclei.

2.4.1 Afferents inputs: Sensory Inputs

The amygdala receives all modalities sensory inputs: olfaction, vision, auditor, gustation and viscera, and somatic sensation (Sah et al, 2003).

Olfactory information reaches the amygdala through a unique direct access that may explain why the amygdala play a key role in social behaviour and social recognition. The olfactory bulb sends direct projections to different amygdaloid structures. The main olfactory bulb makes dense monosynaptic contacts with to the nucleus of the olfactory tract, periamygdaloid cortex and the anterior cortical nucleus (Price, 1973; McDonald, 1998). The accessory olfactory bulb projects to the bed nucleus of the accessory olfactory tract, the medial nucleus and the posterior cortical amygdala, the deeper amygdaloid nuclei, the basolateral nuclei and the accessory basal nuclei, receive only projections from the piriform cortex and anterior olfactory nucleus (Krettek and Price, 1978; Ottersen, 1982; Luskin and Price, 1983). In addition, the cortical nuclei, the nucleus of the olfactory tract, the periamygdaloid cortex and the medial nuclei, are projections targets of the endopiriform nucleus. (Behan and Haberly, 1999).

Somatosensory projections to the amygdala complex originate mainly from the dysgranular parietal insular cortex, while only few originates from the primary somatosensory areas (Shi and Cassell, 1998). Additional somatosensory information, such as nociceptive information, is transmitted by the medial geniculate and the posterior internuclear nucleus, the pontine parabrachial nucleus and thalamic nuclei (Blanchard and Blanchard, 1972; Blanchard and Collingridge, 1972). The main target of these inputs is the lateral nucleus of the amygdala (Shi and Cassell, 1998; Sah et al, 2003).

Gustatory and visceral information arrive from the anterior and posterior insular cortices and from subcortical structures and both converge in the amygdaloid complex (McDonald, 1998). Projections from the nucleus of the solitary tract ascend by way of the parabrachial nucleus and the thalamic taste/taste visceral relay nucleus. The central nuclei receive both direct projections from the nucleus of the solitary tract and the parabrachial nucleus, while the lateral amygdala receives inputs from the thalamus (Price, 2003; Yasui, Itoh and Sugimoto, 1987).

Auditor and visual inputs arise both from thalamic and high order visual and auditory areas. Cortical acoustic inputs arise from the Te3 while cortical visual inputs arise from the Oc2 area (LeDoux, Farb and Romanski, 1991; McDonald, 1998). The lateral amygdala is the major target of these cortical and thalamic projections but visual inputs terminate also in the central lateral nucleus and the magnocellular basal nucleus (Sah et al, 2003).

The amygdala complex receives afferents from the hypothalamus. The paraventricular nucleus of the hypothalamus contains different populations of magnocellular and parvocellular cells. Those neurons can contain neuropeptides such as oxytocin and vasopressin or neurotransmitters such as acetylcholine, dopamine, TRH, enkephalin, angiotension II and

neurotensin. The projections from the paraventricular nucleus to the medial, lateral and central, amygdala has been mainly associated with oxytocin and/or vasopressin (Pittman, Blume and Renaud, 1981; Pitkänen, 2000)

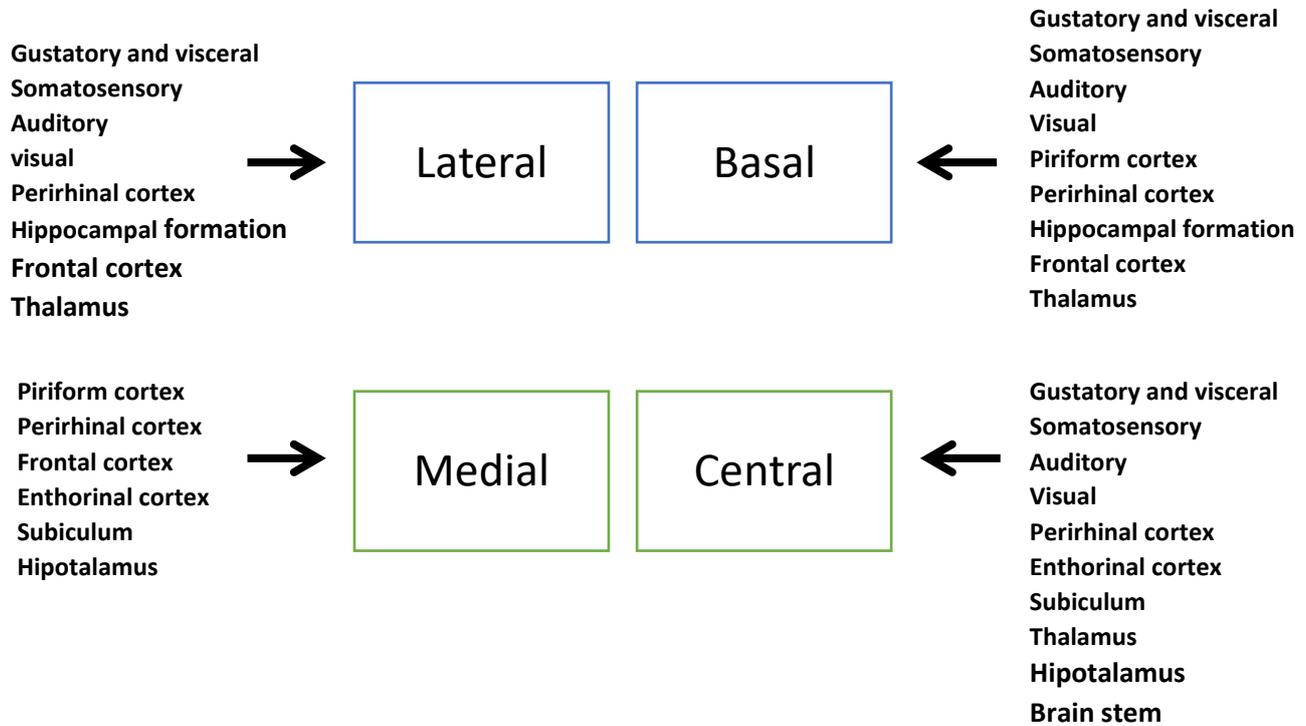


Figure 2.2 Summary of the afferents from the amygdaloid nuclei (Lateral, Basal, Medial and Central). Readapted from Sah et al (2003)

2.4.2 Afferents inputs: Polymodal inputs

The amygdala receives polymodal sensory information from four main areas: prefrontal cortex, perirhinal cortex, entorhinal cortex, parahippocampal cortex and hippocampus (Sah et al, 2003). The prefrontal cortical areas receive inputs from all modalities and many of them are involved in rewarding and behaviours, including social interaction (Uylings, Groenewegen, Kolb, 2003). The primary targets of these dense projections from the prefrontal cortex are the

basolateral nuclei but further afferents reach the accessory basal, the central and the medial nuclei (McDonald, Mascagni and Guo, 1992; Sah et al, 2003).

Amygdala and memory related areas (perirhinal cortex, entorhinal cortex, parahippocampal area and hippocampus) have reciprocal and dense projections (Pitkänen, 2000). The perirhinal cortex provide strong projections to the basolateral nuclei and in lesser extent to the cortical nuclei (Shi and Cassel, 1999). The hippocampus, through the subicular region, sends inputs to most of amygdaloid nuclei but the basolateral amygdala is the main target.

The lateral entorhinal cortex sends most of the monosynaptic projections to the basolateral and the cortical nuclei (Brothers and Finch, 1985; McDonald and Mascagni, 1997). The lateral entorhinal cortex receives strong olfactory inputs from both the olfactory bulb and the piriform cortex and it has been showed that the stimulation of entorhinal cortex deep layers can modulate response to olfactory input in the BLA and to a lesser extent in the piriform cortex (Mouly and Scala, 2006).

In summary, the basolateral amygdala receives all sensory information modalities, including olfactory and somatosensory information, and receives inputs from regions involved in memory, including the hippocampus and the entorhinal cortex, and receives afferents from the prefrontal cortex.

2.4.3 Efferent Connections

The amygdala complex projects to cortical, hypothalamic and brain stem areas.

Cortical efferents from the amygdala are not strong and originate mostly from central and basolateral nuclei. The glutamatergic pyramidal cells in the BLA and in the AB, send

substantial projections to perirhinal cortex, prefrontal and frontal cortex, medial temporal lobe, hippocampus, nucleus accumbens and thalamus (Paré, Smith and Paré, 1995; Petrovich, Canteras and Swanson, 2001; Pitkänen, 2000).

The CeM sends substantial projections to hypothalamus, bed nucleus of the stria terminalis, and to various nuclei in the brain stem, including periaqueductal grey, parabrachial nucleus and nucleus of the solitary tract (Dong, Petrovich and Swanson, 2001; Veening, Swanson and Sawchenko, 1984). These efferents are involved in the response of emotional states, especially fear, and the activation of CeM induces the stimulation of the brain stem areas which control the autonomic and hormonal system (LeDoux, Farb and Romansky, 1991).

The hypothalamus can be divided in the rostral segment containing nuclei involved in ingestive and social behaviours, and the caudal segment containing nuclei involved in exploratory or foraging behaviours (Swanson, 2000). The nuclei in the caudal segment of the hypothalamus are mostly innervated by the medial and capsular division of the central nucleus (Petrovich, Canteras and Swanson, 2001). The medial nucleus, the posterior basal nucleus and the posterolateral cortical nucleus, which are areas receiving several olfactory projections, innervate the rostral part of the hypothalamus which is involved in reproductive behaviour. The medial amygdala projects to oxytocinergic and vasopressinergic neurons in the paraventricular nucleus of the hypothalamus (Petrovich, Canteras and Swanson, 2001). The hypothalamus receives further projections from the bed nucleus of the stria terminalis that is directly innervated from the CeA. The CeA together with the bed nucleus of the stria terminalis also innervate locus coeruleus, substantia nigra, ventral tegmental area, raphe and nucleus basalis (Price, Russchen and Amaral, 1987; Amaral et al, 1992).

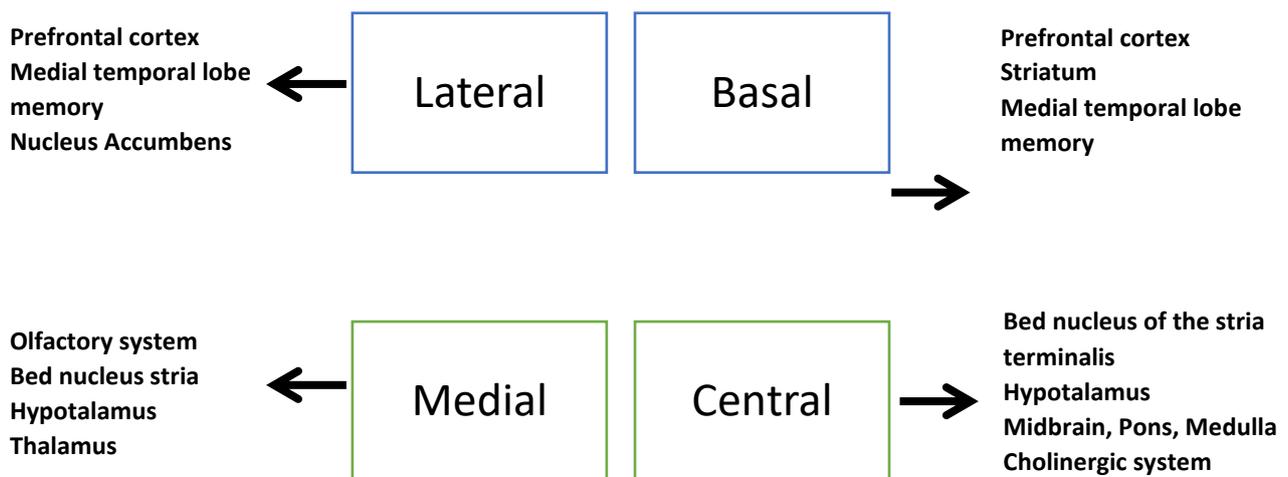


Figure 2.3 Summary of the efferents from the amygdaloid nuclei (Lateral, Basal, Medial and Central). Readapted from Sah et al (2003)

2.4.4 Intra connections in the amygdala

The previous sections suggest high organised projections between cortical and subcortical areas and amygdaloid nuclei: the sensory projections terminate mainly in the lateral amygdala, the entorhinal cortex and the hippocampus afferents mainly terminate in the basal nuclei, and the hypothalamus projections terminate in the central medial, basal nuclei and accessory basal nuclei. However, information entering in a nucleus can be integrated with information entering in a different nucleus. For example, sensory information enters in the basolateral nuclei of the amygdala, where is first processed locally, then integrated with information coming from other sources, such as memory output from the hippocampus, and finally progress to the centromedial nuclei that act as an output station. This integration process takes place thanks to the intra-amygdaloid connections (Pitkänen, Savander and LeDoux, 1997).

Most of the intra-amygdaloid connections originated from the lateral nucleus of the amygdala. LA has high organised rostro-caudal intra-nuclear connections, with the dorsolateral division projecting to the medial division and to the lateral division. The LA lateral division is the entrance for different sensory inputs that are processed in parallel, while the medial division is the entrance of declarative memory inputs coming from hippocampus and entorhinal cortex. The overlap of the projections in the medial division of the lateral amygdala might indicate this could be the site where sensory information is integrated with information coming from past experiences (Pitkänen et al, 1995; Sah et al, 2003). The extra-nuclear connections are descending and are mainly directed to the basal and accessory basal nucleus but lighter projection have been observed also in the capsular part of the central nucleus, the periamygdaloid cortex the central division of the medial nucleus, the posterior cortical nucleus and the lateral division of the amygdalohippocampal area (Pitkänen et al, 1995).

The basal nucleus and the accessory basal nucleus have both rostrocaudal intranuclear connections and extra-nuclear connections. The basal nucleus in rats has three divisions, magnocellular, intermediate and parvocellular. The parvocellular division has heavy reciprocal connection with the magnocellular division and strong connection with the intermediate division, but the intermediate division projects weakly to the parvocellular division (Savander et al, 1995). The basal nucleus extra-nuclear projections are mainly directed to the central and medial nuclei, and to the nucleus of the olfactory tract, the anterior amygdaloid area, the anterior cortical nucleus and the amygdalohippocampal area (Savander et al, 1995). The accessory basal nucleus sends projection to the medial division of the lateral and central nuclei, to the amygdalohippocampal area and to the posterior cortical nucleus (Savander et al, 1995).

Intra-nuclear connections in the central nucleus are unidirectional inputs mainly from the lateral division to the medial and capsular division; the only exception is the light reciprocal projection between the capsular and the medial division. The intermediate division does not project to any of the other divisions of the central nucleus. Intra-amygdaloid inputs from other nuclei are mainly directed to the medial and capsular division while the projections back to the other nuclei are meagre. Extra-amygdaloid information from cortical and subcortical sources enters in the lateral divisions. The lateral division is also the main output to the other brain area. This dense intra- and extra-nuclear network in the central nucleus, specifically in the lateral division, suggests that this area may integrate different sources of information entering in the central nucleus. In addition, the weak projections to the others amygdaloid nuclei suggest that the central nucleus executes the responses evoked by the other amygdaloid nuclei that innervate the central nucleus (Jolkkonen and Pitkänen, 1998)

Chapter 3: Anatomy and Connections of the Piriform Cortex

3.1 Anatomy

The piriform cortex (Pir) is the largest area of the primary olfactory cortex that extends along the ventral and lateral surface of the forebrain (Neville and Haberly, 2003). Although from a cytoarchitectonic point of view the piriform cortex seems homogeneously organised, it is divided into an anterior and posterior part. This division is mainly based on the thickness of layer III and the presence of the overlying lateral olfactory tract which runs along the surface of the anterior piriform cortex (Luskin and Price, 1983; Löscher and Ebert, 1996). Ekstrand et al (2001), found the evidence of a third area in the piriform cortex called ventrorostral anterior piriform cortex that has cytoarchitecture, immunocytochemical markers and connection that differs from the anterior piriform cortex

3.1.1 Layers and cellular morphology

While neocortical areas are six-layered, the piriform cortex, like others phylogenetically old structures have a trilaminar organization (Haberley and Price, 1978). Layer I, the plexiform layer, contains the pyramidal cell apical dendrite from deeper layers, and very few interneurons or globular cells. Layer I can be further divided into sublamina Ia, that is the most superficial part of the piriform cortex, containing the projections from the olfactory bulb, and Ib that contains the afferents from other neurons in the piriform cortex and from others olfactory cortical areas (Löscher and Ebert, 1996). The two sublamina differ for the origin of the fibre

that take synaptic contact in this area: in the sublamina Ia cells contact afferents from the olfactory bulb, in sublamina Ib cells synapse with extrinsic fibres from the primary olfactory cortex. Sublamina b also contains intrinsic cortico-cortical association fibres (Price, 1973). The layer II is a compact layer that mostly contains pyramidal cell bodies. Pyramidal cells are glutamatergic and represent the predominant cell type in the piriform cortex and their cell body is predominant in lamina IIb. Other cells have been observed in this layer, such as the semilunar cells that are pyramidal-like cells lacking of basal dendrites, mostly observed in sublamina IIa and the glutamatergic globular cells (Haberly and Feig, 1983, Martinez et al, 1987). Layer III contain a moderate dense amount of pyramidal cell bodies and association fibres (Litaudon, Datiche and Cattarelli, 1997). In this layer have been observed pyramidal cells, which gradually decrease in number with depth, GABAergic multipolar cells, which on the contrary are homogeneously distributed, and globular cells (Haberly et al, 1987; Haberly, 1983). Adjacent to layer III is the endopiriform nucleus also referred as layer IV since it overlies rostro/caudally along the all dimension of the piriform cortex and contract numerous reciprocal connection with this area (Krettek and Price, 1977). The dorsal region of the endopiriform nucleus is mainly characterised by packed multipolar cells (Tseng and Haberly, 1989) while the ventral region has more diffusely arranged cells (Krettek and Price, 1977).

3.2 Afferent and efferent connections

3.2.1 Connections with the olfactory structures

The piriform cortex is a unique part of the brain involved in olfactory experiences. In fact, the Pir has extensive connections with other regions which are part of the olfactory network, including anterior olfactory nucleus, olfactory tubercle, lateral amygdala nucleus,

periamygdaloid cortex, orbitofrontal cortex, insula cortex and the anterior part of the entorhinal cortex (Carmichael, Clugnet and Price, 1994; Shipley and Reyes 1991). While other cortical regions receive olfactory information through a thalamic pathway, the piriform cortex receives projections directly from the olfactory bulb via the lateral olfactory tract (Price, 1973). **Figure 3.1** shows a general scheme of how olfactory information is transferred from the main olfactory bulb to the piriform cortex and the other structures.

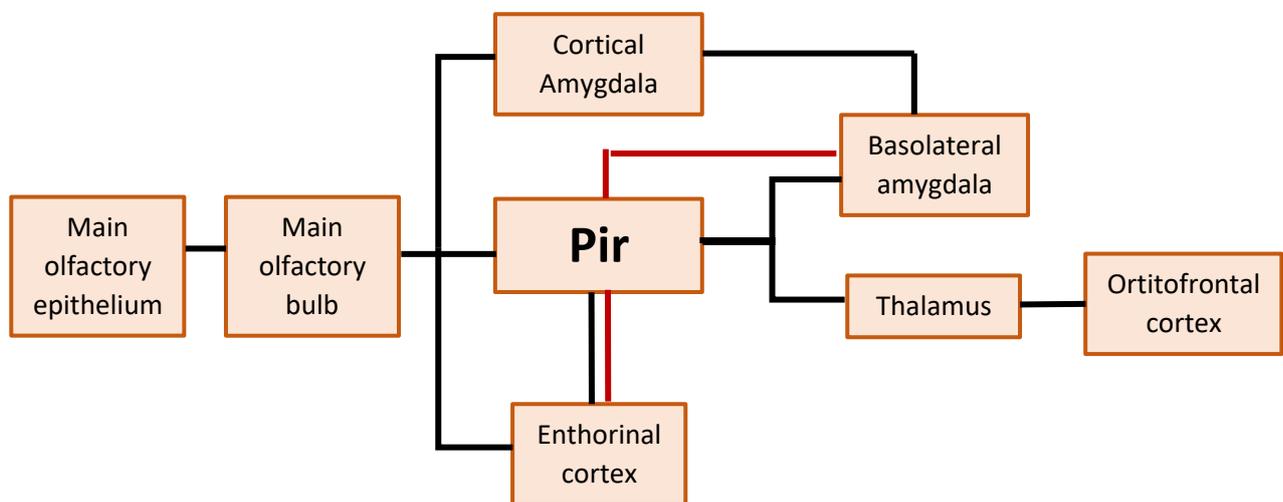


Figure 3.1. General scheme of the connections of the piriform cortex with other brain structures.

3.2.2 Connections with the olfactory bulb

The pyramidal cells in the piriform cortex receive direct axonal projections from the mitral cells of the olfactory bulb (Price and Powell, 1970). Mitral cell axons terminate exclusively in layer Ia of the Pir Ctx spreading across the entire surface without a proper spatial pattern (Luskin and Price, 1982). Rennaker and colleagues (2007), using single cell recordings,

observed that various pyramidal cells, dispersed along the anterior piriform cortex, fired to a same given odour. These results are supported by c-fos studies revealing that a single odourant activates pyramidal cells widely distributed without an obvious topographical organization (Illig and Haberly, 2003). However, tracing experiments show that mitral projections are not completely broad (Scott et al, 1980), in fact a small area of the olfactory bulb projects broadly to large areas in the piriform cortex, and at the same time each small area of the piriform cortex receives inputs from broadly distributed areas in the olfactory bulb. Thus, a single mitral cell has synaptic contacts with different pyramidal cells and a single pyramidal cell receives synaptic information from more than one mitral cell (Litaudon, Datiche and Cattarelli, 1997). These synapses show an asymmetric organisation typical of glutamatergic excitatory synapses (Collins and Howlett, 1988). The Tufted cells in the olfactory bulb appear to project only to a specific region of the piriform cortex: the ventrorostral anterior piriform cortex (Ekstrand et al, 2001).

Both the anterior and the posterior piriform cortex project back to the olfactory bulb. These projections provide a feedback control in fact they terminate mostly on GABAergic granule cells interneurons which will control olfactory bulb excitability. Luskin and Price (1983) using different retrograde tracers observed that the posterior piriform cortex produced a large number of labelled cells in all the olfactory bulb, in particular in the medial and lateral parts where mitral cells are densely packed (Luskin and Price, 1983). On the contrary, the posterior piriform cortex projects back to a very small number of tufted cells. Luskin and Price (1983) pointed out that, depending on the tracers used, the anterior piriform cortex produced few or substantial labelled cells in the anteromedial part of the olfactory bulb. Even if previous studies claimed a random input organization (Ghosh et al, 2011), in a new study, adjacent neurons in the Pir have

be found to project to similar regions of the granule cell layer of the olfactory bulb showing a spatially clustered pattern (Padmanabhan et al, 2016).

3.2.3 Connections with the amygdala

The unique reciprocal connections between the amygdala and the piriform cortex may have the role to incorporate hedonic or associative information with the representation of odours (Illig and Wilson, 2009). Projections from the amygdala to the piriform cortex have been traced in different studies (Majak and colleagues, 2004; Canteras et al, 1992; Luskin and Price, 1983; Petrovich et al, 1996). Only selective nuclei of the amygdala project to the piriform cortex. The most predominant projections originate from the basolateral nuclei, especially from the medial rostral half of the medial division of the lateral nucleus. The projections from the basal nucleus are from the magnocellular division and they terminate in layers II and III of the anterior and posterior Pir (Majak and colleagues, 2004). The magnocellular division and the rostral aspect of the parvocellular division of the accessory basal nucleus terminate in the ventral aspect of the anterior and posterior piriform cortex. More projections to the anterior and posterior Pir are provided by the periamygdaloid cortex, specifically from the periamygdaloid and sulcus subfield (Majak and colleagues, 2004; Luskin and Price, 1983). The posterior cortical nucleus and in less amount the anterior cortical nucleus, provide moderate inputs to the posterior piriform cortex (Majak and colleagues, 2004; Canteras et al, 1992). The amygdalohippocampal area instead sends very light projections to the piriform cortex (Majak and colleagues, 2004; Canteras et al, 1992). The most substantial projections terminate in the posterior Pir while projections to the anterior Pir are light in density. Projections all terminate in Pir layer Ib and III, the only exceptions are projections from the basal nucleus that terminate in layers II and III.

The piriform cortex projections back to the amygdala are mostly sent from the posterior piriform cortex (Haberly and Price, 1978).

3.2.4 Connections with the entorhinal cortex and the hippocampus

Pyramidal cells in the piriform cortex send direct projections to both lateral entorhinal cortex and ventral subiculum via the lateral olfactory tract (Luskin and Price, 1983). Both anatomical and electrophysiological studies have shown that the entorhinal cortex receives olfactory information not only from the piriform cortex, but also directly from the olfactory bulb. Furthermore, the lateral entorhinal cortex projects back to the posterior piriform cortex and to the endopiriform nuclei (Luskin and Price, 1983), receive projections and project back to the hippocampus and projects to the amygdala (Yatrasmitraaiv et al , 2003). On this purpose, Mouly and Scala (2006), showed that paired-pulse stimulation of the entorhinal cortex resulted in a short interval duration inhibition of pyramidal cells in anterior and posterior piriform cortex and basolateral amygdala. Thus, the lateral entorhinal cortex transmits olfactory information from the piriform cortex to hippocampus and amygdala and back to the piriform cortex (Swanson and Kohler, 1986; Insausti, Herrero and Witter, 1997).

3.2.5 Connections with other structures

The piriform cortex has further connections with structures not directly related to olfactory processing such as thalamus, hypothalamus and neocortical areas but that still offer a significant contribution to areas involved in processing olfactory information.

The Pir is directly connected to the lateral hypothalamus. The connections originate mostly from the endopiriform nucleus and the deepest layer of the Pir, while very few inputs originate from cells in layer I that receives direct information from the olfactory bulb (Price, 1995).

The most ventral part of the piriform cortex is connected in a triangular system with the mediodorsal thalamic nucleus and the neocortex. The Pir sends inputs to the central aspect of the mediodorsal thalamic nucleus, from which olfactory information is transferred to the olfactory-related neocortical areas, to the lateral orbitofrontal cortex and to the posterior agranular insular cortices. The neocortical areas send inputs in return to the mediodorsal thalamic nucleus. Since the mediodorsal thalamic nucleus receives inputs from the piriform cortex and the neocortex, this nucleus represent the designed area where olfactory and neocortical pattern can be simultaneously compared (Litaudon, Datiche and Cattarelli, 1997; Ray and Price, 1992).

The piriform cortex has reciprocal connections with the frontal cortex that goes beside the trans-thalamic pathway mentioned above. Datiche and Cattarelli (1996) found that the Pir is topographically organized, with the anterior piriform cortex projecting and receiving back fibres in the ventrolateral and lateral orbital areas and the anterior part of the agranular insular cortex; and the posterior piriform cortex directly connected with the infralimbic area and the posterior of the agranular insular cortex (Datiche and Cattarelli, 1996). Interestingly, the insular cortex receives a direct projection from the piriform cortex and the mediodorsal thalamic nucleus. In addition, the caudal part of the Pir is directly connected with the infralimbic cortex which may be involved in the elaboration of emotional and visceral responses to olfactory cues (Litaudon, Datiche and Cattarelli, 1997)

3.3 Intra connections in the piriform cortex

The highly organized connective circuitries in the piriform cortex, provide a pathway by which the representation of a stimulus becomes distributed in parallel to almost all the Pir. In contrast to the other cortical areas where each stimulus has a topic projection, inputs to the Pir are rather diffuse. Axons running to the lateral olfactory tract contact a large number of spatially distributed cells and at the same time, pyramidal cells in the Pir take contacts with a large number of other cells distributed in all cortex. This circuit is high organised with the activation proceeding from anterior to posterior and from the surface to the deeper layers (Löscher and Ebert, 1996). Once the olfactory bulb axons in the lateral olfactory tract are activated by odorant cues, pyramidal cells, in the piriform cortex's layers II and III, and horizontal cells in the piriform cortex's layer I, receive excitatory inputs (Hasselmo and Bower, 1990; Tseng and Haberly, 1989). Pyramidal cells axons, which run along layer III, provide excitatory collateral inputs to surrounding GABAergic multipolar cells and to apical dendrites of distant pyramidal cell in layer Ib (Luskin and Price, 1983; Haberly and Presto, 1986). Multipolar cells in layer III and horizontal cells in layer I make contact with the dendrites or the somata of pyramidal cells in layer II or contact other GABAergic cells and inhibit their activity (Haberley et al, 1987; Haberley and Fig, 1983). Those cells are GABAergic and make contact with pyramidal cells to modulate the firing rate through GABA_A and GABA_B receptors. GABA_A receptors are located in the pyramidal cells' somata and provide a powerful inhibition, GABA_B receptors are found in pyramidal cells' dendrites and they provide a slow inhibition (Haberly et al., 1987; Tseng and Haberly, 1988). Horizontal cells are directly activated by the olfactory bulb and they are thought to provide feedforward inhibition while multipolar cells are activated by other pyramidal cells and they are probably involved in feedback inhibition (Haberly, 1990; Löscher and Ebert, 1996).

Chapter 4 The Neurobiology of Social Recognition

4.1 Social recognition

Social recognition memory of conspecific identity is crucial to establish social relationships and to form stable social groups. This makes social memory a prerogative for all organisms living in a complex social system requiring the ability to recognise friends from predators, mates from intruders, offspring from strangers, and in monogamous species it is required to form the pair bond (Paul, Corsello, Tranel, & Adolphs, 2010). The term social memory includes a variety of type of learning and memory probably sub-served by distinct anatomical and neurochemical circuits in the brain. This chapter will focus on the social recognition of conspecifics, the social memory related to the storage of information about specific individuals which allows the identification of the same upon subsequent encounters. To a large extent this chapter will summarize the key findings obtained with the social recognition paradigms in rodents, including the factors that modulate the formation and expression of this type of social memory, and the neurobiological mechanisms involved.

4.2 Species mechanism to encode social information

According to Ferguson, Young and Insel (2002), social information encoding may be influenced by different sensory cues in different species. For example, humans and nonhuman primates base social recognition mostly on visual and auditory signals. In the human and monkey brain, the temporal lobe represents a critical visual association region to recognise

faces. Indeed, neurons of the temporal lobe in both the species appear to differentiate faces from objects and from novel and familiar stimuli, (Fried, Katherine et al, 1997; Gothard, battaglia et al, 2007; Leonard, Rolls, Wilson and Baylis, 1985). Always in the temporal lobe (amygdala), Mosher, Zimmermand and Gothard (2014) identified a set of neurons in monkeys that selectively responded to fixations on the eyes of other monkeys and to eye contact. Even if there are evidence that even monkeys and humans likely use olfaction in social recognition, (Porter et al, 1986; Holand and Schleidt, 1997); in rodents social recognition encoding relies mainly in this sensory modality (Paul, 2010; Dantzer et al, 1990; Popik et al, 1991).

Rodents are an example of olfactory orientated mammals, where social recognition memory relies largely on odours, even if auditory and visual cues may have important influence. The two different olfactory systems (main olfactory system and accessory olfactory system) adapted to for discriminating volatile odorants and non-volatile odorants that may convey information related to sex, age, and identity and hierarchy position of the individual. Thor (1979) suggested that in rats two different odours could determine the identity of a conspecific: the “individual odour” and the “androgen odour”. The first is determined by the biochemistry and by the diet; a mix of volatile endogenous metabolites secreted with the urine that could impart an individual specific odour (Singh, Brown and Roser, 1987). The second type of odour is the “androgen odour” a pheromone related odour that appears with puberty and could be associated with social dominance in male rodents. The role of olfaction in providing the main salient cues for social recognition has been confirmed by many following studies. For example, in female prairie vole, a highly social species which exhibit a monogamous mating system, the lesion of the vomeronasal organ disrupt mate-induced pair bonding (Curtis et al, 2001). In rats, vomeronasal lesions result in only temporary loss of the ability to recognize a familiar individual (Bluthe and Dantzer, 1993), suggesting that more than one path is involved in social

memory, while lesions of the olfactory bulb blocked social recognition (Dantzer et al, 1990). Similarly, Popik et al (1991) assessed social memory manipulating the amount of olfactory information. The social recognition memory was impaired in anosmic rats and by the use of washed animals as social stimuli. Washing the social stimuli seemed to diminish the olfactory characteristics, thus reducing social memory formation. Finally, urine or soiled bedding alone is sufficient to form a memory for an individual signature scent (Sawyer et al, 1984). All these results confirm the importance of the nature of olfactory cues in social recognition of a conspecific.

Although olfaction is without doubt one of the most important sensory cue in rodent social recognition, it has been reported that the ultrasonic vocalization, emitted and heard by mice and rats, may as well be involved in this form of memory. Ultrasonic vocalizations appear to be important in different aspects of rodent social interaction including the mother-offspring interaction, sexual arousal, bonding, and even to communicate social hierarchy (Hofer, 1996; Bowers et al, 2013; Brudzynski, 2009; Wesson, 2013). The relevance in social recognition memory is that vocalizations may have an individual variability important for the individual identification. A sex difference have been already observed in pups, where males separated from their mother, emit a larger number of ultrasonic vocalization which are lower in frequency and amplitude when compared to female pups' vocalization. This has been suggested having a role in pups retrieval since mothers tended to retrieve first male pups and then female pups (Bower, et al 2013). Furthermore, ultrasonic vocalization may even been used as index of social memory in female mice, since the number of vocalization emitted tend to decrease when mice are re-exposed to a familiar conspecific (Moles, 2007). The spectrographic analysis of the ultrasonic vocalization may in future be relevant to improve the quality of the rodent social memory testing.

4.3 Social recognition Paradigms

Social recognition memory can be assessed in the laboratory due to the natural tendency of rodents to investigate an unfamiliar conspecific over a familiar one. Most of the tests used today to investigate short-term social recognition capacities are variants of the habituation/dishabituation paradigm proposed by Thor and Hollaway (1982). Generally, the social stimuli used in the social recognition test are unrelated juveniles or ovariectomised (OVX) females belonging to the same strain. This offers the advantage of avoiding aggressive and sexual behaviour between animals, even if the use of juvenile stimuli is inconvenient for long-term studies of social recognition (Ferguson, Young and Insel, 2002). In the habituation/dishabituation paradigm, the experimental animal is first exposed to an unfamiliar social stimulus or its partial cues (urine or soiled bedding). This phase, called habituation phase, is followed by a second or multiple re-exposure to the same social stimulus, now familiar, where recognition memory is indicated by a reduction in investigation time. This less amount of time spent investigating the same social stimulus in the re-exposure trials, cannot simply represent habituation to the presence of a social stimulus. In fact, in a third phase, the dishabituation phase, the experimental animal is exposed to a novel conspecific which is supposed to trigger an intense investigation indistinguishable from the habituation trial. The different duration time spent in social investigation between the first and the second encounter with the same social stimulus or between the encounter with the familiar stimulus and the novel stimulus, is used as index of social recognition memory. The duration of interaction and the number of repeated trials and the interval between the exposures of the experimental animal to the same individual are the variables that can be manipulated to investigate treatments that are able to prolong or inhibit the normal recognition response (Ferguson, Young and Insel, 2002).

Another variant of this social recognition test is the social discrimination paradigm (Engelmann et al, 1995). Like in the habituation/dishabituation test, this paradigm comprises a first phase (habituation phase) where the experimental animal is first exposed to an unfamiliar conspecific. In the second phase, the experimental animal is simultaneously exposed to two stimuli animals, the now familiar stimulus, which was presented in the habituation phase, and a novel stimulus. This variant of the social recognition test has several advantages: first, there is a reduction in the number of sessions for a given experimental series that may affect the motivation for social investigation; second, the simultaneous presentation of both the familiar and novel conspecifics offer an internal control; and finally, in pharmacological studies, this version of the task allows for the separation memory-related effects from other behavioural effects (Gabor et al, 2012). In some cases, the animals are left to freely engage in the apparatus (home cage, or a familiar environment different from the home cage), whereas in other studies, the experiments is set up in a three-chamber apparatus where the social stimuli are restricted in cups. The restriction of the stimuli animal simplifies the analysis of the preference measurements, however the experimental animal does not get direct access to the social stimuli, and this may be a disadvantage because direct body contact and sniffing may be necessary to properly evaluate social recognition memory. (Engelmann et al, 2011).

4.4 Factors that may influence social recognition in rodents

Many factors tend to influence the strength of memory in a social recognition paradigm: species, sex, age and stress.

Memory performances are species dependent and it is well known that in a social recognition paradigm, mice have a long-term memory far superior in comparison to rats. In fact, mice can

remember familiar conspecifics for days after a single 5 min exposure (Kogan et al, 2000, Ritcher et al, 2005). On the contrary, rats' social memory appears to last for only 30-60 min after a single 5 min encounter (Popik, Woltering et al, 1991); but the long-term consolidation can occur after 1-hour exposition with another juvenile (Gur, Tendler and Wagner, 2014). The nature of this difference in memory retention may be related to differences in olfaction between rats and mice. Noack et colleagues (2010) exposed mice and rats first to repeated trials with the same conspecific juvenile, then to volatile and non-volatile fraction of the juvenile olfactory signature. Interesting in mice, but not in rats, the presentation of the only volatile component of the juvenile conspecific scent was sufficient to form short- and long-term recognition memory. Furthermore, the exposure to a juvenile conspecific in mice increased the activation of both the main and the accessory olfactory bulb. Rats instead, needed the access to the non-volatile fraction of the olfactory signature of the social stimulus itself to form at least the short-term memory for the social stimulus. This has been proven by the fact that the social interaction with a juvenile conspecific increased the activation only in the accessory olfactory bulb where the processing of non-volatile odorants supposedly occurs. In few words, differences between the two species appear to be based on differences in processing volatile and non-volatile olfactory cues of the social stimulus.

Even if the majority of studies in social recognition memory involves male subjects which create a general limitation in understanding the biological basis of social recognition, (Beery and Zucker, 2011), it has been shown, at least in rats, that there are indeed sex differences in social recognition memory. Females rats, for example, exhibit lower levels of baseline investigatory behaviour. In fact, despite females showing a strong interest in juvenile social stimuli, the investigatory behaviour of female rats are shorter when compared to the investigatory behaviour of male rats (Bluthé and Dantzer , 1990; Dumais et al, 2016). However,

females displayed stronger social recognition than males. In fact, male rats appear to remember a familiar conspecific approximately for 45 minutes, while in female rats, social recognition memory lasts up to 120 minutes. Even if sex-differences in social recognition appear not to be influenced by the ovarian cycle, since females in any phase of the oestrus cycle show a better social recognition memory compared to males, estrogen may be involved in social memory. Female mice lacking the estrogen receptors (α and β) showed social memory impairment (Choleris et al, 2003). Similarly, the estrogen supplement in ovariectomised mice improved social memory (Tang et al, 2005). Clearly, the neurobiological differences between male and female rodents appear to contribute to the differences in social recognition performance.

There are numerous studies in rodents which show how aging is accompanied by a decline in various learning performances. In a similar way, social recognition memory is influenced by aging, in both rats and mice (Terranova et al., 1994). In fact, 22-24 months old rats show reduced social recognition of juvenile or ovariectomised females compared to 3 months old rats (Guan and Dluzen, 1994; Prediger et al., 2006). So also aging, together with the factors mentioned above, influence social recognition memory, however, it is plausible that the list is not restricted to these factors.

4.5 Mechanisms underlying social recognition: Brain areas involved in social recognition

Pharmacological treatments, lesion studies, immediately early genes expression or optogenetic activation have been paired with the habituation/dishabituation paradigm and its variants to underlie the involvement of specific brain regions in social memory.

Despite its variability, individual recognition in rodents as well as in most mammalian species is determined by the contribution of different brain regions which include many limbic areas:

amygdala, hippocampus, piriform cortex, lateral septum (LS), nucleus acumbens (NAc), bed nucleus of stria terminalis, and several hypothalamic areas (Ferguson et al, 2001; Samuelsen and Meredith,2011, Gothard, battaglia et al, 2007)

4.5.1 Role of the hippocampal formation in social recognition

Since social recognition is a type of memory, successful memory trace retrieval has to be based on intact acquisition of new information. C-Fos is an immediate early gene that is normally transcribed in response to neuronal activation, and thus conveniently used in immunohistochemistry studies to highlight the activation of specific brain regions following a behavioural test, in this case, social recognition paradigm. The first social encounter with an unfamiliar social stimulus represents the acquisition phase of social recognition. C-Fos studies performed immediately after the first encounter in rodents revealed an overlapping of c-Fos activation patterns in different brain regions: as expected main and accessories olfactory bulb (MOB and AOB), since as said before social interaction in rats and mice is mainly based on odorant cues, lateral septum, medial nucleus of the amygdala (MeA), medial preoptic area and piriform cortex (Ferguson et al., 2001; Samuelsen and Meredith, 2011; Ritcher et al., 2005; Engelmann, 2009). All these results concur to suggest the lack of involvement of the dorsal hippocampal areas.

Similar results have been confirmed by cytotoxic lesions confined in the hippocampus. In rats, lesions of both the dorsal and the ventral hippocampus, including the four CA subfields and the dentate gyrus, did not affect social recognition memory after 30 minutes following the exposure to a juvenile conspecific stimulus (Bannerman et al., 2001; Squire, 2006). However, other areas of the hippocampal formation could support this form of memory. In fact,

Bannerman et al., (2002) showed that retro-hippocampal lesions which included the entorhinal cortex and the subiculum, produced mild social recognition memory impairment in rats. Similarly, transections of the fimbria, which convey projections to and from the hippocampal formation, impaired social recognition memory and this is accompanied by a lack of social interest (Maaswinkel et al., 1996).

Despite the fact that some previously cited studies in rats failed to show any contribution of the hippocampus to social memory (Ferguson et al., 2001; Samuelsen and Meredith, 2011; Bannerman et al., 2001; Squires, 2006), studies in mice elicited that the role of the hippocampus is not only limited to processing spatial information, but also social information. Kogan et al., (2000), for example, found that bilateral lesions of the hippocampus in mice do not affect short-term social recognition memory but it disrupts social recognition at 30 min after the first encounter.

The discrepancy in the results discussed above, clearly challenges the interpretation and the understanding of the role the hippocampus plays in social recognition memory. A possible interpretation is that the hippocampus and its different subareas may only be temporally involved in the social memory process, which like other types of memory includes acquisition, consolidation and retrieval of the information. These different phases in social memory processes are dependent on two stages of the protein synthesis, one starting 3 hours after training and one starting 6 hours after training (Richter et al., 2005; Kogan et al., 2000) and the different hippocampal subareas may only give a specific time-dependent contribution in the memory phases. In fact, different studies show the involvement of two selective hippocampal subareas in social recognition, CA1 and CA2 (Phan et al., 2012; Uekita and Okanoya, 2011; Zinn et al., 2016; Stevenson and Caldwell, 2014; Hitti and Siegelbaum, 2014). The CA2 appear

to be critical in social recognition since the inactivation of CA2 pyramidal cells caused a critical loss in social memory leaving intact other form of memory like spatial and contextual memory (Hitty and Siegelbaum, 2014). The dorsal CA1 region seems to play a major role in the consolidation of social information, and the CA2 region serves as a link between the CA1 and the CA3, suggesting that probably all the hippocampus is involved in social recognition (Zinn et al., 2016; Sekino et al., 1997).

At a first look, the findings in the hippocampus show a certain discrepancy, but taken together those data may suggest that a certain brain region may be only temporally involved in acquisition, consolidation and retrieval encoding of social recognition (Kooji and Sandi, 2012), and the hippocampus appears to mainly play a role in memory consolidation of social recognition memory (Cammorata et al., 2008).

4.5.2 Role of amygdala in social recognition

According to Cammorata and colleagues (2008), the hippocampus works in parallel with the amygdala to process many types of memory information. The role of the amygdala in social memory can be easily explained by anatomical data. First, the olfactory information, which is the salient cue in social recognition, has unique direct access to the amygdala, which seems to act as a major site for the integration of both the main and the accessory olfactory pathway (Price, 1973; McDonald, 1998). In fact, the amygdala receive volatile olfactory information through the olfactory bulb and the olfactory piriform cortex (Mouly and Scala, 2006) and non-volatile olfactory information directly from the vomeronasal organ (Kevetter & Winans, 1981). Finally, the amygdala directly projects to the ventral and dorsal hippocampus and vice versa, modulating different aspects of social rodent behaviour, such as social interaction with a

conspecific (Felix-Ortiz and Tye, 2014; Katayama et al., 2009; Kirkpatrick et al., 1994; Wang et al., 2006). Thus, it is not surprising that different studies showed an overlapping c-Fos activation of the amygdala after a first exposure with a OVX female or a juvenile (Ferguson et al., 2001; Samuelsen and Meredith, 2011; Ritcher et al., 2005; Engelmann, 2009; Ophir et al., 2009).

Two of the amygdala nuclei appears to play a major role in social recognition: the medial amygdala and the basolateral amygdala. Samuelsen and Meredith (2011) found that the exposure to any biologically relevant stimulus, such as female urine or cat collar, can result in a general pattern of immediate-early gene expression in the MeA of male mice, but only the exposure to the female conspecific urines resulted in activation of the dorsal posterior MeA. Ferguson and colleagues (2001) using c-Fos immunoreactivity in wild-type mice found that the social recognition task, among other areas, induced the activation of the cortical amygdala and the medial amygdala. This activation of the medial amygdala was not observed in oxytocin knock-out mice suggesting that the role of the MeA in social recognition may be dependent on oxytocin, a prosocial neuropeptide. The results obtained in mice are comparable to the ones in hamsters, where the MeA exhibited differential c-Fos expression depending on the category of the stimulus, reproductive signals or conspecific competitive/territorial signals (Meredith and Westberry, 2004).

However, lesion studies appear to show some discrepancy between species. For example, a lesion study in female hamster, showed that MeA was critical in discriminate opposite-sex odour but not same-gender odour (Petrulis et al., 1999). On the opposite, MeA lesions in male mice impaired social recognition for a same-gender conspecific, and reduced oxytocin, a neuropeptide involved in social interaction in hypothalamus and plasma (Wang et al., 2014).

Perhaps, this discrepancy may be explained by differences in the location of the lesion. In fact, Maras and Petrulis (2006) showed there are functional differences between the anterior MeA and the posterior MeA, even if both type of lesions eliminated the preference for the opposite-sex odours in male hamsters. In fact, while selective lesion of the anterior MeA increased investigation for the conspecific odours independently by the sex, the lesion of the posteriodorsal MeA reduced the investigation only for opposite-sex odours. Considering that this unusual behaviour following the lesion of the posteriodorsal or anterior MeA was not caused by deficits in odour discrimination, taken together, these results suggest that while the posteriodorsal MeA may regulate the motivation to approach a sexual stimulus, the anterior MeA may have the role to direct this motivation toward an appropriate target (Petrulis, 2009).

In mice, the involvement of the MeA in social recognition has been proved also by electrophysiological and pharmacological studies. Using single cell recordings in anaesthetised mice, Bergan and colleagues (2014) observed similar neural responses to female and male urine in the olfactory bulb, but the opposite-sex conspecific urine induced a higher response in the medial amygdala (Bergan and colleagues, 2014). The MeA appear to be involved in processing the non-volatile information of a conspecific, as suggested by a study where lidocaine was directly injected into MeA (Noack et al., 2015). Interesting, when the injection was performed before the retrieval phase of a social discrimination task, social recognition was impaired. Instead when the lidocaine was injected before the learning session (acquisition phase), the animal showed intact social recognition memory. This data may suggest that the MeA is not involved in the acquisition phase of the conspecific odour information (Noack et al., 2015).

The basolateral amygdala has been investigated considerably less than the MeA, even if there are numerous evidences that suggest the involvement of the basolateral amygdala (BLA) in

social interaction. For example, Felix-Ortiz and Tye (2014) showed that the inhibition or the stimulation of the amygdala axon terminals in the ventral hippocampus respectively increased and decreased social interaction in the resident intruder test and in the three-chamber sociability test. Similarly, Katayama and colleagues (2009) showed, in rats, the involvement of the BLA in social interaction, but in their study the firing activity was positively correlated with the amount of social interaction with another conspecific. However, how the BLA is involved in social interaction is still unknown, and only few studies in rodents tried to understand if the BLA is involved in social recognition of a conspecific, and lesion studies appear to be contradictory. For example, Maaswinkel and colleagues (1996) using a typical habituation/dishabituation social recognition test, reported that lesions of the BLA in rats did not impair the ability to recognise a familiar from a novel conspecific. On the contrary, in mice, disruption of either medial or basolateral amygdala, impaired social recognition of conspecific but not flavour recognition (Wang et al., 2014), indicating a specific role of both these nuclei in social memory but not general olfactory recognition. At a first look, this may suggest that the contradictory results in this two lesion studies could be in part due to species differences, rats in the first study and mice in the second one. However, a recent study in rats reported that the local administration of a protein synthesis blocker to the BLA completely blocked long-term social recognition memory with no effect on short-term memory (Zinn et al., 2016). The main difference between the two studies in rats (Maaswinkel et al., 1996; Zinn et al., 2016) appear to be the length of the familiarization period and of the inter-trial period between the first encounter and the testing phase. In fact, while in the Maaswinkel and colleagues study the habituation phase lasted only 5 minutes and the testing animal was reintroduced to the same familiar animal or a novel animal after a short period of time (5 min), in the case of the Zinn's study, the familiarization phase lasted for 1h and the animals were tested immediately after the first encounter, short-term memory, and the day after the first encounter, long-term memory.

In few words, the two studies found the same results: BLA is not involved in the short-term social recognition memory but Zinn and colleagues reported the involvement of the BLA in the long-term social recognition memory.

4.5.3 Role of the piriform cortex in social recognition

The piriform cortex is another area where neuronal activation have been observed following an encounter with a conspecific (Ferguson et al, 2001; Richter et al, 2005) and could have a relevant role in social recognition of the conspecific identity. The piriform cortex receives direct projections from the main olfactory bulb (Chapuis et al, 2013; Wilson and Sullivan, 2011), and it is directly involved in odour cues processing (Price and Powell, 1970; Chapuis, 2013; Wilson and Sullivan, 2011). This means that the piriform cortex is involved also in processing olfactory cues for the recognition of conspecific. In humans, olfactory cues alone are sufficient for the recognition of other members of our species suggesting that olfaction may supplement visual and auditory cues used in human conspecific like kin recognition. In fact, adult human kin can accurately discriminate between the odours of their sibling even after 1-30 months separation, and recognise their own odour and the one belonging to their partner when presented together with the odour of a stranger of the same sex and age (Porter et al, 1986; Holdand Schleidt, 1977; Russell, 1976). These evidences show how smell alone is sufficient to recognise the familiar smell of another individual of the same species; the main question is to understand if the piriform cortex is simply processing odorant information or it may be involved in the more complex circuits of social recognition memory. To explore this concept, it is important to understand the connection of the piriform cortex with other brain regions.

The strong connection between piriform cortex and amygdala suggests that the role of the piriform cortex cannot be only related to odour processing. Kondoh et al, (2016) showed that a small area comprising less than 5% of the piriform cortex, called the amygdalo-piriform transition area, contained neurons which, following the exposition to the scent of a predator, could activate CRH-neurons and induce increase in blood-stress hormones. The further involvement of the piriform cortex in more than odour processing has been shown by other recent studies. The piriform cortex, together with the amygdala has also been shown to be part of the neural circuits of lactating rat brain. In fact, suckling-induced release of oxytocin shows the activation of the piriform cortex (Febo and Ferris, 2014) showing a close functional interaction between oxytocin and olfactory functions.

The piriform cortex is strongly connected with input and output fibres to the hypothalamus. The paraventricular and supraoptic nuclei of the hypothalamus releases oxytocin and vasopressin responsible for modulating a wide range of functions and emotions, and is involved in social recognition memory. On this point, there is a relevant article recently published in the *Journal of Neuroscience*, which identified regions where the pro-social neuropeptide oxytocin may influence maternal behaviour (Mitre et al, 2016). One of the most interesting aspects is that the piriform cortex was the only area where female rats differed from male rats in the expression of oxytocin receptors. The second interesting aspect is that the differences between male and female was observed also in virgin females suggesting that the increased expression of oxytocin receptors compared to males is not only observed during the maternal-transition period. These data acquire even more relevance when considering that female rats have a better social recognition memory compared to male rats (Engelman, 1998). This means that not only the piriform cortex is involved in maternal behaviour, but the strong connections with areas involved in social recognition and emotions together with the expression of oxytocin receptors,

may suggest that the piriform cortex does something more than processing olfactory information and perhaps having a direct role on the social recognition of a conspecific identity. The strong connection with areas such as the entorhinal cortex and orbitofrontal cortex (Howard et al, 2009; Illig, 2005) supports the idea that the piriform cortex may be involved in social memory. A connection with these areas may be important to the recall of odorant information. Data presented by Ross and Eichenbaum (2006), reveal that the role of the hippocampus is important in the initial phase of the memory of a socially transmitted food preferences, while the role of the hippocampus in memory retrieval decreases with time. Instead, there is an increased involvement of orbitofrontal cortex, entorhinal cortex and piriform cortex in successively later retrieval tests, supporting the idea that memory consolidation is required to transfer the memory processing to olfactory cortical areas (Gottfried, 2009). This means that the piriform cortex supports permanent memories for the social transmission of a food preference, and this may apply to other types of social memory.

4.6 Mechanisms underlying social recognition in rodents: Neuropeptides involved in social recognition

Prior research has established a functional role in social recognition for two closely related neuropeptides, arginine vasopressin (AVP) and oxytocin (OXT), and their associated receptors. OXT and AVP are produced in different parvocellular nuclei of the hypothalamus and other extra-hypothalamic areas, which project to a variety of brain regions. It is not a coincidence that most of the neural structures, involved in social recognition memory, mentioned in the previous section (lateral septum, hippocampus, amygdala, and medial preoptic area), are the

ones where AVP and OXT have been found (Ophir et al, 2009; Popik & van Ree 1991; van Wimersma Greidanus and Maigret, 1995).

The crucial role of these two neuropeptides has been widely investigated using different kind of approaches, starting from pharmacological studies to the use of genetically modified rodents. AVP's involvement in social memory is relatively straightforward. For example, systemic or intracerebroventricular (ICV) infusion of AVP or other AVP-derived peptides immediately after the first encounter with a social stimulus enhanced the memory for that social stimulus (Dantzer et al 1987; Bluthé and Dantzer, 1992; Sekiguchi e van Ree, 1991). This effect was reversed by the use of an AVP antagonist (Dantzer et al 1987). A similar result was observed in Brattleboro rats where the deficiency in AVP impaired the recognition of a conspecific encountered 30 min earlier and was rescued by the administration of AVP in the lateral septum (Engelmann and Landgraf, 1994; Feifel et al 2009). Knock out mice for AVP receptors, V1a or V1b, showed social recognition deficits (Wersinger et al, 2002; Wersinger et al, 2004); but, V1a receptors KO animals did not manage to habituate to a familiar conspecific even after multiple presentations (Wersinger et al, 2004). Those data shows that AVP is important for social recognition memory and is nly mediated by V1AR.

OXT appears to have a dose-dependent effect on social recognition. Low doses injected systemically or ICV produce a facilitator effect (Popik, Vetulani, & van Ree, 1992, Benelli et al, 1995) while high doses of OXT produce amnesic effects (Popik and Vetulani, 1991; Dantzer et al, 1987). According with these results, the ICV infusion of an OXT antagonist, immediately after the acquisition phase, affected social memory (Lukas et al, 2013; Samuelsen and Meredith, 2011). The advent of OXTKO mice further established the essential role of OXT in familiarity recognition. Male and female OXTKO mice showed social recognition

impairment (Ferguson et al, 2000; Choleris et al 2003) which was prevented by ICV injection of OXT 10 min before but not after the initial exposure with the social stimulus (Ferguson et al, 2001). These studies suggest that the release of OXT facilitate the acquisition phase but not consolidation and retrieval.

In line with the studies on the immediate early genes activation, amygdala, lateral septum and piriform cortex are areas implicated in AVP and OXT –mediated social memory. The amygdala was found to be an important site of action for AVP and OXT activity in the social recognition memory formation. The MeA, in rodents, is particularly rich in OXT receptors and V1a and V1b receptors (Arakawa et al 2010, Veinante and Freund-Mercier, 1997). Amygdala lesions in mice caused a reduction of OXT level in hypothalamus and plasma and deficits in social recognition (Wang et al, 2015). The infusion of OXT directly in the MeA mimicked the beneficial effects of OXT on social memory that were observed after ICV infusion (Ferguson et al, 2001), while, the local use of an OXY receptors antagonist impaired social recognition in both female and male mice (Choleris et al 2007; Ferguson et al, 2000). The present findings are consistent with the results obtained in Male OXYKO mice which present a similar impairment in the social recognition of conspecifics. In fact, the injection of oxytocin in this area restored the social memory deficits in these OXT knock out animals (Ferguson et al, 2001). Similar findings were reported in rats, although some species-specific differences may apply. In a recent study, Lukas and colleagues (2013) found that OXT in the MeA of male mice is only essential to recognise female stimuli, showing one more time that this area could be involved only in discriminating opposite-sex odours. Finally, Wang and colleagues (2015) reported that the impaired learning and memory in amygdala lesioned mice was reversed by systemic OXT administration. While the effect of OXT in the amygdala is well established, there is limited evidence for the activity of AVP in this area. One of the few findings are from

Arakawa and colleagues (2010). Their data provide evidence that AVP receptors modulate avoidance behaviour for an unhealthy conspecific odour, while the direct infusions of AVP receptor antagonists in the MeA blocked the recognition in juvenile but not in adult mice. Even if the precise nature of the action of oxytocin and AVP in the amygdala remains unknown, those findings demonstrate that the presence of OXY in the MeA is necessary during the initial social exposure for the proper processing of the olfactory formation and the development of the social memory (Ferguson et al, 2001) while AVP could regulate the avoidance response to conspecific cues.

The lateral septum is another region where AVP and OXT have a relevant role in different species of rodents. In various strain of rats, AVP directly injected in this area, immediately after the first exposure to a social stimulus, improved social memory of a conspecific in both the habituation/dishabituation paradigm and the discrimination paradigm (Appenrodt, Juszczak and Schwarzberg, 2002; Engelmann and Landgraf, 1994). Always in rats, social recognition was enhanced when OXT was infused directly in the lateral septum (Gabor et al 2012). In V1aR knock out mice, the re-expression in the lateral septum of V1a receptors could rescue the social recognition impairment (Bielsky et al, 2005). Lateral septum OXT receptor knock down mice have deficits in social recognition but normal social behaviour (Mesic et al, 2015). Finally, a study on prairie voles, a rodentia species which shows monogamous behaviour and social bond, reported that a reduction of OXT receptors and an increase of V1a receptors into the lateral septum, correlates to poor performance in recognition of a female conspecific (Ophir et al, 2009).

Chapter 5: Material and Methods

This chapter describes the methods common to the two electrophysiological experiments featured in Chapters 7, 8, 9, 10 and 11. Any details specific to individual experiments are described within those chapters.

5.1 Ethics

All experiments and procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and EU directive 2010/63/EU. Experiments were performed under Dr Colin Lever's Home Office project licence (PPL 40/2935) and a personal licence held by Francesca Pibiri (86E2E979).

5.2 Subjects

Six adult female Lister Hooded rats were used in the following experiments, weighing between 200 and 350g at time of surgery. Animals lived in their home cage groups until they were selected for surgery and, after surgery, were housed individually in cages [53 x 42.5 x 20 cm]. Room illumination was set to a 12h/12h light/dark cycle that started with lights on at 11pm. Water and foods were provided ad libitum.

5.3 Recording apparatus

5.3.1 Recording electrodes

Electrodes were constructed using HM-L insulated platinum-iridium wire (90% platinum, 10% iridium) (California Fine wire, Grover Beach, CA). The electrodes were configured into a tetrode formation, which is considered to improve the quality of the signal discrimination based on the spatial position of the neurons (Recce and O'Keefe, 1989; Harris et al., 2001). Tetrodes were all made from 25 μm or 17 μm wire electrodes with a length of 18 mm; the ends were taped together to create a loop which was then draped over a rod. The loop and the taped ends were then held together with a bent weighted needle, to create 2 loops and 4 pieces of wire. The wires were then twisted together using a magnetic spinner at a pitch of 2 turns/ mm and cut to form a tetrode. An alcohol flame burner was used to strip the insulation from the upper ends of the strands that remained untwisted.

5.3.2 Microdrives

The twisted part of four tetrodes were loaded into a moveable 16-channel 'poor-lady' microdrive (Axona Ltd., St. Albans, UK). See Figure 5.1 for a diagram of a standard microdrive (**figure 5.1**). The not more insulated upper ends of each tetrode were twisted around 4 solid-core steel posts on the microdrive in a way that each tip in the tetrode constitutes a single recording channel. Conductive silver paint (Farnell) was used to ensure continuous electrical contact between the electrode and the metallic posts. The electrodes around the post were protected with nail varnish.

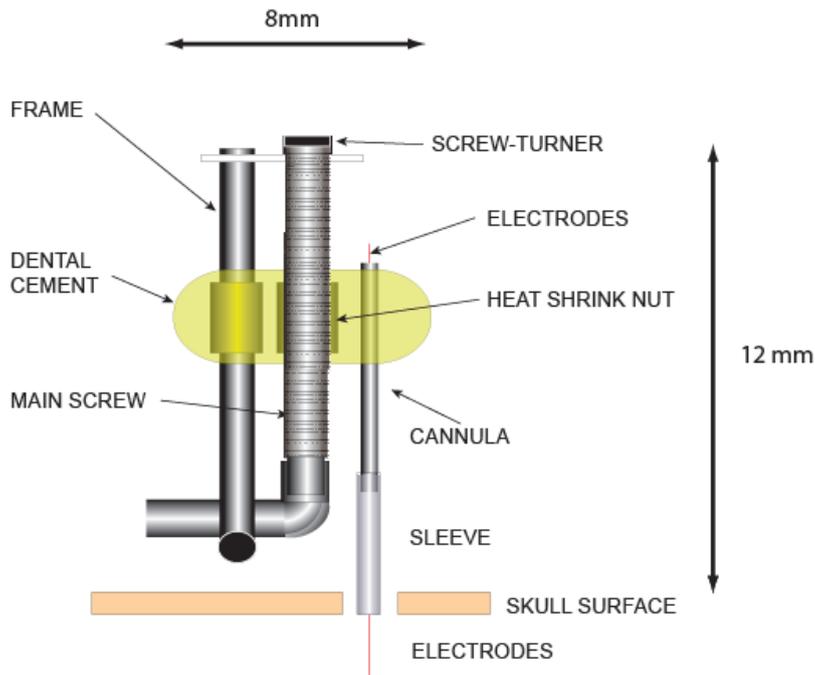


Figure 5.1 Diagram showing a 'poor-lady' microdrive and its main features. The microdrive was fixed to the skull using dental acrylic. A full turn of the screw advances the cannulas and tetrodes by 200 μm . Adapted from a diagram by John Huxter.

The tips of the tetrodes were cut with precision surgical scissors to ensure their ends followed a rhomboidal pattern separated by no more than the diameter of the wire. This allows, in general, for all 4 electrodes of the tetrode to measure the activity of the same cell but with different amplitudes. This allows single cell isolation by plotting the recorded amplitude of one electrode against the one recorded by another electrode of the tetrode. Each microdrive was loaded with four tetrodes resulting in 16 channels recording cell activity. Almost the full lengths of the four tetrodes were super-glued together to give a close and strengthened configuration ($<0.5\text{ mm}$).

Each rat used in this thesis was implanted with 2 microdrives, one in each hemisphere. The 2 metal 'feet' of the drive were cemented to the skull to stabilise the implant. The microdrive' framework allows the cannula and the loaded tetrodes to move up or down by rotating the threaded post using the screw turner. One full anticlockwise turn of the screw (360°) moves

the tetrodes 200 μm down. To protect the cannula and tetrodes, a sleeve 1-1 was fitted over them. During surgery, this sleeve was pulled down and fixed to the skull with dental cement.

5.4 Surgery

The microdrives were implanted using a deep anaesthesia induced by a combination of oxygen (flow rate: 3 litre/min) and isoflurane (3% of the gas volume). Twenty minutes before to start the surgery, the rat was administered with an analgesic [buprenorphine (Vetergesic), Reckitt Beckinsler, Hull, UK, 0.4ml-0.8ml, s.c.] and an antibiotic [enrofloxacin (Baytril), Bayer, Newbury, UK, 0.3ml, s.c.]. The top of the head was then shaved, and once the breathing was stable under anaesthetic, usually within 10 minutes, the animal was fitted into the stereotaxic frame via restraining ear bars. Vaseline was applied to protect the eyes and to prevent them drying out, while a topical antiseptic, Betadine, (Seton HealthcareLTD, Oldham, UK) was applied to the incision site.

An incision was made along the midline of the skull, and the skin was retracted to expose the skull and to give a sufficient view of both the bregma and lamda brain plate joins.

A small burr drill was used to make 7 holes in the skull in which stainless steel screws (3mm diameter, Precision Technology Supplies, East Grinstead, UK) were screwed into the holes to aid the adherence of the cement. One of the screw was attached to a connector pin that serves as an electrical ground. Following this, a trephine drill was used to make two holes (1.5-mm diameter), one over each hemisphere. The placement of these holes was determined by the tetrode co-ordinates (co-ordinates for each rat shown in Table 5.1). Both the dura and pia were removed and the surface of the brain was kept moist with a saline soaked sterile cotton bud. The microdrives were stereotaxically positioned to the target coordinates and slowly lowered

into the brain. The protective sleeve around the cannula was then lowered to rest upon the surface of the brain with the aim of protecting the exposed tetrodes. The sleeve-brain junction was then covered in sterile Vaseline. To ensure a stable implant the microdrives were affixed to the skull using dental cement around the sleeve, drive feet, screws and skull. Each drive ground wire was carefully soldered to the ground screw and cemented to prevent the animal detaching the ground connections. A plastic screw was cemented to the back of the implant to protect the ground wires and to allow the connection to the headstage to the implant by a crocodile clip.

Table 5.1: Target implant co-ordinates for all rats. Co-ordinates for the implants were selected based on Paxinos and Watson, (2007). The table gives the rat number, hemisphere; L left and R right, the target area amygdala (A), piriform cortex (Pir). The co-ordinates are given in mm behind bregma, anterior-posterior (AP) along the midline and medial-lateral (ML) from the midline and dorsal-ventral (DV) from the surface of the brain. The last column shows the supposed DV coordinates of the implant before animals were perfused.

Animal code	AP	ML	DV	DV before perfusion
426	-1.9	±4.1	-7	R -7.28
429	-1.9	±4.1	-7.0	L -7.35
438	-2.3	±4.8	R -7.7 L 7.8	R -8.2 L -8.3
451	-2.3	±4.8	-7.8	R -8.15 L -8.1
452	-1.9	±4.8	-7.1	R-7.7
464	-1.9	±4.8	-7	L -8.25 R -8.2

5.5 Recording techniques

Axona recording system (Axona Ltd., St.Albans, UK) allowed for simultaneous recording of extracellular action potentials (spikes) from individual neurons (units), EEG or local field

potential (LFP) and 2D animal positionin. Each of these measures was individually time stamped, at different rates.

Each subject was connected to the recording system by a head-stage amplifier which could be plugged into the microdrive. The head-stage cables were light and very flexible, thereby enabling the animal to move around freely. The implanted electrodes were AC-coupled to these amplifiers. The lightweight wires were 2-3 metres long and connected the head-stage to a preamplifier.

5.5.1 Position Tracking

The rat's head position, orientation and running speed was tracked using two arrays of small, infrared light-emitting diodes (LEDs) mounted on the head-stage and centred above the rats' skull. The two sets of LEDs were separated by 5 cm, with one of the set brighter and more widely projecting than the other. The differential brightness and size allows the recording software to distinguish accurately between the two light groups and use this information to infer the head direction of the rat in space. The two LED sets were monitored using an infrared camera attached to the ceiling of the room and positioned above the middle of the testing arena (DACQUSB, Axona, St Albans, UK).

Offline analysis defined the point equidistant to the two LEDs groups as the position of the rats head (TINT, Axona, St Albans, UK). Position was sampled at a 50 Hz rate. Running speed was calculated from this 50-Hz position tracking data, with 400 s boxcar smoothing.

5.5.2 EEG

The EEG signal was amplified 5000-7000 times, band pass filtered at 0.34–125 Hz and sampled at 250 Hz. This means the raw signal passed through a low-pass filter (0.34 to 125 Hz), which rejected (attenuated) frequencies outside this range. The origin and gain (amplification) of the EEG signals were set during the screening that preceded every recording session and were maintained throughout the day.

5.5.3 Single-units

For each implant, there were 16 independent extracellular electrophysiological recording channels. Signals on the channels dedicated to single-cell recording were amplified (10,000–20,000) and band-pass filtered (500 Hz-7 kHz). All the channels of a given tetrode were recorded differentially: for the four channels of each tetrodes, a single channel from a different tetrode within the same hemisphere was used as a reference channel, and its signal was subtracted from the active one. The reference channel was generally selected as a channel that had little activity to avoid spurious addition of unit activity to the original channel. This referencing allowed to remove most of the background activity (usually generated by the animal moving, chewing, grooming or other artefacts) and thus increased the signal readability.

Each channel was continuously monitored at a sampling rate of 50 kHz (largely enough for the timescale of spikes, in ms). Action potentials were stored as 50 points per channel (1 ms, with 200 s pre-threshold and 800 s post-threshold) whenever the signal from any of the pre-specified recording channels exceeded a given threshold (c.61% of baseline to ceiling value). For example, if the maximum recorded value was 150 μ V (microvolts), the signal was recorded if it exceeded 90 μ V as set by the 61% threshold on at least one of the four channels. Gains and

references for each channel were set each day before recording and kept identical for all trials of that day.

Screening for cell activity began approximately 1 week post-surgery. The animal was screened whilst on a holding platform. Electrodes were slowly lowered (typically in steps no larger than 100 μm , usually in 2 x 50 μm steps per day).

5.6 Materials

5.6.1 Apparatus

The following experiments were carried out in a battle-grey painted 40 cm x 40 cm x 50 cm high wooden box, which was placed on top of a square platform elevated 70 cm from the floor. The arena was placed directly below two cameras, one was the infrared camera (see **figure 5.2**) for tracking the LEDs on the head-stage of the animal, the other one was a digital video camera for behavioural recordings. The size of the apparatus was established in order to increase the number of contacts between the subject rat and the social stimulus during trials with the social condition.

5.6.2 Laboratory layout

During screening and testing the room was dimly lit with a lamp to the south of the environment. During screening and between trials the rats rested upon the holding platform located to the west of the testing environments. This was a shallow wooden platform (35cm x 35cm) containing woodchip bedding. The platform had ridged edges (5cm high 5cm thick)

raised approximately 70cm off the floor. Rats were always passively transported by the experimenter into the test environment from west to east.

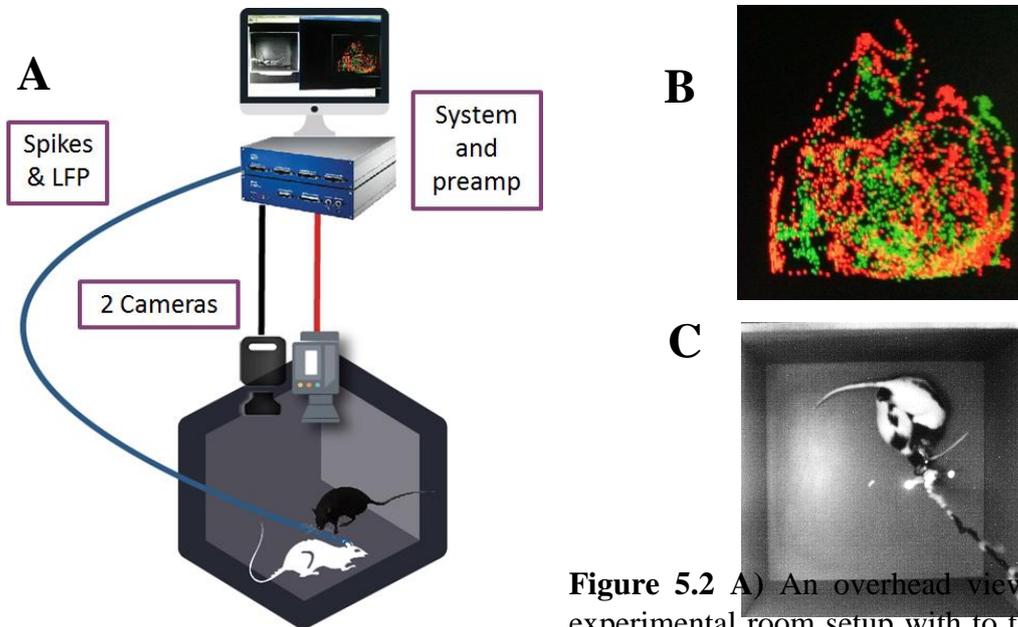


Figure 5.2 A) An overhead view of the experimental room setup with to the left a side on view of recording arena. The two cameras were synchronised with the electrophysiological recordings and had different function. One camera recorded the behaviour during the recording, the other camera was tracking the position and the orientation of the head-stage of the animal. B) The Head position tracker. C) Time-stamped behavioural image

5.7 Experimental procedures

5.7.1 Cell screening

Rats were allowed one week of post-surgery recovery before screening. Rats were handled in a towel which was used to restrain them in order to connect the recording system to the microdrive on the head of the rat. Electrode recordings were monitored, while the rat was placed in the platform. Cell screening then began by lowering the tetrodes down the dorso-ventral axis from their initial implant position just above the amygdala or piriform cortex, in search of active pyramidal excitatory cells or inhibitory interneurons.

In general, larger steps (electrodes were moved in 25-100 μm steps, with a maximum movement of 100 $\mu\text{m}/\text{day}$) were used until spikes (pyramidal cells or interneurons) were observed. This process preceded all experimental manipulations. Upon finding active cells, the rat was returned to its home cage until the following day to ensure that the tetrodes were stable. In fact, due to tissue drag, electrodes keep traveling through the brain after being moved. Recordings only started once the tetrodes were deemed stable (*i.e.* recorded activity was similar across successive trials). No experimental recordings were made on days when the electrodes were moved. For this reason, electrode activity was monitored daily until active cells appeared. Recording sessions were carried out without knowing the exact position of the electrodes, when one or more pyramidal cells or interneurons were observed.

5.7.2 General procedure

The rat was passively transported into the laboratory and placed upon the holding platform, located to the west of the recording environments. The rat's implant was then attached to the head-stage for cell screening. For all testing trials, the rat was placed (from laboratory west to east) into the environment facing laboratory east. Recording began within 0-3 seconds once the rat was placed into the environment, and ended about 5-10 seconds before the rat was taken out of the environment for being placed upon the holding platform.

5.7.3 Testing sessions

Each testing session was aimed to test the activity of cells, in the amygdala or in the piriform cortex, in succeeding trials. The number of trials in each session was dependent on the number of the conditions tested and on the number of the controls used during the session. No more than three conditions were used in the daily session. Each condition was repeated 6 times and

trials were organised in a semi randomised order where the same condition was not repeated consecutively for more than two trials.

The experimenter walked around the environment during the trials, in order to immediately stop the trial in case of head-stage problems that could incur during social trials. The experimenter was able to monitor the behaviour taken by the subject rat and in case by the social stimulus during the trial, through the video captured by the camera synchronised with the recording software (DACQ USB, Axona, St Albans, UK).

5.8 Data processing

5.8.1 Spike clustering

Once the data had been acquired, off-line analysis was necessary to identify individual cells via spike clustering and to characterise the LFP recordings. The custom-made software TINT (Tetrode Interface, Axona Ltd) was used to separate spikes in clusters, to combine position, unit and LFP data, and to calculate speed and direction, as well as basic cell properties.

Given the close proximity of the four tips in a tetrode (channels), four simultaneous action potentials can be recorded from the same tetrode (one on each electrode channel). The software compared the relative amplitude of each spike across all four channels to perform a triangulation of the signal in space, assuming that the extracellular medium was homogeneous. The amplitude recorded by each channel was plotted against the amplitude of the same cell recorded by another channel in the tetrode. This produced 6 individual scatter plots where spikes were represented as a collection of points. This scatter plot contained multiple cell recordings, where spikes from the same cell formed distinctive clusters. The differing peak-to-

trough amplitudes (the difference between the maximum and minimum voltages of the action potential) of individual cells leads to each cell's cluster to have a different profile across the plots, lending to discrimination between cells. This is due to the tetrode design. Even if two neurons are spatially very close, their position relative to the tips of the tetrode will differ, entailing slight differences in the recorded action potentials from each cell. Therefore, the spikes from these cells will occupy a different position on the cluster plots.

5.8.2 Cell identification

Tetrodes can record the spikes emitted by a large number of nearby cells, which can be masquerade by general 'noise' and occasionally by artefacts generated by other sources as electrical devices. TINT was used to assign these distinctive clusters to single cell clusters by drawing ellipsoid polygons around well isolated clusters.

The basic method behind cluster-cutting requires the experimenter to draw a polygon around a cluster to define the cell. The main parameter for cluster cutting is the peak-to-trough amplitude using the amplitude plots mentioned above. The cluster shape for pyramidal cells is generally ovoid because these neurons tend to fire in bursts, with the spike amplitude decreasing with each subsequent action potential of the spike train (Ranck, 1973). However, clusters for other cellular forms may not be so clearly elliptical. The benefit of having 6 plots comparing the 4 channels means that if a cell isn't well isolated on one scatter plot it may be more isolated on another (**figure 5.3**)

Not all cells were well isolated and easy to 'cut' on the basis of amplitude (A). In these cases, cells were identified and separated on the basis of a second parameter; waveform shape. This

is the time interval (and slope) between the peak and trough of the waveform. In order to do this TINT can generate a graph plotting the voltage of spikes at a particular time point (V_t) or a combination of both V_t and A. To reduce error, clusters that did not appear to be well-isolated were ignored. Once all the desired spikes had been selected, noise and erroneous spikes which did not belong to the cell could be removed using the same methods.

In total, approximately 13000 clusters from 27 sessions from 6 rats were isolated for the dataset described in the present thesis.

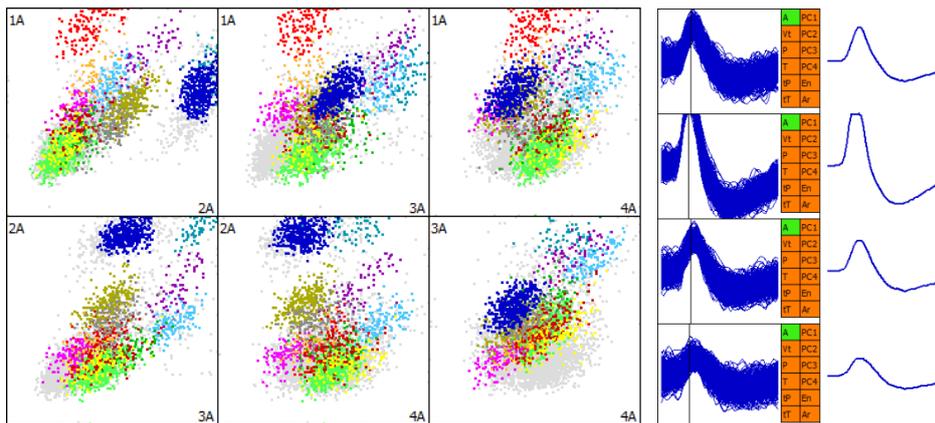


Figure 5.3. Examples of cluster-cutting from rat 438. Each cluster (colour) is assumed to represent a different neuron (*left*). Each cluster plot illustrates the amplitude (A) of the spikes on two channels simultaneously; six plots are necessary to represent all the pairs of channels. The right window displays for one cluster, the waveforms of all their spikes and their average next to them. The tetrode shown is tetrode 1 (session 101014).

5.8.3 Recording stability

A good stability of signal was often observed across trials/ days, manifested by the clusters occupying a similar position on the respective cluster plots. If too much change in the global pattern of activity (i.e., most clusters occupied a noticeably different position in the cluster plots) was noticed between trials the cell was considered to have moved away from the tetrode and the cell was not considered in the analysis. Clusters were cut based on one or more reference trials. This was normally the first trial recorded, normally a baseline trial, and the first social trial. Clusters were isolated (cut) using this trial, and this cut was then used as a template to cut the other recording trials. This was primarily a template of action for the experimenter to refer to.

5.9 Waveform analysis

Waveforms were calculated for each cell. These are presented in the basic properties figures in the Results Sections (chapter 8, 9 and 10). To investigate differences in waveform amplitude and interval; the peak-to-trough measurements were looked at (Figure 3.12.1A). These were taken from the negative peak to the positive peak (Figure 3.12.1B). In the basic properties figures in the results section the peak amplitude are given the highest positive-to-negative or negative-to-positive amplitude (μV). And the waveform interval was given as the negative peak-to-trough interval (μs). Cells were classified as pyramidal cells, interneurons or axons on the base of the waveform interval (Robbins et al, 2013). Generally, in the hippocampus, pyramidal cells have a longer waveform interval ($>300 \mu\text{s}$) and a long-lasting hyperpolarization. Interneurons have a shorter waveform interval ($<300 \mu\text{s}$) and a shorter

hyperpolarization period. Axons exhibit a brief hyperpolarization period followed by a brief depolarization, with in average a waveform interval of 150 μs .

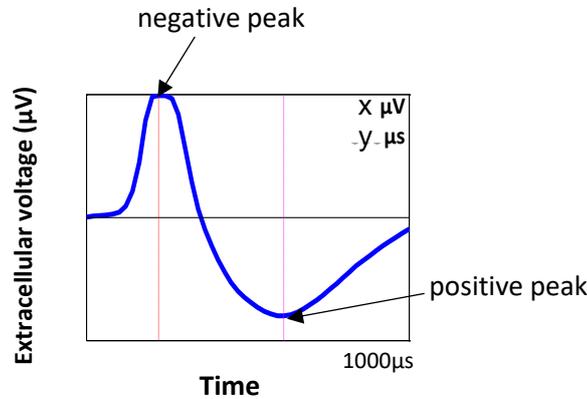


Figure 5.4 Waveform illustration. The X axis shows the time, the Y axis shows voltage. The waveform amplitude and interval statistics are given in the bottom right the waveform amplitude and interval are taken from the negative peak to the positive peak.

5.10 Parameters of cells included in the analysis

To be considered in the analysis neurons had to presents 3 main features. First, high stability across trials during the testing session. This means that if the neuron showed changes in the global patten activity, i.e. the cluster occupied a different position in the cluster plot, or there were waveform variety across trial, was discharged from the analysis. A neuron was considered for the analysis only when the number of spikes in at least one of the trial in the testing session was ≥ 50 (in 180 seconds, i.e. 0.28Hz). Finally, only neurons with waveform interval $>150 \mu\text{s}$ were considered.

5.11 Data Analysis and Statistics

The firing rate of each cell was calculated for each trial. The number of spikes recorded in each trial was divided by the duration of the trial (180s). For many cells, the distribution of the firing rates over 12 trials did not conform to a normal distribution. To compare mean firing rate between two conditions (Base vs Fam, Fam vs Nov or Fam1 vs Fam2), cells considered in the analysis were statistically analysed with a two-related sample non-parametric analysis, the Wilcoxon test. The non-parametric Wilcoxon test represents a more cautious approach because of the lower sensitivity but when data come from a skewed distribution, like in this case, this test can reach higher power compared to the t-test (Krzywinski & Altman, 2014).

Chapter 6: Behavioural Methods

6.1 General procedure

The implanted animal was transported passively from her home cage to the testing room and placed upon the holding platform. The testing session started immediately after the screening. The testing session comprised a set of multiple trials interspersed by inter-trial period. Electrophysiology and behaviour were recording during each trials. When the trial required the presence of a stimulus inside, either social (familiar or novel rat) or unsocial (object, Sphero, bedding), the stimulus was placed inside the apparatus 3 minutes before the trial started.

6.1.1 Trial length and inter-trial

The trial length was 3 minutes to encourage the social interaction with novel or familiar conspecifics. Trial length was relatively short, in order to avoid excessive social habituation, and to try to ensure that levels of social engagement persisted throughout the session. The inter-trial interval was 8-10 minutes. The inter trial interval was at least eight minutes to ensure any trace-related firing was sufficiently diminished to be able to observe between-condition differences between successive trials. On the other hand, testing days could often be very long, and a short inter-trial could reduce the amount of time the subject animal was out from his home cage.

6.1.2 Conditions

The number of trials in each session was depending on the number of the conditions tested and on the number of the controls used during the session. The first trial of the day aimed to

acclimatise the implanted animal to the arena, and it was always a baseline, represented by alone time in the apparatus. **Table 6.1** shows the summary of the conditions used in the sessions.

Table 6.1 Summary of the three conditions used in these experiments

Baseline (Base)	In the Baseline condition, the implanted animal was placed alone inside the arena with no other stimulus inside (social or non-social). The baseline condition was repeated for 6 trials.
Social Familiar (Fam)	In the Familiar condition, the implanted animal was free to interact with a social familiar stimulus. The familiar condition was repeated for 6 trials with the same familiar social stimulus. In the case of the Fam vs Nov session, only one familiar social stimulus was used. In the case of Fam vs Fam sessions, 2 different familiar social stimuli were used (Fam1 and Fam2), and each of the two-familiar condition was repeated 6 times.
Social Novel (Nov)	In the Novel condition, the implanted animal was free to interact with a novel social stimulus. The novel condition was repeated for 6 trials and in each trial a different novel animal was used to keep the novelty status (Nov 1-6).

6.2 Methods for Social familiar vs Non Social sessions (aka ‘Base vs Fam’)

6.2.1 Pre-testing session

Stimulus rats were housed in same/sex group of 2-4 per cage. Familiar animals were typically sisters of the implanted rat. In a couple of cases, sessions involved younger rats which were not sisters of the implanted rat. Using sisters and younger rats was meant to limit the potential for negative social interactions. Immediately prior to testing, the familiar experimental subject was marked by horizontal lines on the tail and placed alone in a holding cage for 1 hour. This pre/test social deprivation is a standard procedure to increase baseline levels of social

interaction. Before the testing session, the familiar stimulus was exposed to the implanted animal for the familiarization session.

6.2.2 Arena familiarisation

In order to guarantee the familiarity with the arena, the implanted animal and the social stimulus were exposed to the arena in 2 familiarization sessions separated by a 24h delay. Twenty-four hours before testing, the implanted animal was connected to the recording system and placed alone inside the apparatus for three 3-minutes trials (familiarization session day 1, **figure 6.2, C**). The familiarization session in day 2 comprised two 3 minutes' trials, 1 hour before the testing session (**figure 6.3, C**).

During the arena familiarization session in both day 1 and day 2, the stimulus rat was placed into the testing apparatus for two 5-minutes trials (**figure 6.2, A** and **figure 6.3 A**). Furthermore, during the testing session, consisting of multiple trials, the familiar stimulus used in the trial was placed 5-3 minutes in the testing apparatus before the trial started.

6.2.3 Social familiarisation

To guarantee the familiarity and ensure standardisation the implanted animal was repeatedly exposed to the same familiar stimulus. The familiarization period consisted of two sessions separated by a 24h delay. The first session comprised three 5 minutes' trials (inter-trial interval 8 minutes) where the implanted animal was free to interact with the social stimulus inside the testing arena. The second session comprised two 5 minutes' trials, 1 hour before the testing session. Immediately before the testing session, the social stimulus was placed alone in a

holding cage for one hour. This pre/test social deprivation is a standard procedure to increase baseline levels of social interaction. When the experimental animal was exposed to the same familiar stimulus for two consecutive days, the familiarization session consisted of a single session: two 5 minutes' trials, 1 hour before the testing session.

This Social Familiarisation procedure was used for all the familiar rats in all the three types of sessions described in this thesis: Familiar vs Baseline, Familiar vs Novel, Familiar-1 vs Familiar-2 (**figure 6.2, C** and **6.3, C**).

6.2.4 Testing session

The present testing session was designed to pre-investigate the role of the piriform cortex and the amygdala and their role in social behaviour. This experimental session comprised two conditions, Baseline and Familiar. During the social familiar condition (Fam) the implanted animal was exposed to the same familiar social stimulus for 6 trials. Social trials were interspersed with baseline trials (Base), where the implanted animal was alone inside the squared arena. The two conditions (Fam and Base) were repeated 6 times (6 three minute trials), for a total of 12 trials. **Figure 6.4** shows a schematic representation of the trial sequence and timing of a testing session.

6.3 Methods for Social familiar vs Social novel sessions (aka ‘Fam vs Nov’)

6.3.1 Pre-testing Session

Novel and Familiar stimuli rats were housed in same/sex group of 2-4 per cage. Novel animals were always younger than the experimental rat to limit the numbers of negative social interaction. Immediately prior to testing, each novel and familiar experimental subject was marked by horizontal lines on the tail and placed alone in a holding cage for 1 hour. This pre/test social deprivation is a standard procedure to increase baseline levels of social interaction.

During the testing session, each condition was repeated for 6 trials, with a total of 12 trials, when the experimental animal was exposed to two conditions (familiar stimulus versus novel stimuli) or with a total of 18 trials, when two consecutive trials with a social stimulus (one novel and one familiar) were interspersed by a baseline trial in which the implanted rat was placed alone in the apparatus.

Before the testing session, the familiar stimulus was exposed to the implanted animal for the familiarization session. Novel social stimuli encountered the implanted animal for the first time during the testing session. Since in testing session, the novel condition comprised of six trials, six different novel social stimuli were used to keep the novelty status.

6.3.2 Familiarisation Session

The arena familiarization and the social familiarization sessions in case of the implanted animal are the same described in section 6.2.2 and 6.2.3 respectively.

The novel animal was similarly exposed to 2 familiarization sessions (day 1 and day 2) and in both days, novel animals were exposed to the testing arena for a single 5-minutes trial. During the testing session, consisting of multiple trials, the novel or the familiar stimulus used in the trial was placed 5/3 minutes in the testing apparatus before the trial started to allow the acclimatization before testing.

Figures 6.2 (A, B, C) and 6.3 (A B, C) show a schematic representation of the trial sequence and timing of the familiarization sessions in day 1 and day 2 for both the social stimulus and the implanted animal.

6.3.3 Testing Session

The present experimental session was designed to pre-investigate the role of the piriform cortex and the amygdala and their role in social recognition. For these experiments, I adapted the Crawley's sociability and preference for social novelty protocol, leaving the implanted animal the free choice to interact with the social stimulus. This test, uses the natural tendency of rats to explore more novel social stimuli compared to familiar ones, to study social recognition memory (Dantzer et al, 1988, Crawley, 2004). The novel and the familiar conditions were repeated 6 times, for a total of 12 trials when the sessions included only two conditions, **social familiar** and **social novel**, and 18 trials when the social trials were interspersed with baseline trials, where the subject animal was placed alone into the square box. During the **social familiar** condition the implanted animal was exposed to the same familiar social stimulus for 6 trials, while during the **social novel** condition it was exposed to a novel social stimulus in each of the 6 trials (**figure 6.1**).

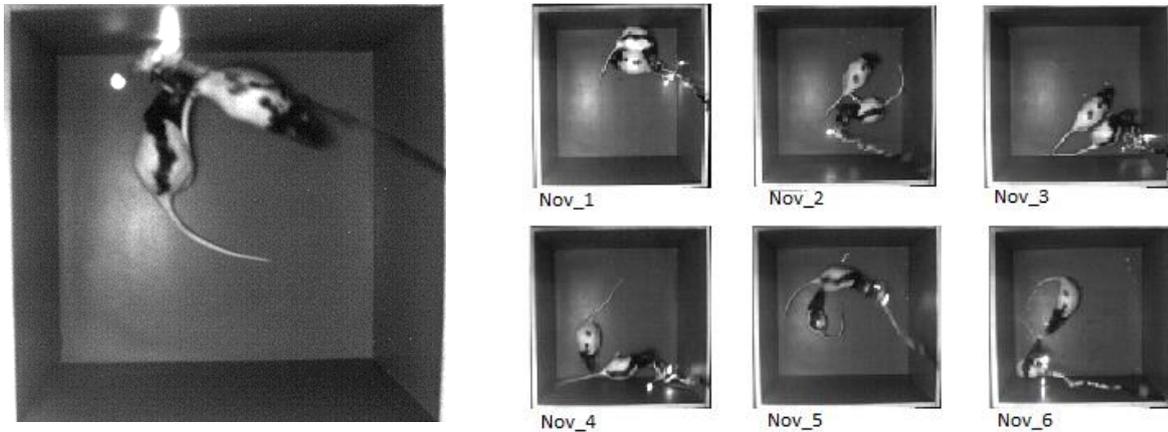


Figure 6.1. The basic premise of the Social Familiar vs Social Novel paradigm.

On the left an image picked from the social familiar condition. The implanted animal is exposed to the same familiar social stimulus for 6 trials. On the right 6 images picked from the social novel condition, where the implanted animal is exposed to a different novel social stimulus (1-6) in each of the 6 trials.

6.4 Social Familiar 1 vs Social Familiar 2 sessions (aka ‘Fam vs Fam’)

6.4.1 Pre-testing session

Stimulus rats were housed in same/sex group of two per cage. Except for one session (464_270816), the familiar animals were always sisters of the implanted animal. The implanted animal and her two sisters were caged together until the day of the surgery. In session 464_270816, the familiar rats were also sisters, but they were not sisters of the implanted rat. These sisters were younger than the implanted rat.

Immediately prior to testing, each familiar experimental subject was marked by horizontal lines on the tail and placed alone in a holding cage for 1 hour. This pre/test social deprivation is a standard procedure to increase baseline levels of social interaction.

Before the testing session, the two familiar stimuli were exposed to the implanted animal for the familiarization session.

6.4.2 Familiarisation session

The arena familiarization session and the social familiarization session in the case of both the implanted animal and the social stimuli, has been already described in section 6.2.2. and 6.2.3 respectively.

6.4.3 Testing session

The present experimental session was designed to pre-investigate the role of the piriform cortex and the amygdala and their role in social recognition. Since the idea was to understand how specific social discrimination might get, the familiar stimuli used in the sessions were sisters and they were caged together; thus, highly similar on several dimensions. Each sister was presented to the implanted animal for 6 trials, for a total of 12 trials, or 18 trials when the social trials were interspersed with baseline trials, when the implanted animal was placed alone into the square box.

6.5 Probe trials

In some sessions, the standard social/non-social-trials were supplemented by probe trials with the attempt to narrow down interpretation. The probe trials used in these studies included: familiar and novel objects, familiar and novel bedding, Sphero. Table 6.2 shows a brief description of the probes used.

During the testing sessions, the probes were placed inside the arena minutes before the trial started. Sphero was placed centrally in the arena and the biological motion started once the trial started. Novel objects and novel bedding were used only in Fam vs Nov sessions.

Table 6.2 Brief description of the probes used in the experiments.

<p>Object</p>	<p>Still object which was placed at the centre of the arena before the trial started. The object was a cylinder 8 cm high, 4 cm diameter The photo shows the familiar object used in these experiments.</p>	
<p>Bedding</p>	<p>Familiar object containing bedding (familiar or novel) with urine and excrements taken from the cage of social stimuli. The novel bedding contained the bedding taken from a cage of conspecifics that the implanted animal never met. The familiar bedding contained urine and faeces of the familiar conspecific used in the trial. When two familiar conspecifics were used, the bedding contained the mix of urine and excrements of both animals.</p>	
<p>Sphero</p>	<p>Sphero is a white robotic ball made of polycarbonate that weighs 168 g, and has a 7.4 cm diameter. Sphero can be controlled by a smartphone and it is capable of rolling around and reach 0.9 meters/sec</p>	

6.5.1 Familiar object and Sphero

The same stimulus object was used throughout all sessions and all rats to guarantee the familiarity of the object, and ensure standardisation. The object was a cylinder 8 cm high, 4 cm diameter.

A Sphero ball (version 2.0) was used in order to probe to what extent any cellular activation might be mimicked by biological motion. The diameter of the Sphero ball was 7.4cm. The

same Sphero ball was used throughout all sessions and all rats to guarantee the familiarity of the object, and ensure standardisation.

The familiarization period consisted of two sessions separated by a 24h delay (**figure 6.2, C**, and **6.3, C**). The first session comprised two-three 5 minutes' trials (inter-trial interval 8 minutes) where the implanted animal was given time to familiarise with Sphero and its motion. The second session comprised two 5 minutes' trials, 1 hour before the testing session. Before each trial, Sphero was cleaned with hot water and 70% alcohol.

6.5.2 Bedding

The object was a plastic cylinder with holes all along the walls to allow the rat to get access to the bedding stimuli. The bedding was placed inside the cylinder. The same stimulus object was used throughout the experiments, to guarantee the familiarity of the object. Before each exposure, the object was cleaned with hot water followed by 70% alcohol. The object was carefully dried before the soiled bedding was placed inside. The bedding was always freshly soiled, and it was collected 5-10 minutes before the bedding trial started and in sessions where two repeat bedding trials were run, the bedding was replaced for the second exposure.

The familiarization period consisted of two sessions separated by a 24h delay. The first session comprised of three 5 minutes' trials (inter-trial 8 minutes) where the implanted animal was given time to familiarise with the object located in the centre of the apparatus. The second session was comprised of two 5 minutes' trial, 1 hour before the testing session. During the familiarization period, fresh soil bedding was placed inside the familiar object.

The familiar bedding comprised of urine and excrements collected from the cage containing the familiar rat/rats used on the day of the experiment. The implanted animal was exposed to

the familiar bedding only during the testing session. The soiled bedding used had been exposed to the familiar rat/rats for at least 24 hours.

The novel bedding comprised of urine and excrements collected from the cage containing rats never previously used during the experiments. The implanted animal was exposed to the familiar bedding only during the testing session. The soiled bedding used was exposed to the novel rats for at least 24 hours.

Familiarization session

Day 1

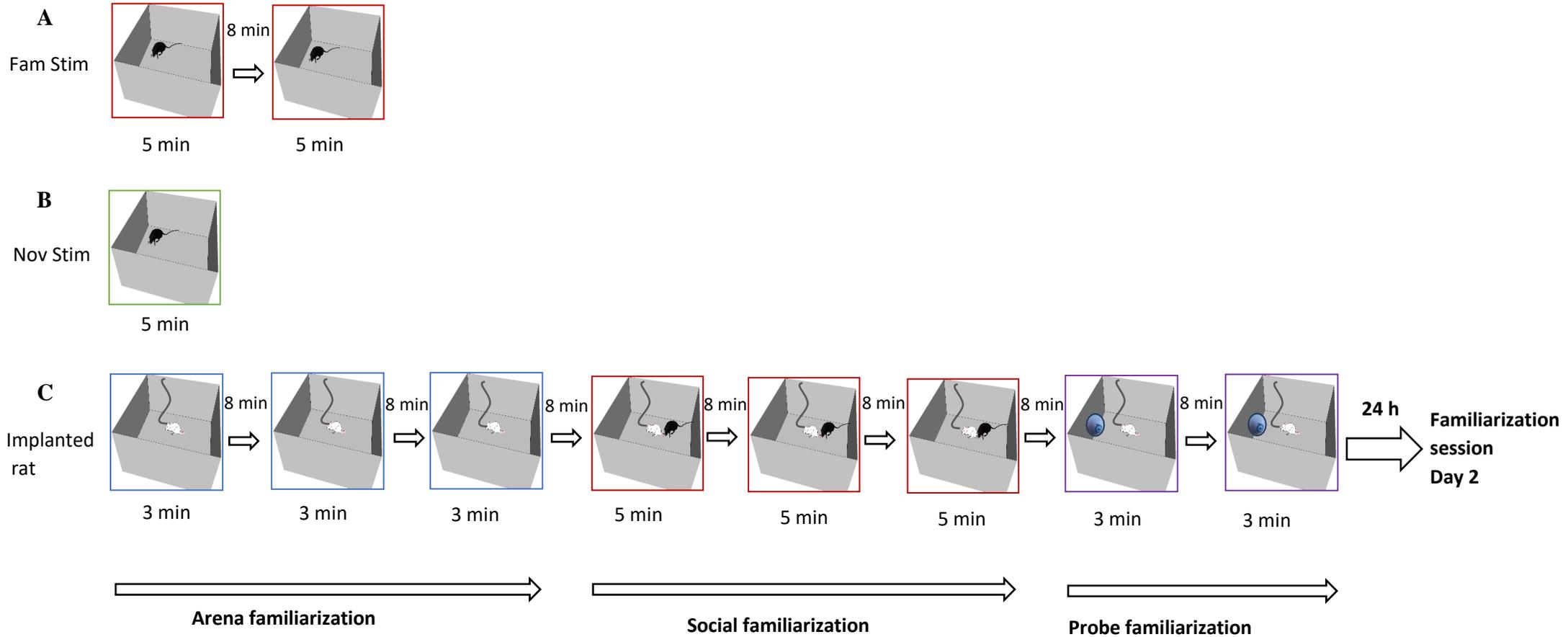


Figure 6.2. Diagram of familiarization session, day 1. Schematic representation of the trial sequence and timing, where each block corresponds to a 3-5 min trial, while the inter-trial period between consecutive trials is 8 minutes. (**A, B and C**) Both the social stimuli (familiar, red, novel, green) and the implanted animal (in blue) familiarized with the arena. **C**) The implanted animal was further exposed to the social stimulus for the ‘social familiarization’ (familiar, red), and to the control stimulus (probe familiarization) that was planned to be used in the testing session (violet). The familiarization session day 1, was followed 24 h later by the familiarization session day 2.

Familiarization session

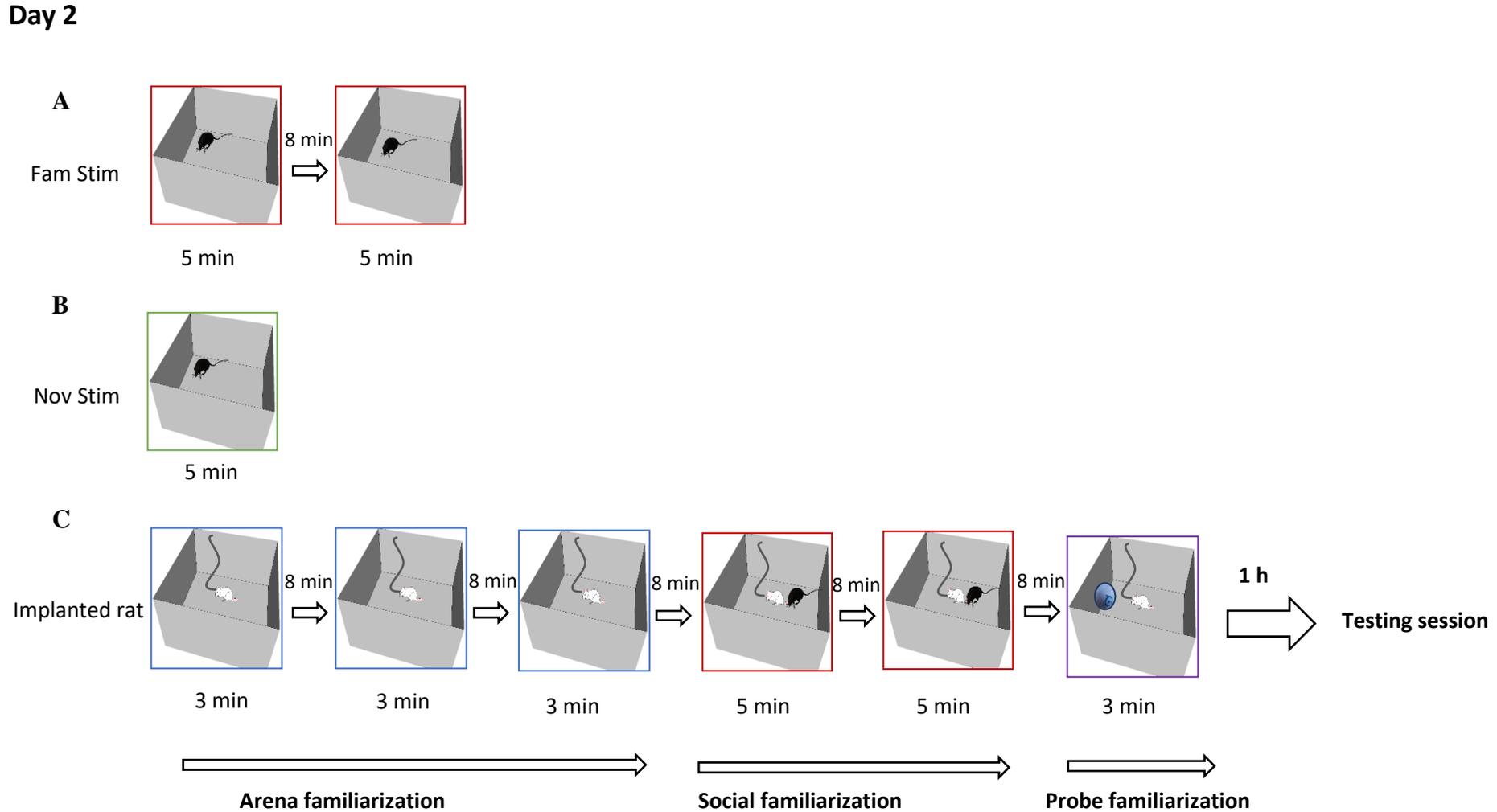


Figure 6.3. Diagram of familiarization session, day 2. Schematic representation of the trial sequence and timing, where each block corresponds to a 3-5 min trial, while the inter-trial period between consecutive trials is 8 minutes. (**A**, **B** and **C**) both the social stimuli (familiar, red, novel, green) and the implanted animal (in blue) were exposed to a familiarization period for the arena. **C**) The implanted animal was further exposed to the social stimulus (for the ‘social familiarization’, red), and to the control stimulus (probe familiarization) that was planned to be used in the testing session (violet). The testing session started 1h after the familiarization session day 2.

Testing session

Day 2

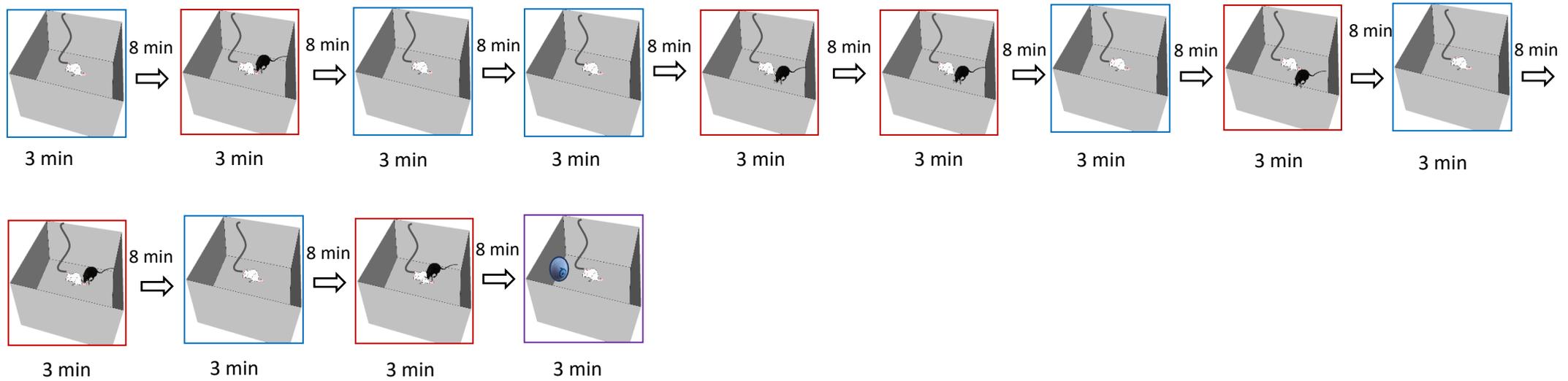


Figure 6.4. Diagram of Base vs Fam testing session. Schematic representation of the trial sequence and timing of a 2 condition testing session, where each block corresponds to a 3 min trial, while the inter-trial period between consecutive trials is 8 minutes. The blue boxes represent baseline trials, meaning that the implanted animal was alone inside the apparatus, the red boxes represent social trials, meaning that the implanted animal was free to interact with a social stimulus. Each condition, in this case Base and Fam, is repeated for 6 trials for a total of 12 trial in the session. The last trial (violet) is a probe trial. Probe trials were presented in some session with the aim to narrow down interpretations.

6.6 Oestrus cycle stages: the use of the Vaginal Smear

The reproductive cycle of female rats is called the oestrous cycle and its stages are characterized as proestrus, oestrus, metestrus (or Diestrus I) and Diestrus (or Diestrus II) (Freeman, 1988). The ovulation occurs from the beginning of proestrus to the end of oestrus (Schwartz, 1964).

Samples were collected immediately after the testing session using a micropipette (200 μ l). The tip of the micropipette was inserted into the vaginal orifice, with no more than 1 cm of penetration. Then, 200 μ L of distilled water was flushed inside twice and the contents placed on a slide. The smear was evaluated immediately, fresh and unstained. A standard microscope was used to evaluate the vaginal smear.

The identification of the oestrus cycle through the vaginal smear involved the characterization of few distinctive cells type, often in combination, that typically correlate with the status of the vaginal mucosa, uterus and ovaries (Goldman et al 2007).

Proestrus can be identified in the smear by the presence of round nucleated epithelial cells organised in clusters. In a standard 4-days cycle, proestrus lasts for a day and it is followed by the oestrus phase that can persist for one or two days. In this second phase, the oestrus phase, the sample tends to present a predominance of cornified cells and a large presence of needle-like cells. The metoestrus (or dioestrus I) is the next phase, and is a period of transition between the oestrus phase and the Dioestrus (II), where the vaginal smear is characterised by the co-presence of leukocytes and cornified and round epithelial cells. During the Dioestrus (II) phase, leukocytes are predominant or even exclusive. The smear may also show some small clumps of nucleated epithelial cells.

6.7 Behavioural scoring

Behaviours in social trials was recorded using a camera synchronised with the electrophysiological recordings. Early sessions were recorded using a Streampix camera. Later sessions were recorded using the Axona DacqTrack system. Social trials were analysed off-line in form of digitized motion images and behaviour was scored. Time spent by the implanted animal investigating the social stimuli, during each of the twelve 3-minutes exposures, were used for calculations. The Behavioural scoring was done blind to trial status by a collaborator (Ricardo Bindi, University of São Paulo). Different behavioural parameters were considered in this analysis, **table 6.3** shows a brief description of the behavioural parameters used in the analysis. Any aggressive encounter between animals was immediate cause for terminating the experiment.

6.8 Data analysis

To understand if the implanted animal could discriminate between familiar and novel conspecific or between 2 different sisters from a behavioural point of view, The different parameters described in table 6.3 were analysed to identify any behavioural differences between the 2 social stimuli used in each testing session. When behavioural parameters met the normal distribution were analysed with paired simple t-test, when did not, the non-parametric Wilcoxon test was applied.

To understand if social interaction could contribute to changes in firing, the firing rate of the cells recorded in each session was analysed in relation to the different behavioural parameters considered in the analysis. Pearson correlation was used for cells which firing rate was

normally distributed. Cells that did not show a normal distribution were tested using the non-parametric Spearman correlation

Table 6.3. Description of the behavioural parameters considered in the analysis.

Anogenital sniffing	Time spent by the implanted animal sniffing and touching with visage the anogenital region of the social stimulus. This behaviour is always recorded as active time.
Face to face contacts	Time spent by the implanted animal sniffing the facial region of the social stimulus. Occasionally the implanted animal can be in a non-active time because the social stimulus can engage in face to face without the implanted animal moving or investigating the social stimulus.
Other contacts (Body Contact)	This parameter includes the amount of time the implanted animal is touching/ sniffing or been touched/sniffed by the social stimulus, in any body part, excluding facial recognition and anogenital areas. This behaviour can be either active or passive.
Dominant	Time spent by the implanted animal dominating the social stimulus. The social stimulus is on a belly-up roll; the animal rolls onto his back, after receiving a nip or a bite on the rump. This behaviour is always recorded as active time.
Total contacts	This parameter sums all together the parameters described above. It represents the time in a trial spent by the implanted animal in close proximity to the social contacts, independently by who was approaching the other, the implanted animal or the social stimulus.
Active time	Time spent by the implanted animal proactive exploring the social stimulus and engaging in social behaviours. Whenever the animal ceases to look for the social stimulus the active time stops.

Chapter 7 Histology

This chapter will provide the reader with information on of the histological methods used across all studies presented in this dissertation, also included are details on the histological examination of results for each experimental animal considered in this thesis, except one rat (464), for which the brain was accidentally disposed by of university staff.

7.1 Histological methods

After completion of the experiment, each rat was sacrificed with an overdose of sodium pentobarbital [(Euthalal), Merial, Harlow, UK; 1ml i.p.] and perfused trans-cardially with saline solution, followed by 4% paraformaldehyde. Once the brain was extracted, it was stored in 4% paraformaldehyde; before the histology the brain was transferred into a distilled water solution containing 30% of sucrose until the brain sunked to the bottom of the tube (usually 1-3 days). The brain was sliced coronally into 40 μm thick sections, mounted onto gelatin-coated slides and stained using Cresyl-Violet Acetate (Sigma) Nissl-stained solution which facilitated localisation of the tetrode tracks and tips. The target implant co-ordinates are given in **table 5.1** in chapter 5.

7.1.1 Tetrode configuration reconstruction

Once the implant was removed from the brain, the tetrodes were analysed in relation to their position in the skull, to estimate how the tetrodes were configured inside the brain. The ‘bubble test’, was used to establish the position of each tetrode in the skull. The ‘bubble test’ consists in passing a current through each electrode of the 4 tetrodes in a microdrive, into saline water.

The formation of bubbles in the tips of a specific tetrode, as a result of electrolysis at the cathode and anode, allowed to locate the specific tetrode among the 4 tetrodes that were loaded in the microdrive and link that tetrode to the electrophysiological recordings. Once established, the location of the specific tetrode, the possible location of the tetrode in the brain was estimated. For example, how was the tetrode positioned in relation to the other tetrodes? Was the tetrode more anterior? **Figure 7.1 A** shows the implant from 438, with part of the skull still attached to it, while **figure 7.1 B** shows the established tetrode configuration. In the example, tetrode 1 is the most anterior. This means that looking at the series of brain sections from animal 438, in an antero-posterior order, I should expect to observe first the track from tetrode 1.

7.1.2 Identification of the recording site

Once stained, the coronal 40 µm thick brain sections, were visualised through a microscope, and sections presenting any sign of the tetrodes' track were digitalised at 4X magnification. In general, between 15-20 sections presented part of the tetrodes track. In fact, due to both the angulation of the tetrodes in the brain and the angulation of the brain when sliced, the track often appeared fragmented in different sections. The most ventral track was considered to be the location of the tetrodes tips when the animal was sacrificed. However, the tetrodes were normally moved along the dorsoventral axes (DV) among the different sessions. For this reason, a 2mm ruler was 4X magnified and digitalised to reconstruct the position of the tetrodes' tip following the tetrodes movements. Various aspects were considered to reconstruct the position of the tips of the tetrodes:

- 1) The most ventral track of the tetrode was supposed to correspond to the last DV coordinates before animal was sacrificed.
- 2) The approximate location of the tetrodes during each recording session was established considering the DV coordinated of the tetrodes in each session considered

- 3) The brain is expected to shrink by approximately 20% after fixation. The shrinking was considered when the location of the tetrodes' tips was calculated.

In **figure 7.2** an example is provided of how the location of the tetrodes was calculated. The 4X magnified and digitalised 2mm ruler was positioned on the section, close to the tips marks. Session 438_031014 was considered in this example; the DV coordinate at the time the animal was sacrificed was -8.2 mm. The DV coordinate at the time of the session considered was 7.9 mm. This means that at the time of the recording session tetrodes were 0.3 mm more dorsal compared to the most ventral track. Considering the anticipated brain shrinkage (20%), the estimated tetrode location during that recording session should be 0.24 mm more dorsal than the bottom of the track in the session.

Once the position of the electrodes tips was established in the sections, the histological landmarks highlighted with the Cresyl-Violet staining were considered to help identifying recording sites. The electrode tip locations were examined and determined on the base of the anatomic arrangement of the amygdala and piriform cortex, as depicted in the stereotaxic atlas of Paxinos and Watson (2007); and precise landmarks in the brain. The main landmarks used were the optic tract and the basolateral amygdala. The basolateral amygdala was highlighted with the Cresyl-Violet staining and consists of a roughly triangular area bordered laterally by the external capsule, caudomedially by the lateral ventricle and rostromedially by the stria terminalis.

7.2 Problems

Even if the attempts to reconstructing the recording position have been extremely cautious, they can still lead to erroneous judgments. A simple case of misleading judgment can be cause

by the brain resistance to electrode movements. As described in chapter 5 section 5.3.2, the microdrives allow the cannula and the loaded tetrodes to move up and down along the dorsoventral axes. A full turn anticlockwise move the tetrodes down of 200 μm . Electrodes keep traveling through the brain after being moved. Normally, no recordings were done in the 24 hours after tetrodes were moved to allow tetrodes to move down and acquire a stable position. However, It may be possible that due to drag tissue, or microglia immunoreaction around the tetrodes and the tips of the electrodes, the cannula can get blocked or not fully moved the predicted amount (i.e. 200 μm for 1 full turn anticlockwise). This may lead to miscalculation errors since the 200 μm , (160 μm , if considered 20% of brain shrinking) will be still considered for histological examination. Another misleading examination can be caused by damage of brain tissue when the electrodes were pulled out of the brain after the animal was perfused. Even if the electrodes were gently removed, the possibility that damage occurred to the tissue surrounding the electrode tract cannot be excluded. Another potentially misleading judgement can be the association of a certain tetrode to a specific brain section. In fact, even if it is possible to reconstruct the tetrode configuration post-mortem once the implant was removed, there are not clear evidence that show how the tetrodes were really configured when in the brain. Finally, since multiple steps were carried out to locate the position of the tetrodes in the brain, also the experimental error at any step cannot be excluded. In few words, the histological reconstruction, as described in this chapter, can lead to erroneous judgments. For example, for this reason, it is not possible to establish for certain which layer of the piriform cortex the recordings were taken from.

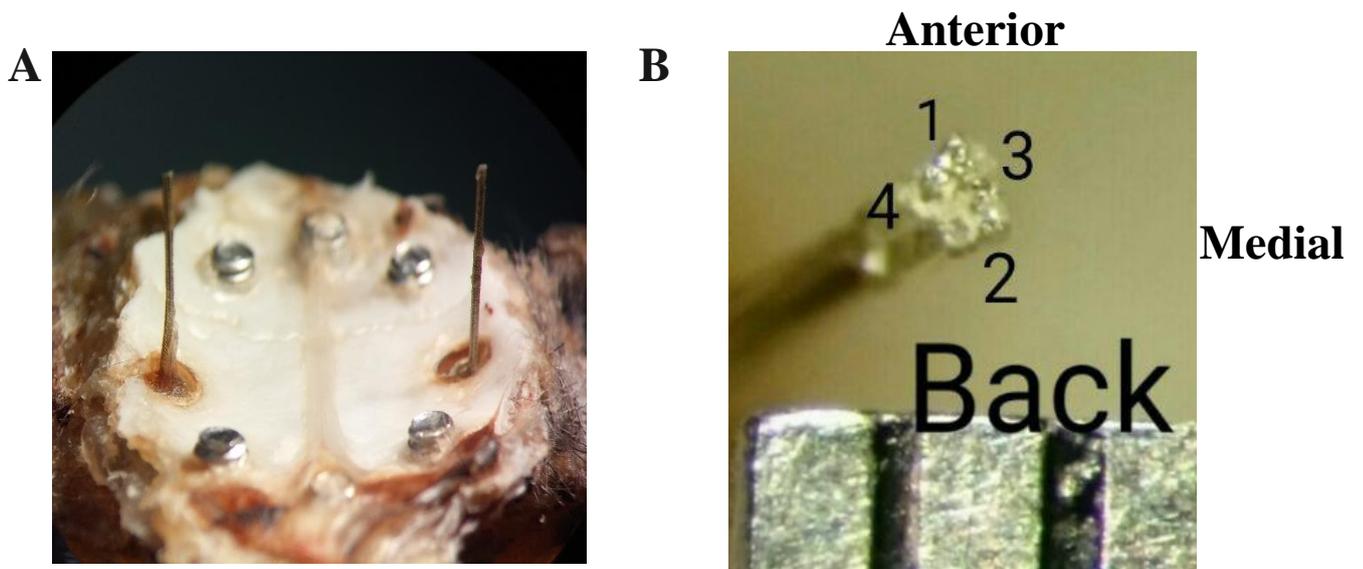


Figure 7.1 Reconstruction of tetrodes configuration. (A) shows the tetrodes previously inserted in the brain of animal 438. (B) shows the tetrodes configuration reconstruction from rat 438, right hemisphere. The ‘bubble test’ allowed to number the tetrodes (1-4) and to locate the 4 tetrodes in anterior-posterior and medial-lateral axes, between each other. The position of the ruler, allowed to broadly estimate the dimension of the electrodes, suggesting that the track of the tetrodes’ tips, all together, should not be over 500 μm .

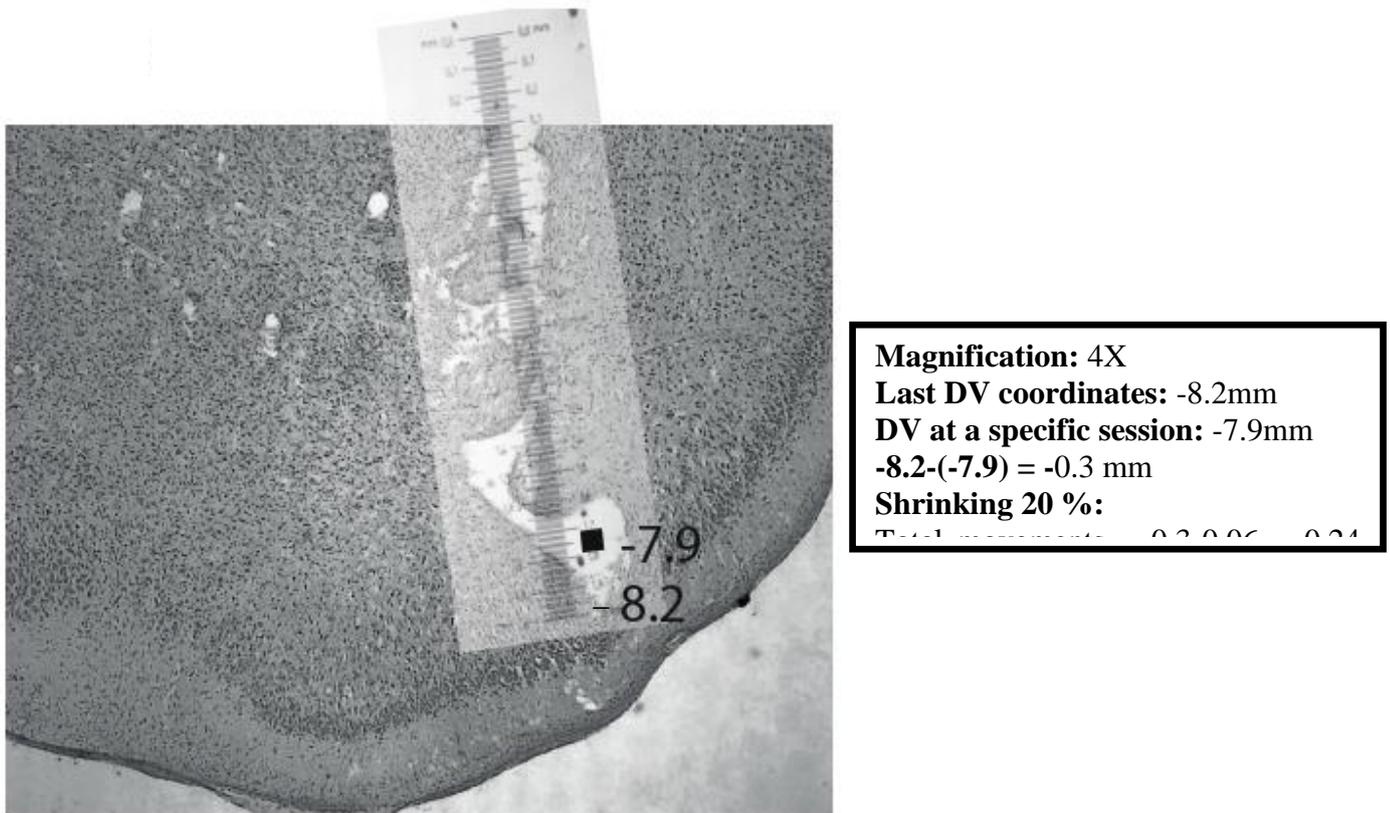


Figure 7.2 Reconstruction of tetrode position during a specific session (438_031014). The photo of the histology and the ruler were both taken at 4X magnification. The number -8.2 represent the last DV coordinates before the animal was sacrificed, for this reason it was assumed that -8.2 correspond to the bottom of the tetrode track. The DV coordinated of the implants at the time of session 438_031014, were 7.9. The black square represent the estimated location were the cells from tetrode 8 were recorded during session 438_031014.

7.3 Piriform Cortex

Histological examination of 3 out of 6 animals confirmed that tetrodes were implanted in the piriform cortex. As explained in the previous paragraph, the histology reconstruction can lead to erroneous judgement, for this reason it is not possible to establish precisely from which layer of the piriform cortex the recordings were performed. Even if the histological examination was not possible in the case of rat 464, AP and ML coordinates used for 464 including the DV at the time of the recording sessions suggests that the tips of the electrodes were in the piriform cortex.

438

Figures 7.3-7.8 show the representative location of the recording tetrode tips in animals 438 across different testing sessions. **Figure 7.3-7.5** show the electrodes' track in the right hemisphere, **figure 7.6-7.8** show the electrodes' track in the left hemisphere. Presumably, most of the recordings were performed from layer 3. **Table 5.1** in chapter 5 shows the DV coordinates at the time of surgery and the supposed DV coordinates at the time of the animal perfusion. Electrodes were moved in total 500 μm down compared to the initial position, in both Left and Right hemisphere. The cells analyzed in this thesis from animal 438 were recorded in the right hemisphere between DV -7.7 and DV -8.0, in the left hemisphere between DV -7.8 and DV -8.1.

426

Histological examination of rat 426, confirmed that tetrodes were implanted deeply into layer 4 of the piriform cortex, so called endopiriform nucleus. All cells were recorded in the right hemisphere and tetrodes were moved down in total of about 300 μm in respect to the initial DV. All the testing sessions analysed were recorded between DV -7050 and DV -7225.

451

Figures 7.9 and 7.10 show the representative locations of the recording tetrode tips in animals 451, respectively in the left and in the right hemisphere, across different testing sessions. The cells analysed from animal 451 were all recorded at DV 7.8.

7.4 Amygdala

Histological examination of 2 out of 6 animals confirmed tetrodes were implanted in the amygdala. Animal 452 had the tetrodes implanted in the basolateral amygdala (**figure 7.11**). The cells analysed during the two testing sessions 040615 and 290715, were recorded at DV 7.6.

Animal 429 had the tetrodes implanted in the central amygdala (**figure 7.12**). Only one recording session (1201614) was recorded from this animal at DV 7.35.

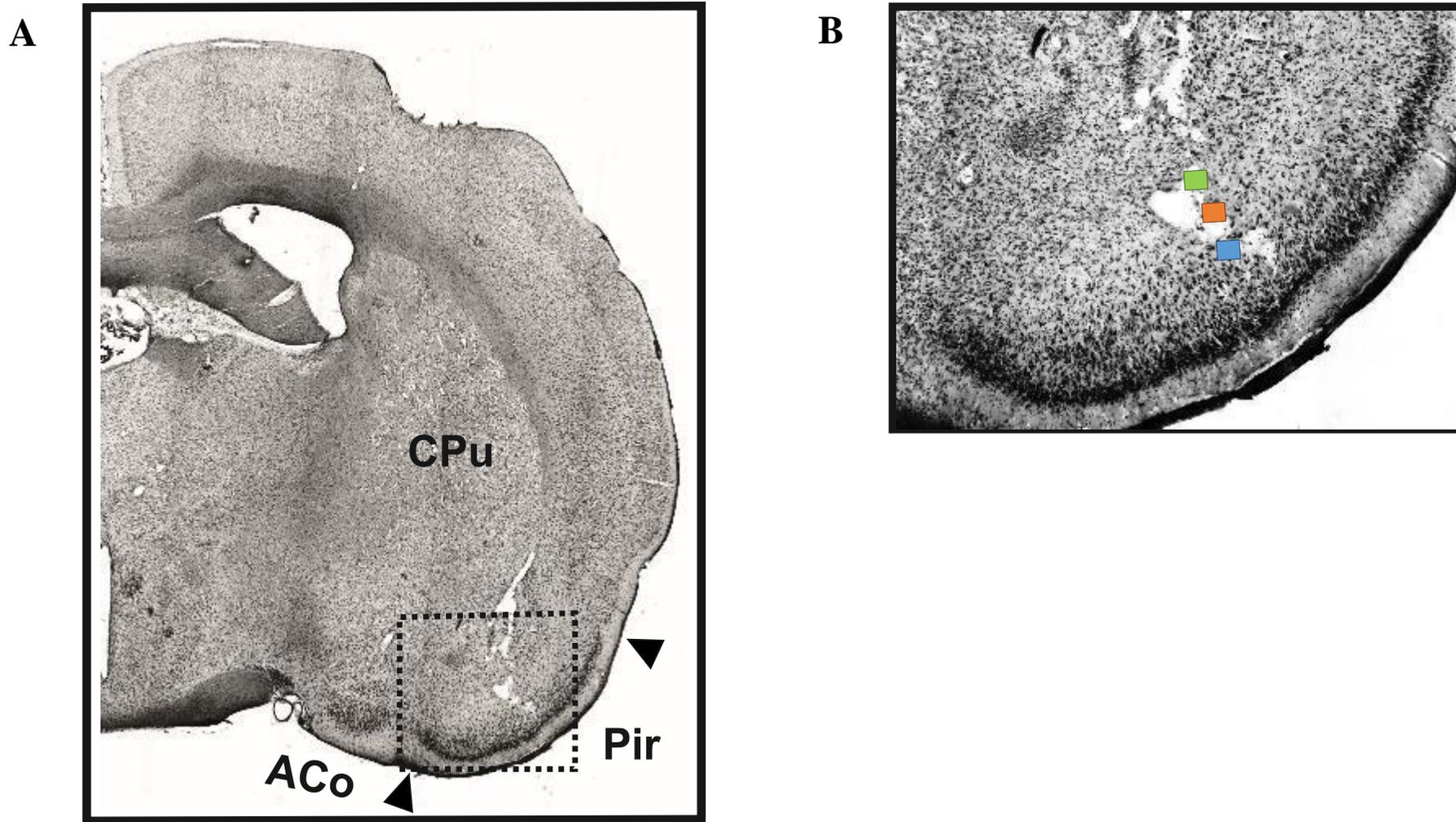


Figure 7.3 Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438. The coronal section shown is from Bregma -1.65. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom track of one of the tetrodes, in the piriform cortex. The green square represents the supposed position of the tetrode during sessions 290814, 160914 and 040914, the orange square the supposed position of the tetrode in session 031014 and 101014.

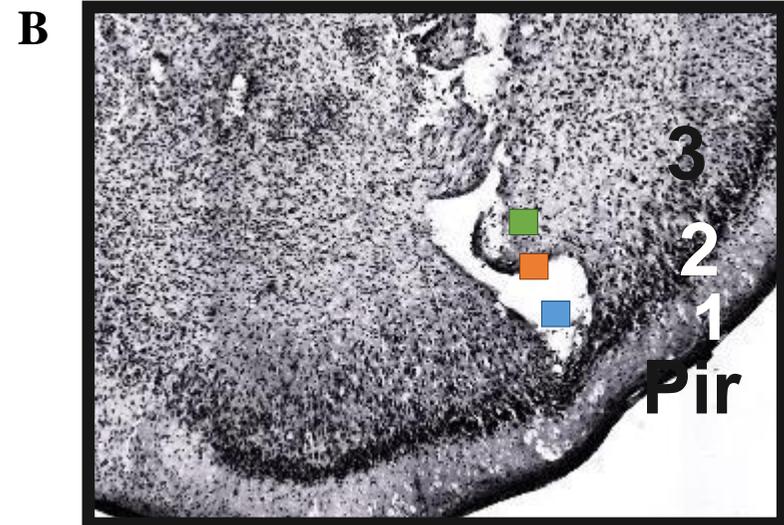
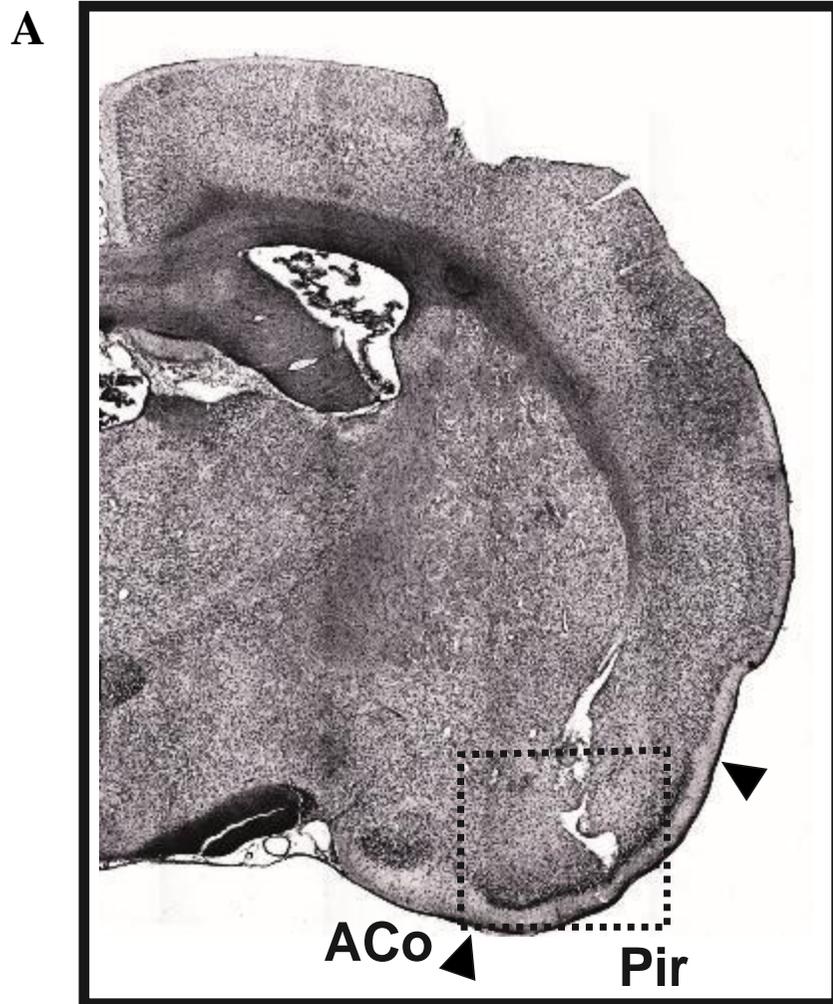


Figure 7.4. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438. The coronal section shown is from Bregma -1.75. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode track in the piriform cortex; the green square represents the location of cells recorded in sessions 290814, 160914 and 040914, the orange square represents the location of cells recorded in sessions 031014 and 101014 and the blue square represent the location of cells recorded in session 101214

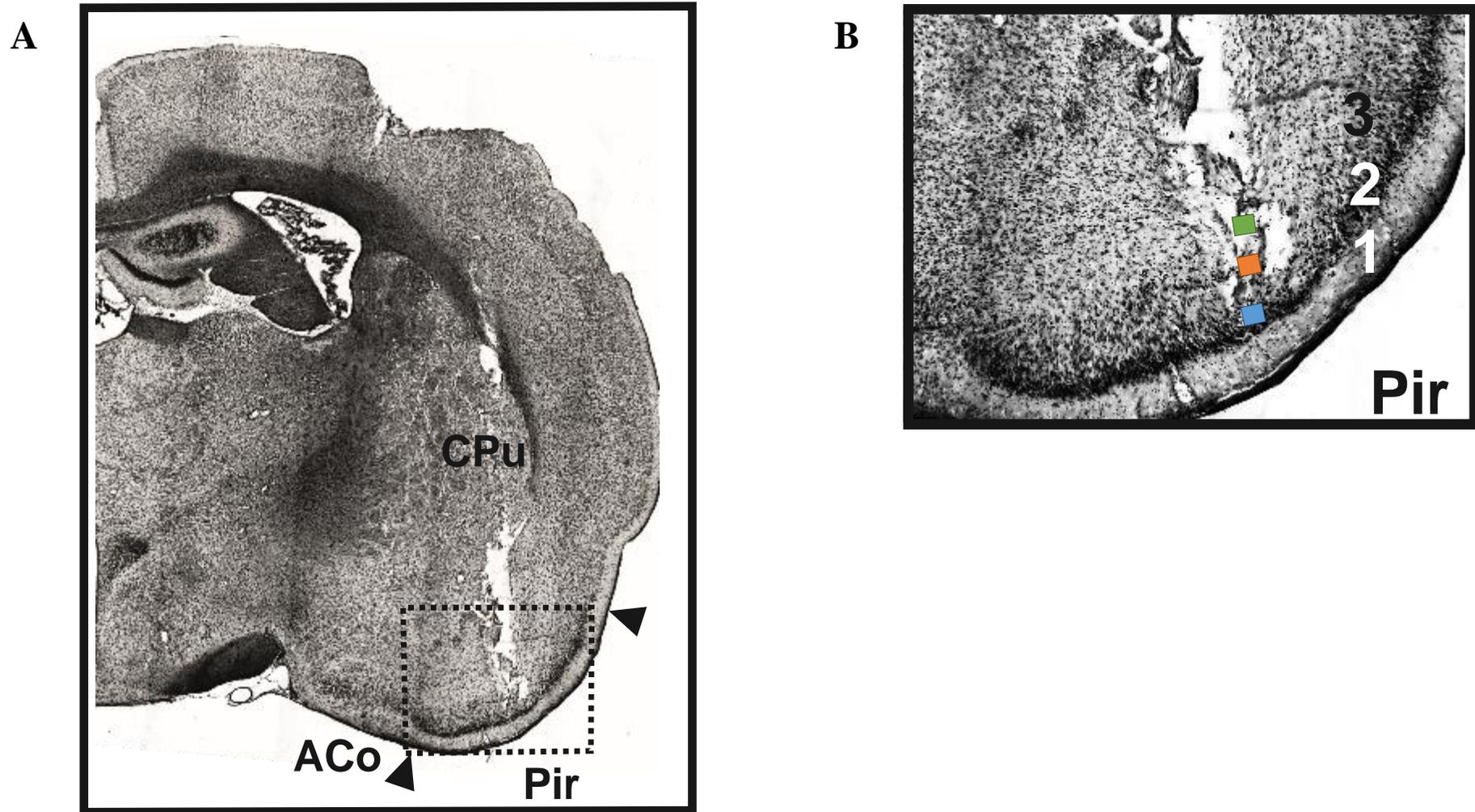


Figure 7.5. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438. The coronal section shown is from Bregma -1.90. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode track in the piriform cortex; the green square represents the location of cells recorded in sessions 290814, 160914 and 040914, the orange square represents the location of cells recorded in sessions 031014 and 101014 and the blue square represent the location of cells recorded in session 101214

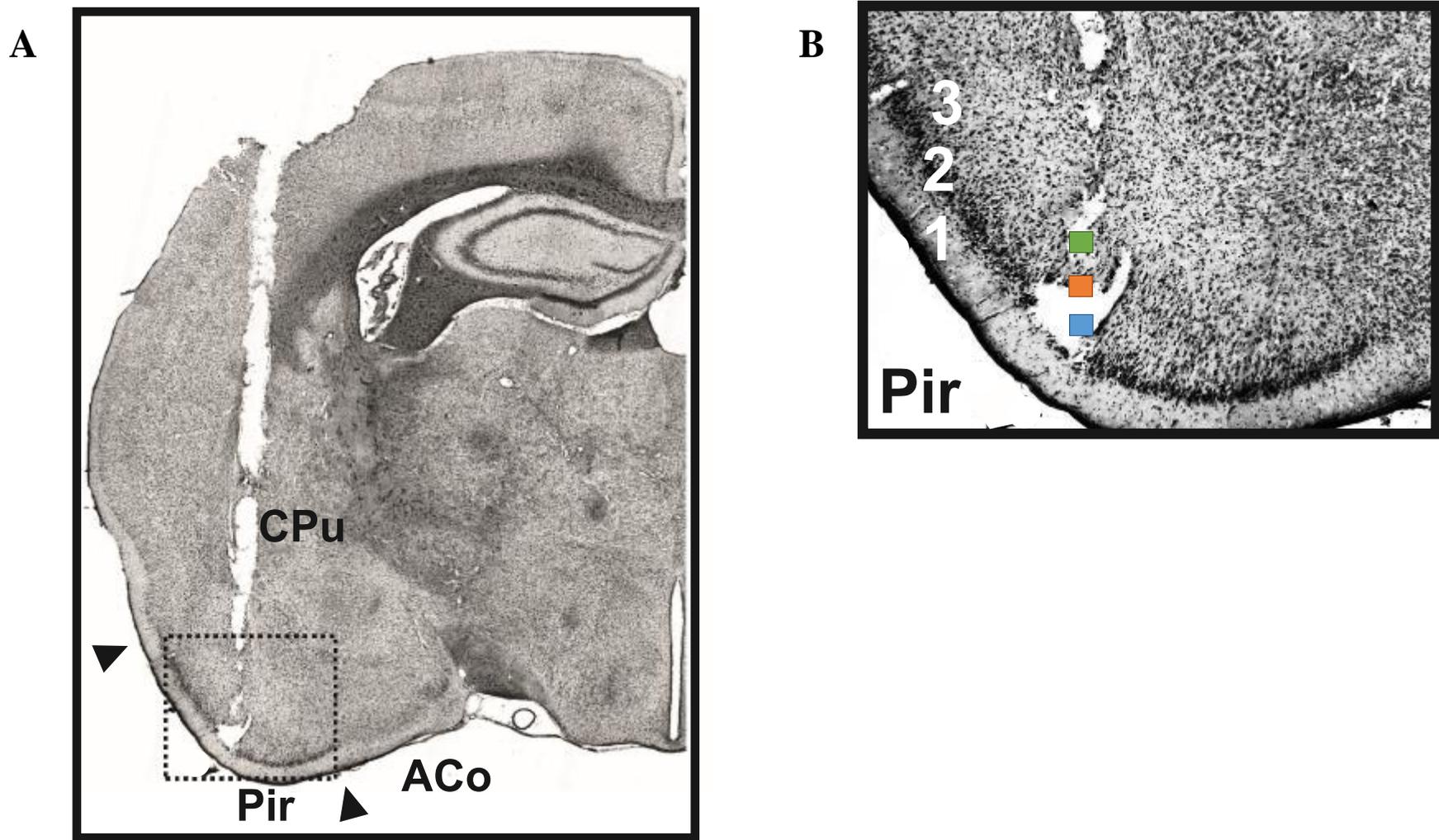


Figure 7.6: Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438, left hemisphere. The coronal section shown is from Bregma -2.0. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode tracks in the piriform cortex, tetrode 2; the green square represents the location of cells recorded in sessions 290814, 160914 and 040914, the orange square represents the location of cells recorded in sessions 031014 and 101014 and the blue square represent the location of cells recorded in session 101214

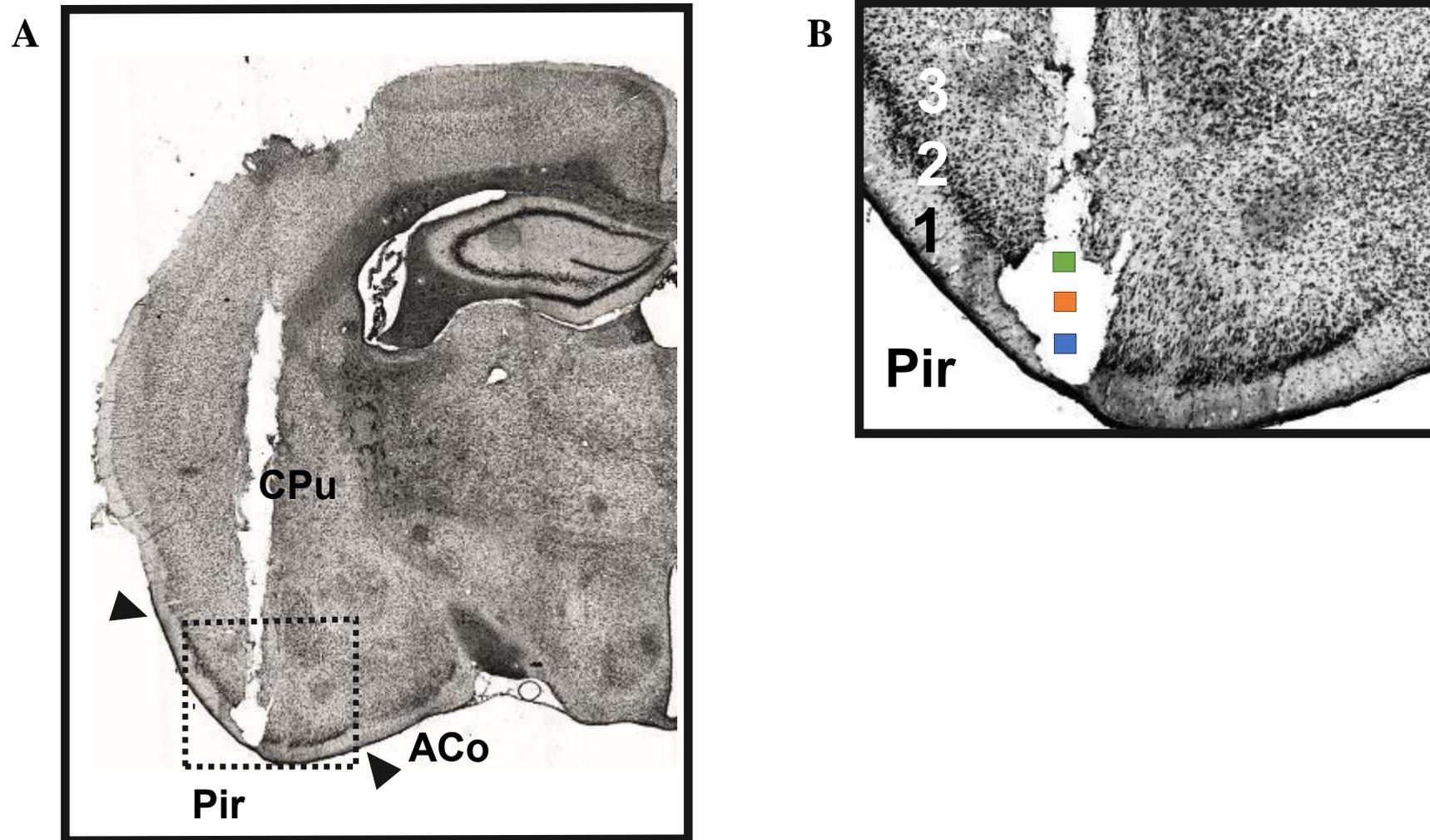


Figure 7.7. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438, left hemisphere. The coronal section shown is from Bregma -2.1. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode track in the piriform cortex, tetrode 1; the green square represents the location of cells recorded in sessions 290814, 160914 and 040914, the orange square represents the location of cells recorded in sessions 031014 and 101014 and the blue square represent the location of cells recorded in session 101214

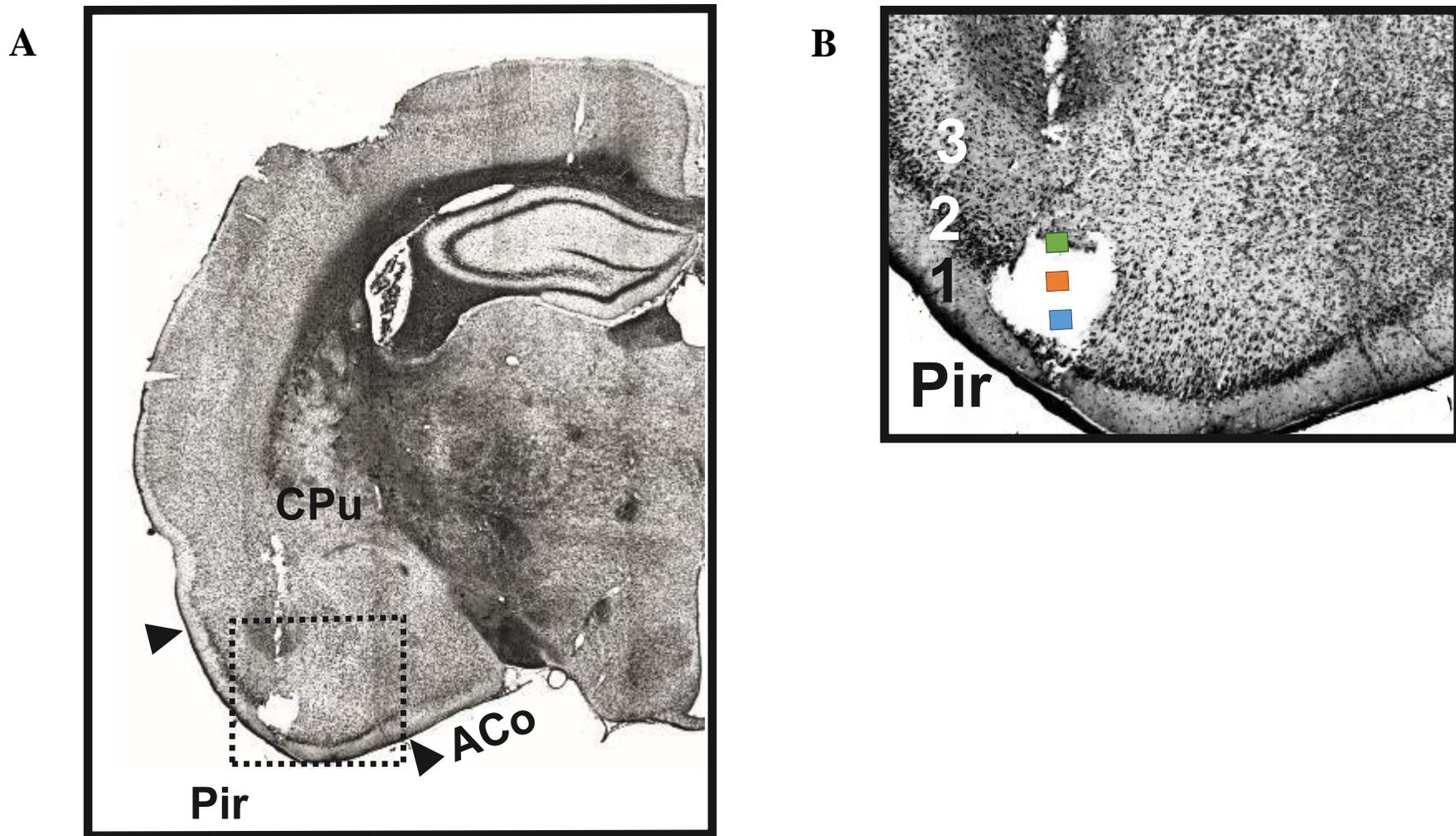


Figure 7.8. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 438, left hemisphere. The coronal section shown is from Bregma -2.4. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode track in the piriform cortex, tetrode 3; the green square represents the location of cells recorded in sessions 290814, 160914 and 040914, the orange square represents the the location of cells recorded in sessions 031014 and 101014 and the blue square represent the location of cells recorded in session 101214

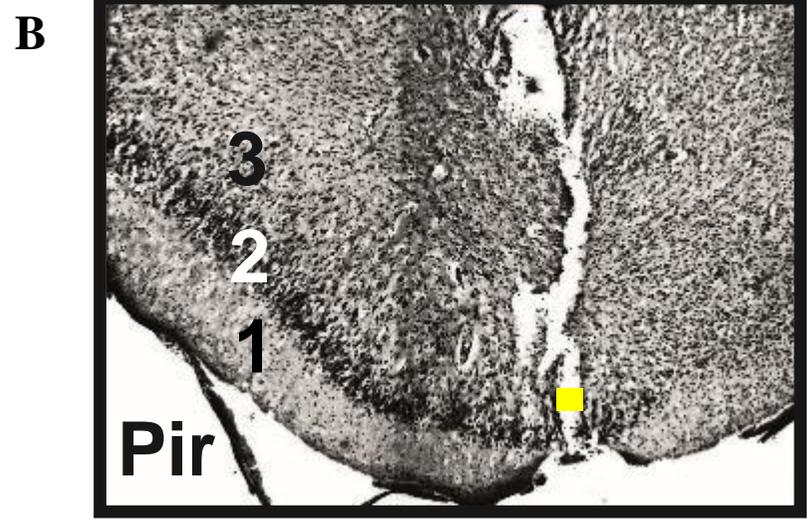
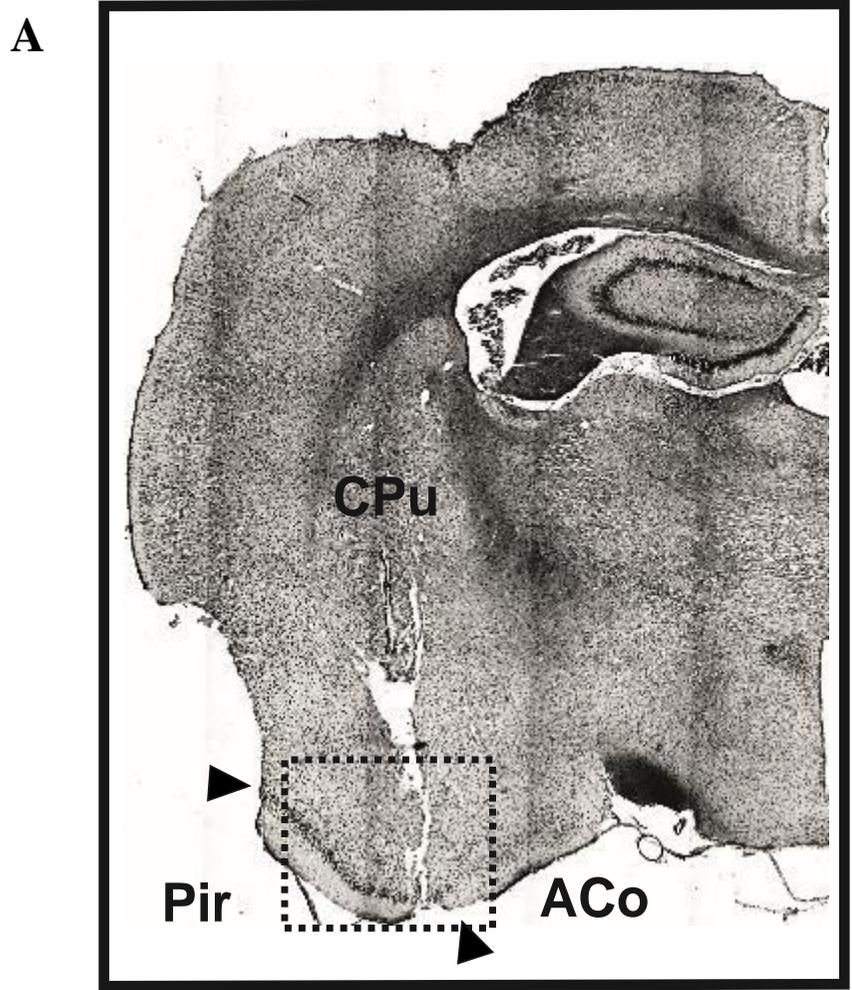


Figure 7.9. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 451, left hemisphere. The coronal section shown is from Bregma -1.9. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetrode track in the piriform cortex, tetrode 1; the yellow square represents the location of cells recorded in session 080415.

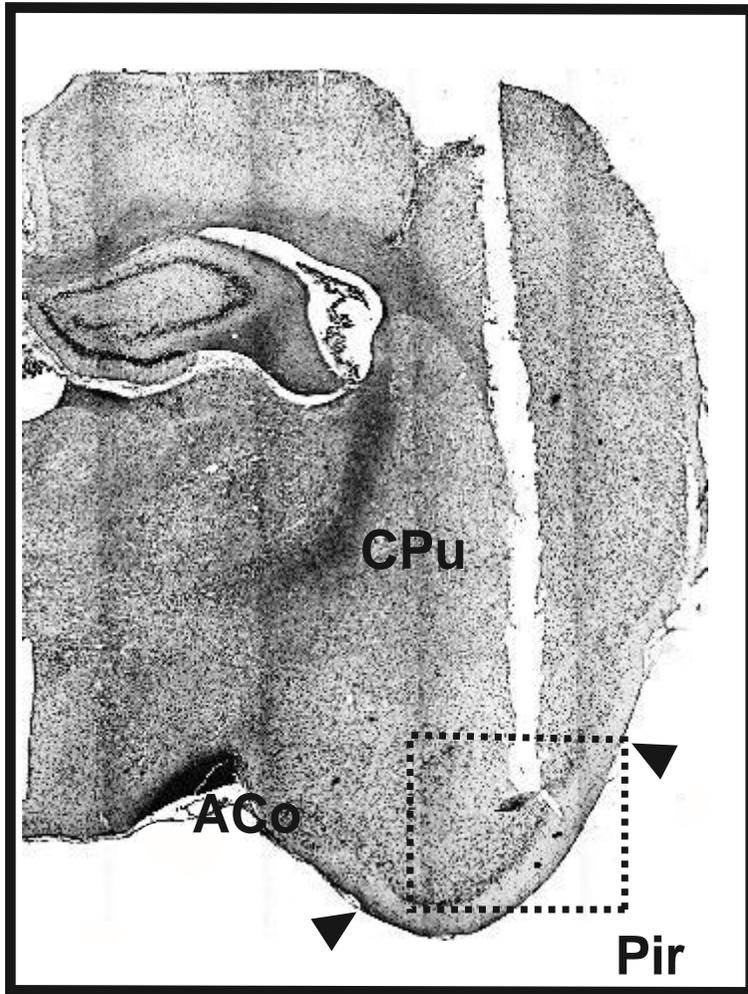
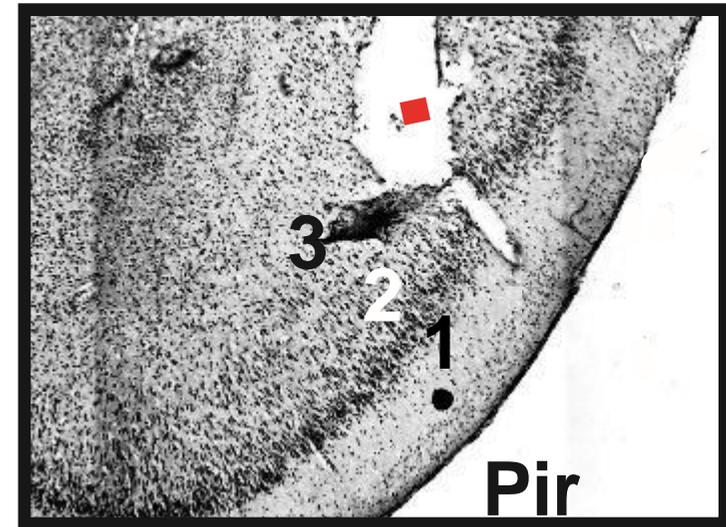
A**B**

Figure 7.10-. Location of cell-recording electrodes. A) shows the progression of the electrode track into the piriform cortex in Rat 451, right hemisphere. The coronal section shown is from Bregma -1.90. Arrows indicate the portion of the piriform cortex (Pir) in the section. B) shows the bottom of one of the tetraode track in the piriform cortex, tetraode 5; the yellow square represents the location of cells recorded in session 080415.

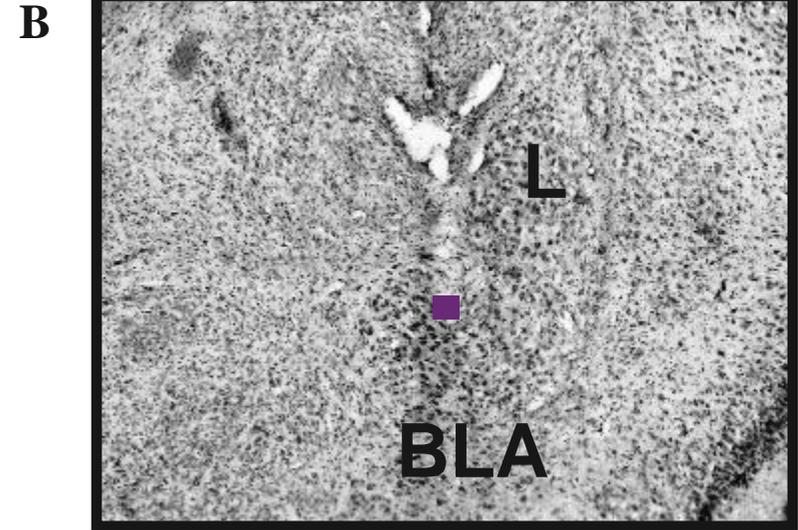
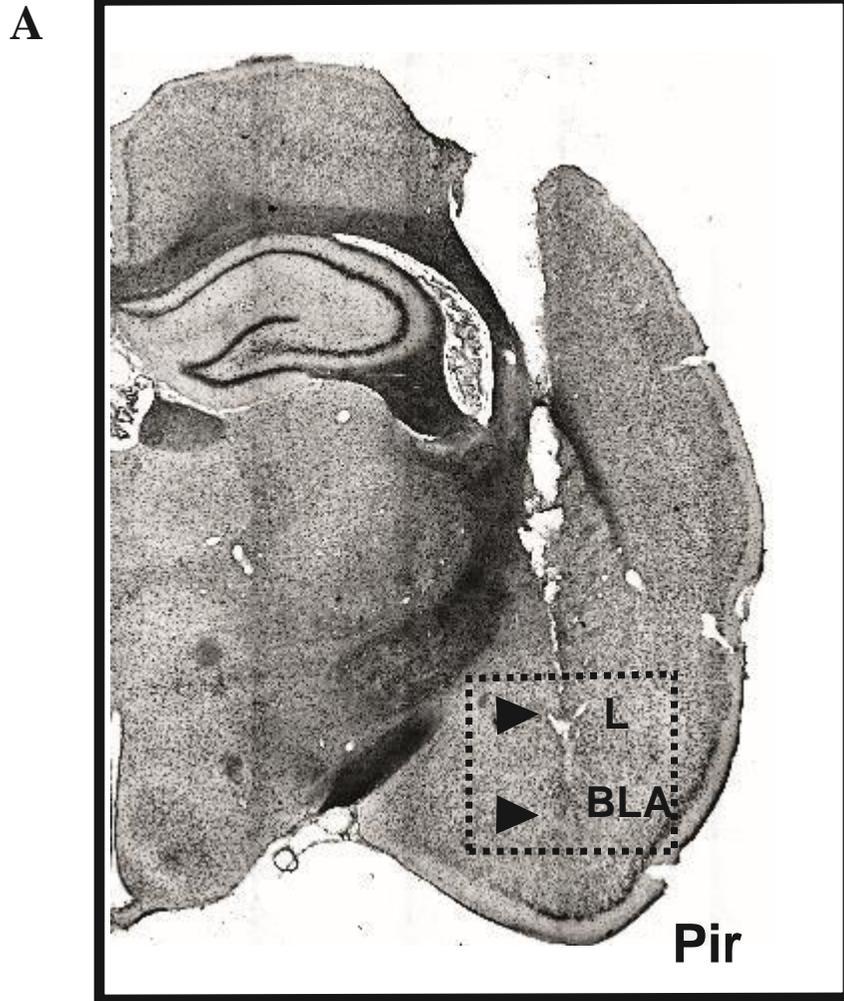


Figure 7.11 Representative location of cell-recording electrodes in the basolateral amygdala (BLA). A) shows the progression of the electrode track into the basolateral amygdala (BLA) in Rat 452, right hemisphere. The coronal section shown is from Bregma -2.1. Arrows indicate the portion of BLA in the section. B) shows the bottom of the electrode track in the piriform cortex; the purple square represents the supposed location of cells recorded in sessions 040615 and 290715.

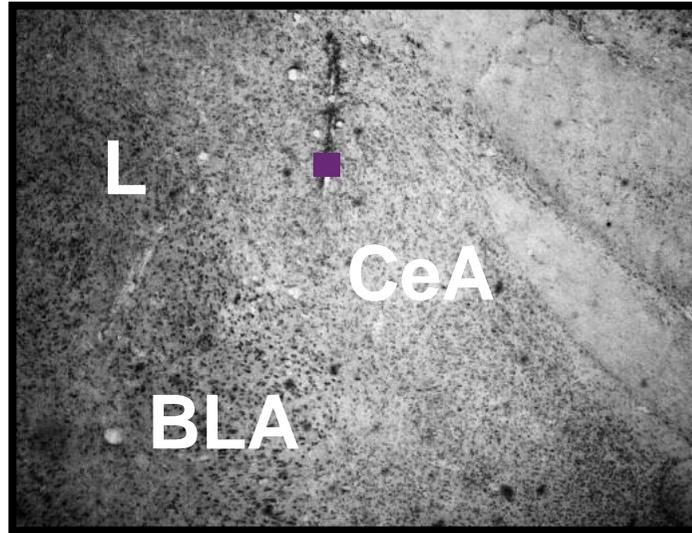


Figure 7.12 Representative location of cell-recording electrodes in the basolateral amygdala (BLA), coronal section shown is from Bregma -2.2. the image shows the bottom of the electrode track in the central amygdala; the purple square represents the supposed location of cells recorded in sessions 120614

Chapter 8: Social-Specific Cells

8.1 Experimental rationale

This is the first of a series of results chapters which attempt to address the gaps in our understanding of the neurological basis of social cognition, with a focus on social identity coding. Across 4 chapters, I will present data of 854 cells recorded for an average of 15 trials, where I cut on average 13000 clusters. Of those cells, 91 cells were recorded from the amygdala of two rats. The rest of the cells were recorded from the piriform cortex: 287 from layers 1-3, and 57 from the endopiriform nucleus, widely considered as the 4th layer of the piriform cortex by anatomists such as Swanson and Price (Swanson and Petrovich, 1998; Krettek and Price, 1977).

Specifically, the present chapter aimed to examine the role of neurons recorded in piriform cortex and amygdala in social interaction with a familiar conspecific, whose findings have inspired the social recognition experiments that will be presented later in chapter 8, 9 and 10. The idea of focusing on the amygdala is easy to understand, since the amygdala has been linked to autism (e.g. Baron-Cohen et al, 2000) and at many levels to social interaction and social recognition in rats (Choleris et al, 2007, Wang and colleagues, 2014). The focus on the piriform cortex can be explain by 4 important reasons. First the importance of olfaction in social recognition and emotional memory, in rodents as well as in humans, make the primary olfactory cortex one of the best candidate for being involved in social interaction (Kippin et al, 2003; Krusemark et al, 2013). Second, even if the olfactory input is dominant (Price, 1973), the piriform cortex is strongly interconnected with the amygdala (Majak et al, 2004) and the

orbitofrontal cortex (Illig et al, 2005). Third, the piriform cortex of female rodents, who are superior compared to males in social cognition and motivation (Engelmann, 1998), exhibits a higher density of receptors for the social neuropeptide oxytocin (Mitre et al, 2016). Indeed, in the study presented by Mitre and colleagues (2016) , the piriform cortex was the only region showing higher oxytocin receptor density in females compared to males. Finally, social identity coding for an individual conspecific may rely on circuit s pattern-separation and completion. The anatomical organization of the piriform cortex strongly suggests the presence of such circuits, and in particular the strong interconnection between pyramidal cells across all the piriform cortex, makes this brain area ideal to support pattern completion (Haberly, 2001; Barnes and Wilson, 2008; Franks et al, 2011, Hasselmo et al, 1996). However, almost nothing has been done in terms of neural recordings in the basolateral amygdala and the piriform cortex during a naturalistic, unconstrained social interaction that does not involve sex or aggressive behaviour. This first chapter will present an overview of the neural activity recorded in amygdala and piriform cortex trying to set up the basic knowledge for the next chapters that will focus on the role of piriform cortex in social identity coding.

8.2 Summary of the experimental procedure

As described in the method section, Lister-Hooded female rats were implanted with microdrives both the left and right piriform cortex or amygdala and habituated over a prolonged period to singularly encounter a familiar social stimulus in a small square box (40×40×50cm). During the social familiar condition (Fam) the implanted animal was exposed to the same familiar social stimulus for 6 trials. Social trials were interspersed with baseline trials (Base), where the implanted animal was alone inside the squared arena. The two conditions (Fam and Base) were repeated 6 times (6 three minute trials), for a total of 12 trials. Some of the sessions,

included trials where the subject animal was further tested for probes conditions, such as bedding from familiar or novel rats, familiar or novel objects, and Sphero, an electronically controlled ball intended to mimic biological motion. As shown in **table 8.1** cells were recorded from a total of 15 sessions: 7 sessions from three rats implanted in the piriform cortex, 5 sessions from one rat implanted into the endopiriform nucleus (deep Piriform) and 3 sessions from one rat implanted into the amygdala. Since the endopiriform nucleus is considered the 4th layer of the piriform cortex, in this chapter, the cells recorded from the endopiriform nucleus will be considered as part of the piriform cortex. The pie graphs in **figure 8.1** show the percentage of Social-Specific Cells recorded in the amygdala and piriform cortex

Table 8.1 Summary of cells considered for the Fam vs Base sessions. Four rats were implanted in the piriform cortex and 2 in the amygdala.

Animal code	Session	Track location	Total cells recorded	Total cells considered	Social-Specific Cells
426	160314	Deep Pir	16	11R	5R
426	220314	Deep Pir	15	6R	2R
426	080414	Deep Pir	12	5R	2R
426	070514	Deep Pir	7	3R	2R
426	090514	Deep Pir	7	1R	NS
438	290814	Pir	35	16L+14R	2L+5R
438	160914	Pir	68	42L+15R	11L+4R
451	170315	Pir	90	7L+55R	4L+14R
464	180516	Pir?	7	7R	4R
464	230616	Pir?	25	8L+16R	4L+7R
464	280616	Pir?	34	15L+17R	1L+2R
464	070716	Pir?	28	24R	4R
Total piriform ctx cells			344	N=262	N=73 (28%)
429	120615	CeA	2	2	1
452	040615	BLA	51	27L+8R	17L+3R
452	290715	BLA	38	17L+8R	9L+5R
Total amygdala cells			91	N=61	N=34 (57%)

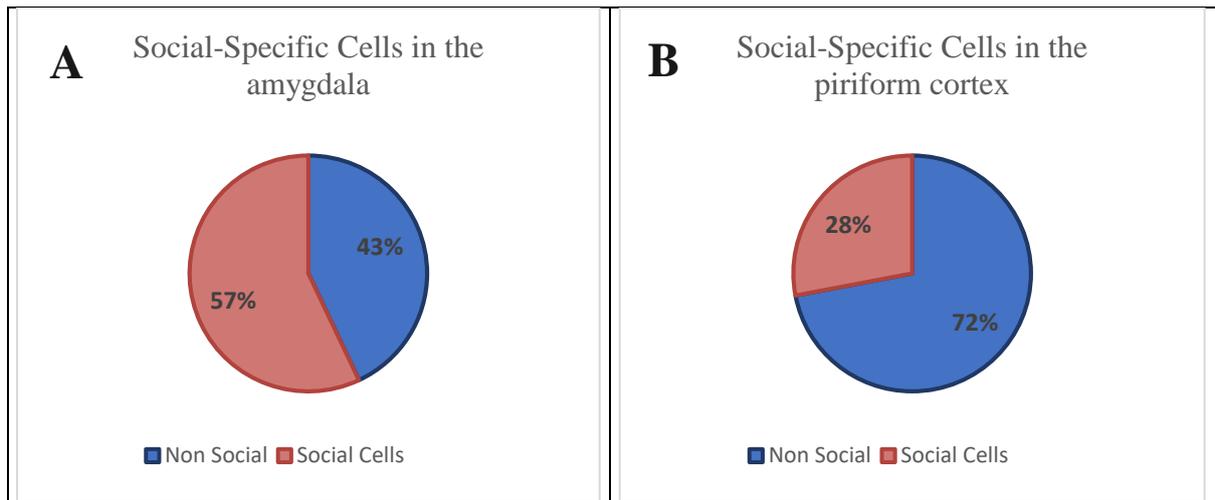


Figure 8.1. Percentage of Social-Specific Cells recorded in the amygdala (A) and in the piriform cortex (B).

8.3 Cells recorded in the BLA

8.3.1 Most cells recorded from the basolateral amygdala are influenced by the presence of a social familiar stimulus

Eighty-nine cells were recorded from the amygdala of two rats during the 3 sessions listed in **table 8.1**. A total of 61 out of 91 neurons were considered in this analysis because followed the main parameters described in the method section (Chapter 5), and were identified as pyramidal cells or interneurons with high stability across trials. Since not all cells' firing rate showed a normal distribution, in order to analyse all cells in a consistent manner I used a two-related sample non-parametric analysis, the Wilcoxon test. The non-parametric Wilcoxon test represents a more cautious approach because of the lower sensitivity but when data come from a skewed distribution, like in this case, this test can reach higher power compared to the t-test (Krzywinski & Altman, 2014). **Figure 8.2** shows the graphs for two representative cells

recorded from the basolateral amygdala which exhibited a significant change in firing when the implanted animal was paired with a familiar conspecific. With this approach, 34 cells out of (57%) were found to show a significant change in firing in trials where the implanted animal was exposed to a familiar conspecific compared to trials where the implanted animal was alone inside the arena [16/34, $z < -2.2$ $p < 0.03$]. Of these 34 cells, 22 (65%) increased the firing rate during social trials and 12 (35%) decreased the firing rate in social trials .

Two cells were recorded in the central amygdala and one of the two cells showed firing suppression during the social trials. The same cell has been recorded also for the familiar novel session. This cell will be further analysed in chapter 8.

I use this graph format throughout the thesis: for each neuron, the left side shows firing rate median for the non-social (Base) and social (Fam) conditions, and the right side shows the averaged waveform.

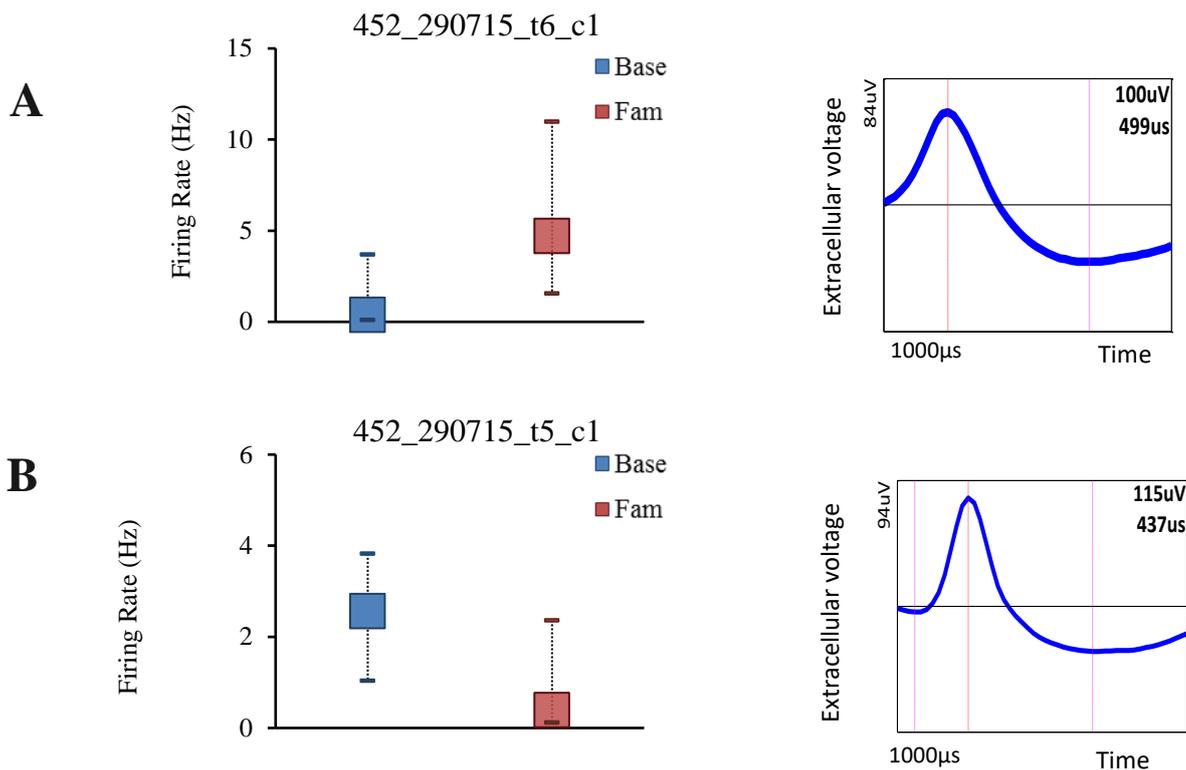


Figure 8.2 Key characteristics example of cells recorded in the basolateral amygdala, whose firing rates showed significant change for the social familiar condition. For both cells, left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Base, blue; Fam, red), the horizontal lines represent the maximum and the minimum values in each condition. The right panel shows the cell waveform. (A) Graph of one of the 22 cells which increased the firing during the social condition trials. (B) Graph of one of the 12 cells which decreased the firing during the social condition trials. In the waveform illustration, the X axis shows the time, the Y axis shows the voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

8.3.2 Interpreting cell firing changes

A clear majority of basolateral amygdala neurons (56%) showed altered firing in the presence of a familiar rat. Relative to baseline firing rates, about two-thirds of basolateral amygdala neurons increased their firing rate, with one-third decreasing rate. There are many factors that could account for changes in firing rate. While behavioural scoring offers the best avenue to constrain interpretation, it should be noted that scoring was burdensome, and it seemed appropriate to focus scoring upon the ‘Familiar vs Novel’ and ‘Familiar vs Familiar’ sessions

described in later chapters. This means that any understanding of this non-social vs social type session must remain preliminary. Here, the main interpretation comes from analysis of average trial speed, probe trials, and comparison to piriform cortex neurons. The primary global interpretation is that changes in firing reflect changes in motivation or cognition. They could also simply reflect changes in arousal. One approach to considering the effect of arousal is to examine average trial running speed. Average trial speed can be interpreted in two ways: an index of general arousal; and more strictly as a locomotion variable well-known to positively correlate with neurons throughout the hippocampal formation (e.g. O'Keefe et al, 1998; Lever et al, 2003;). Hippocampal place cells increase their firing rate at higher speeds, and the hippocampus and basolateral amygdala are strongly bidirectional connected. Accordingly, it was important to examine average trial speed as a variable.

8.3.3 Speed analysis

The firing rate of cells that showed a significant change in firing rate when the implanted animal was paired with a familiar stimulus, was further analysed in relation to running speed. Standard multiple regressions were run with average speed and Social status (Baseline = 0, Familiar = 1) as predictor variables for firing rate. These showed that Social status significantly predicted firing rates for 16 of the 34 cells, (Beta values ≥ 0.6 , $p \leq 0.05$). For 4 of the 16 cells the firing rate was predicted from both the Running speed and the Sister status (Beta values ≥ 0.6 , $p \leq 0.05$), Running speed did predict the firing rate of 12 cells (Beta values ≥ 0.6 , $p \leq 0.05$). In other words a majority of cells that showed a significant beta value in this underpowered analysis changed their rates because of the presence of a social animal.

This analysis is conservative in two respects. Firstly, the analysis is underpowered: 12 trials do not permit detection of low correlations, and power is further reduced in a multiple regression, such that the beta value needs to be at least 0.60 to be statistically significant. Secondly, more problematically, there may be not enough variance to disentangle potential confound between novelty and speed. The classic expectation in behavioural literature, and as shown here in many trials, would be that there is much more arousal and locomotion in trials where the implanted animal is paired with a social stimulus, relative to no social stimulus.

Where this is true, social status and locomotion will be highly correlated, and given there are only 12 trials considered in the session, there is insufficient variance to establish robust predictor status to either one of these variables. It is notable that in both sessions 040615 and 290715, the correlation between social status (Baseline = 0, Familiar = 1) and average running speed was high (040615, $r = 0.70$, $p = 0.002$; 290715, $r = 0.62$, $p = 0.017$).

8.4 Piriform neurons are influenced by the presence of a social familiar stimulus

Three hundred and thirty-four cells were recorded from the superficial layer ($n=277$) and the deep layer ($n=57$) of the piriform cortex from four rats in the 12 sessions listed in **table 8.1**. In total 262 out of 334 neurons were considered in this analysis because followed the main parameters described in the method section (chapter 5 Material and Methods), and were identified as pyramidal cells or interneurons with high stability across trials. Axons and cells firing less than 50 spikes were discarded from the analysis.

Since not all cells' firing rate showed a normal distribution, in order to analyse all cells in a consistent manner I used a two-related sample non-parametric analysis, the Wilcoxon test.

Figure 8.3 shows the graphs two representative cells recorded from the piriform cortex which exhibited a significant change in firing when the implanted animal was paired with a familiar conspecific.

With this approach, 72 cells out of 277 (28%) were found to show a significant change in firing in trials where the implanted animal was exposed to a familiar conspecific compared to trials where the implanted animal was alone inside the arena [44/72, $z < -2.2$ $p < 0.03$]. Of these 72 cells, 50 (69%) increased the firing rate during social trials and 22 (31%) decreased the firing rate in social trials.

In comparison to the basolateral amygdala data, the proportion of socially-altered cells was lower (Piriform 28% (72/277) vs BL Amygdala 56% (34/61). These were recorded from different animals, so this difference could simply reflect the source variance from different animals. This seems unlikely however, since the proportion of socially-altered cells never reached a majority of the sample in piriform rats (**426**: 42% (11/26); **438**: 33% (22/87); **451**: 29% (18/62); **464**: 27.5% (22/80).

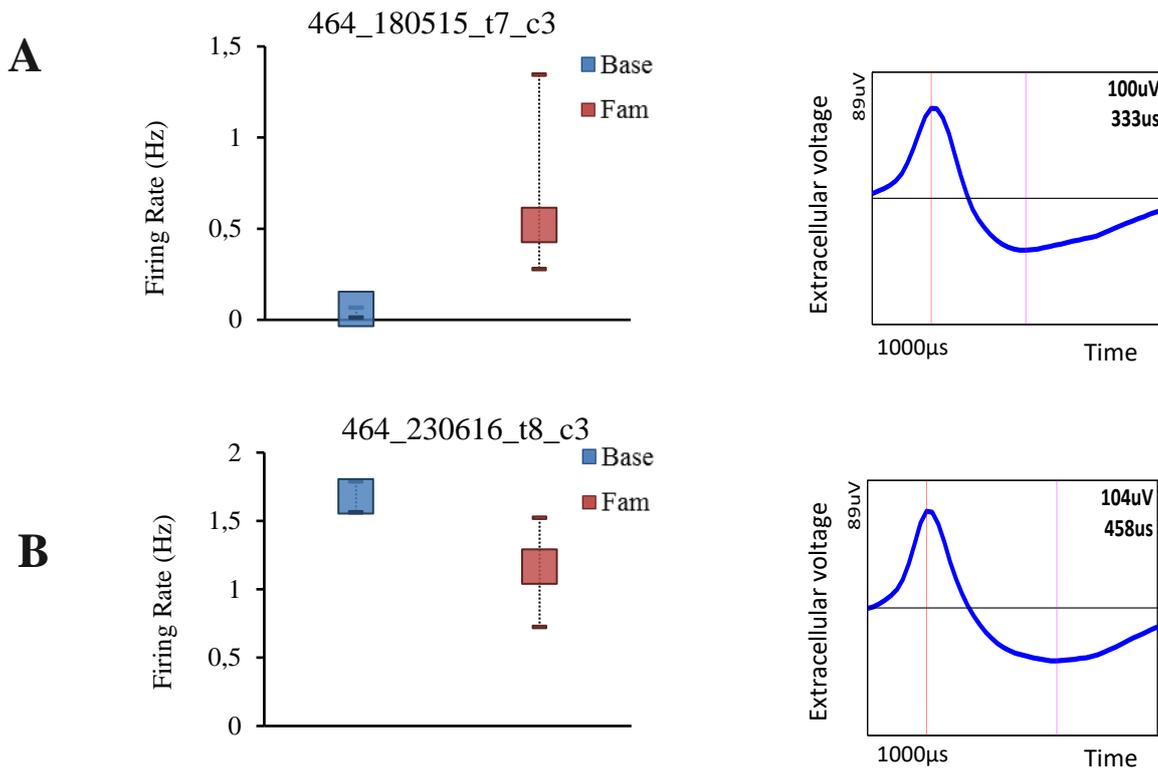


Figure 8.3 Key characteristics example of cells recorded in the piriform cortex, whose firing rates showed significant change for the social familiar condition. For both cells, left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Base, blue; Fam, red), the horizontal lines represent the maximum and the minimum values in each condition. The right panel shows the cell waveform. (A) Graph of one of the 50 cells which increased the firing during the social condition trials. (B) Graph of one of the 22 cells which decreased the firing during the social condition trials. In the waveform illustration, the X axis shows the time, the Y axis shows the voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

8.5 Probe trials

In some sessions, the standard social/non-social-trials were supplemented by probe trials with the attempt to narrow down interpretation. Even if performing statistical analysis on these probe trials was not possible due to the lack of multiple trials, the use of controls allowed to further understand the nature of the firing rate. In some sessions, the experimenter introduced a familiar object inside the testing box (see method section on chapter-section- for further information) to understand if neurons activity could be driven not only by familiar social

stimuli, but also stimuli of other nature like still objects. In other sessions, the experimenter used familiar bedding (see method section on chapter-section- for further information) attempting to understand if volatile olfactory cues were sufficient to trigger the firing rate in a similar way to rat-present trials.

8.5.1 Amygdala

At the end of the 12 trials in session 290715, the implanted animal (452) was exposed to a familiar object. Two out of 14 *neurons* exhibited firing rates that were higher than the mean of the Social trials in the whole-animal session , or were close to this mean (defined as “[Familiar object rate] \geq [Social mean – 0.5 Standard Deviation]). This means that only the firing rate of 14% of cells in the amygdala replicate the conspecific pattern in presence of the familiar object. **Figure 8.4** shows 2 simultaneously recorded cells; only one of them (B) increased the firing rate when exposed to the familiar conspecific and the familiar object.

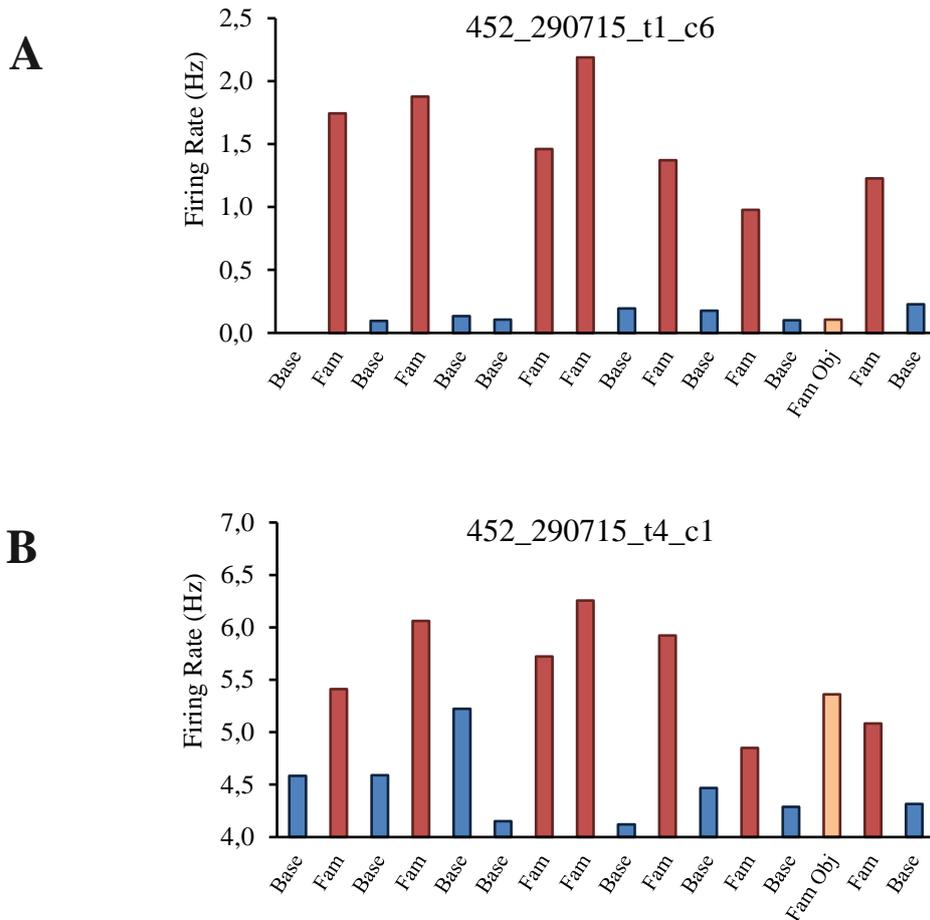


Figure 8.4: cells simultaneously recorded in the left basolateral amygdala showing different response in the familiar object trial. A) Cell 451_290715_t1_c6 increased the firing during social trials but not during baseline trials or the familiar object trial. (B) Cell 451_290715_t4_c1 increased not only during social trials but also in the familiar object trial.

8.5.2 Piriform cortex

At the end of the 12 trials in sessions 180516, 280616 and 070716, the implanted animal (464) was exposed to a still familiar stimulus (Fam Obj) or to a moving familiar stimulus (Sphero the ball). Sphero is a ball that could be controlled with a phone app and it had the aim to mimic the presence of a moving interacting stimulus in the arena (see chapter 6 Behavioural Methods) In these three sessions, the firing rate of 11 neurons discriminates between social trials and non-social trials.

Familiar object

Two out of seven *neurons* (28%) exhibited firing rates that were close to this mean (defined as “[Familiar object rate] \geq [Social mean – 0.5 Standard Deviation]).

Ball (Sphero)

Two out of 11 *neurons* (18%) exhibited firing rates that were higher than the mean of the Social trials in the whole-animal session or were close to this mean (defined as “[Ball] \geq [Social mean – 0.5 Standard Deviation]).

Bedding

In sessions 230616, 280616 and 070716, at the end of the 12 trials, the implanted animal (464) was exposed to bedding containing urine and excrements of the familiar social stimulus used in the session. In these three sessions, the firing rate of 18 neurons (13 social higher neuron) discriminate between social trials and baseline trials. Four out of 13 Social-higher *neurons* (30.7%) exhibited firing rates that were higher than the mean of the Social trials in the whole-animal session or were close to this mean (defined as “[Bedding] \geq [Social mean – 0.5 Standard Deviation]). Nine out of 13 did not repeat the conspecific pattern observed in the whole trial. However, we can be sure that this was not due to inadequate bedding stimuli (e.g. staleness) because these 9 neurons were simultaneously recorded with neurons which replicate the pattern of the whole conspecific trial. **Figure 8.5 A** shows a cell which increased the firing rate when the implanted rat (464 in the example) was exposed to the social stimulus and to the familiar bedding, but not when exposed to the ball (Sphero). **Figure 8.5 B** shows a cell which firing increased only in presence of the familiar social stimulus.

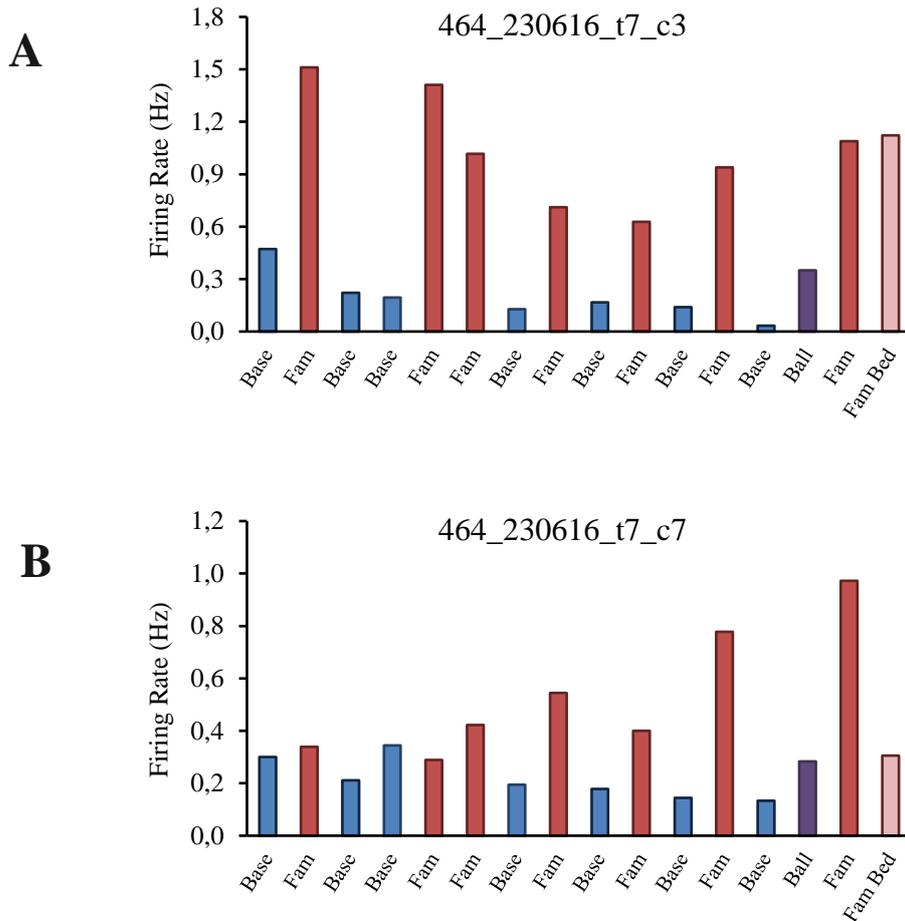


Figure 8.5. Two simultaneously recorded cells which responded differently when exposed to the familiar bedding. (A) Cell 464_230616_t7_c3 increased the firing when the implanted animal was exposed to the familiar conspecific (Fam) and to the familiar bedding (Fam Bed) but not when exposed to Sphero (Ball). (B) cell 464_230616_t7_c7 increased the firing only in social trials but not in the familiar bedding trial or Sphero.

8.6 Discussion

The present chapter was designed to pre-investigate the role of the piriform cortex and the amygdala and their role in social cognition. While the next chapters will focus on the role of the piriform cortex in coding a conspecific social identity, here I gave a first introduction on the neuronal response of piriform cortex neurons when exposed to a familiar conspecific. At the same time, I recorded cells from the amygdala which is strongly inter-connected with the

piriform cortex and the hippocampus (Mouly and Scala, 2006; Majak et al, 2004; Sah et al, 2003) to examine how these neurons were influenced by the encounter with a familiar conspecific. In summary, my data showed that 28% of the neurons recorded from the piriform cortex exhibited a change in the firing rate when the implanted animal was exposed to a familiar conspecific. Furthermore, most cells (57%) recorded from the amygdala of a single rat showed a firing excitation or firing suppression in rat-present trials.

Clearly, the unconstrained interaction used in these experiments open the door to different interpretation with a focus on the general arousal that the encounter with a conspecific can produce in comparison to baseline solitary trials inside the arena. On the other hand, the freely social interaction with a familiar conspecific allowed a naturalistic approach that has barely been tried before with neural recordings, permitting to observe how the piriform cortex and the amygdala neurons fully works in social interaction.

8.6.1 The role of the amygdala in social interaction

Two of the experimental sessions presented in this chapter, aimed to study the neuronal response of amygdala neurons in response to a familiar conspecific. The amygdala has been widely linked to negative emotions, such as fear. However, there is a considered amount of studies which show the importance of the amygdala in processing positive emotions, in particularly the one related to positive reward, and social interaction is considered a positive reward, specially, in the case of isolated animals. Previous works have repeatedly linked the amygdala to social behaviour, in both human and non-human primates, and rodents (Adolphs et al, 1998, Felix Ortix and Tye, 2014, Katayama et al, 2009). However, very few studies focused on the neuronal response of the amygdala during social interaction with a conspecific.

What is known is that the amygdala in monkeys, has different types of neurons: neurons which respond specifically to a certain sensory stimulus (to visual, auditory or somatosensory), neurons that are multimodal, and neurons which respond only to a specific biologically relevant stimulus, such as visual stimulus associated with a certain type of food, and neurons which respond selectively to faces (Baxter and Murray, 2002). It may be the case that the firing discrimination observed between baseline and familiar social trials, in a similar way to the amygdala in non-human primates, may be the response of neurons to specifically biological stimuli, in case of my experiment, the smell and the vision of a familiar social stimulus. In fact, the amygdala is linked with an array of multiple subcortical and cortical structures such as the nucleus accumbens, the medial and the orbitofrontal cortex and sensory cortex like the piriform cortex, which may explain the social firing discrimination between the social and the non-social condition.

A further interpretation of my findings comes from the use of probe trials. Neurons in the amygdala that were recorded in sessions with a familiar object as well as social vs non-social trials showed heterogeneous response in presence of a familiar object. Again, similarly to what has been observed in monkeys, in rodents, cells in the amygdala may have different roles. In fact, some cells were excited independently by the nature of the stimulus, the familiar object or the familiar conspecific, others instead were excited only in presence of the familiar object, but more interestingly, some cells were excited only by the familiar social stimulus. This may further strength the thesis that amygdala neurons are involved in positive rewards and there are neurons in the amygdala that respond to a specific biological relevant stimulus, and maybe specifically for that conspecific.

For future studies, would be interesting to see if the activity of neurons that fires selectively in response to the familiar social stimulus, can be modulated manipulating the affective significance of the social stimulus, similarly to what has been already done in monkey for food.

Another interpretation of my results comes from the fact that the amygdala has been often linked with general arousal. The speed of an animal is often related with the arousal state, and previous studies showed that spontaneous and sensory-evoked activity in the cortex is highly state-dependent (Vinck et al. 2015). For example, in mice, locomotion can increase the firing rate of cells in the primary visual cortex (Keller et al, 2012), and place cells in the hippocampus increase the firing in the place field in relationship to the animal 'speed (McNaughton et al., 1996; O'Keefe et al, 1998). The presence of a conspecific inside the arena creates an aroused state that may not be observed when the animal is alone inside the apparatus and it may be represented by the increase of locomotion. In fact, the speed of the animal in the three sessions, is correlated with the trial conditions, meaning that there was an effect on locomotion when the implanted animal was paired with the familiar conspecific. Nevertheless, the change in firing in almost half of the social-discriminative cells was related to the social condition but not to the speed, suggesting that general arousal alone may not explain the changes in firing observed when the animal was paired with a familiar conspecific at least in those cells.

Despite a different experiment set up, the results I presented in this chapter are partially in line with previous findings from Katayama and colleagues (2009). In fact, Katayama and colleagues found that the majority of cells recorded in the amygdala shows an alteration in the firing activity when the implanted animal is exposed to a novel conspecific. However, they found that social exposure caused firing excitation in the 49% of the recorded cells and firing suppression in only 3% of the cells. In my findings instead, following the exposure with a

familiar conspecific, while 37% of the cells increased the firing activity, a much larger proportion of the cells, namely 20%, showed firing suppression. A first possible explanation for the percentage differences in cells that showed firing suppression may be given by the different set up used. In the Katayama set up, the implanted animal was exposed to a single 1.30h trial (1h baseline, 30 minutes social) where the last 30 minutes of alone time in the arena were compared with 30 minutes of social time with a novel conspecific. While the first 30 minutes of baseline were discharged because considered acclimation, they only considered the last 30 minutes of baseline, when the animal had already explored the arena, and when the arousal levels were low. For this reason, it is believable that inserting a novel animal in the arena induced a global arousal with consequent excitation of amygdala neurons. The Katayama comparison rests on a design that is far from optimal. On the other hand, in my study, the multiple-trial set up and the semi randomised order of the trial conditions minimised the arousal problem. A second possible explanation on this discrepancy, may be given by novelty, since in the Katayama set up the implanted animal was paired with a novel conspecific. In fact, it is well known that emotional arousal, for novelty, enhance the amygdala activity (Blackford et al, 2010). Finally, a third explanation may be given by the familiarity of the implanted animal with the social stimulus. It may be the case that some cells in the amygdala reduce the firing in presence of a very familiar conspecific. I will further discuss this point in the next chapter (chapter 8), but here I want to point out a cell recorded from the central amygdala which showed a lower firing during social trials compared to baseline trials, but it robustly increased the firing rate when exposed to novel social stimuli (see Chapter 8.x below). This may suggest that the amygdala contributes in coding the information of familiar and novel conspecifics.

Clearly much further work needs to be done to understand the role of the amygdala in social interaction. In future, would be interesting to understand if the firing rate correlates with the

behavioural scoring. Another interesting aspect would be to further investigate the role of conspecific familiarity always in relation to the firing rate, using a multi-trial approach where the implanted animal gets exposed to two different familiar conspecifics.

8.6.2 The role of the piriform cortex in social interaction

The social/non-social experiments in this chapter were designed to produce a first analysis of neurons recorded from the piriform cortex and their response to the multi-modality sensory inputs which the free behavioural encounter with a conspecific may provide. Rodent social interaction is mostly driven by odorant olfactory cues and each odorant activates a subpopulation of neurons in the piriform cortex (Stettler and Axel, 2009). The role of the piriform cortex in olfaction (Barnes et al, 2008; Wilson and Sullivan, 2011) is only one of the reasons of why this structure is one of the best areas where recording during rodent social interaction. In fact, the piriform cortex is strongly interconnected with areas involved in social cognition such as the amygdala and the orbitofrontal cortex (Majak et al, 2004, Illig et al, 2005). Furthermore, female rats which show better social motivation and social cognition compare to males (Markham, 2007, Engelmann, 1998), have a higher density of receptors for the pro-social neuropeptide oxytocin (Mitre et al, 2016). These strongly suggest that the piriform cortex is one of the areas strongly involved in social interaction. Even if the piriform cortex has been widely studied for its role in odorant olfactory cues including from an electrophysiological point of view, on my knowledge there are not studies that provide neuronal recordings from a naturalistic unconstrained social interaction. On the contrary, most of the studies which involve the recordings from piriform neurons are carried out on anaesthetised animals. Recording from anaesthetised animals can be preferable since it simplifies technical problems of electrical recording, and it gives the possibility to control physiological variables. However, it is also

true that recording neurons in the piriform cortex from anaesthetised animals only allow to observe how the neurons process olfactory cues without taking in consideration that the piriform cortex is strongly interconnected with other brain areas. For example, Gottfried and colleagues (2004) showed that if an object during the encoding phase is paired with an odour, during the retrieval phase there will still be observed the activation of the piriform cortex even if in this phase the object is not paired with the smell. This is further confirmation that the piriform cortex is not just processing odour cues and the use of anaesthetised animals limit the understanding of this olfactory primary cortex. On this purpose, the unconstrained social interaction, permits to have the full experience with a specific social stimulus which is not just olfaction, but it comprises multi-set of information from other sensory stimuli such as auditory, visual, gustatory and somatosensory.

My findings, probably for the first time, showed that cells in the piriform cortex are responsive during social interaction with a familiar conspecific. Even more interestingly, while some cells were responsive both to the conspecific and to the conspecific' odours, other cells were responsive only to the conspecific. This may suggest that some cells are not purely olfactory related, but may require the multi-sensory modality inputs for the conspecific representation.

A first important consideration that needs to be taken into account is that, even considering just the olfactory cues (not somatosensory, visual, auditory and gustatory), the social stimulus is a mixture of multiple odorants, including volatile and non-volatile odorant cues. The exposure to a single odour, only activates a small ensemble of neurons, but the same neurons can respond to multiple odorants (Stettler and Axel, 2009; Miura et al, 2012). This does not mean that the neural pattern representation for a mixture of odorant is the sum of the neural pattern representation for each odorant; otherwise this would result in a massive global neuronal activity and overlapping patterns. In fact, Stettler and Axel (2009) showed that 40-60% of the

cells that showed a response for a single odour, failed to respond to a mix of odours, and the ones which were still responsive to the mix, showed a diminished response.

Similarly, my findings showed data the mixture of odour of a social stimulus cause in the 8.4% of the case, suppression of the neural activity compared to non-social trials. This is in line with the most credited hypothesis which sees individual cells in the piriform cortex integrating the inputs from multiple glomeruli, resulting in the activation or suppression of cells that did not show a response to the individual component of a mix (Stettler and Axel, 2009). Suppression can be easily explained by the strong inhibitory local feedback from layer 1, that scale with excitation (Franks et al, 2011) and may be essential to normalise the function, maintaining active only a certain number of neurons independently by the mixture of odorants (Stettler and Axel, 2009).

Finally, the neural recording I presented in this chapter are not just a mixture of multiple odorants, but it is the mix specific for a single individual, like a signature. It is plausible that during social recordings different piriform subpopulation of neurons were working together as ensembles to code the olfactory information for that specific individual. In fact, it has been widely demonstrated that the segregated organization observed in the olfactory bulb, is dispersed in the piriform cortex where different odorants activate unique ensembles of cortical neurons (Stettler and Axel, 2009). This may suggest that in the primary olfactory cortex there may not exist an equivalent of the ‘grandmother cell’, but neurons may work together to give the odour representation of that specific animal. In few words, cells which did not show any significant effect for the social condition, may still work together with other neurons to code the conspecific signature and recognise a specific familiar animal. Clearly, further analysis needs to be done to fully understand the role of piriform cortex neurons in encoding the

conspecific signature, but in the next chapters I will further analyse the role of the piriform cortex in social recognition.

Chapter 9: Social Novel-vs-Familiar Specific Cells

9.1 Experimental rationale

The present chapter aims to analyse the role of piriform cortex in social interaction with another conspecific. In chapter 8, I showed cells recorded from the basolateral amygdala and the second and third layer of the piriform cortex, which changed the firing activity during the presence of a familiar female rat. Results obtained in the amygdala can be easily explained by the role of the amygdala not only in rodents but also in primates including humans, in facial recognition, emotion recognition, arousal and social interaction. The interesting findings obtained in the piriform cortex require instead a further analysis to better interpret the role of this olfactory paleocortical region in social interaction. The role of the piriform cortex in processing olfactory cues and the relevance of olfaction in rodents as well as humans in social recognition, are the theories that support the experiments carried out in the present chapter. The basic idea of social and non-social trials, was adapted to interpret the role of the piriform cortex in the context of social interaction. For this reason, social trials in this experimental chapter will include not only familiar social stimuli but also novel social stimuli. The use of novel and familiar conspecifics was relevant to understand if odorant cues could elicit piriform neurons independently by the social stimuli used, or if familiar and novel odorant cues could be discriminated by piriform neurons showing an involvement of this area in the complex circuit of social recognition memory. With the attempt to constrain interpretation of experimental findings, electrophysiological recordings were supplemented by social behavioural scoring, the use of probe trials and the by the identification of the vaginal smear of the social stimuli used.

This chapter, will provided evidences of neurons in the piriform cortex which could discriminate between familiar and novel conspecific.

9.2 Summary of the experimental procedure

As described in the method section, three Lister-Hooded female rats were implanted with microdrives in both the left and right piriform cortex and habituated over a prolonged period to singularly encounter a familiar social stimulus or a novel social stimulus in a small square box (40×40×50cm). Each condition was repeated 6 times (i.e. six trials each lasting three minute), for a total of 12 trials when the sessions included only two conditions, **social familiar** and **social novel**, and 18 trials when the social trials were interspersed with baseline trials, where the subject animal was placed alone into the square box. During the **social familiar** condition the implanted animal was exposed to the same familiar social stimulus for 6 trials, while during the **social novel** condition it was exposed to a novel social stimulus in each of the 6 trials. Some of the sessions included trials where the subject animal was further tested in probes conditions, such as familiar or novel bedding, familiar or novel objects, or Sphero. As shown in **table 9.1** cells were recorded from 6 different sessions.

Table 9.1 Summary of cells considered for the Fam vs Nov sessions in three of the rats implanted in the piriform cortex and one rat implanted in the amygdala

Animal code	Session	Conditions	Track location	Total cells recorded	Total cells considered	Social novel-vs-familiar specific cells
429	120614	Fam, Nov	CeA	2	2	1
Total amygdala				2	2	1
438	040914	Fam, Nov	Pir	42	8 R +18 L	3 R +2 L
438	101214	Base, Fam, Nov	Pir	35	8 R +14 L	2 L
451	080415	Base, Fam, Nov	Pir	37	13 R +6 L	2 L
464	010716	Fam, Nov	Pir?	34	17 R +15 L	5 R +3 L
464	260716	Fam, Nov	Pir?	38	32 R	10 R
Total piriform ctx				186	132	27 (20.5%)

9.3 Cells Recorded in the amygdala

9.3.1 One of two cells recorded in the amygdala was influenced by the presence of a novel social stimulus

Two cells were recorded from the central amygdala of one rat during a single 12-trials session listed in **table 9.1**. The two cells were considered in the analyses because followed the main parameters described in the method section, and were identified as pyramidal cells with high stability across trials. The two cells were analysed using Wilcoxon test since variables violated criterions of normal distribution. One of the two cells analysed strongly increased the firing rate when the implanted animal was exposed to a novel conspecific ($z=-2.201$, $p=0.028$, **figure 9.1**).

9.3.2 Interpreting cell firing change

One amygdala cell out of two show altered firing in the presence of novel rats. There are many factors that could account for changes in firing rate. The primary global interpretation is that changes in firing reflect changes in motivation or cognition. Here, the main interpretation comes from analysis of average trial speed, and by the fact that the same cell whose firing rate was higher during the novel trials, was earlier tested for the 'Baseline vs Familiar' session (see chapter 8).

Average trial speed can be interpreted in two ways: an index of general arousal; and more strictly as a locomotion variable well-known to positively correlate with neurons throughout the hippocampal formation (e.g. O'Keefe et al, 1998; Lever et al, 2003). Hippocampal place cells increase their firing rate at higher speeds, and hippocampus and amygdala are strongly bidirectionally connected. Accordingly, it was important to examine average trial speed as a variable. A standard multiple regression was run with average speed and novel status (Familiar= 0, Novel= 1) as predictor variables for firing rate. These showed that both social novelty status as well as average speed significantly predicted the firing rates of this social-novelty altered cell, (Novel status: beta value = 0.65, $p=0.01$; Average speed: beta value = 0.5, $p \leq 0.028$). Furthermore, a paired simple t-test showed that the session did not show any significant difference in terms of average speed (cm/s) between the novel and the familiar condition ($t(5)=0.19$, $p=0.86$). In other words, the cell that showed a significant beta value in this underpowered analysis changed its rates because of the presence of the novel social stimulus.

The classic expectation in behavioural literature, would be that novel social stimuli elicit higher arousal and motivation compared to familiar social stimuli. Accordingly, the cell presented in

figure 9.1, increased the firing in trials where a novel conspecific was inside the apparatus. However, it would have also been expected that a familiar stimulus elicits higher arousal and motivation compared to alone time (baseline trials). If this cell was driven by motivation and arousal, I should expect the cell increasing the firing during the social trials, compared to baseline. However, it seems unlikely that motivation was driving the firing rate of this cell since the same cell showed firing suppression during familiar social trials, when compared to novel trials and baseline trials. On the other hand, the strong increased firing activity that the cell showed in the presence of novel animals may reflect the encoding of the memory trace for the novel stimulus, in a similar way as pyramidal cells' burst in the hippocampus is the key for the LTP and the subsequent memory formation (Buzsaki et al, 1989, Yiu et al, 2014).

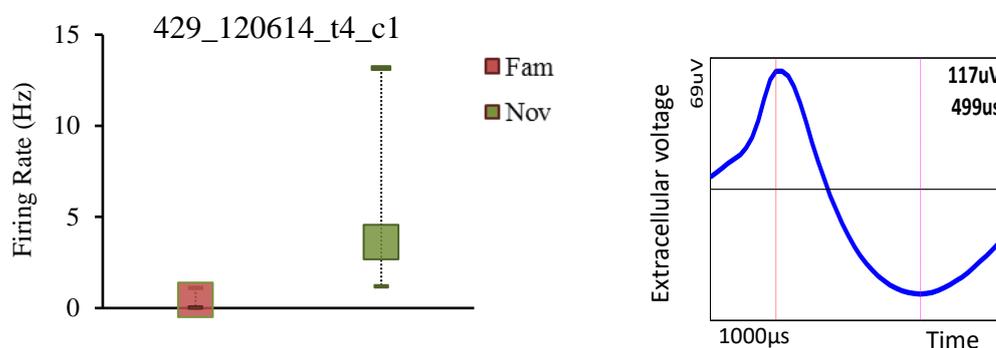
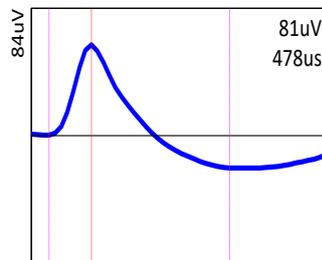
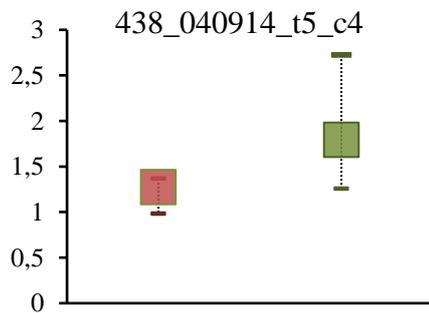
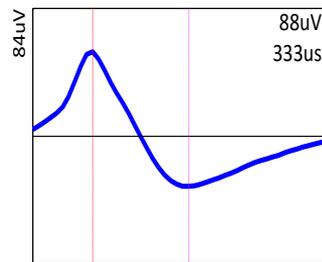
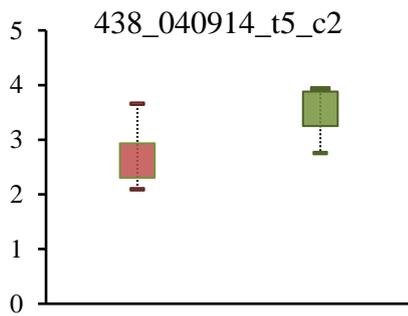
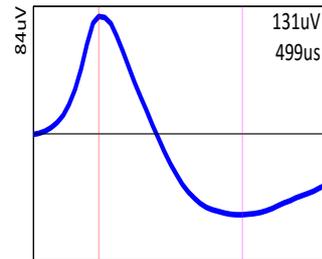
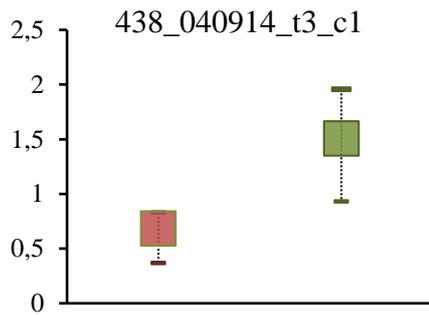
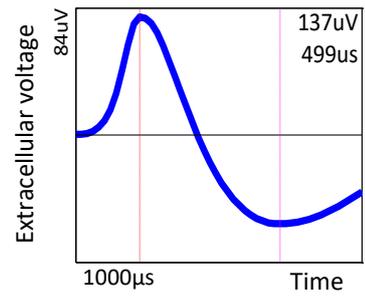
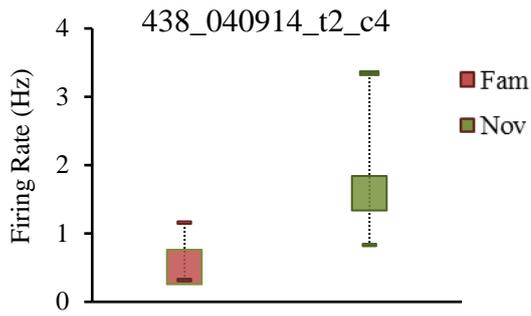


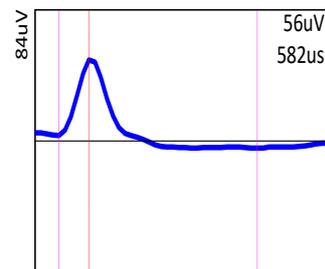
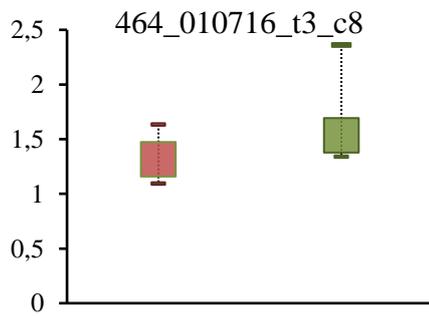
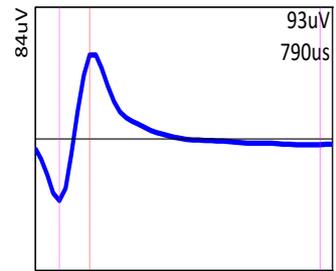
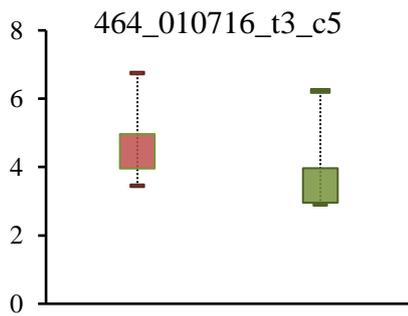
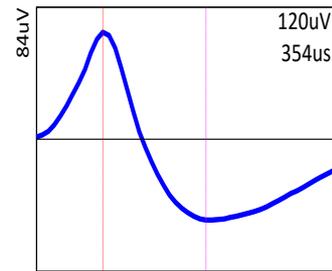
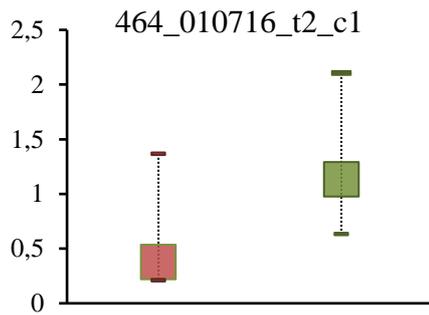
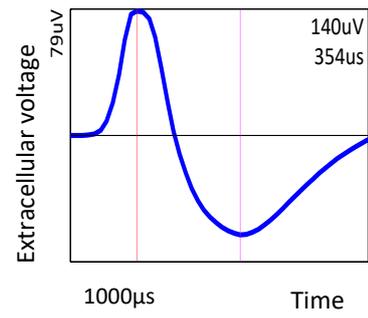
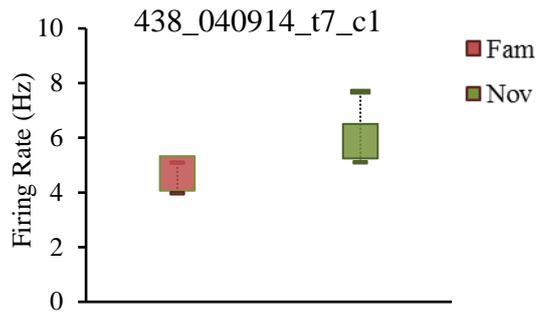
Figure 9.1 Key characteristics of the cells recorded in the central amygdala, whose firing rate showed preferential firing for novel conspecific. Left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Fam, red; Nov, green), the horizontal lines represent the maximum and the minimum firing rate values in each condition. In the illustrations of the waveform, the X axis shows the time, the Y axis shows voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

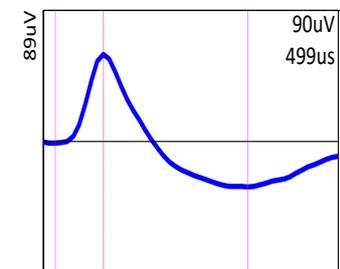
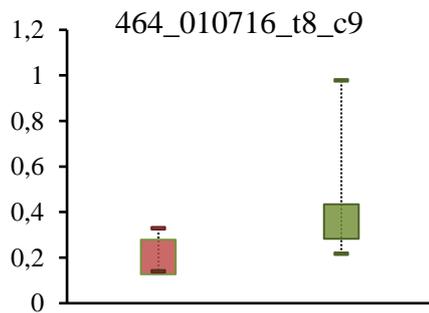
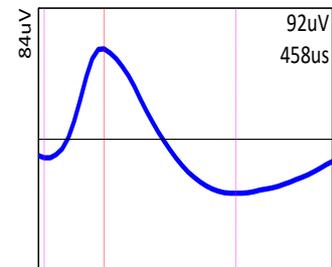
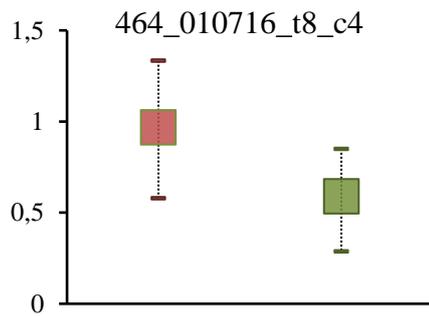
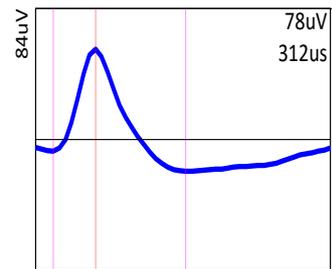
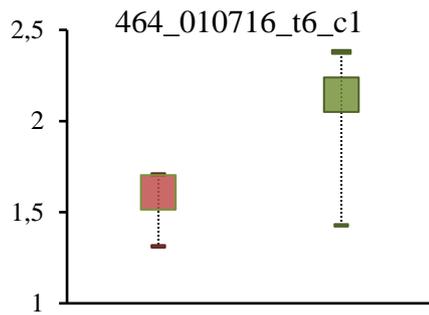
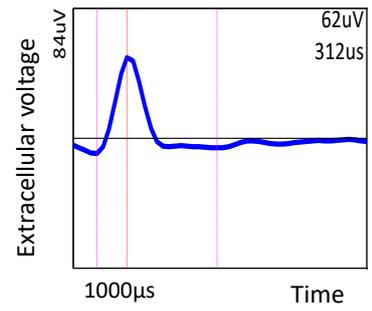
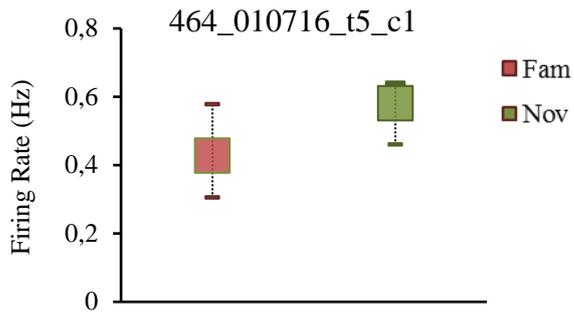
9.4 Cells recorded in the piriform cortex

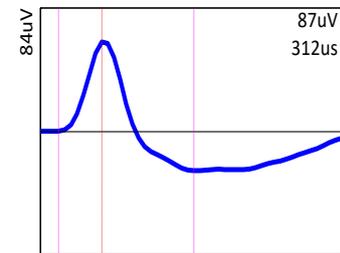
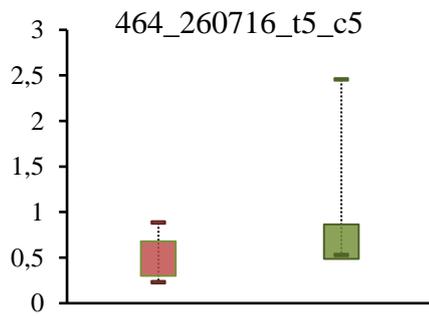
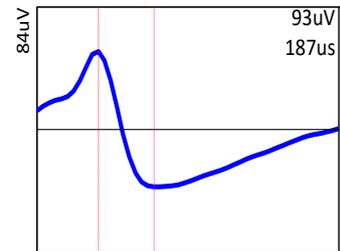
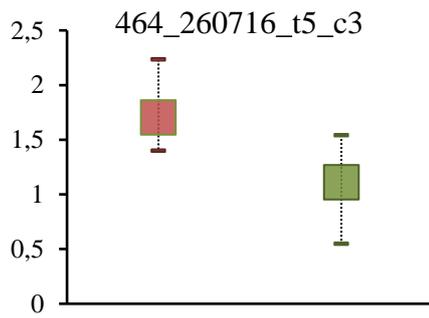
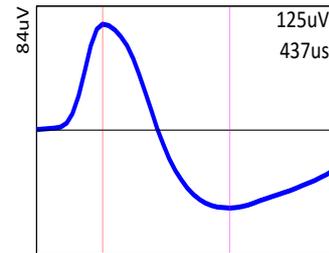
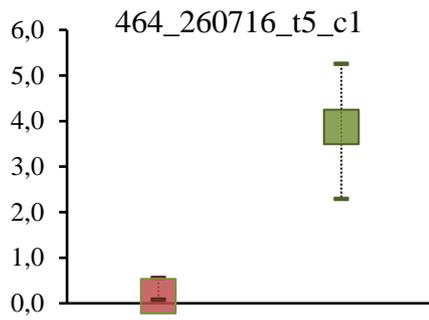
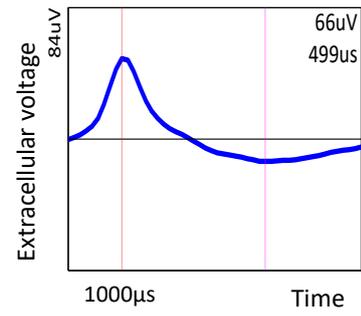
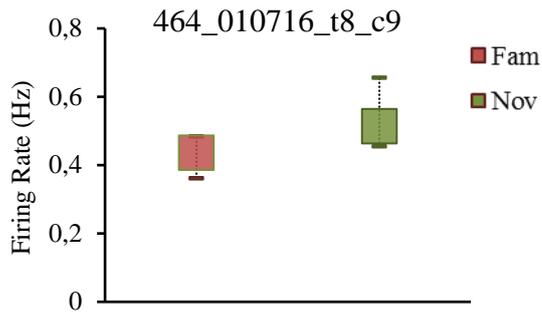
One-hundred and eighty-six cells were recorded from the piriform cortex of three rats during the 5 trial sessions listed in **table 9.1**. A total of 132 neurons were considered in this analysis because followed the main parameters described in the method section, and were identified as pyramidal cells or interneurons with high stability across trials. Of the 132 cells analysed, 90 cells were recorded during the two-condition sessions (Fam vs Nov; **figure 9.2**), while the rest of the cells (n=41) were recorded during the eighteen-trial session where familiar and novel trials were interspersed with baseline trials (**figure 9.3**).

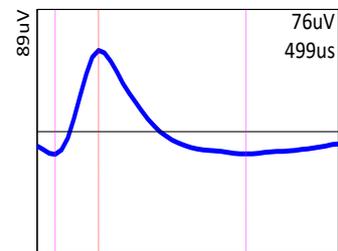
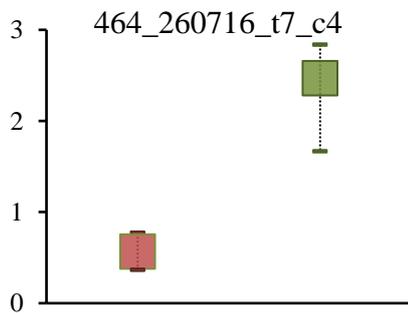
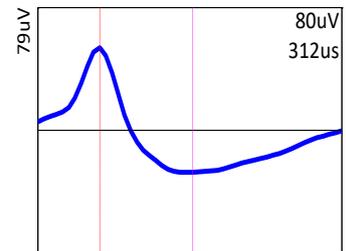
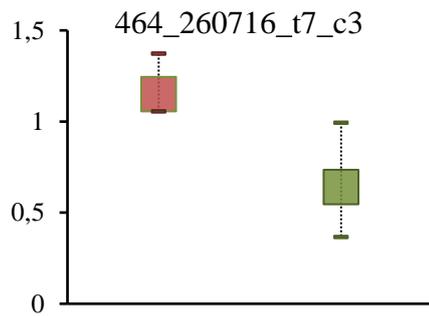
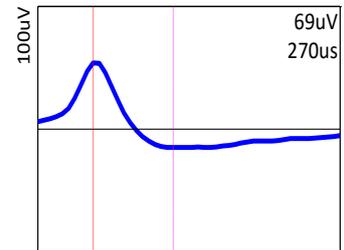
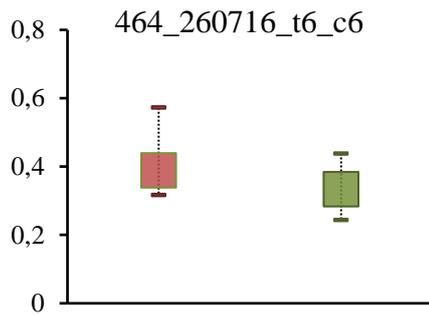
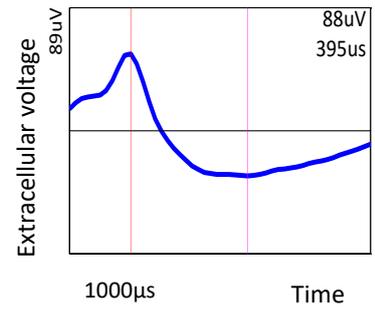
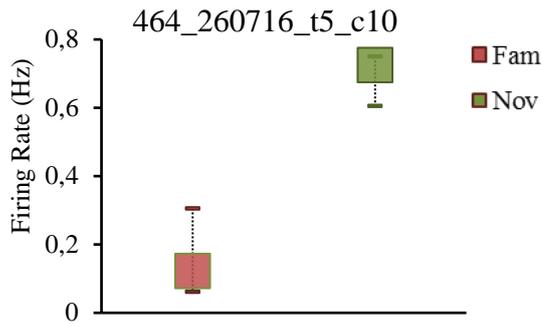
In order to analyse all cells in a consistent manner, only the Novel-Familiar conditions (Fam and Nov) were considered, excluding the baseline condition in the case of the three-conditions-sessions, and analysed all cells using a non-parametric test. The non-parametric Wilcoxon test generally represents a more cautious approach than the paired-t test because of lower sensitivity but when data come from a skewed distribution this test can reach higher power compared to the t-test (Krzywinski & Altman, 2014). With this approach, 27 cells out of 132 (**figure 9.2 and 9.3**) were found to show a significant change in the firing for the novel or the familiar condition. The results are shown in (**table 9.2**). Among the 3 rats, the proportion of novelty altered cells was somewhat different (438: **14.6%** (7/48); 451: **10.5%** (2/19), 464: **27.7%** (18/65)). Perhaps, these differences could reflect the source of variance from different animals. Another possibility, since the lack of histological evaluation in case of rat 464, is that the recordings from this rat may have been from deep piriform cortex.











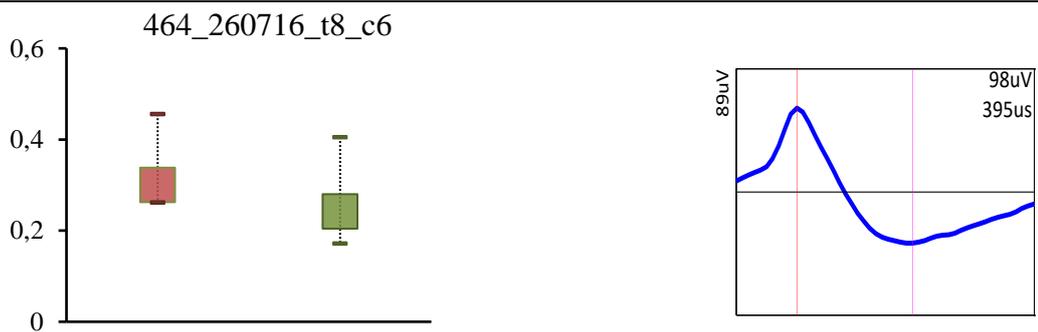
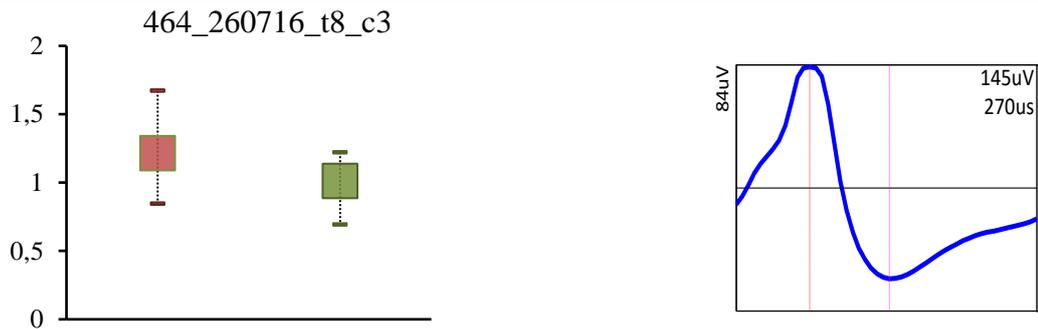
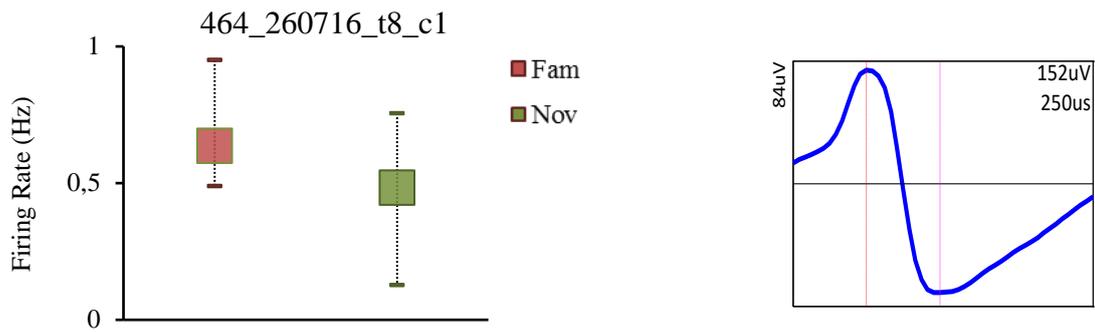


Figure 9.2 Key characteristics of the 23 cells recorded in the two-conditions sessions, whose firing rate showed preferential firing for the familiar or the novel conspecific. . For each cell, left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Fam, red; Nov, green), the horizontal lines represent the maximum and the minimum firing rate values in each condition. In the illustrations of the waveform, the X axis shows time, the Y axis shows the voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

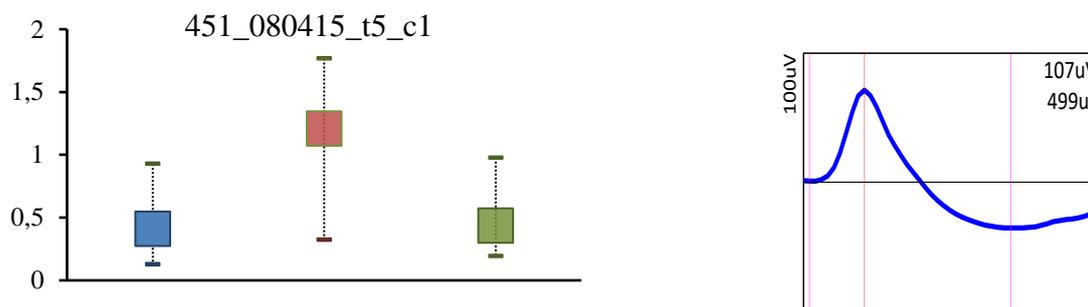
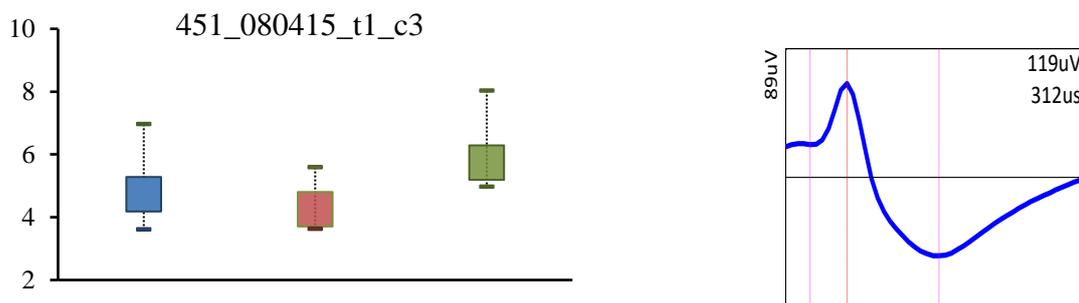
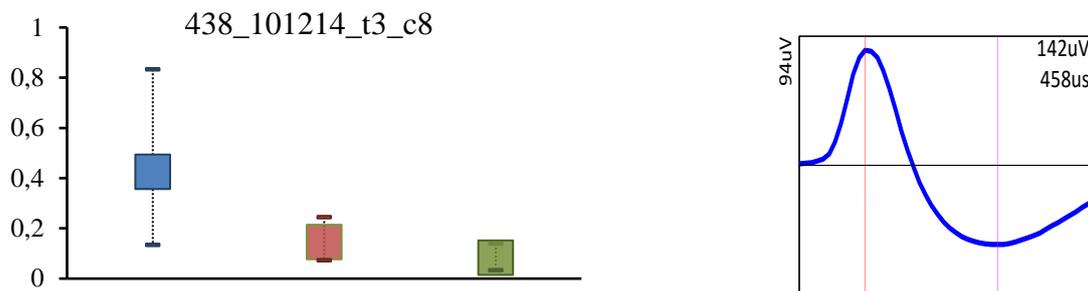
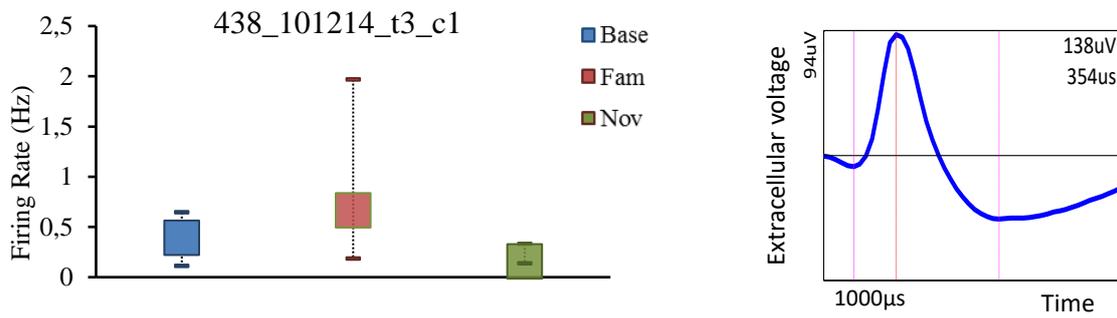


Figure 9.3. Key characteristics of the 4 cells recorded in the three-conditions sessions, whose firing rate showed preferentially high or suppressed firing for the familiar conspecific or the novel conspecifics. For each cell, left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Base, blue; Fam, red; Nov, green), the horizontal lines represent the maximum and the minimum firing rate values in each condition. The right panel shows the cell waveform. In the illustrations of the waveform, the X axis shows the time, the Y axis shows the voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

9.4.1 Novel-familiar discriminative neurons

Twenty-seven (20.6%) out of 131 cells considered in the analysis showed a distinctive significant change in firing between the novel and the familiar conditions. Of those, 20 were recorded during the 12-trial sessions and they represent about the 26% of the population of cells considered in the two conditions analysis. The other 4 neurons were recorded during the 18-trial sessions and represent the 10% of the total cells considered in the three conditions analysis. Notably, some of the sessions included both novel-higher neurons and familiar-higher neurons recorded simultaneously. Sixteen of the 27 cells, representing the 12.2% of the population of cells considered, increased the firing rate when exposed to a novel conspecific (novel-higher neurons), while 11 cells (8.4%) increased the firing when exposed to a familiar conspecific (familiar-higher neurons) (**figure 9.4**). **Table 9.2** shows the p values of the Novel-vs Fam discriminative cells.

Percentage of Nov-vs-Fam-Specific Cells

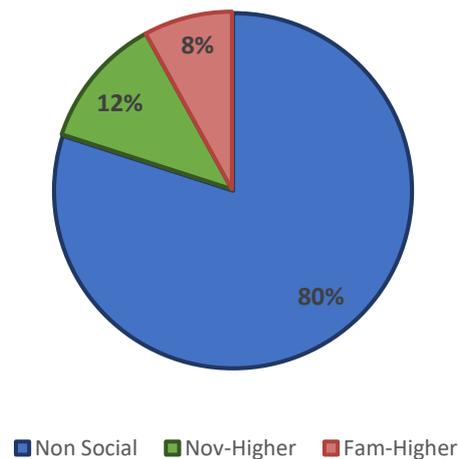


Figure 9.4. Percentage of Nov-vs-Fam Specific Cells recorded in the in the piriform cortex. In red the percentage of Familiar-Higher neurons (Fam-Higher) which increased the firing activity in presence of a familiar conspecific, in green the Novel-Higher neurons (Nov-Higher) which increased the firing activity in presence of a novel conspecific. In blue the percentage of cells whose firing did not discriminate between familiar and novel conspecifics.

Table 9.2 Table showing the firing rate (mean± s.e.m.) of the 27 significant cells that discriminate between novel and familiar conspecifics. In red background are shown the familiar-higher neurons, in green the novel-higher neurons.

Cell code	Mean firing rate ± s.e.m. (Hz)	Z (n=6)	p values
438_040914_t2_4	Fam= 0.7 ± 0.14; Nov= 1.8 ± 0.41	-2.201	0.028
438_040914_t3_1	Fam= 0.7 ± 0.07 Nov= 1.4 ± 0.17	-2.201	0.028
438_040914_t5_2	Fam= 2.8 ± 0.21 Nov= 3.4 ± 0.18	-1.992	0.046
438_040914_t5_4	Fam= 1.4 ± 0.1 Nov= 1.9 ± 0.2	-1.992	0.046
438_040914_t7_1	Fam= 4.8 ± 0.16 Nov= 6.1 ± 0.35	-2.201	0.028
438_101214_t3_c1	Fam= 0.8 ± 0.3 Nov= 0.2 ± 0.03	-2.201	0.028
438_101214_t3_c8	Fam= 0.2 ± 0.03 Nov= 0.1 ± 0.02	-2.201	0.028
451_080415_t1_c3	Fam= 4.5 ± 0.4 Nov= 6.0 ± 0.5	-1.992	0.046
451_080415_t5_c1	Fam= 1 ± 0.2 Nov= 0.5 ± 0.1	-2.201	0.028
464_010716_t2_c1	Fam= 0.5 ± 0.18 Nov= 1.29 ± 0.23	-2.201	0.028
464_010716_t3_c5	Fam= 4.7 ± 0.49 Nov= 4.1 ± 0.53	-2.207	0.027
464_010716_t3_c8	Fam= 1.33 ± 0.08 Nov= 1.6 ± 0.15	-2.201	0.028
464_010716_t5_c1	Fam= 0.43 ± 0.04 Nov= 0.55 ± 0.03	-2.201	0.028
464_010716_t6_c1	Fam= 1.6 ± 0.06 Nov= 2.05 ± 0.15	-1.992	0.046
464_010716_t8_c4	Fam= 0.97 ± 0.11 Nov= 0.62 ± 0.09	-2.201	0.028
464_010716_t8_c9	Fam= 0.22 ± 0.03 Nov= 0.45 ± 0.11	-2.201	0.028
464_010716_t8_c14	Fam= 0.43 ± 0.02 Nov= 0.53 ± 0.03	-2.207	0.027
464_260716_t5_c1	Fam= 0.2 ± 0.1 Nov= 3.7 ± 0.5	-2.201	0.028
464_260716_t5_c3	Fam= 1.8 ± 0.1 Nov= 1.0 ± 0.2	-2.201	0.028
464_260716_t5_c5	Fam= 0.5 ± 0.1 Nov= 1 ± 0.3	-2.201	0.028
464_260716_t5_c10	Fam= 0.1 ± 0.04 Nov= 0.7 ± 0.02	-2.201	0.028

464_260716_t6_c6	Fam= 0.4 ± 0.05 Nov= 0.3 ± 0.03	-2.032	0.042
464_260716_t7_c3	Fam= 1.2 ± 0.1 Nov= 0.6 ± 0.1	-2.201	0.028
464_260716_t7_c4	Fam= 0.6 ± 0.1 Nov= 2.3 ± 0.2	-2.201	0.028
464_260716_t8_c1	Fam= 0.7 ± 0.1 Nov= 0.4 ± 0.1	-2.023	0.043
464_260716_t8_c3	Fam= 1.2 ± 0.1 Nov= 1.0 ± 0.1	-2.207	0.027
464_260716_t8_c6	Fam= 0.34 ± 0.032 Nov= 0.26 ± 0.032	-2.032	0.042

9.4.2 Waveforms of Social Novel-familiar discriminative cells

The tetrodes implanted in the three rats (438,451 and 464) targeted the piriform cortex, detailed histological information are given in chapter 7. The extracellularly-recorded waveforms of the 27 novel-familiar discriminative cells from the channel showing the largest peak-to-trough amplitude had mean peak-to-trough latencies of 380 ± 19 microseconds and mean peak-to-trough amplitude of 102 ± 6 microvolts. For three of the 27 cells a manual correction was necessary to perform the descriptive analysis (464_010716_t5_c1, 464_010716_t3_c8, and the axon-like waveform 464_010716_t3_c5). In general, these long peak-to-trough latencies and peak-to-trough amplitudes are consistent with novel-familiar discriminative cells being principal cells (pyramidal cells in the piriform cortex).

9.5 Speed analysis

The paired t-test was run to determine whether differences in firing between the two groups (Nov and Fam) could be related to differences in Running Speed means. **Table 9.3** shows the

Mean \pm s.e.m (cm/s) of the 2 groups (Nov and Fam) in the 5 sessions analysed. The analysis showed that only in session 464_010716 the Running Speed mean was significantly higher during trials with novel conspecifics ($t(5)=-8.4$, $p<0.001$), (**table 9.3**).

The firing rate of familiar-novel discriminative neurons was examined in relation to average trial running speed. Considering the relation of the firing with the average trial speed is one approach to seeing if alterations in firing rate may also reflect change in arousal state. Standard multiple regressions were run with average speed and Novelty status (Fam and Nov) as predictor variables for firing rate. These showed that Novelty status significantly predicted firing rates for 11 of the 27 cells, (Beta values ≥ 0.6 , $p \leq 0.05$), with 5 of the 11 cells showing beta values of ≥ 0.8 and p values of ≤ 0.005 . It should be noted that this analysis is conservative, with beta values having to be 0.6 or more to be statistically significant. Moreover, it is notable that 4 of the 5 sessions contained at least one cell that showed significant relationship to novelty status in these multiple regressions. Interestingly, *running speed did not significantly predict firing rates in any of the 27 cells* (Beta values ≤ 0.1 , $p > 0.05$). In other words, although a secondary modulation of firing rates cannot be ruled out, arousal or locomotion was not a likely primary driver of the novel-vs-familiar specific firing patterns.

Table 9.3 Table showing the Running speed (mean± s.e.m.) between novel (Nov) and familiar (Fam) conditions of the 5 sessions considered in the analysis.

Session	Mean ± s.e.m	t	P value
464_010716	Fam= 5.8 ± 0.2 Nov= 7.7 ± 0.2	-8.4	<0.0001
464_260716	Fam= 14.2 ± 1.8 Nov= 15.0 ± 0.9	-0.5	0.6
451_080415	Fam= 10.9 ± 1.4 Nov= 11.0 ± 1.0	-0.1	0.9
438_101214	Fam= 10.1 ± 0.7 Nov= 9.1 ± 1.4	0.5	0.6
438_040914	Fam= 9.5 ± 1.1 Nov= 9.1 ± 0.4	0.4	0.7

9.6 Behavioural correlation

The firing rate of 27 cells has been shown to be driven by one of the social conditions (Fam or Nov). To constrain interpretation, the present work is aided by blind behavioural scoring. As explained in the method chapter during the testing phase, a camera was synchronised with the electrophysiological recordings to further analyse the nature of the novel-familiar firing discrimination. Four of the sessions shown were included in the behavioural analysis: 040914, 080414, 010716, 260716. Sessions were analysed singularly, since differences in behavioural performance have been observed in each session. Twenty-five of the 109 cells recorded in these 4 sessions showed novel-familiar-discriminative firing rate. The firing of the 25 cells was analysed to find possible correlation with the time spent in total social interaction, or specifically with anogenital sniffing, face to face contacts, general contacts, dominance behaviour, that are important aspects of rodent social interaction, and with the time spent

actively exploring the other social stimulus (active time). Interestingly, only 5 of the 25 cells had a firing rate correlated with one of the behavioural parameters. Specifically, three cells were correlated with active time (464_010716_t2_c1, $\rho = 0.615$, $p=0.03$; 464_010716_t3_c8, $\rho=0.566$, $p=0.05$; and 464_260716_t5_c5, $\rho=0.606$, $p=0.04$) were correlated with active time, one cell, 464_260716_t5_c1 ($\rho=0.573$, $p=0.05$) was correlated with total social contacts and two cells, 464_260716_t7_c4 and 464_260716_t5_c5, were correlated with anogenital contacts ($\rho=0.617$, $p=0.03$; $\rho=0.606$, $p=0.04$). These findings suggest that for at least 20 of the cells, the firing rate was not driven by the social exploration of the social stimuli. Behavioural analysis will be further discussed in chapter 11.

9.7 Probe trials

In some sessions, the standard social-trials were supplemented by probe trials with the attempt to narrow down interpretation. Even if performing statistical analysis on these probe trials was not possible due to the lack of multiple samples, the use of controls allowed to further understand the nature of the firing rate. In some sessions, the experimenter introduced a familiar or a novel object inside the testing box (see chapter 6 section 6.3 for further information) to understand if novel or familiar stimuli, like an object, were effective at triggering the novelty pattern or the familiar pattern seen in presence of familiar and novel social stimuli. In other sessions, the experimenter used bedding taken from the cage of the novel or familiar rat (Behavioural Methods, chapter 6 for further details) attempting to understand if volatile olfactory cues were sufficiently effective at triggering the social specific pattern seen in presence of the conspecific itself.

I selected all neurons that were shown to be statistically discriminatory and where probe trials were done at the end of the recording session, and divided these neurons into two categories: *Novel-social higher*, or *Familiar-social higher* neurons.

In case of the bedding, a total of 10 *Novel-social higher* neurons were taken from two Novel-Vs-Familiar Social sessions from rat 464 (sessions 010716, 260716). Eight *Familiar-social higher* neurons were taken from the same two Novel-Vs-Familiar Social sessions just mentioned.

In case of the objects, one *Novel-social higher* neuron was taken from one Novel-Vs-Familiar Social sessions from rat 451 (session 080415). One *Familiar-social higher* neuron was taken from the same Novel-Vs-Familiar Social sessions just mentioned and two were taken from one Novel-Vs-Familiar Social sessions from rat 438 (session 101214).

9.7.1 Novel-social higher neurons

In bedding-probe trials, eight out of 10 Novel-social higher neurons replicated the firing pattern observed in presence of the conspecific itself, firing higher for the bedding of the novel rat than the bedding of the familiar rat. Six of these eight neurons (i.e **figure 9.5**) exhibited firing rates that were higher than the mean of the novel-social trials in the whole-animal session, or were close to this mean (defined as “[Novel bedding rate] \geq [Novel/Social mean – 0.5 Standard Deviation]). This would suggest that volatile odorants present in the urine/faeces of bedding were generally sufficient to detect the presence of novel conspecifics. For the two (of ten) Novel-social higher neurons where the bedding did *not* mimic the response for the novel conspecific (**figure 9.6**), we can be sure that this was *not due to inadequate bedding stimuli*

(e.g. staleness) since these two neurons were simultaneously recorded with four Novel-social higher neurons that did respond to the bedding . This means that at least some bedding-non-replicating neurons were not just attributable to some failure of the bedding as a sensory triggering cue, but it could indicate that the odorant cues in the bedding were not sufficient to replicate the pattern observed the whole-animal session.

In object-probe trials, the Novel-social higher neuron did not replicate the firing pattern observed in presence of the conspecific itself (**figure 9.7 A**), meaning that the firing rate was not close to this mean (defined as “[Novel object rate] \geq [Novel/Social mean – 0.5 Standard Deviation]). This may suggest that the Novel-higher neuron was not simply responding to novel stimuli, but changes in firing were reflecting the presence of the conspecific inside the arena.

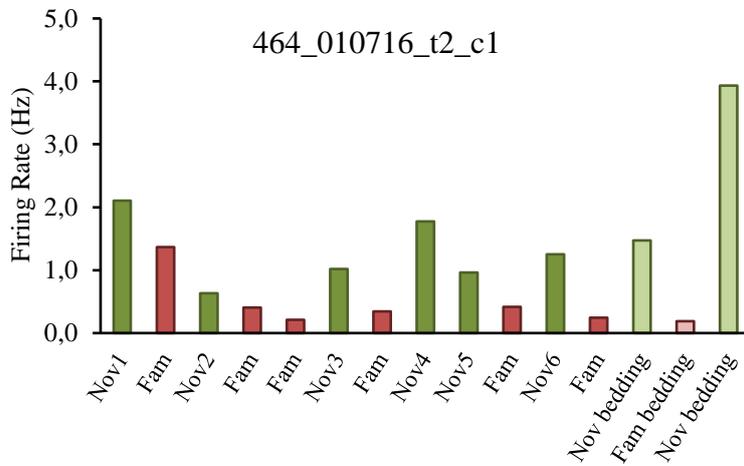
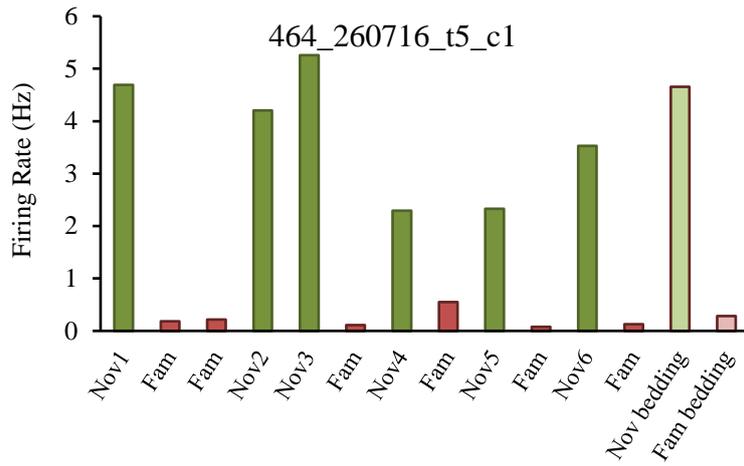


Figure 9.5. Graphs of two Novel-social higher neurons. The x axes show the trial conditions, the y axes shows the firing rate (Hz). The bedding-probe trials replicated the pattern observed in whole-animal session.

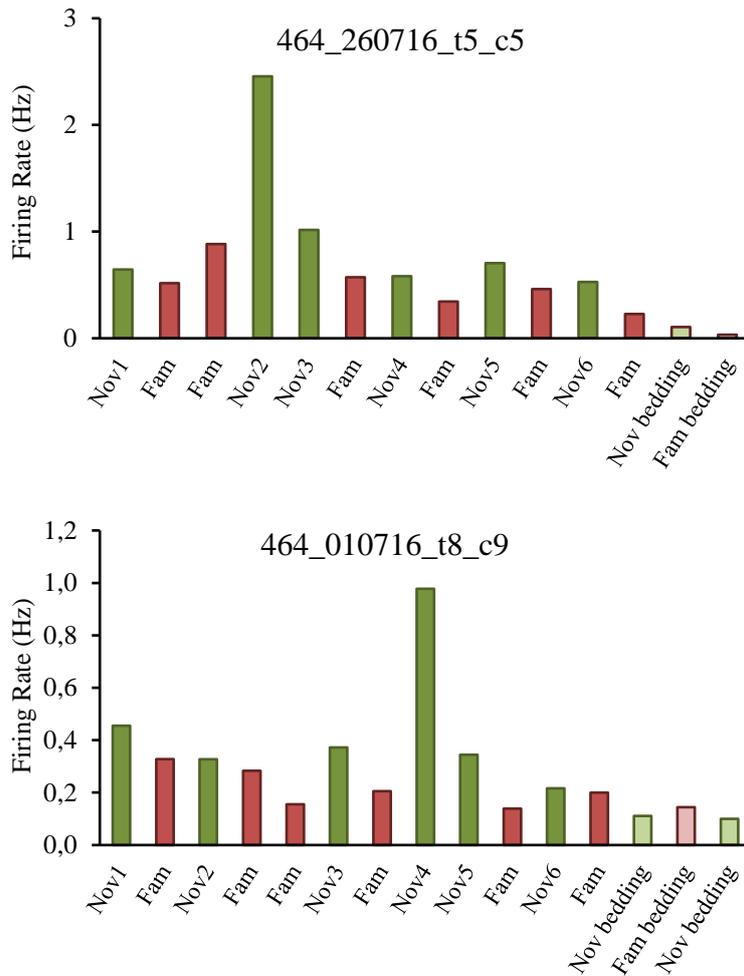


Figure 9.6. Graphs of two Novel-social higher neurons. The x axes show the trial conditions, the y axes show the firing rate (Hz). The bedding-probe trials did not replicate the pattern observed in whole-animal session.

9.7.2 Familiar-social higher neurons

In bedding-probe trials, only three out of eight *Familiar-social higher* neurons exhibited similarly-higher firing rates to bedding containing odorants of familiar animal. Two of these three neurons exhibited firing rates to the familiar-bedding that were *higher than the mean* of the familiar-social trials in the whole-animal session; the other did not and was not *close to this mean* (defined as [Familiar bedding rate] \geq [Familiar/Social mean – 0.5 Standard

Deviation]) (**figure 9.8**) . The non-replicated pattern of six out of 8 neurons was *not due to inadequate bedding stimuli* (e.g. staleness) since they were simultaneously recorded the three Familiar-social higher neurons that did respond to the bedding (**figure 9.9**). This would suggest that, for the majority of *Familiar-social higher* neurons, volatile odorants present in the urine/faeces of bedding were generally **not** sufficient to detect the presence of familiar conspecifics, even though these conspecifics were highly familiar. It may be plausible that the bedding-non-replicating neurons were not just attributable to some failure of the bedding as a sensory triggering cue, but it could indicate that the odorant cues in the bedding were not sufficient to replicate the pattern observed the whole-animal session.

In object-probe trials, the Familiar-social higher neuron did not replicate the firing pattern observed in presence of the conspecific itself (**figure 9.7 B**), meaning that the firing rate was not close to this mean (defined as “[Familiar object rate] \geq [Familiar/Social mean – 0.5 Standard Deviation]). This may suggest that the Familiar-higher neuron was not simply responding to familiar stimuli, but changes in firing were reflecting the presence of the conspecific inside the arena.

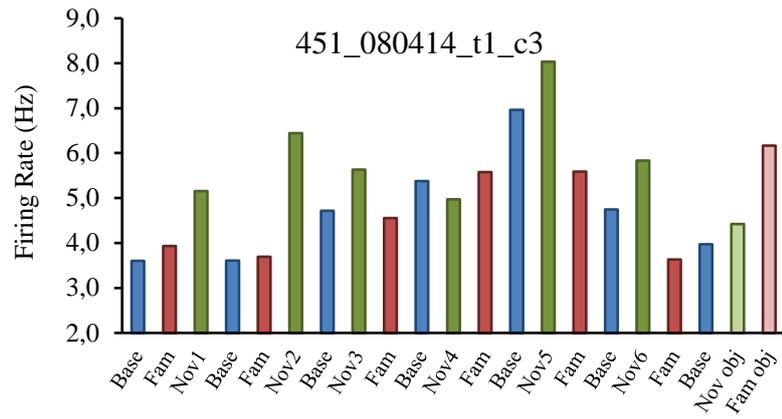
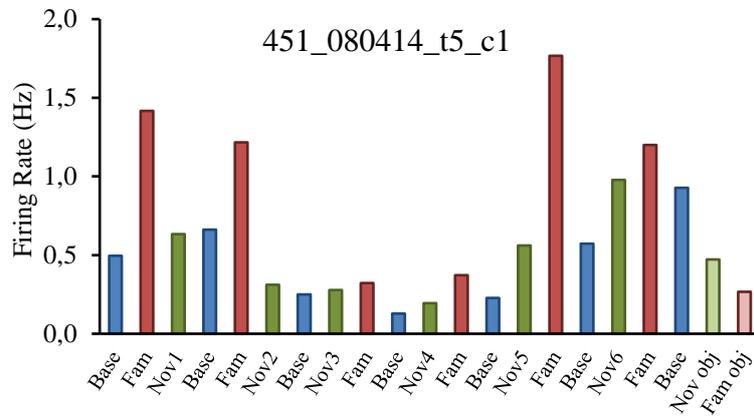
A**B**

Figure 9.7. Graphs of two social higher neurons. The x axes show the trial conditions, the y axes shows the firing rate (Hz). A) **Novel social higher neurons:** the object-probe trials did not replicate the pattern observed in the whole-animal session. B) **Familiar social higher neurons** The object -probe trials did not replicate the pattern observed in whole-animal session.

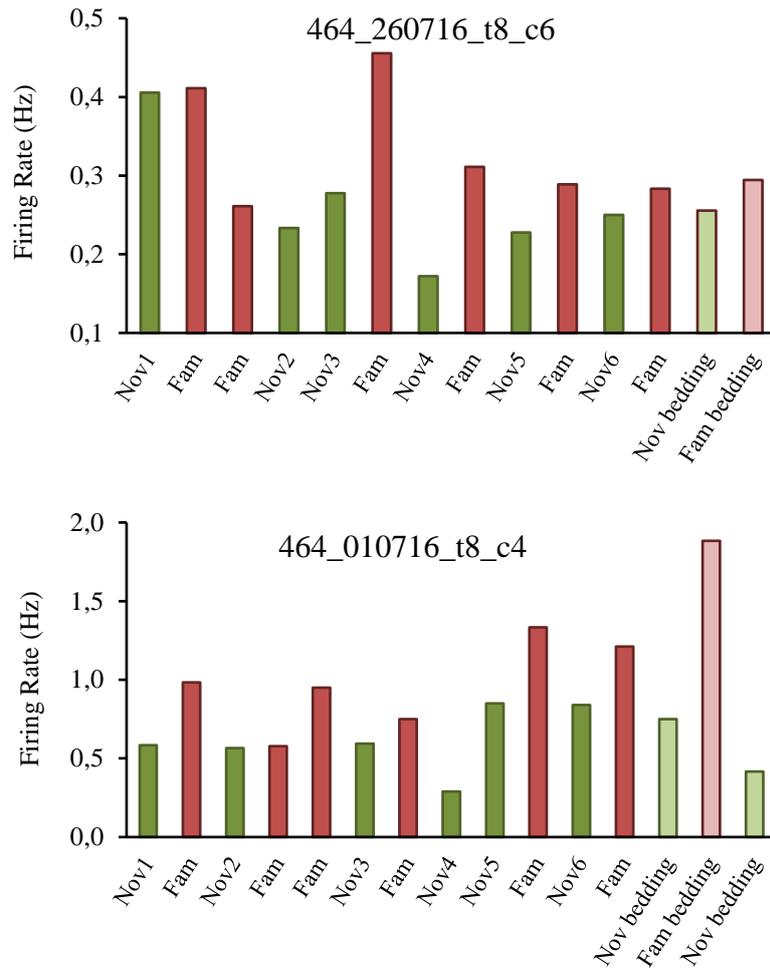


Figure 9.8. Graphs of two Familiar-social higher neurons. The x axes show the trial conditions, the y axes shows the firing rate (Hz). The bedding-probe trials replicated the pattern observed in whole-animal session.

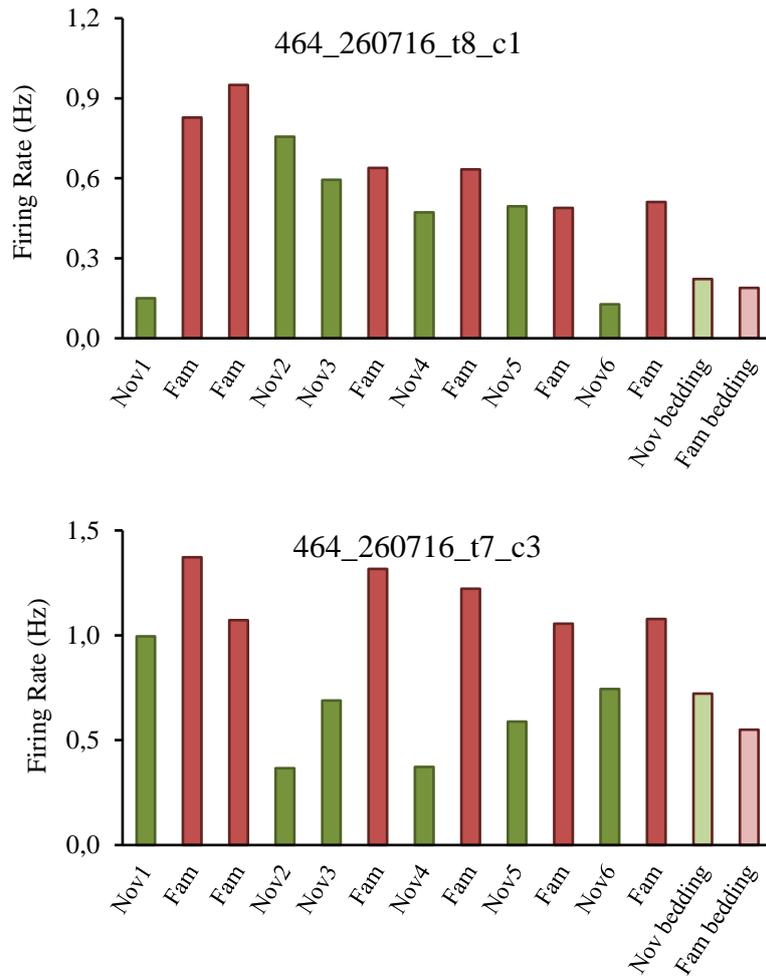


Figure 9.9. Graphs of two Familiar-social higher neurons. The x axes show the trial conditions, the y axes shows the firing rate (Hz). The bedding-probe trials did not replicate the pattern observed in whole-animal session.

9.8 The firing rate of piriform neurons is not driven by pheromones.

The use of female social stimuli potentially adds a further confound variable for this experimental manipulation, because of concern that the difference in firing rate observed in familiar and novel-higher neurons may be due to the different hormonal cycle in familiar and novel social stimuli. For 2 of the sessions considered in the analysis, samples were collected after the testing phase and the oestrus cycle was identified through the vaginal smear and involved the characterization of few distinctive cells type as described by Goldman and colleagues (2007) (see Behavioural Methods chapter 6). In both the sessions considered (010716 and 260716) there were two novel social stimuli that were the same oestrus stage of the familiar stimulus (**Table 9.4**).

Table 9.4 Oestrus characterization of the social stimuli in two of the sessions considered in the analysis

Sessions	Fam	Nov1	Nov2	Nov3	Nov4	Nov5	Nov6
010716	Oestrus	Metoestrus	Oestrus	Metoestrus	Oestrus	Metoestrus	Metoestrus
260716	Proestrus	Dioestrus	Oestrus	Proestrus	Proestrus	Dioestrus	Oestrus

In session 010716, the novel stimuli Nov2 and Nov4 were in oestrus like the social familiar stimulus, and in session 260716 Nov3 and Nov4 were both in proestrus like the familiar social stimulus (Fam). This means that the firing rate of the cells tested in these two sessions is not driven by difference in hormonal cycle. In **figure 9.10** an example of one of the cell that increase the firing rate when exposed to the novel social stimuli, independently by the hormonal phase.

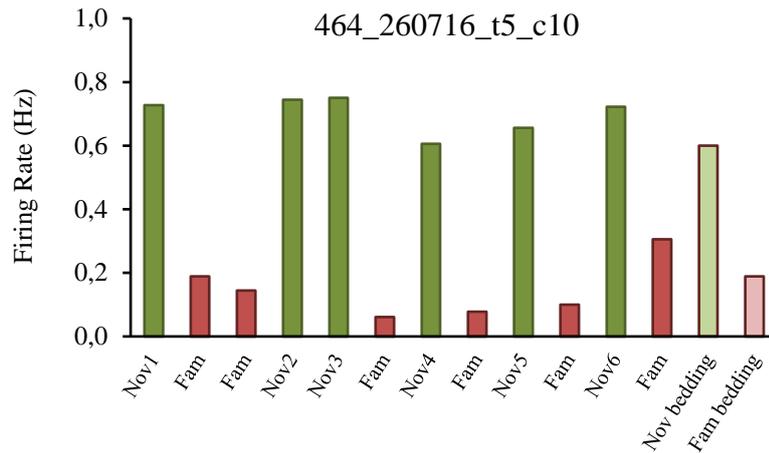


Figure 9.10. Example of cell that increase the firing rate when exposed to novel social stimuli. independently by the hormonal phase. Nov3 and Nov4 were in the same oestrus phase of the familiar social stimulus (Fam).

9.9 Discussion: Piriform cortex

The present chapter represents the first attempt to examine the piriform cortex in the context of social recognition memory. The experiments discussed in chapter 8 showed, for the first time, that neurons in the piriform cortex were responsive during unconstrained social interaction with another conspecific, and while some cells were responsive to both the conspecific and the conspecific bedding, other cells, were responsive only to the presence of the conspecific itself. These data, due to the sacrificing controlled cue in favour of a naturalistic social interaction, leave open more than one interpretation. Firstly, changes in firing could have just been reflecting changes in the arousal state since the social interaction with a conspecific is supposed to be more exciting than alone time in the arena (baseline). Secondly, the piriform cortex may respond to multi-sensory inputs (olfactory, gustatory, auditor, somatosensory and visual) that only the unconstrained social interaction may provide, since rats were free to explore each other and engage social contacts. Thirdly, piriform neurons may contribute to

social identity coding of individual conspecific. This theory is supported by the anatomical characteristics of the piriform cortex. The present chapter provided a further step in the understanding of the role of piriform neurons in social interaction providing evidences that 20.5% of neurons could discriminate between familiar and novel conspecifics. Of these novel-familiar discriminative neurons, 40.7% were familiar-higher neurons, firing higher during familiar trials, while 59.2% were novel-higher neurons, and they robustly fired higher during novel trials. This discussion section, aims to narrow down interpretation and trying to explain why the role in piriform cortex in social identity coding may be a possible explanation for these findings.

Previous studies appointed that behavioural state such as different sleeping phase, walking or even anaesthesia influence the electrophysiological activity in the cortex (Haider et al, 2012; Steriade et al, 2001). Specifically, the speed of the animal appears to strongly influences the firing activity of cells in the primary cortex, with the strong increased activity of interneurons (Keller et al, 2012; Niell and Stryker, 2010; Polack et al, 2013), and in the hippocampus (e.g. O'Keefe et al, 1998; Lever et al, 2003). Certainly, the exposure to a novel rat, may produce a higher general arousal state compared to the exposure to a familiar rat, represented with increased exploration of the novel animal (Dantzer et al, 1988). However, in my findings, the change in firing was never related to the average speed of the animal, while a high portion of cells appear to have a strong correlation with the social-novelty status of the trial. Even if considering speed, a measure of arousal can be limiting, since speed may not be the best behavioural expression of emotional arousal, my findings suggest that general arousal alone may not explain the difference firing activity when the implanted animal was exposed to a novel conspecific.

My findings, probably for the first time showed that there are cells in the piriform cortex that are discriminating between familiar and novel conspecifics, and these firing differences appear not to be related to differences in social interaction. In fact, 80% of the cells considered in the behavioural analysis did not show correlation with any of the behavioural social parameters considered in the behavioural scoring. Behavioural differences in social interaction in relation to the firing rate of the cells are further discussed in chapter 11. The behavioural scoring offered the advantage to understand if social exploration may drive the firing of cells in the piriform cortex, however future analysis may consider observing the firing relation with a micro-behavioural analysis.

Another interpretation of my data comes from the oestrus characterization for the social stimuli. It may be claimed that the differences in the response observed in familiar and novel animals may be driven by different hormones produced in different phases of the oestrus cycle. However, it is unlikely that the firing discrimination between novel and familiar conspecific may be due to difference in the oestrus stage since at least in two of the sessions considered (see **table 9.4, figure 9.10**) the social familiar is in the same oestrus stage of the social novel stimulus. The cells firing was following the individual conspecific pattern (familiar-higher neurons or novel-higher neurons) independently by the oestrus stage of the social stimulus.

However, it is notable that 61% of the neurons which discriminated novel and familiar rats responded similarly to the presence of just the relevant conspecific's bedding. In this case, it shows that the activation of volatile odour associated with the conspecific was sufficient to drive the neuron in the socially-specific way (e.g. higher to novel rats). It may be plausible that the replication of the pattern observed in the whole animal trial, may reflect pattern

completion, meaning that a few odorants are sufficient to reproduce the mnemonic pattern completion-like process in a similar way as occurs in the hippocampus with place cells. In these cases, these few odorants were sufficient to reactivate the conspecific firing pattern

Of the familiar-novel specific neurons, 39% of the neurons did not replicate the firing response observed in the presence of the social stimuli. These bedding-non-replicating neurons cannot simply be considered a failure of the bedding as a sensory triggering cue since these neurons were simultaneously recorded with bedding-replicating neurons. This may suggest that volatile odours were not a major contributor of the firing of these neurons.

It cannot be ruled out that the firing response during presence of the conspecific of its bedding can be reflecting perception without any memory. However, my findings are in line with previous studies which support the theory that the piriform cortex may be involved in learning response (Karunanayaka et al, 2015, Gottfried et al, 2014). In these studies, the presentation of an object or a visual cue was paired with a smell in the encoding phase. In the retrieval phase, the participants were only exposed to the visual information but not to the olfactory information. Nevertheless, the authors observed a strong activation of the piriform cortex in the retrieval phase when olfaction was not involved. In a similar way, my findings are showing that cells in the piriform cortex can sometimes need something more than the conspecific bedding for reactivate the conspecific pattern, while in other cases they may require only olfaction. While my results are informative and by themselves cannot exclude that the re-activation of the piriform cortex is purely sensory, there are different studies which support the idea that the piriform cortex has strong connections with areas such as the amygdala and its similarities with the hippocampal CA3 may be an associative cortex involved in pattern completion, in which social recognition may rely (Cleland and Linster,

2003; Haberley, 2001; Hasselmo et al, 1986; Hasselmo et al, 1991; Hasselmo and Bower, 1993).

Clearly, these findings even if represent a step forward in our understanding of the piriform cortex, need further work to narrow down interpretation. In fact, even if it has been suggested that the piriform cortex may work as an associative memory and accordingly, neurons appear to discriminate between novel and familiar conspecifics, it may be the case that these cells were just responding to sensory cues. The role of the piriform cortex in social interaction and its possible involvement in social recognition memory will be further investigated in chapter 9.

Chapter 10: Sister-Specific Cells

10.1 Experimental rationale

The present chapter aimed to further examine the role of piriform neurons in the context of social recognition memory, already started in chapter 9. Chapter 9, provided evidence that 20% of neurons in the piriform cortex could discriminate between novel and familiar conspecifics. Even if the nature of this discrimination is not completely understood, one plausible interpretation is that piriform neurons may contribute to recognition between conspecifics. However, the use of novel and familiar conspecifics in this paradigm leaves open more than one interpretation. Firstly, changes in firing rate may have just been reflecting changes in the arousal state due to the presence of an unknown social stimulus. Finally, novel rats tend to be explored more compared to familiar rats and the nature of this firing discriminations, may conceivably be related to differences in social interaction between novel and familiar conspecifics. Trying to narrow down interpretation, this chapter examined piriform neurons in response to two familiar social stimuli which were similar in many aspects since they were two sisters living in the same cage, and even sharing the same food. This is important because individual-specific odour signatures reflect diet as well as genetics, and local environmental influences. Can piriform neurons discriminate between two sisters?

10.2 Summary of the experimental procedure

As described in the method section, two Lister-Hooded female rats were implanted with microdrives in the right piriform cortex or both the left and right piriform cortex and habituated over a prolonged period to singularly encounter two different social female familiar stimuli (Fam 1 and Fam 2) into a small square box (40×40×50cm). Since the idea was to understand how specific social discrimination might get, the familiar stimuli used in the sessions were sisters and they were caged together; thus, highly similar on several dimensions. Each sister was presented to the implanted animal for 6 trials, for a total of 12 trials, or 18 trials when the social trials were interspersed with baseline trials, when the implanted animal was placed alone into the square box. Some of the sessions, included trials where the subject animal was further tested for more control conditions, such as the effect on cells of familiar bedding or Sphero. As shown in **table 10.1**, cells were recorded from 7 different sessions.

Table 10.1. Summary of cells considered for the Fam vs Fam sessions in two of the rats implanted in the piriform cortex. In all these sessions, the two familiar rats were sisters. In all sessions except 464_270916, the sisters were also the sisters of the implanted rat. In the 464_270916 session, the stimulus sisters were 48 weeks old, while the implanted rat was 5 weeks old.

Animal code	Session	Conditions	Total cells recorded	Total cells considered	Sister-specific cells
438	031014	Base, Fam1, Fam2	49	7 R +25 L	3 L+2R
438	101014	Base, Fam1, Fam2	48	6 R +33 L	8 L+1R
464	200616	Base, Fam1, Fam2	10	4 R	NS
464	240716	Fam1, Fam2	32	31 R	7 R
464	270716	Fam1, Fam2	27	24 R	6 R
464	280716	Base, Fam1, Fam2	43	27 R	1 R
464	270916	Fam1, Fam2	24	10	1L 1R
Total			N=233	N=167	N=30 (18%)

10.3 Electrophysiological recordings

Two hundred and thirty-three cells were recorded from the piriform cortex of two rats (one confirmed, one supposed to be in the piriform cortex) during the 7 trial sessions listed in **table 10.1**. In total, 167 neurons were considered in this analysis because followed the main parameters described in the method section (Material and methods chapter 5), and were identified as pyramidal cells or interneurons with high stability across trials. Of the 167 cells analysed, 65 cells were recorded during the two-condition sessions (Fam1 vs Fam2), while the rest (n=102) were recorded during the eighteen-trial session where the 12 trials with the sisters were interspersed with baseline trials.

In order to analyse all cells in a consistent manner, only the sister conditions (Fam1 and Fam2) were considered, excluding the baseline condition in the case of the three-conditions-sessions, and analysed all cells using a non-parametric test. The non-parametric Wilcoxon test generally represents a more cautious approach than the paired-t test because of lower sensitivity but when data come from a skewed distribution this test can reach higher power compared to the t-test (Krzywinski & Altman, 2014). With this approach, 30 cells out of 167 showed sister-specific firing.

10.3.1 Sister specific firing

Thirty of the 167 cells considered in this analysis (18%), showed sister firing discrimination, meaning that cell firing rate was specific for one of the two familiar sisters, Fam1 or Fam2. The p and z values of the 30 cells is reported in **table 10.2**. Of those 30 cells, 15 were recorded during the 18-trial sessions (**figure 10.1**), while the other 15 cells were recorded during the

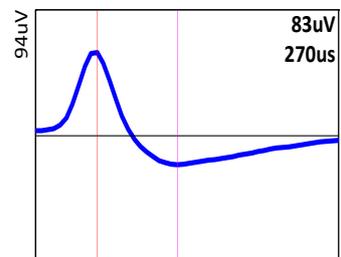
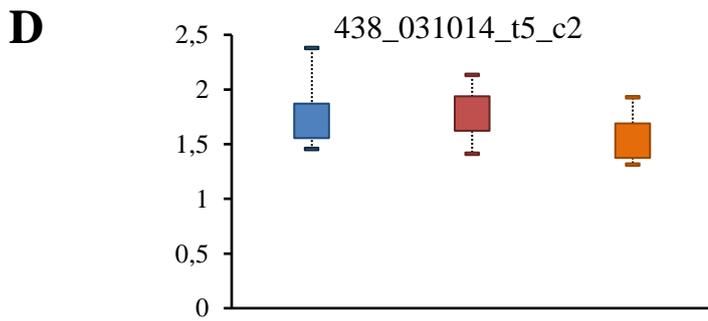
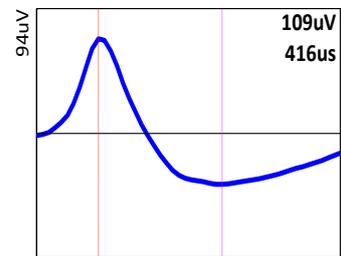
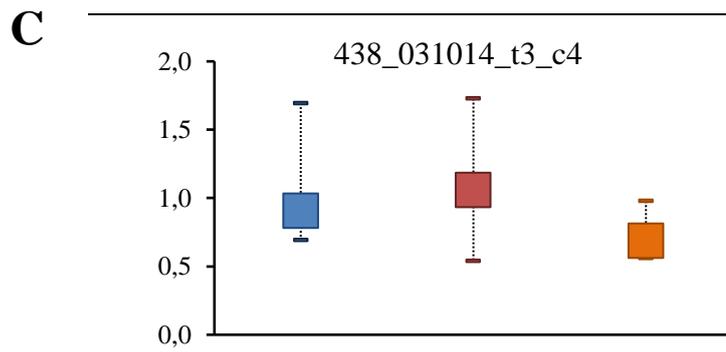
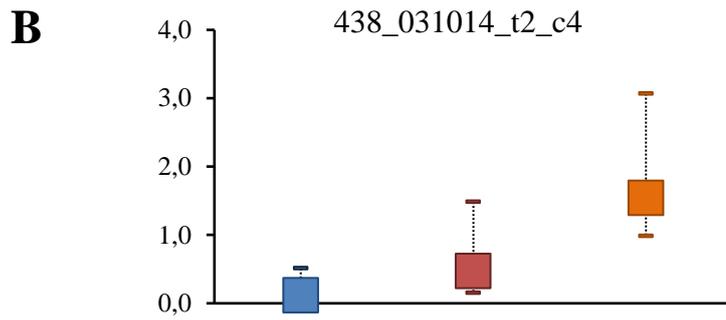
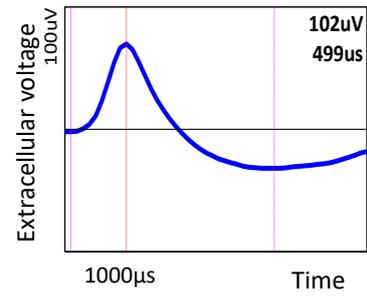
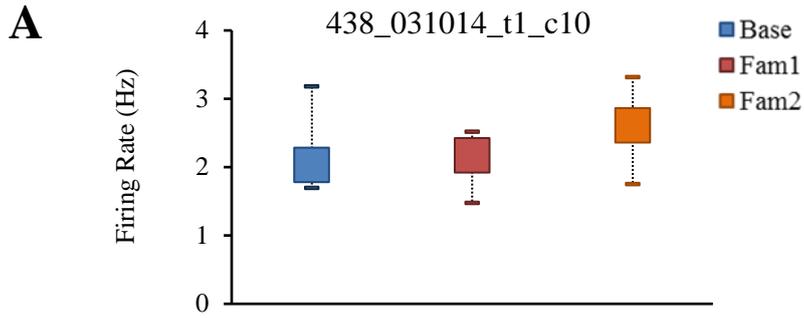
two-condition sessions (**figure 10.2**). **Figures 10.1 and 10.2** show the firing rate median of the cells in the three and two condition sessions that exhibit a significant change in firing when the implanted animal was paired with one of the two sisters. In one of the reported sessions, 200616, none of the 4 analysed cells (2% of the total analysed data) showed a relationship between firing rate and the encounter with one of the familiar social stimulus (Fam1 or Fam2). Among the 2 rats, the proportion of sister-specific firing patterns was similar (438: 19.7% (14/71); 464: 16.7% (16/96)).

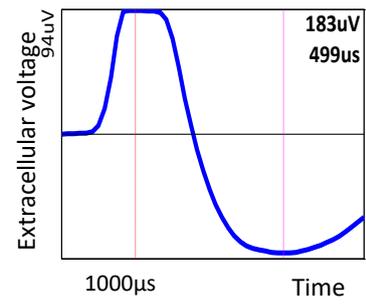
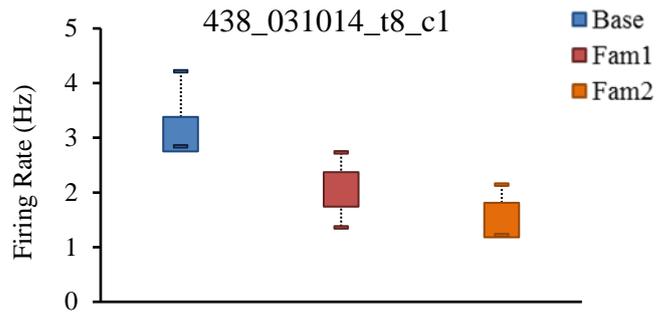
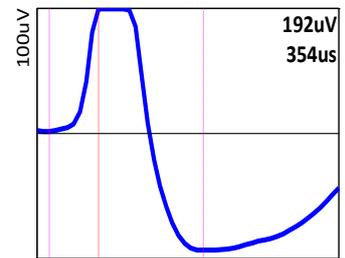
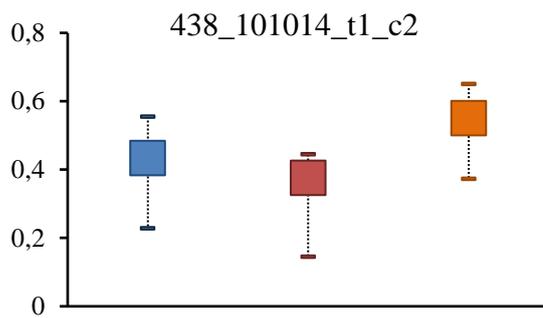
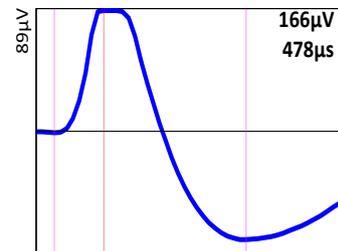
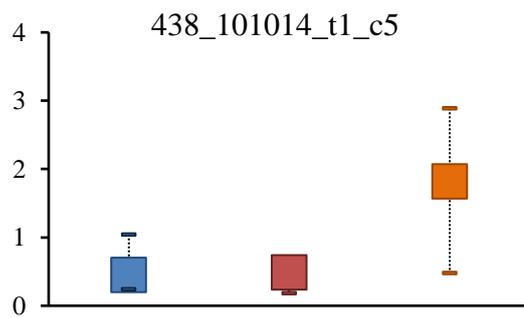
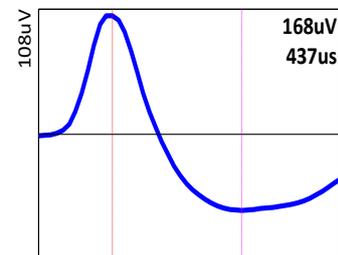
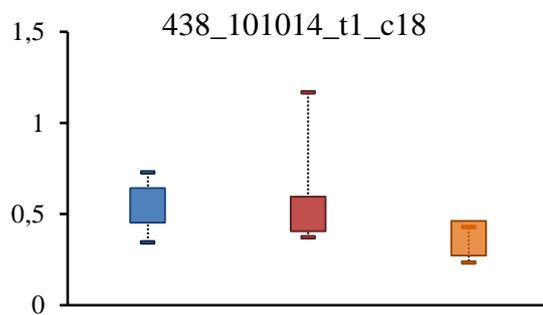
10.3.2 Waveforms of sister-specific cells

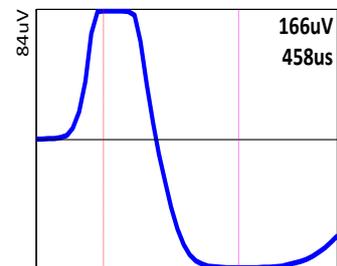
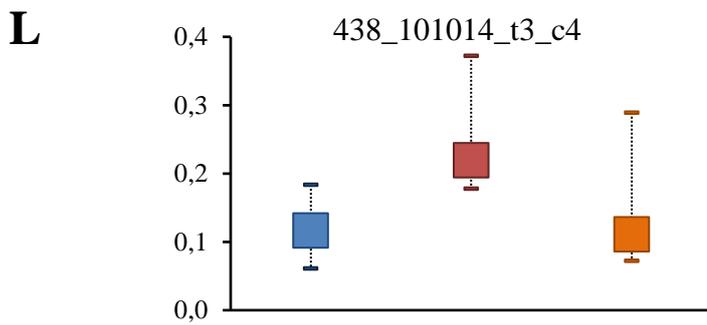
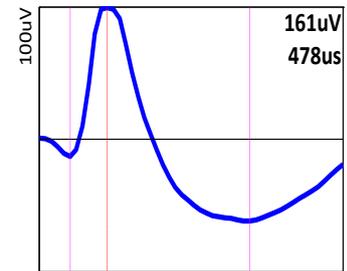
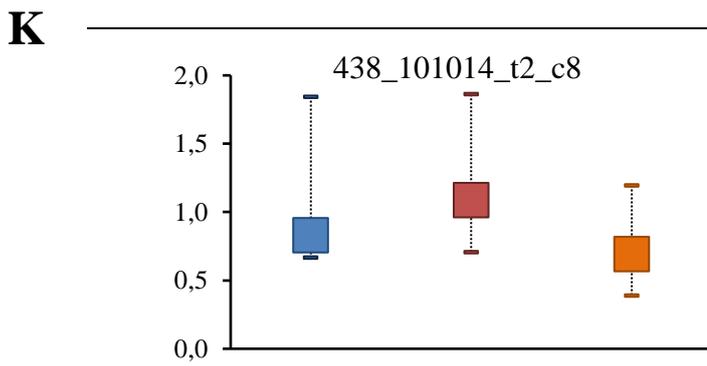
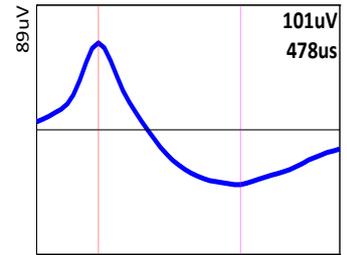
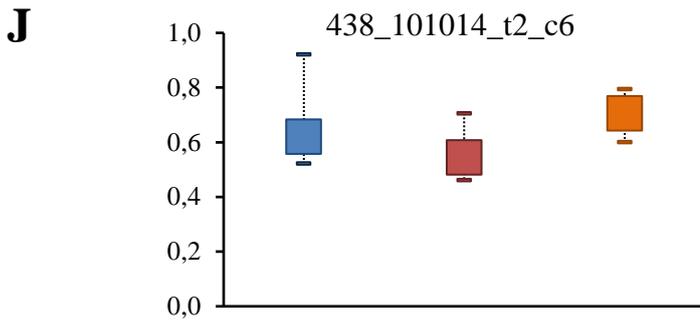
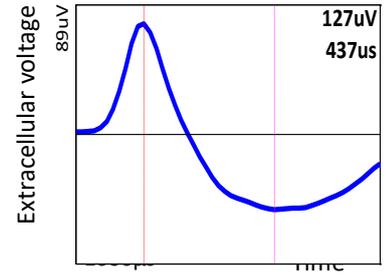
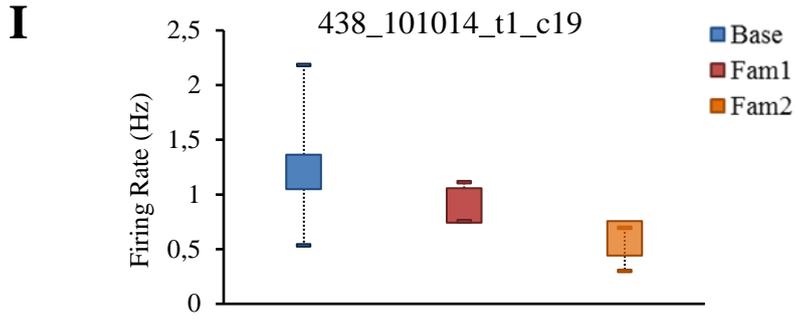
The tetrodes implanted in the two rats (438 and 464) targeted the piriform cortex; detailed histological information are given in chapter 7. The extracellularly-recorded waveforms of the 30 sister specific cells, from the channel showing the largest peak-to-trough amplitude, had mean peak-to-trough latencies of 385 ± 17 microseconds and mean peak-to-trough amplitude of 119 ± 7.4 microvolts. For two of the 30 cells a manual correction was necessary to perform the descriptive analysis (464_270716_t7_c8, and the axon-like waveform 464_240716_t8_c11). Overall, these long peak-to-trough latencies and peak-to-trough amplitudes are consistent with sister specific cells being principal cells (pyramidal cells in the piriform cortex). **Figures 10.1-10.2** show the waveform of the 30 Sister-Specific cells; the X axis shows the time, the Y axis shows voltage.

Table 10.2 reports the p values of the sisters-specific cells.

Session	Cell code	Z values	P values
438_031014	t8_1	-1.992	0,046
	t5_2	-2.201	0,028
	t1_10	-2.201	0,028
	t2_4	-2.201	0,028
	t3_3	-1.992	0,046
438_101014	t1_2	-1.992	0,046
	t1_5	-1.992	0,046
	t1_18	-2.201	0,028
	t1_19	-2.201	0,028
	t2_6	-2.201	0,028
	t2_8	-2.201	0,028
	t3_4	-1.997	0,046
	t4_3	-2.207	0,027
	t5_3	-1.992	0,046
464_240716	t8_c4	-2.023	0,043
	t8_c5	-2.023	0,043
	t8_c10	-2.023	0,043
	t8_c11	-2.023	0,043
	t6_c3	-2.023	0,043
	t7_c8	-2.023	0,043
	t5_c8	-2.023	0,043
464_270716	t5_c1	-1.992	0,046
	t6_c3	-2.226	0,026
	t7_c1	-1.992	0,046
	t7_c8	-2.201	0,028
	t8_c4	-2.201	0,028
	t8_c6	-2.201	0,028
464_280716	t8_c1	-1.992	0,046
464_270916	t6_c5	-2.201	0,028
	t3_c20	-2.207	0,027



E**F****G****H**



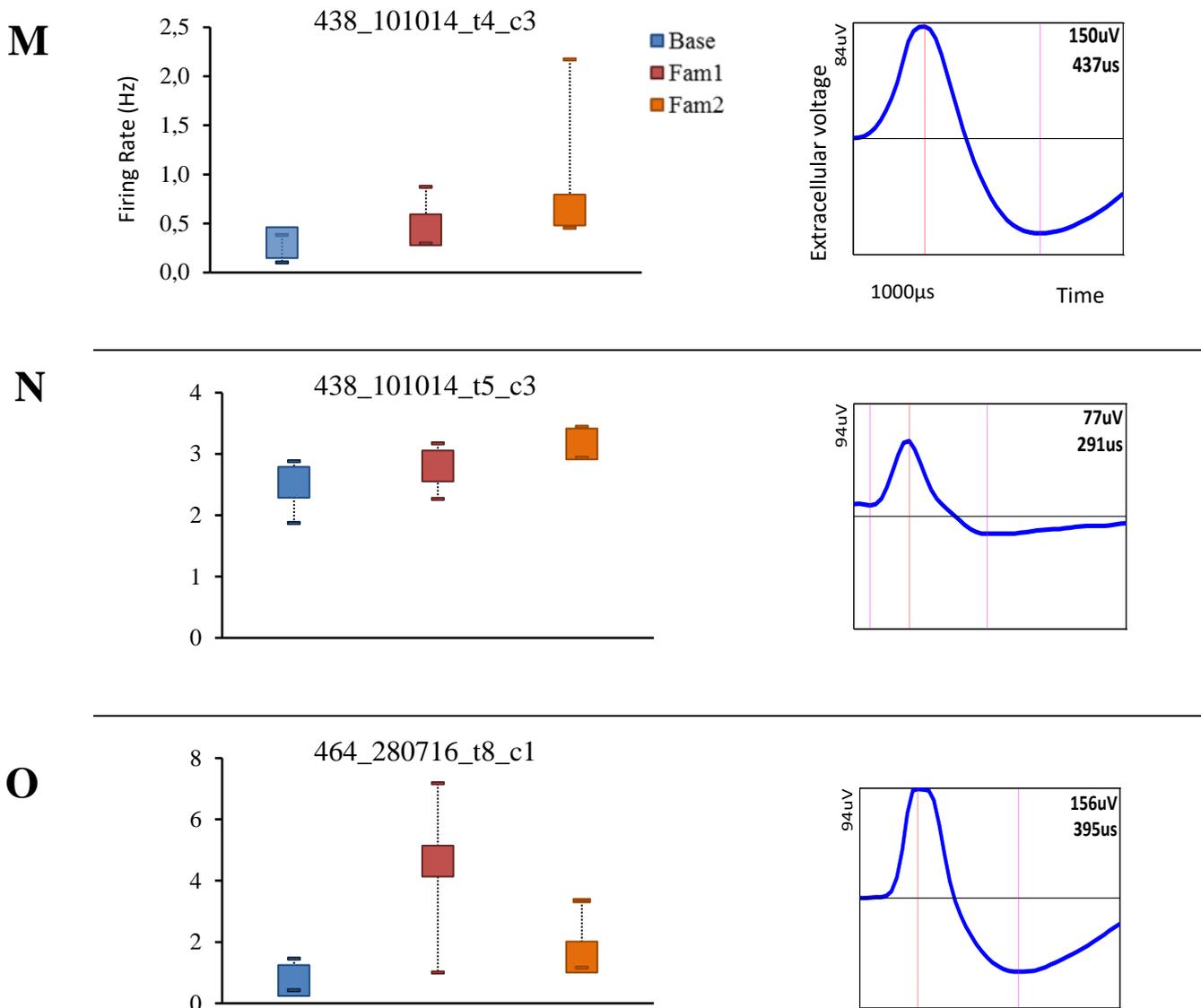
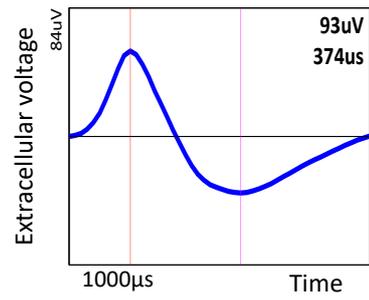
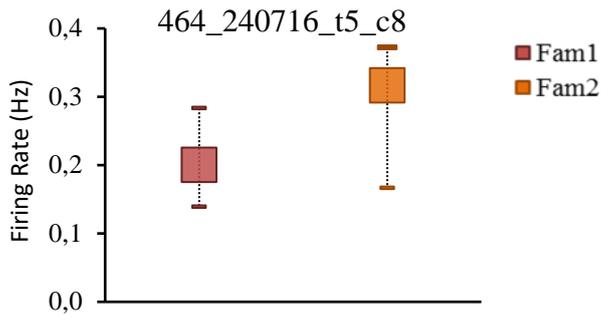
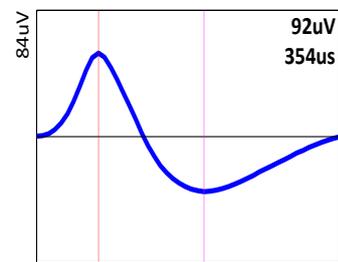
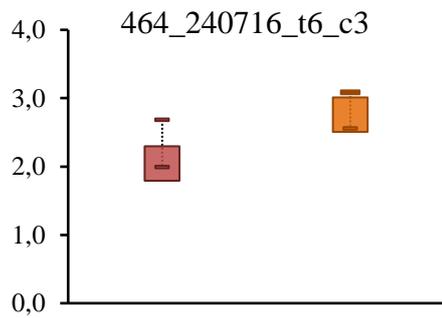
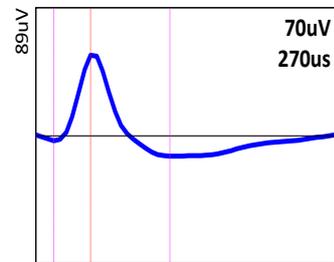
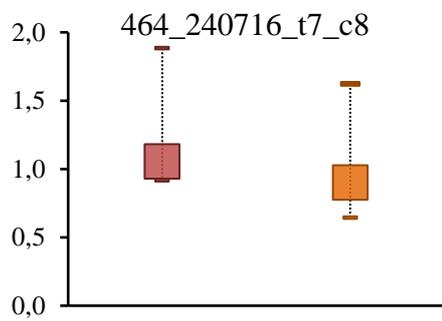
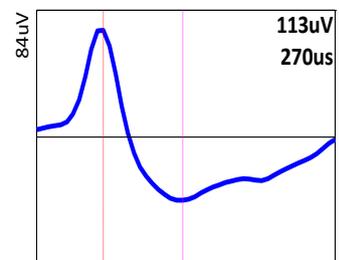
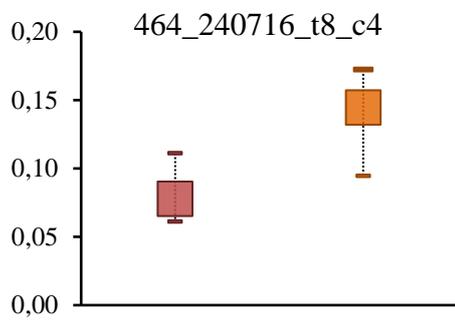
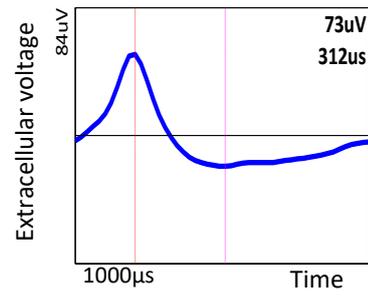
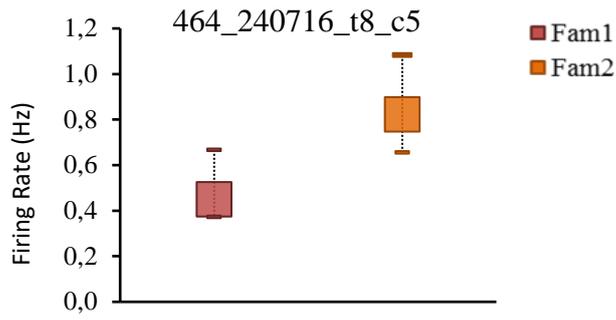
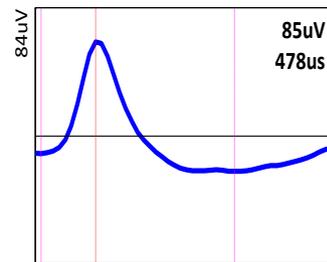
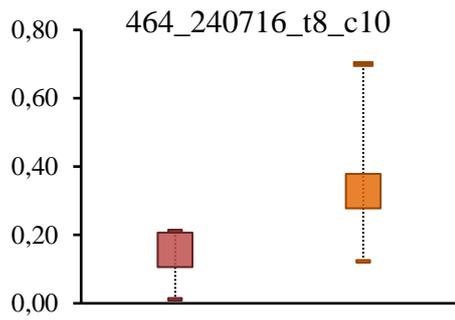
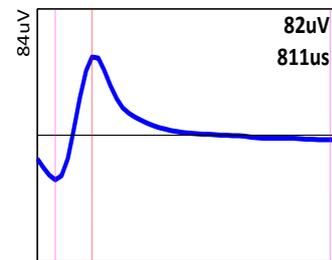
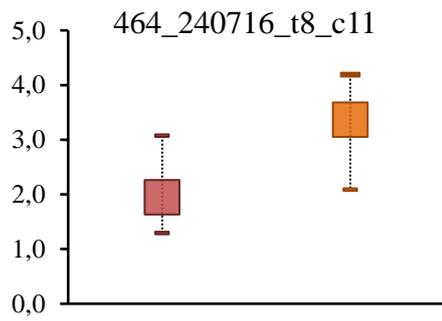
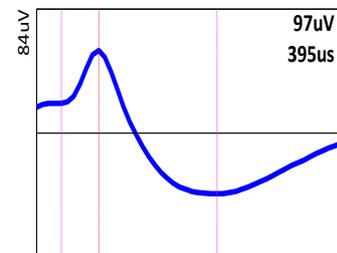
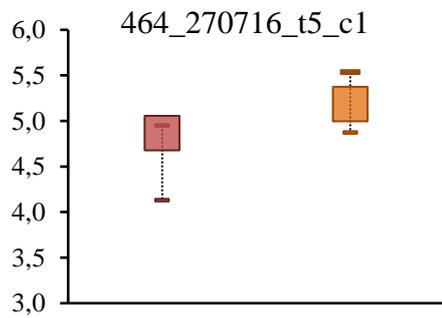
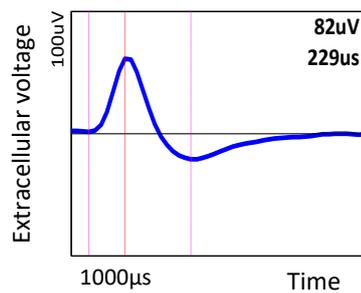
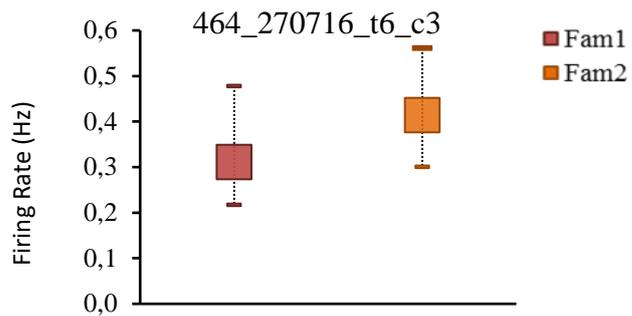
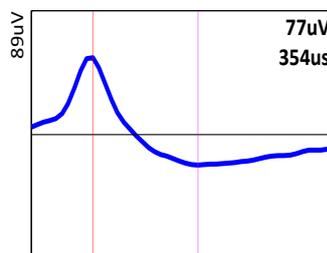
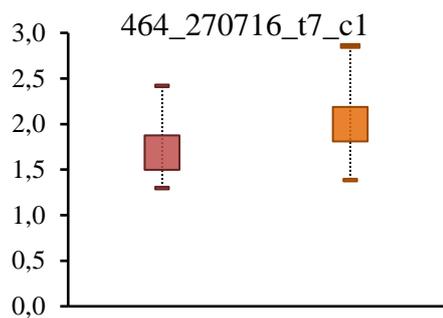
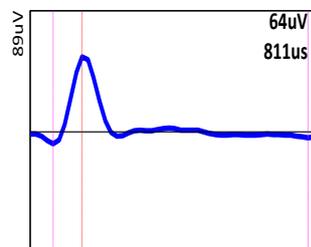
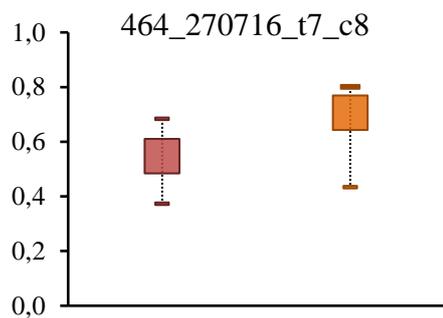
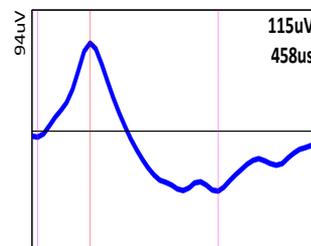
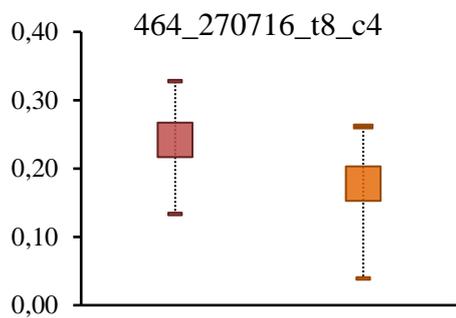


Figure 10.1. Key characteristics of the 15 Sister-Specific cells recorded in the three conditions sessions, i.e. neurons that showed preferential firing for one familiar sister. For each cell, left panel shows bar graphs of firing rate. The squared bars represent the median value for each condition (Base, blue; Fam1, red; Fam2, orange), the horizontal lines represent the maximum and the minimum values in each condition. The right panel shows the cell waveform: the X axis shows the time, the Y axis shows voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

A**B****C****D**

E**F****G****H**

I**J****K****L**

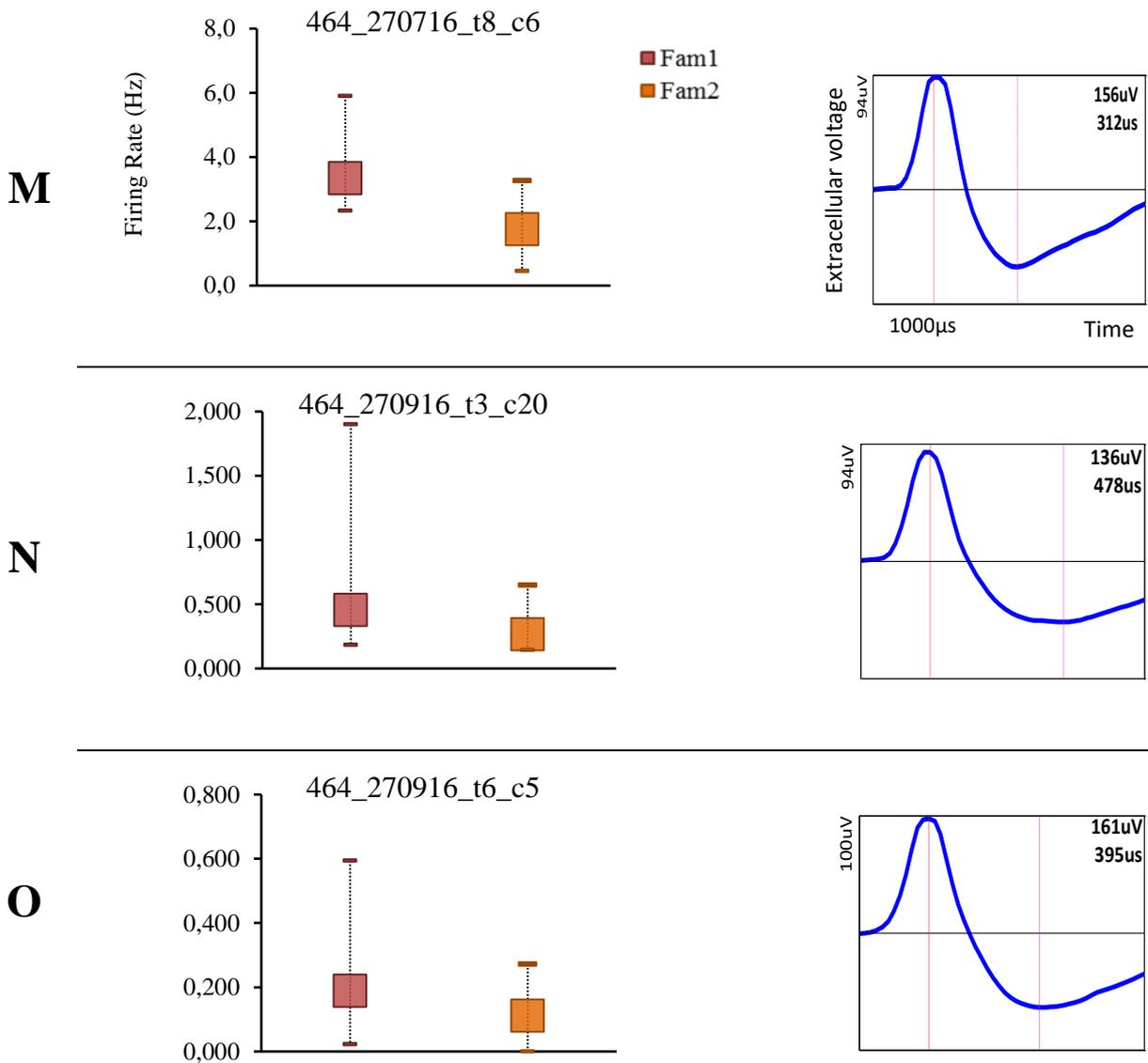


Figure 10.2. Key characteristics of the 15 Sister-Specific cells recorded in the two conditions sessions, i.e. neurons that showed preferential firing for one familiar sister. For each cell, left panel shows bar graphs of firing rate. The squared bars represent the median value for each condition (Fam1, red; Fam2, orange), the horizontal lines represent the maximum and the minimum values in each condition. The right panel shows the cell waveform: the X axis shows the time, the Y axis shows voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

10.4 Social non-specific firing

I next analysed the 102 neurons recorded in the three-condition sessions (031014, 101014, 200616, 280716). 12 cells (13.7%) exhibited significantly different firing during *both* social-condition trials (Social cells), compared to the non-social condition trials (baseline). The pie chart in **figure 10.3** compares the percentage of Social cells and Sister-specific cells with non-social cells. Seven of the 12 cells increased the firing rate when the implanted animal was paired with a familiar conspecific, while the remaining five significantly reduced firing during social trials. **Figure 10.4** shows the firing rate median, of one Social cell recorded during a three-condition testing session, that exhibit a change in the activity during both social trials (Fam1 and Fam2).

Percentage of Sister-Specific Cells

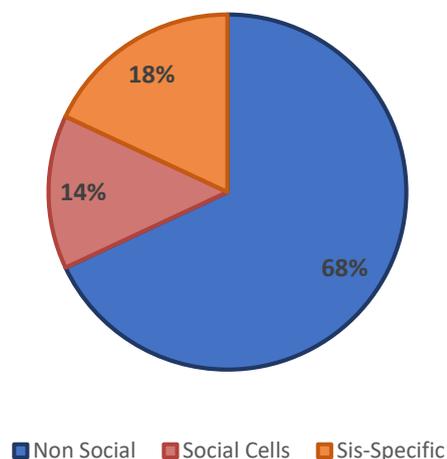


Figure 10.3. Percentage of Nov-vs-Fam Specific Cells recorded in the in the piriform cortex. In orange the percentage of Sister-Specific neurons (Sis-Specific) which increased the firing activity in presence of only one sister, in red the Social neurons (Social cells) whose increased the firing activity in presence both sister. In blue the percentage of Non Social cells (Non Social) whose firing appear not related to the presence of a social stimulus.

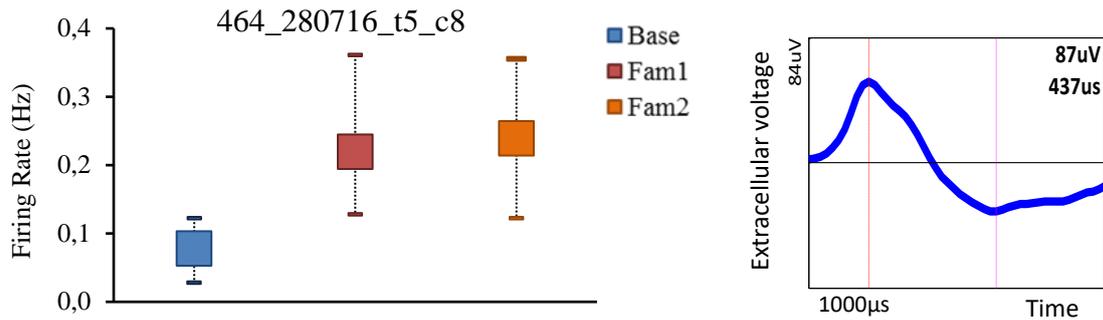


Figure 10.4. Key characteristics of a ‘social cell’, i.e. neuron that showed preferential firing for the social trials compared to baseline. Left panel shows bar graphs of firing rate. The squared boxes represent the median value for each condition (Base, blue; Fam1, red; Fam2, orange), the horizontal lines represent the maximum and the minimum values in each condition. The right panel shows the cell waveform: the X axis shows the time, the Y axis shows voltage. The waveform peak-to-trough amplitude and interval statistics are given in the top right. The waveform amplitude and interval are taken from the negative peak.

10.5 Speed analysis

The firing rate of cells that showed a significant change in firing when paired with only one of the two familiar stimuli was examined in relation to the average trial running speed. Previous studies showed that spontaneous and sensory-evoked activity in the cortex is highly state-dependent (Haider et al, 2013, Steriade et al, 2001) and considering running speed could be an approach to examine the effect of the arousal. For this reason, cells that showed sister-specific firing were further analysed in relation to speed and testing condition (Fam1 and Fam2). The firing rate of cells that showed a significant change in firing when the implanted animal was paired with a familiar stimulus, was further analysed in relation to running speed. Due to technical error, data from 270716 was excluded from the speed analysis. Standard multiple regressions were run with average speed and Social status (Fam and Base) as predictor variables for firing rate. These showed that Sister status significantly predicted firing rates for

12 of the 24 cells, (Beta values ≥ 0.6 , $p \leq 0.05$). For 3 of the 12 cells the firing rate was predicted from both the Running speed and the Sister status (Beta values ≥ 0.6 , $p \leq 0.05$), but in all the 3 cases, the Sisters status appeared to be a better predictor. Running speed alone did not significantly predicted firing rates in any of the 30 cells (Beta values ≤ 0.1 , $p > 0.05$). Since the implanted animal is equally familiar with the two sisters (Fam1 and Fam2), there should not be a significant difference in locomotion between the two conditions. Where this is true, social status and locomotion will not be correlated. It is notable that in the 5 sessions 031014, 101014, 240716, 280716 and 270916, the correlation between sister status (Fam1= 0, Fam2 = 1) and average running speed was low ($r \leq 0.49$, $p > 0.05$). However, even if the variance may be sufficient to establish predictor status to either one of these variables, this analysis is conservative because underpowered.

The paired t-test was run to determine whether differences in firing between the two groups (Fam1 and Fam2) could be related to differences in Running Speed means. **Table 10.3** shows the Mean \pm s.e.m (cm/s) of the 2 groups (Fam1 and Fam2) in the 5 sessions analysed. The analysis showed that only in session 464_240716 the Running Speed mean was significantly higher during trials with novel conspecifics ($t(5)=4.5$, $p=0.01$), (**table 10.3**). This result is in line with the results above described suggesting that the Running speed cannot fully justify the sister-sister discriminative firing

Table 10.3 Table showing the Running speed (mean± s.e.m.) between Fam1 and 2 conditions of the 6 sessions considered in the analysis.

Session	Mean ± s.e.m	t	p values
438_031014	Fam1= 9.2 ± 1.1 Fam2= 7.6 ± 1.1	1.5	0.2
438_101014	Fam1= 7.9 ± 1.5 Fam2= 8.6 ± 0.6	-0.7	0.5
464_270916	Fam1= 5.9 ± 0.7 Fam2= 6.7 ± 0.6	-1.1	0.3
464_200616	Fam1= 7.0 ± 1.0 Fam2= 6.7 ± 0.4	0.3	0.8
464_240716	Fam1= 7.1 ± 0.7 Fam2= 5.3 ± 0.9	4.5	0.01
464_280716	Fam1= 5.8 ± 1.0 Fam2= 6.3 ± 0.7	-0.5	0.7

10.6 Behavioural correlation

The behavioural scoring of the social trials, offers the best avenue to constrain interpretation that could account for changes in firing. Here, the main interpretation come from the correlation of social behavioural parameter with the firing rate of cells which discriminate between the two sisters. As reported in the method section above, the experimenter measured different behavioural parameters: time spent in anogenital sniffing, face to face contacts, other contacts and dominance. Each parameter was analysed individually and combined as total social contact. The amount of time spent by the implanted animal in the proactive exploration of the social stimulus was also considered. Since only 4 sessions were considered for the behavioural analysis, a total of 21 cells out of 30 were analysed in relation to the behavioural parameters

above mentioned. Interestingly, only 4 out of 21 cells showed firing correlation with at least one of the behavioural parameters, while the firing rate of 17 out of 21 cells was not correlated to any behavioural parameters. This may suggest that the firing rate of these sister-specific cells was driven by the presence of a specific sister inside the arena, independently of the social behavioural exploration in that trial. The behavioural analysis of the 4 sessions will be further discussed in chapter 11.

10.7 Probe trials

To further understand the relationship between the activity of the cells and the firing specificity for one of the two familiar social stimuli, the implanted rat was exposed to probe trials at the end of the session 280716. **Figure 10.5** shows the firing rate of the single cells which discriminated the two sisters in session 28/07/16. This cell showed firing excitation in presence of both the sisters but, the response was stronger for sister Fam1. Even if performing statistical analysis on these probe trials was not possible due to the lack of multiple samples, the use of controls such as familiar bedding and Sphero helped to interpret the nature of the firing changes. The bedding was taken from the cage containing both the familiar rats (Behavioural Methods, chapter 6 for further details) and the aim was attempting to understand if volatile olfactory cues were sufficiently effective at triggering the social specific pattern seen in presence of the conspecific itself. Sphero, is an interacting robotic ball controlled through a phone app, and the aim was attempting to understand if biological motion, mimicking the presence of a conspecific inside the arena, could reproduce a similar excitation seen in presence of the sisters.

The graph shows that neither the bedding of the two familiar conspecifics nor Sphero (Ball) were close to this mean defined as “[Familiar bedding rate] \geq [Familiar/Social mean – 0.5 Standard Deviation]).

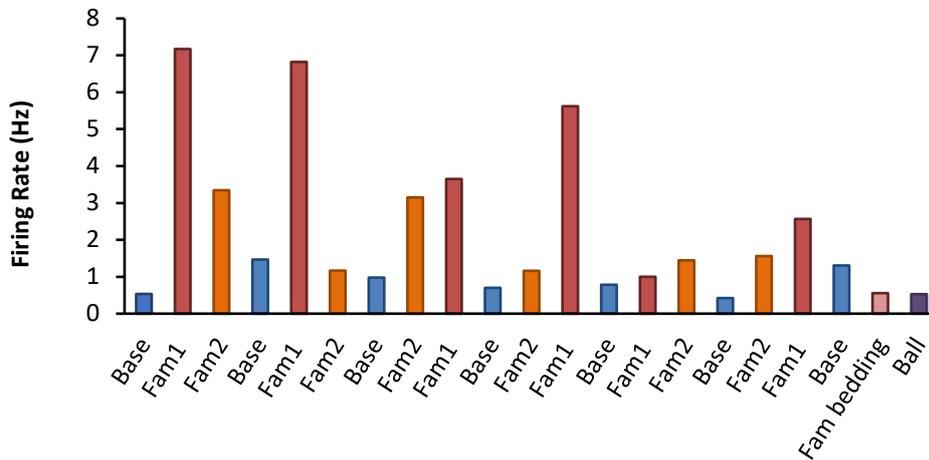


Figure 10.5. Firing rate of cell 464_280716_t8_c1 in each trial. The red bar show the firing rate of the cell when the implanted rat was paired with Fam1. Orange bars show the firing rate when the implanted animal was paired with Fam2. Blu bars show the firing rate of the cells during baseline trials. Pink and Purple bars show the firing rate of the cell in two other probe conditions, in order familiar bedding and Sphero (the interacting ball).

10.8 Firing pattern of the Sister-specific cells in relation to baseline trials.

Fifteen of the 30 sister-specific cells were recorded during the three-conditions sessions. Baseline trials represent a further control which allowed to better interpret the nature of the firing discrimination between the two sisters, and how the firing pattern was altered in presence of the sisters compared to a baseline pattern. Broadly speaking, did the presence of the sisters induce firing suppression or firing excitation of the cells?

Statistical differences between the firing rate means of each cell in the three conditions sessions was analysed using the Friedman test, followed by a post-hoc analysis where the Wilcoxon test

was run to identify significant difference in firing between the baseline and the two familiar conditions (Fam1 and Fam2). Friedman test revealed that 12 out of 15 cells showed significant differences in firing in the three conditions, $\chi^2(2) \geq 6.3$, $p \leq 0.042$. The firing rate of 4 out of 12 cells was significantly affected by both sisters' trials when compared to the baseline condition. Two of the 4 cells showed firing excitation during sisters' trials (figure 10.1, B and O); 1 showed firing suppression during both trials with sister Fam1 and trials with sister Fam2 (figure 10.1, E), and 1 showed firing suppression for one of the sister and firing excitation for the other sister (figure 10.1, N). The firing rate of 6 out of 12 cells was altered only in the presence of 1 sister, while the mean of the firing rate for the other sister, was closed to the mean of the firing rate of the baseline trials. Due to the small sample of the data presented here, Posthoc corrections were not applied to this analysis. Then, the possibility must be considered that the multiple comparisons increased the possibility of type I errors, and since the multiple comparison included three groups, overall, the probability of getting Type I error has increased from 5% to 14.3. Ideally, it would be necessary to control the risk of family-wise errors by correcting the level of significance for each test, such that the probability of a Type I error was decreased to at least 5% across the multiple comparison. Given the small sample, however, these data are not conducive to the application of the conservative options due to the overall lack of statistical power. Also, due to the limited range of significant p-values, using conservative methods, such as the Bonferroni corrections, would increase the probability of Type II errors, thereby increasing the chance of rejecting an existing difference in the data. Since these data represent an entirely novel set of experiments, which were exploratory in nature, the sample size is relatively small, with only 6 trials considered in each condition. For these reasons, applying conservative corrections on these data presented here could risk false conclusions due to washing out these primary data. While in this thesis, due to the small sample, the only aim of the analyses presented was to understand the direction of the firing pattern in

the presence of the two sisters, future works need to increase the sample size to minimize the possibility of family-wise errors

10.9 The firing rate of piriform neurons is not driven by pheromones.

The use of female social stimuli adds further potential confounds for this experimental manipulation. Pheromones are molecules that on the base of previous studies, are thought by some to be processed differently to other volatile molecules, and the piriform cortex has been considered in some studies not to play a role in pheromone processing (Kippin et al, 2003; Kelliher et al, 1999). The present section aims to answer two questions: 1) to what extent can cells in the piriform cortex recognise two very similar sisters? The two sisters were similar from many points of view (genetic, diet, environment), and similarity in the oestrus cycle would suggest a high sensibility of piriform neurons in recognise between two conspecifics, 2) could changes in firing reflecting a specific stage in the oestrus cycle?

For seven of the sessions considered in the analysis, samples were collected after the testing phase and the oestrus cycle was identified through the vaginal smear and involved the characterization of few distinctive cells type as described by Goldman and colleagues (2007) (details in chapter 6 Behavioural Methods) (**table 10.4**).

In session 438_031014 the two sisters (Fam 1 and Fam2) were both in the stage dioestrus. This suggest that even if the two sisters presented the same oestrus stage, 5 cells in the piriform cortex were still showing firing discrimination between the two sisters.

It is unlikely that the firing discrimination between the two sisters may be caused by differences in the oestrus stage. In fact, at different oestrus stage, like in session 101014, the firing rate of cell 438_101014_t3_c4 appear to be driven by sister Fam1 (**figure 10.1, L**) and the firing rate

of a simultaneously recorded cell 438_101014_t4_c3 appear to be driven by sister Fam2 (figure 10.1, M).

Table 10.4 Oestrus characterization of the implanted animal and the familiar stimuli in the sessions considered in the analysis

Sessions	Implanted animal	Fam1	Fam2
438_031014	Proestrus	Dioestrus	Dioestrus
438_101014	Proestrus	Proestrus	Dioestrus
464_200616	Proestrus	Oestrus	Proestrus
464_240716	Dioestrus	Oestrus	Metoeestrus
464_270716	Oestrus	Proestrus	Oestrus
464_280716	Metoeestrus	Oestrus	Metoeestrus
464_270916	Oestrus	Proestrus	Oestrus

10.10 Discussion

The present chapter aimed to study the function of the piriform cortex from a naturalistic point of view and contribute to further understand the role of these neurons in social discrimination between two conspecifics. In the previous chapters, 8 and 9, I analysed and discussed which factors can influence the changes in firing in the piriform cortex. The naturalistic approach used in the present study, leave open more than one interpretation since it sacrifices the controlled cues delivery in favour of unconstrained social interaction. For example, changes in firing may reflect change in arousal state. Also, the contribution of the multisensory inputs cannot be excluded via freely interaction between the implanted animal and the conspecific. However, the findings examined in chapter 9 may support the theory that neurons in the piriform cortex may contribute to the social recognition of a conspecific, since 20.5% of neurons in the piriform cortex could discriminate between familiar and novel social stimuli.

In line with finding discussed in chapter 9, the present chapter showed that a similar percentage of neurons (18%) changed the firing in response to two familiar social stimuli. If from a point of view, changes in firing rate between novel and familiar conspecifics could have been related to difference in the arousal state for a completely novel conspecific inside the arena, the fact that piriform neurons can also discriminate between two familiar animals (two sisters) may suggest that arousal state may not be involved in this firing discrimination.

Accordingly, the firing discrimination between the two sisters appears not to be related to running speed or biological motion. Examining the average trial running speed is one approach to considering the effect of arousal, and from a certain point of view it can be considered an index of general arousal; and more strictly as a locomotion variable well-known to positively correlate with neurons throughout the hippocampal formation (O'Keefe et al, 1998; Lever et al, 2003; Kropff et al, Nature, 2015). However, my findings showed that the firing of neurons which discriminated between the two sisters, was never correlated to the speed alone, while the firing of 50% of the cells appeared to predict the presence of a specific sister inside the arena.

Another interpretation of these findings comes from the probe trial with Sphero, a moving ball controlled with a phone app, which could be said to mimic the biological motion of a conspecific inside the arena. The biological motion and the arousal created by the continuous interaction with Sphero, did not produced the similar excitation pattern, observed in the case of the two sisters, suggesting that general arousal and quasi-biological motion may not be the major contributor in the firing discrimination observed between the sisters.

Similar to the results presented in chapter 8, where the social/non-social experiments aimed to provide an initial analysis of neuronal firing pattern in the piriform cortex, sister-specific

neurons showed both firing suppression and excitation in presence of a specific sister, when compared to the firing recorded in the baseline trials. In some cases, the cell showed firing excitation for one sister and firing suppression for the second sister. If we consider the model of exposure to multiple odorants in anaesthetised animals (Stettler and Alex, 2009), the firing suppression could be related to the fact that a rat is represented by a mixture of odorants from different parts of the body. In the piriform cortex, a certain familiar conspecific may be represented as odour-mix (if we only consider the olfactory stimuli) which suppresses the firing of certain neurons and excite the firing of other neurons. A complementary interpretation is that even if each cell *per se* appear to show a specific pattern in presence of a sister, the identity of a conspecific is likely coded by the unique ensemble of excited or suppressed neurons. In fact, cells simultaneously recorded, showed different firing pattern in response to the same conspecific, excitation or suppression, and if what we are observing is memory coding, it is plausible that the identity of a conspecific is coded by multiple-member set of neurons, i.e. a ‘cell assembly’

Considering the firing of the neuron recorded in session 280716, it showed considerably excitation for both the sisters compared to baseline trials, but fired strongly in response to one sister compared to the other one. However, when the implanted animal was exposed to the bedding of the two sisters, the firing rate of this cell, failed to replicate the pattern observed in presence of the sisters. Unfortunately, the fact that only one neuron recorded in this session discriminated between the two sisters makes it difficult to be conclusive about sufficient olfactory cues. The candidate interpretation is that the cell responded to bodily scents or other bodily cues in physical-interaction trials that were not present in the urine/faeces of the bedding trial. It cannot be ruled out however, that the non-replicated pattern for the bedding could also be related to the fact that it contained scents *from both the sisters*; but if this was the case, it

would still be expected the firing to increase, since the firing increased compared to baseline, with both the sisters. Another interpretation is that the neuron was showing a neuron-specific response to the conspecifics and not a general one. Accordingly, in the findings examined in chapter 9, together with neurons that only responded to bedding in a manner similar to social trials, there were neurons that only responded to the conspecific, suggesting that this non-response to the bedding may not be attributed to the failure of the bedding as a sensory triggering cue. Instead, it may be plausible that for this cell, the volatile odours of the conspecifics were not the major contributor to the increase in the firing.

The main interpretation of these findings come from the behavioural scoring. My findings, showed that there are cells in the piriform cortex that are discriminating between two similar familiar conspecifics, and these firing differences appear not to be related to differences in social interaction. In fact, the majority of sisters' discriminative neurons do not show correlation with any of the behavioural social parameters, suggesting that differences in the time spent in close contact or in proactive exploration of the other conspecific, may not contribute to the firing differentiation. The behavioural scoring offered the advantage to understand if social exploration may drive the firing of cells in the piriform cortex, however future analysis may consider observing the firing relation with a microbehavioural analysis.

Thor in 1979, suggested that the representation of a conspecific is defined by two different odours. The 'individual odour', determined by diet, genetic and environment (Singh and Roser, 1987) and the 'androgen odour', a pheromone related odour. The two familiar animals presented in this study, were sisters leaving in the same cage and eating the same food, meaning that they shared genetic, diet and environmental space. It may be expected that two sisters leaving so close to each other, and eating the same food, may present similar odorant cues (and

possible similar appearance) and still be recognised as different individual by another conspecific. Then considering the theory that the piriform cortex could be involved in memory encoding, it is not surprising that cells in the piriform cortex could discriminate between similar sisters. Regarding the ‘androgen odour’, it is unlikely that the firing discrimination between the two sisters may be caused by differences in the oestrus stage, in fact when the two familiar were both in the stage dioestrus, five cells could still discriminate between the two conspecifics. Furthermore, discriminative cells were observed in different phases of the oestrus cycle. In few words, differences in the oestrus cycle appear not to be the major contributor for changes in firing between the sisters.

Even if the possible contribution of arousal and multisensory inputs cannot be excluded, the findings here reported suggest that the piriform cortex may contribute to social identity coding of individual conspecifics. The next chapter will provide an overview of the social activity during the recording sessions analysed in the present chapter and chapter 9 with the attempt to narrow down interpretation on the nature of piriform cortex neurons’ firing, such as the sociability of the social stimulus, or other aspects of the social interaction.

Chapter 11: Behavioural analysis

11.1 Experimental rationale

Chapter 9 and 10 provided evidences that neurons in the piriform cortex can discriminate between novel and familiar conspecifics and even more interestingly, between two familiar conspecifics. This chapter will provide an overview of the social activity during the recording sessions analysed in chapter 9 and 10, with the attempt to narrow down interpretation on the nature of this conspecific discrimination. Given that piriform cortex predominantly receives olfactory inputs, and olfaction contributes to social motivation and social recognition memory, I took account of social parameters like anogenital sniffing and face to face contacts. In fact, the direct investigation toward the anogenital region and the face are important to acquire odour information, and could potentially involve the activation of neurons in the piriform cortex. Other parameters analysed are summarised in chapter 6, section 6.8. Broadly speaking, this chapter aimed to answer 3 questions:

- 1) Were there differences in behavioural interaction between novel and familiar social trials?*
- 2) Were there differences in behavioural interaction between the two familiar social trials?*
- 3) Are social discriminatory firing patterns better explained by aspects of social behaviour?*

The third question merits micro-behavioural analyses well beyond the scope of the current thesis. Perhaps the most appropriate analysis would involve an assumption-free, machine-learning approach where the data input would be the spike train on the one hand, and the visual stream of the behavioural record (in 20-ms segments) on the other. The approach taken here

was certainly much simpler. Since the previous analysis in chapters 9 and 10 take place at the trial level (e.g. cell fires significantly higher in trials with novel rats than in trials with familiar rat, or cell fires significantly higher in trials with familiar-1 sister than in trials with familiar-2 sister), correlations were at the trial level (n = 12 per session).

Behavioural results and the possible correlation of behaviours with the firing activity will be first analysed by sessions and finally summarised and discussed later in this chapter. Broadly speaking, as we shall see at least in the simple analysis offered here, the possibility that socially-discriminating cells were better explained by behaviour than by socially-related cognition or motivation was true for only a small minority of socially-discriminating cells.

11.2 Summary of the experimental procedure

As described in the method section, Lister-Hooded female rats were implanted with microdrives both the left and right piriform cortex and habituated over a prolonged period to singularly encounter a familiar social stimulus or a novel social stimulus into a small square box (40×40×50cm). Each social condition was presented to the implanted animal for 6 trials, for a total of 12 trials. During the social familiar condition the implanted animal was exposed to the same familiar social stimulus for 6 trials, while during the social novel condition was exposed to a different novel social stimulus in each of the 6 trials. Social trials were analysed off-line in form of digitized motion images and behaviour was scored. Time spent by the implanted animal investigating the social stimuli, during each of the twelve 3-minutes exposures, were used for calculations. Different behavioural parameters were considered in this analysis: time spent in anogenital sniffing, face to face contacts, other contacts, dominance, total social contacts and active contact. **Table 6.3** in chapter 6 shows a brief description of the

behavioural parameters used in the analysis. The firing rate of cells recorded in each session was analysed in relation to the different behavioural parameters to understand if social interaction could contribute to changes in firing. Pearson correlation was used for cells which firing rate was normally distributed. Cells that did not show a normal distribution were tested using the non-parametric Spearman correlation.

11.3 Fam-Nov sessions

Chapter 9 provided evidence that 27 out of 132 neurons could discriminate between novel and familiar conspecifics. Here, I will investigate the nature of this firing discrimination, in relation to social behaviour. Social trials were analysed off-line in form of digitized motion images and behaviour was scored. Four of the sessions shown in **table 11.1** were considered for the behavioural analysis: 040914, 080414, 010716, 260716. I present the data in a session-by-session manner since the implanted rat showed different behavioural performance in each session. One session was not scored, thus leaving 25 social novel-vs-familiar specific neurons available for further analysis. Anomalies in behavioural performances will be discussed in the animal and age of the social stimuli is included in **table 11.1**. Of the 109 cells analysed in the four sessions, 41 (38%) showed firing correlation with at least one of the social behavioural parameters considered in the analysis. Interestingly, only 5 of the 25 social novel-vs-familiar specific neurons showed firing rate correlation with one of the behavioural parameters. This suggests that the firing rate of these novel-familiar discriminative cells was driven by the presence of a familiar or novel conspecific, independently of the social behavioural exploration in that trial.

Table 11.1. Summary of session details considered for the Fam vs Nov sessions in three of the rats implanted in the piriform cortex.

Session	Implanted animal Age	Implanted animal Oestrus cycle	Familiar animal Age	Novel animals 1-6 Age	Track location	Total cells considered	Social novel-vs-familiar specific cells
438_040914	20 weeks	Dioestrus	20 weeks	6-8 weeks	Pir	26	5
451_080415	14 weeks	Proestrus	14 weeks	14 weeks	Pir	19	2
464_010716	32 weeks	Oestrus	32 weeks	22 weeks	Pir?	32	8
464_260716	35 weeks	Oestrus	35 weeks	26 weeks	Pir?	32	10
Total cells						109	25

11.3.1 Rat 464, session 010716

This session included 12 trials (6, Fam1; 6, Novel). The implanted rat (464) spent on average 63% of the time in a trial in close contact with another social stimulus, novel or familiar. However, on average, only 32% of the time in a trial was spent by the implanted animal proactively engaging in social contacts with the social stimulus. Overall, there was no significant difference between the amounts of time (mean \pm s.e.m.) the implanted animal spent in ‘total social contact’ with familiar 117.5 ± 6.8 and novel 108 ± 8.0 social stimuli $t(5)=1.5$, $p=0.2$ (**figure 11.1 A**). However as expected in presence of novel stimuli, the implanted animal proactively interacted more with the novel than the familiar social stimuli (Nov: 86.8 ± 10.2 ; Fam: 27.7 ± 7.2 ; $t(5)=-4.4$, $p=0.007$; **Figure 11.1 E**). As expected, there was a higher level of ‘anogenital sniffing’ in the novel condition (Fam: 3.7 ± 1.7 ; Nov: 12.4 ± 3.7 ; $t(5)=-2.99$, $p=0.03$; **figure 11.1 B**). There were no significant differences in ‘other contacts’ ($t(5)=2.2$, $p=0.075$; **figure 11.1 C**) and in ‘face to face contacts’ ($z(n=6)=-0.14$, $p=0.9$; **figure 11.1 D**). No dominant behaviour was observed in this session.

Table 11.2 shows the list of cells whose firing was correlated with any of the social parameter considered in the analysis. Four cells out of 32 (12.5%) showed a positive correlation with time spent in ‘active’ interaction, i.e. proactive exploration of the social stimulus, novel or familiar. Two cells out of 32 (6%) showed correlation with the time spent in total social contacts, one was positively correlated, one was negative correlated. Four out of 32 (12.5%) cells showed a negative correlation with the time spent in other contacts. The implications of this, i.e. reduced firing with increasing social interaction, are not fully clear. Social interaction could be acting in an inhibitory manner. The firing rate of 9 out of 32 cells (28%) considered in the analysis showed a statistically significant correlation with the time spent in anogenital sniffing. These cells merit much closer attention than was possible in this relatively crude correlation analysis, because the amount of time spent by the implanted animal in this behaviour represent less than 5% of the total trial. None of the cells showed correlation with the time spent in face to face contacts.

11.3.1.2 Social discriminatory firing patterns were not better explained by aspects of social behaviour

During this session, 8 cells (25%) out of 32, showed firing discrimination between familiar and novel rats. Since in this session, the implanted animal proactively interacted more with the novel conspecifics and this was accompanied by an increase in anogenital sniffing and body sniffing, the analysis correlation aimed to understand if the firing activity of these cells could have been related to the behaviour. Two out of 8 cells (464_010716_t2_c1: $\rho = 0.62$, $p = 0.03$ and 464_010716_t3_c8: $\rho = 0.57$, $p = 0.05$) showed a correlation (positive) with the time spent by the implanted animal in proactively interacting with the social stimulus (active time) (see **table 11.2**). However, 6 out of 8 cells appeared not to be correlated with any behavioural

parameter, suggesting that social behaviour was a major contributor for the firing discrimination between familiar and novel conspecifics.

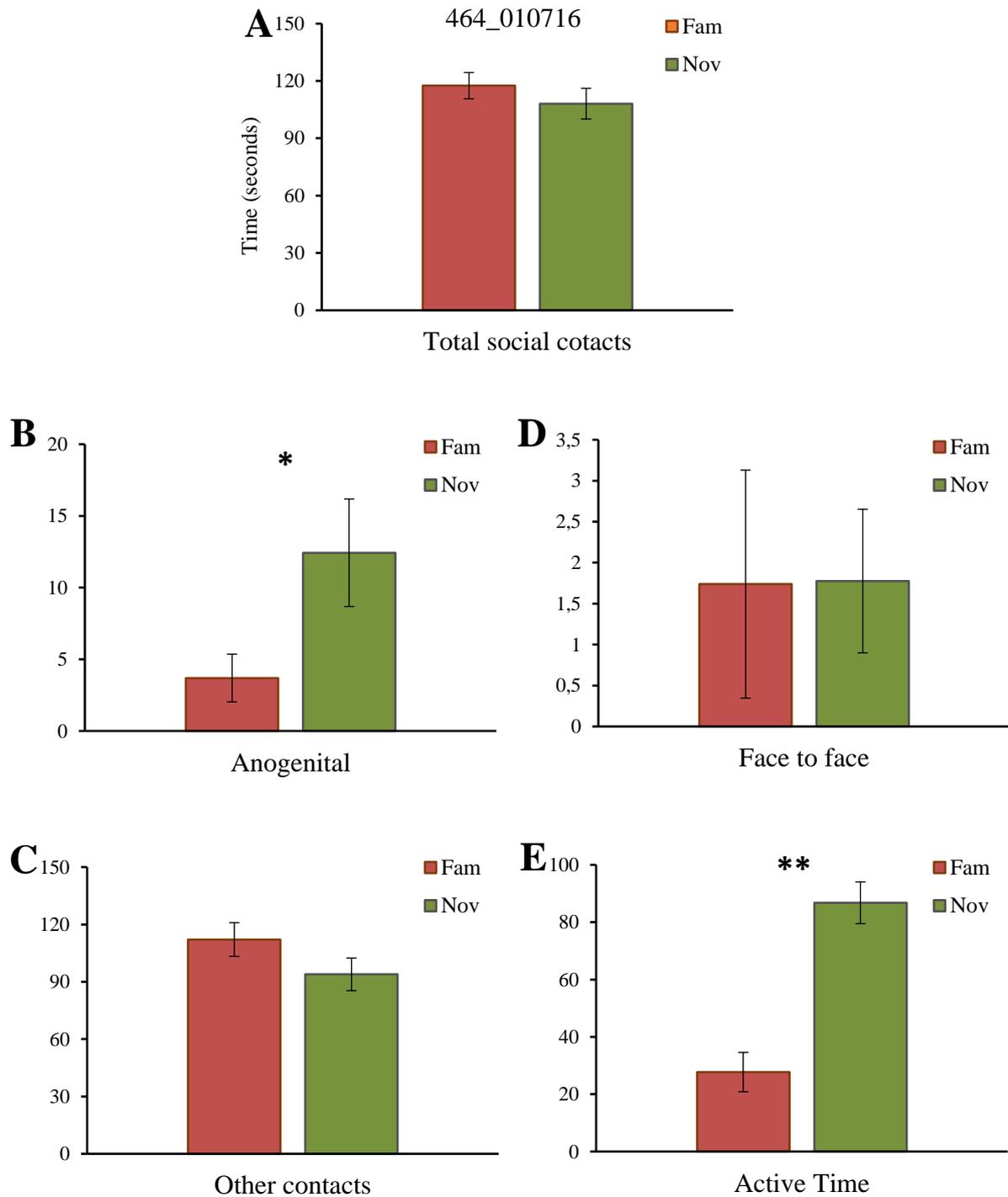


Figure 11.1 Behavioural performance (*mean ± s.e.m.*) of rat 464 during social interaction in session 010716. Graphs show (A) total social contacts ($t(5)=1.5$, $p=0.2$); (B) anogenital contacts ($t(5)=-2.99$, $p=0.03$); (C) other contacts ($t(5)=2.2$, $p=0.075$); (D) face to face contacts ($z(n=6)=-0.14$, $p=0.9$), and (E) active time ($t(5)=-4.4$, $p=0.007$).

Table 11.2: cells that showed significant correlation with some of the behavioural parameters in animal 464, session 010716. Pearson correlation was used for the majority of the cells considered. Cell that did not show a normal distribution (in red) were tested using the non-parametric Spearman correlation

Cell ID	Behavioural Parameter	r/ ρ	p
464_010716_t6_c4	Anogenital	-0.68	0.02
464_010716_t6_c5	Anogenital	-0.7	0.01
464_010716_t6_c6	Total Social	0.573	0.05
464_010716_t8_c1	Anogenital	0.675	0.02
	Other contacts	-0.627	0.029
464_010716_t2_c1	Active Time	0.615	0.03
464_010716_t3_c2	Anogenital	0.714	0.01
	Other contacts	-0.568	0.05
464_010716_t3_c7	Anogenital	0.596	0.04
464_010716_t3_c8	Active Time	0.566	0.05
464_010716_t3_c12	Anogenital	0.669	0.02
	Other contacts	-0.61	0.04
464_010716_t3_c13	Anogenital	0.857	<0.001
	Active Time	0.654	0.02
464_010716_t4_c2	Other contacts	-0.585	0.05
	Total Social	-0.745	0.005
464_010716_t4_c4	Anogenital	0.6	0.04
464_010716_t4_c5	Anogenital	0.751	0.005
	Active Time	0.661	0.02

11.3.2 Rat 464, session 260716

This session included 12 trials (6, Fam1; 6, Novel). The implanted rat (464) spent on average 44% of the time actively engaging with another social stimulus, novel or familiar. Overall, there was no significant difference between the amounts of time (mean \pm s.e.m.) the implanted animal spent in total social contacts with familiar 62.1.5 \pm 16.2 and novel 94.6 \pm 7.5 social stimuli $t(5)=-2.2$, $p=0.09$ (**figure 11.2 A**). However, the implanted rat spent more time proactively

engaging with the social novel stimuli 112.9 ± 19.5 then the familiar stimulus 68.3 ± 25.7 , (Active time: $t(5) = -3.6$, $p = 0.016$; **figure 11.2 E**). Wilcoxon test revealed higher levels of ‘anogenital contacts’ in the novel condition (Fam: 5.02 ± 3.4 ; Nov: 17.5 ± 7.6); $z(n=6) = -3.6$, $p = 0.016$; **figure 11.2 B**), while, no significant difference were observed in face to face contacts ($z(n=6) = -0.4$, $p = 0.7$) between the social familiar (1.6 ± 0.4) and the novel (2.3 ± 1.9) conditions, (**figure 11.2 D**). Furthermore, a paired simple t-test revealed no significance difference between novel and familiar social stimuli, in ‘other contacts’ (Fam: 55.5 ± 14.7 ; Nov: 73.8 ± 7 ; $t(5) = -0.95$, $p = 0.4$; **figure 11.2 C**) No dominant behaviour has been observed in this session.

Table 11.3 shows the list of cells whose firing was correlated with any of the social parameter considered in the analysis. The firing rate of 14 (44%) out of 32 cells considered in the analysis showed a statistically significant correlation with one or more behavioural parameters (total social contacts, anogenital sniffing, face to face contacts, other contacts, active time). Interestingly, in 10 out of 14 cells, the firing rate was correlated with ‘active time’. Active time is time spent by the implanted animal sniffing and touching body parts, including anogenital and facial areas, of the social stimulus. Then it may be plausible that the 10 cells were correlated to the amount of odorant information acquired during this period. This may explain also the firing suppression observed for three cells (3/10) following the increase of proactive social interaction.

11.3.2.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

During this session, 10 cells out of 32 (31%) showed firing discrimination between familiar and novel conspecifics. Given that the implanted animal proactively interacted more with the novel than the familiar conspecifics, and this was accompanied by increase in anogenital

sniffing, here, the analysis correlation aimed to understand if the firing activity of these cells could have been related to the behavioural interaction with the conspecific. Three cells out of 10 (464_260716_t5_c1 and 464_260716_t5_c5 and 464_260716_t7_c4) showed a significant correlation with one of the behavioural parameters mentioned above (see **table 11.3**). Specifically, two cells' firing rates were positively correlated with the time spent in anogenital sniffing, and one was correlated with the total time spent in social contacts. The issue of whether anogenital sniffing can explain the firing rate of these cells needs closer inspection, since the amount of time spent by the implanted animal in this behaviour represents on average 6.2% of the total trial. Seven cells out of 10 did not show correlation with any social behavioural parameter, suggesting that, at least in the case of these 7 cells, social behaviour was not one of the major contributor for the firing discrimination between familiar and novel conspecifics.

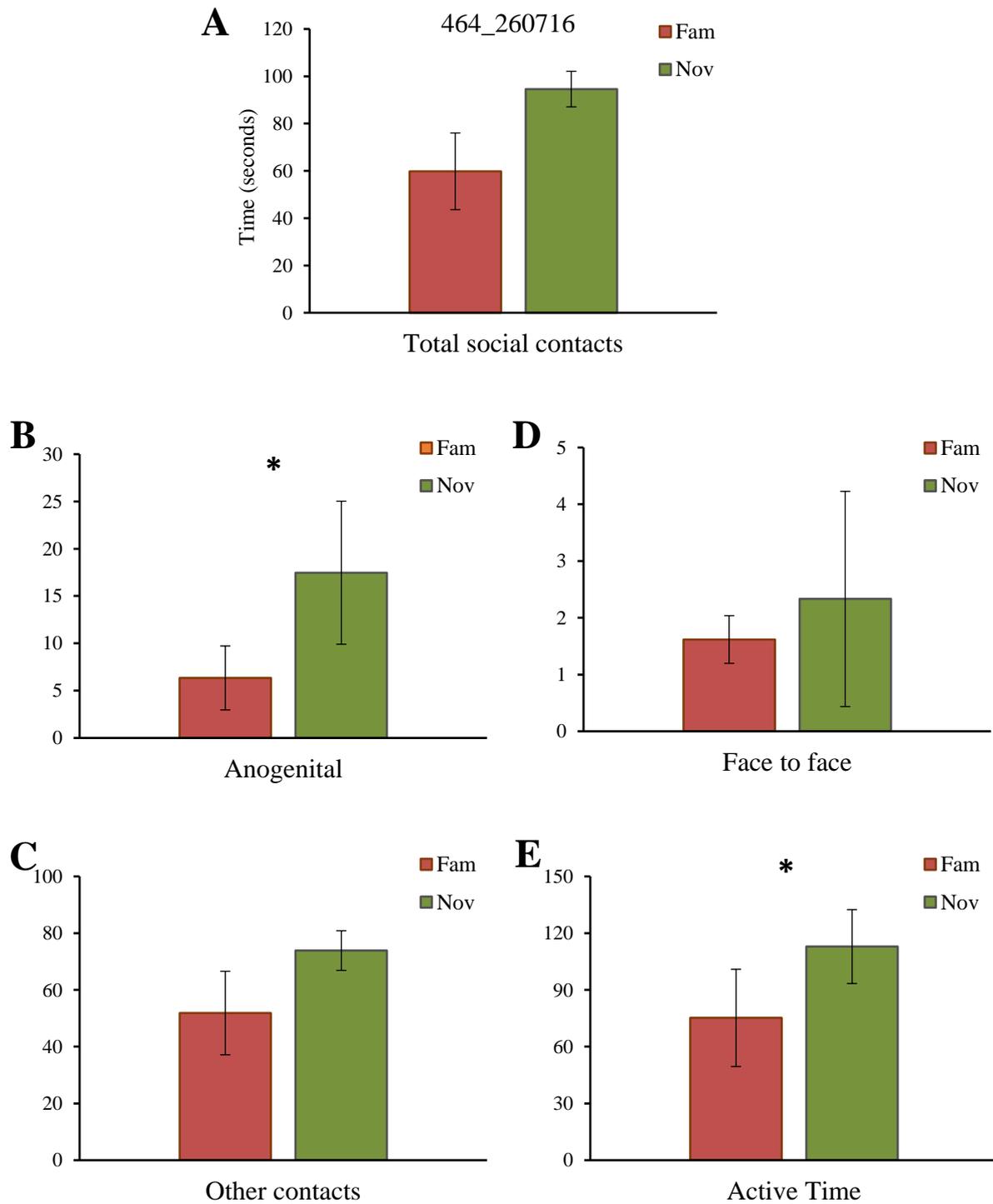


Figure 11.2 Behavioural performance (*mean ± s.e.m.*) of rat 464 during social interaction in session 260716. Graphs show (A) total social contacts ($t(5)=-2.1$, $p=0.09$); (B) anogenital contacts ($z(n=6)=-1.99$, $p=0.046$); (C) other contacts ($t(5)=-0.95$, $p=0.38$); (D) face to face contacts ($z(n=6)=-0.405$, $p=0.7$), and (E) active time ($t(5)=-3.6$, $p=0.016$). No dominant behaviour has been observed in this session

p

Table 11.3: cells that showed significant correlation with some of the behavioural parameters in animal 464, session 260716. Pearson correlation was used for cells whose firing rate was normally distributed. Cell that did not show a normal distribution and correlations with the parameter face to face contacts were tested using the non-parametric Spearman correlation (in red).

Cell ID	Behavioural Parameter	r/ ρ	p
464_260716_t5_c1	Total social contacts	0.573	0.05
464_260716_t5_c2	Face to face	-0.584	0.05
464_260716_t5_c4	Anogenital	-0.605	0.04
	Active Time	-0.604	0.04
464_260716_t5_c5	Anogenital	0.606	0.04
	Active Time	0.658	0.02
464_260716_t5_c7	Active Time	-0.617	0.03
464_260716_t6_c2	Anogenital	0.787	0.002
	Face to face	0.800	0.002
	Active Time	0.765	0.004
464_260716_t8_c4	Total social contacts	0.613	0.03
	Active Time	0.691	0.01
464_260716_t8_c5	Face to face	0.655	0.02
464_260716_t8_c11	Anogenital	0.577	0.05
	Total social contacts	0.613	0.03
	Active Time	0.853	<0.001
464_260716_t7_c4	Anogenital	0.617	0.03
464_260716_t7_c5	Face to face	-0.591	0.04
	Active Time	-0.696	0.01
464_260716_t7_c7	Anogenital	0.588	0.04
	Total social contacts	0.769	0.003
	Active Time	0.844	0.001
464_260716_t7_c9	Total social contacts	0.610	0.04
	Active Time	0.775	0.003
464_260716_t7_c10	Other contacts	0.781	0.003
	Total social contacts	0.812	0.001
	Active Time	0.661	0.02

11.3.3 Rat 438, session 040914

This session included 12 trials (6, Fam1; 6, Novel). The implanted rat (464) spent in average 56% of the time in social interaction with another social stimulus, novel or familiar. However, in average only the 44% of the trial was spent by the implanted rat in proactively exploring the social stimulus. Contrary to the expectation, the time spent in ‘total social contacts’ was significantly higher for the familiar than the novel social stimulus (Fam: 116.3 ± 8.7 ; Nov: 85.7 ± 17 ; $t(5) = 2.5$, $p = 0.05$; **figure 11.3 A**). However, the active time was very similar between the two conditions (Nov, 77 ± 25 ; Fam 80.6 ± 32.2 ; $t(5) = 0.2$, $p = 0.8$), meaning that the implanted animal did not show any motivational preference in interacting with the familiar stimulus, and the difference in ‘total social contacts’ appears to be related to the higher sociability of the familiar compared to the novel rat (**figure 11.3 F**). A Paired simple t-test revealed that the implanted animal spent more time in ‘other contacts’ with the familiar rat than the novel (Fam: 97 ± 7.8 ; Nov 63.5 ± 16.7 ; $t(5) = -3$, $p = 0.03$; **figure 11.3 C**). No significant difference was observed in ‘face to face contacts’ ($z(n=6) = -0.37$, $p = 0.7$), ‘anogenital contacts’ ($z(n=6) = -1.15$, $p = 0.25$), and ‘dominant’ behaviour ($z(n=6) = -0.54$, $p = 0.6$) between the social familiar (face to face contacts: 2 ± 1.5 ; anogenital contacts: 8.6 ± 16.7 ; dominance: 8.7 ± 4.4) and the social novel (face to face contacts: 3.7 ± 1.8 ; anogenital contacts: 13.2 ± 4.2 ; dominance: 5.2 ± 4.7) conditions (**figure 11.3 B, E and D**).

Table 11.4 shows the list of cells whose firing was correlated with any of the social parameters considered in the analysis. The firing rate of 11 (42%) out of 26 cells considered in the analysis showed a statistically significant correlation with one or more behavioural parameters (total social contacts, anogenital sniffing, face to face contacts, other contacts). Seven cells (27%) out of 11 showed a positive or negative correlation with ‘active time’. However, the ‘active

time' between the social novel and social familiar conditions was very similar, suggesting it is unlikely that the firing rate of these cells was driven by the proactively social interaction with a social stimulus.

11.3.3.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

During this session, 5 cells (19%) out of 26, showed firing discrimination between familiar and novel rats, but none of these cells showed a significant relation with the behavioural parameters considered, including 'total social contacts' and 'other contacts'. This may suggest that the firing rate of these social novel-familiar discriminative cells was not driven by the behavioural social interaction with another conspecific.

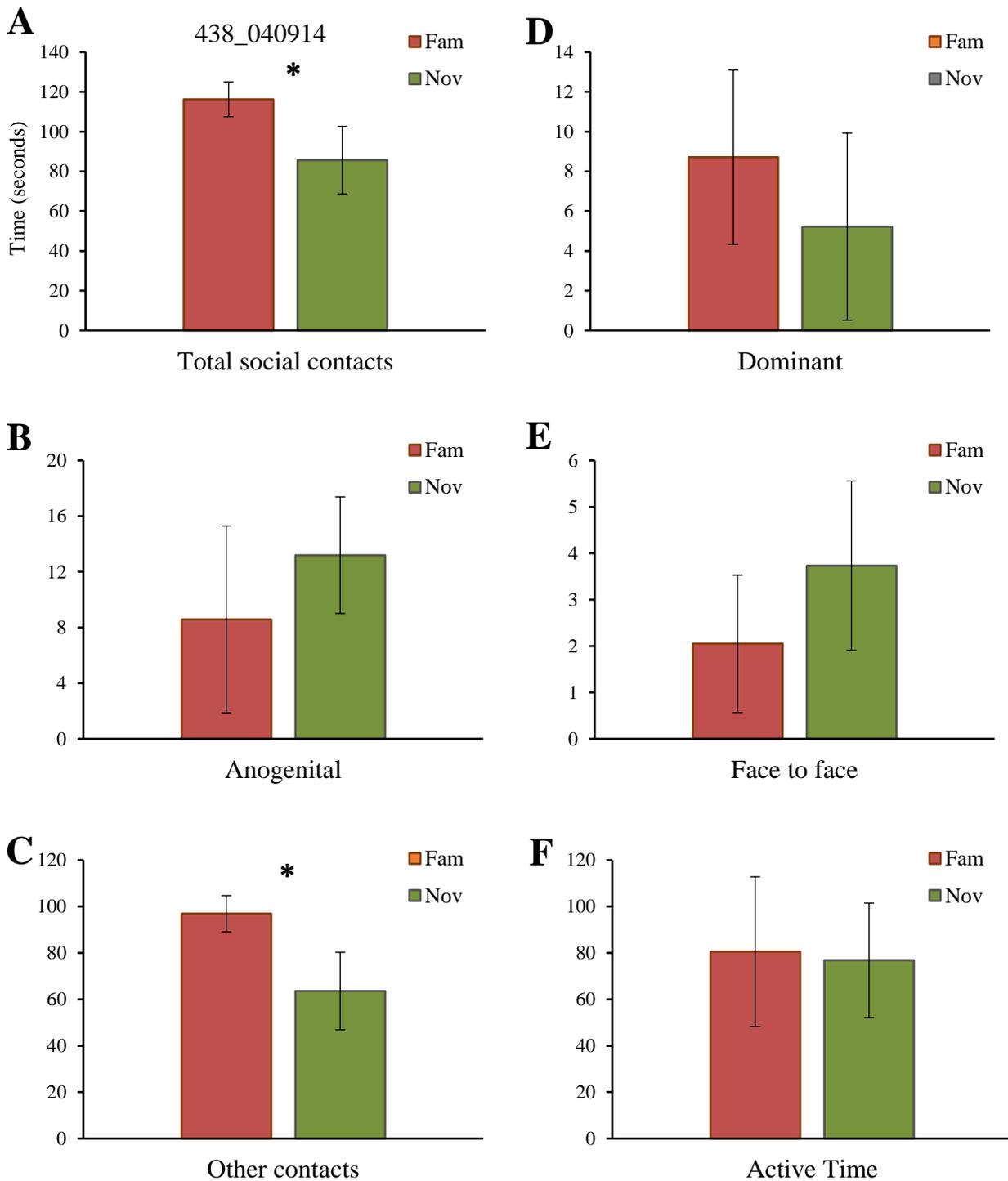


Figure 11.3 . Behavioural performance (*mean ± s.e.m.*) of rat 438 during social interaction in session 040914. Graphs show (A) total social contacts ($t(5)=-2.5$, $p=0.05$); (B) anogenital contacts ($z(n=6)=-1.15$, $p=0.25$); (C) other contacts ($t(5)=-2.97$, $p=0.03$); (E) dominant ($z(n=6)=-0.5$, $p=0.6$); (E) face to face contacts ($z(n=6)=-0.37$, $p=0.7$), and (F) active time ($t(5)=0.2$, $p=0.8$).

Table 11.4: cells that showed significant correlation with some of the behavioural parameters in animal 438, session 040914. Pearson correlation was used for cells whose firing rate was normally distributed. Cell that did not show a normal distribution and correlations with the parameter face to face contacts were tested using the non-parametric Spearman correlation (in red).

Cell ID	Behavioural Parameter	r/ ρ	p
438_040914_t1_1	Active Time	.766	0.004
	Face to face	.637	0.03
438_040914_t2_5	Active Time	.580	0.05
438_040914_t2_6	Anogenital	-.671	0.05
438_040914_t2_8	Anogenital	-.697	0.01
438_040914_t2_14	Dominant	.732	0.007
438_040914_t3_2	Anogenital	-.647	0.02
438_040914_t3_5	Active Time	.679	0.03
	Other contacts	.631	0.04
438_040914_t4_1	Anogenital	-.594	0.04
438_040914_t5_3	Active Time	.744	0.006
	Anogenital	.720	0.008
438_040914_t6_3	Active Time	-.748	0.005
	Dominant	-.667	0.02
Active Time	Active Time	-.795	0.002
	Active Time	-.818	0.001

11.3.4 Rat 451, session 080415

This session included 12 trials (6, Fam1; 6, Nov), however, due to technical errors on the behavioural video recordings, the behaviour for one social-familiar trial was not available, thus only 11/12 trials were considered in the analysis (5, Fam; 6, Nov). The implanted rat (451) spent in average 65% of the time proactively exploring another social stimulus, novel or familiar. Contrary to general expectations, there was no significant difference between the amount of time the implanted animal spent in total social contacts with the social familiar 136.3 ± 5.3 and the novel 120.3 ± 7.5 social stimuli $t(4)=1.5$, $p=0.2$ (**figure 11.4 A**). ‘Active time’, i.e. the amount of time the implanted animal spent actively interacting with the social

stimuli ('active' time), was not significantly different between the two conditions, though the implanted animal on average proactively interacted for about 50% more time with the novel conspecific compared to the familiar one (Fam: 98 ± 23 ; Nov: 149 ± 9 ; active time: $(t(4)) = -2.0$, $p = 0.12$; **figure 11.4 E**). It is plausible that this difference would have been significant if 6, not 5 trials were available for the Familiar condition. No significant differences were observed in 'face to face contacts' ($(z(n=5)) = -1.1$, $p = 0.3$), 'anogenital contacts' ($(t(4)) = 0.02$, $p = 0.98$), and 'other contacts' ($(t(4)) = -2.1$, $p = 0.11$), between the social familiar (face to face contacts, 0.2 ± 0.2 ; anogenital contacts, 9.3 ± 5.1 ; other contact 126 ± 4.3) and the social novel (face to face contacts, 0.8 ± 0.5 ; anogenital contacts, 9.1 ± 3.2 ; other contact 113 ± 3.6) conditions (**figure 11.4 D, B and C**). Data from dominance behaviour were not sufficient to perform any statistical analysis (**no shown**).

Table 11.5 shows the list of cells whose firing was correlated with any of the social parameters considered in the analysis. This analysis aimed to understand if changes in the firing rate of the cells recorded in the session could have been triggered by changes in behavioural social interaction between novel and familiar conspecifics. The firing rate of 7 out of 19 cells considered in the analysis (37%) showed a statistically significant correlation with one or more behavioural parameters (total social contacts, anogenital sniffing, face to face contacts, other contacts). Even if there was not significant difference in 'total social contacts' and 'other contact' between the social novel and the social familiar condition, 4 cells (4/7) were positive correlated with 'total social contacts' while other 2 (2/7) were correlated with 'other contacts'. One cell was correlated with the parameter 'anogenital contacts', and another one with the parameter 'face to face contacts'. The issue of whether anogenital sniffing and face to face contact can explain the firing rate of these cells needs closer inspection, since the amount of

time spent by the implanted animal in these behaviours represents on average less than 6% of the total trial time.

11.3.4.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

In this session, the firing rate of 2 cells (10.5%) out of 19 cells discriminated familiar from novel conspecifics. None of the 2 cells showed a significant correlation with the behavioural parameters considered in the analysis (see **table 11.5**). Furthermore, none of the behavioural parameters considered showed any significant difference between familiar and novel conspecifics, confirming that unlikely the firing rate of the two cells was reflecting changes in social interactions between novel and familiar conspecifics.

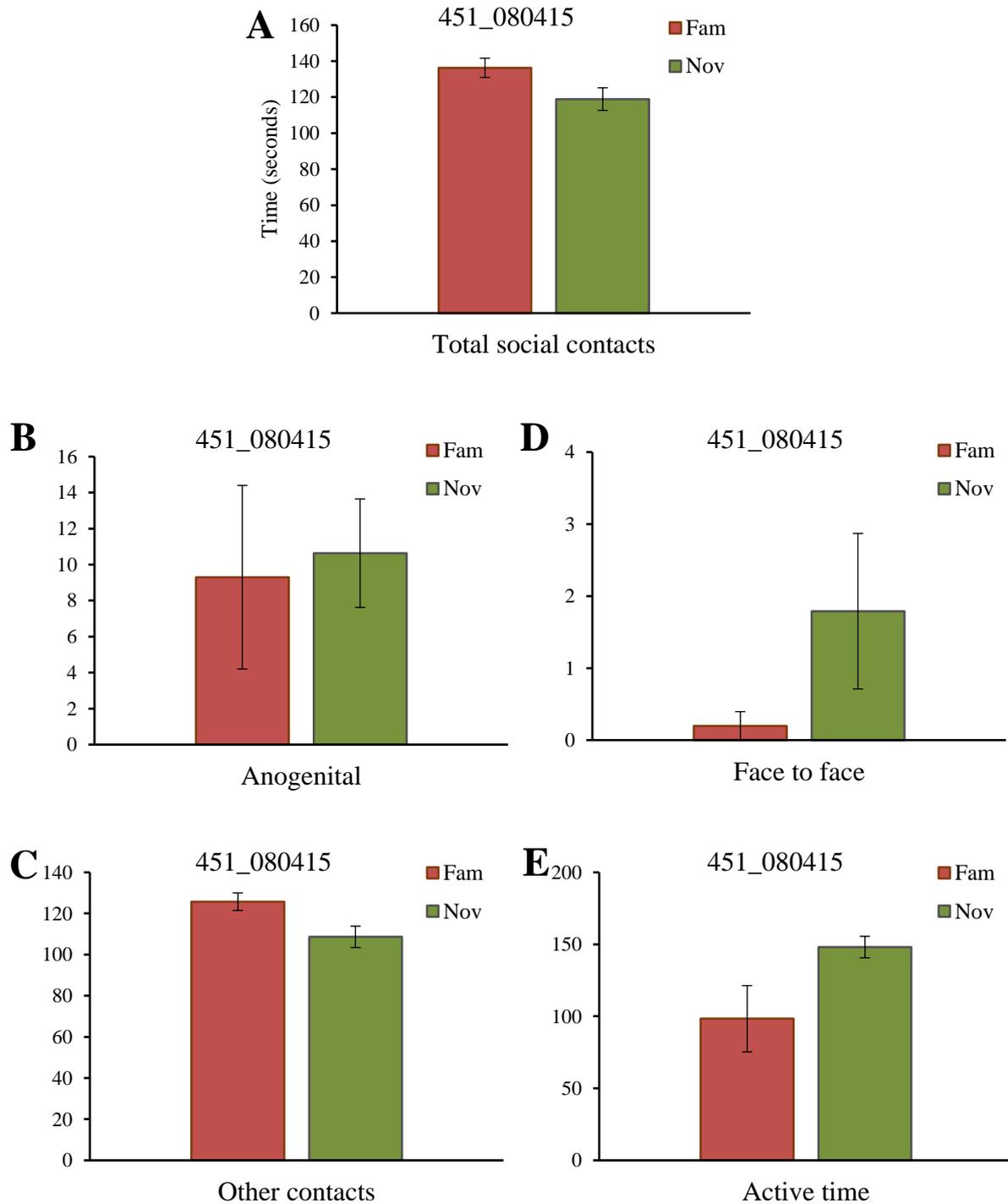


Figure 11.4. Behavioural performance (*mean ± s.e.m.*) of rat 451 during social interaction in session 080415. Graphs show (A) total social contacts ($t(4)=1.5$, $p=0.2$); (B) anogenital contacts ($t(4)=0.02$, $p=0.98$); (C) other contacts ($t(4)=-2.1$, $p=0.11$); (D) face to face contacts ($z(n=5)=-1.1$, $p=0.3$), and (E) active time ($t(4)=-2.0$, $p=0.12$). It should be noted that the behavioural record for one familiar trial was not available.

Table 11.5 : Cells that showed significant correlation with some of the behavioural parameters in animal 451, session 080415. Pearson correlation was used for cells whose firing rate was normally distributed. Cell that did not show a normal distribution and correlations with the parameter face to face contacts were tested using the non-parametric Spearman correlation (in red).

Cell ID	Behavioural Parameter	r/ ρ	p
451_080415_t1_c1	Total social contacts	0.599	0.05
451_080415_t3_c1	Face to face	0.632	0.04
451_080415_t5_c1	Other contacts	0.601	0.05
451_080415_t6_c1	Other contacts	-0.658	0.03
	Active Time	0.702	0.02
451_080415_t6_c2	Total social contacts	0.787	0.004
451_080415_t6_c10	Anogenital	0.723	0.01
	Total social contacts	0.676	0.02
451_080415_t6_c11	Other contacts	0.677	0.02
	Total social contacts	0.642	0.03

11.4 Fam-Fam sessions

In Chapter 10, I showed 30 cells whose firing rate was driven by the encounter with one of the two equally familiar sisters presented in the session (Fam1 or Fam2). Here I will further investigate the nature of the firing activity considering the behavioural performance. Four of the sessions were considered for the behavioural analysis: 031014, 101014, 270716, 280716 (**table 11.6**). The firing rate of cells recorded in each session was analysed in relation to the different behavioural parameters (**table 6.3**, chapter 6) to understand if social interaction could contribute to changes in firing. Pearson correlation was used for cells which firing rate was normally distributed. Cells that did not show a normal distribution were tested using the non-parametric Spearman correlation. Of the 122 cells analysed in these four sessions, 39 (32%) showed firing correlation with at least one of the social behavioural parameters considered in the analysis. Of the 122 cells, 21 showed firing discrimination between the sisters presented in the session. Interestingly, the firing rate of 17 out of 21 cells was not correlated to any behavioural parameters. This may suggest that the firing rate of these sisters' specific cells was driven by the presence of a specific sister inside the arena, independently of the behavioural social interaction in that trial.

Table 11.6. Summary of cells considered for the Fam vs Fam sessions in two of the rats implanted in the piriform cortex.

Session	Implanted animal Age	Implanted animal Oestrus cycle	Fam1 Age	Fam2 Age	Total cells considered	Sister-specific cells
438_031014	24 weeks	Proestrus	24 weeks	24 weeks	32	5
438_101014	25 weeks	Proestrus	25 weeks	25 weeks	39	9
464_270716	38 weeks	Oestrus	38 weeks	38 weeks	24	6
464_280716	38 weeks	Metooestrus	38 weeks	38 weeks	27	1
Total					122	21

11.4.1 Rat 438 session 031014

This session includes 18 trials, but only social trials (n=12) were considered in the analysis. The subject rat (438) spent in average the 80% of the time of social trials proactively exploring another familiar social stimulus. Overall, ‘total social contact’ did not significant differ between the two social conditions ($t(5)=1.3$, $p=0.25$; **figure 11.5 A**). The implanted rat spent a similar amount of time in ‘anogenital sniffing’ ($z(n=6)=-0.11$, $p=0.9$) and in ‘other contacts’ ($t(5)=-1.6$, $p=0.18$) with the two sisters (Fam1 and Fam2), while data from ‘face to face contacts’ were not sufficient to perform any statistical analysis (**figure 11.5 B,C,D**). The subject rat displayed dominance behaviour with the Fam2 sister but not at all with the Fam 1 ($Z(n=6)=-2.023$, $p=0.043$, **figure 11.5D**).

Table 11.7 shows the cells whose firing rate showed a significant correlation with any of the social parameter considered in the analysis. Of the 32 cells considered in the analysis, 10 cells (31%) showed a statistically significant correlation with one of the behavioural parameters considered in the analysis. The firing rate of one cell (1/10) showed a significant negative correlation with the time spent in social interaction (total social contacts) (438_031014_t3_c3, $r=-0.70$, $p=0.01$). Seven cells (out of 10) showed positive or negative correlation with the parameter ‘active time’. The negative correlation may suggest that the firing rate of this cell was not reflecting the general arousal that should be expected following increasing in social interaction. Three cells (3/10) exhibited a significant decreased firing rate in relation to the amount of time spent in anogenital sniffing, and the firing rate of 4 cells (4/10) was significantly correlated with the parameter ‘dominant’. However, it is unlikely that the time spent in ‘anogenital sniffing’ or ‘dominant’ could predict the firing rate of these cells. Since the number

of seconds spent by the implanted animal in this behaviour represent less than 7% of the total trial.

11.4.1.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

During this session, the firing rate of 5 cells discriminated between the two sisters. Two out of five (438_031014_t2_c4 and 438_031014_t3_3) showed significant correlation with at least one of the behavioural parameters. Cell 438_031014_t2_c4 showed firing correlation with time spent in 'dominant behaviour'. However, the average time of 'dominant behaviour' observed was just 7.4 seconds in each trial, and moreover 'dominant behaviour' was only observed for 5 out of the 6 trials with the Fam2 stimulus. Importantly, firing was much higher in all the Fam2 trials for this cell, including the Fam2 trial with no dominant behaviour (Fam2, Trial f firing rate: **1.24 Hz** vs Fam1, Trial e and I firing rate: **0.55 Hz** and **0.36 Hz**.) Thus it is clear that the Fam2-specificity of this cell cannot be primarily explained by 'dominant behaviour', though of course minor modulatory influences related to the very brief dominance episodes cannot be ruled out. Cell 438_031014_t2_c4 increased the firing rate in relation to the increase of 'active time'. In this session, the parameter 'active time' was significantly different between the two conditions (Fam 1 and Fam2), meaning that the implanted animal showed motivational preference for one of the two sisters, exploring more Fam2. This means that it may be plausible that in the case of this cell, changes in firing may reflect changes in proactive exploration of a social stimulus. Cell 438_031014_t3_3 showed negative correlation with different parameters: anogenital contacts, total social contacts, other contacts and active time. Time spent in 'anogenital sniffing' unlikely could reflect the decreasing in firing rate since they are not representative behaviour in the trial. On the other hand, the negative correlation with total social contact and other contacts may suggest that the firing suppression was related to the mix

of olfactory cues due to the close proximity with the social stimulus. In summary, at least 4 cells whose firing discriminate between one of the two sisters, were not correlated to any behavioural parameters suggesting that for these 4 cells, changes in firing were not reflecting changes in social interaction between the 2 familiar stimuli.

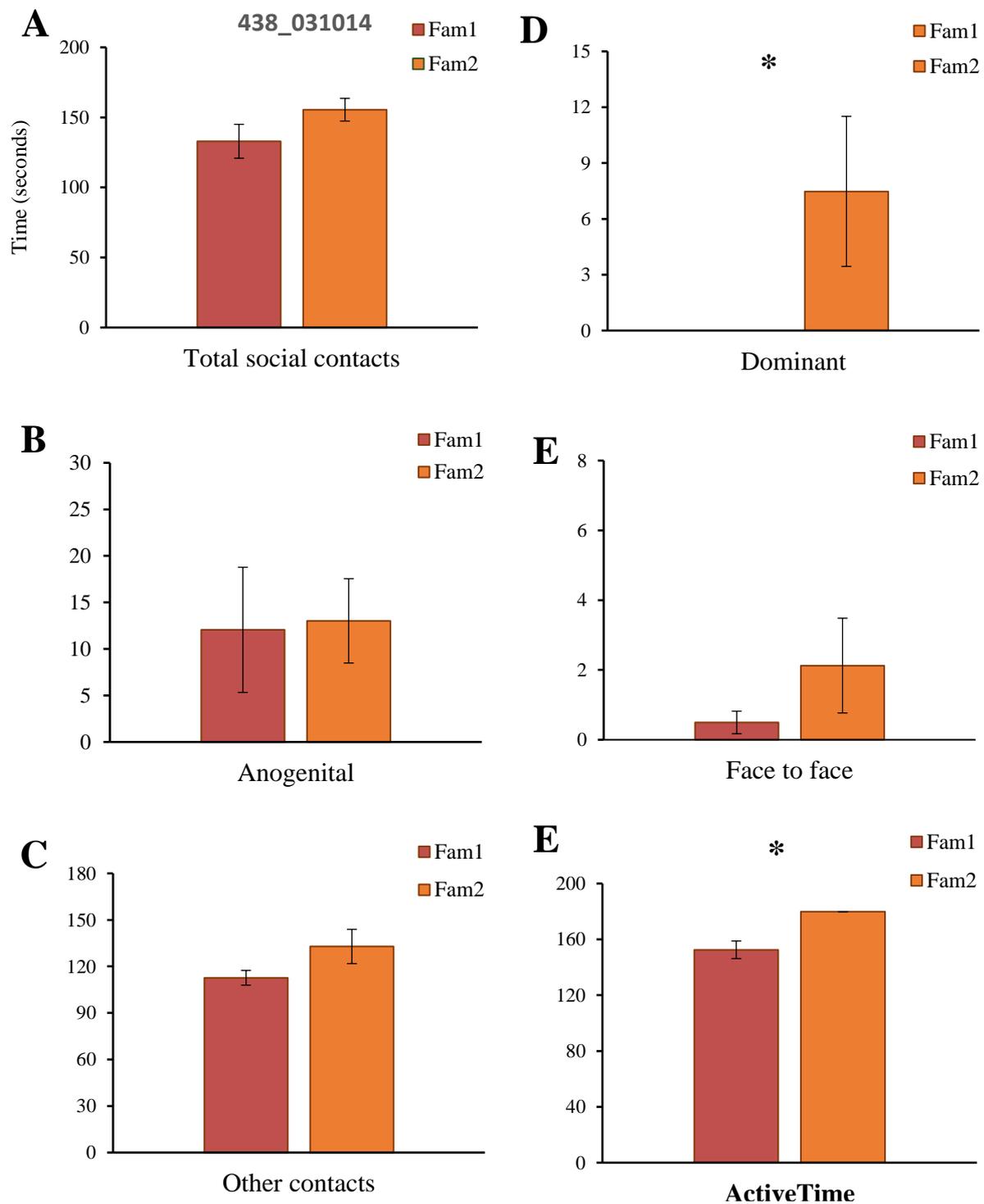


Figure 11.5: Behavioural performance (*mean ± s.e.m.*) of rat 438 during social interaction in session 031014. Graphs show (A) total social contacts ($t(5)=-1.3$, $p=0.25$); (B) anogenital ($z(n=6)=-0.11$, $p=0.9$); (C) other contacts ($t(5)=-1.6$, $p=0.18$); (D) Dominance $z(n=6)=-2.0$, $p=0.043$) (E) and face to face contacts (no statistical analysis was performed); and (F) active time ($z(n=6)=-2.0$, $p=0.043$).

Table 11.7 cells that showed significant correlation with some of the behavioural parameters in animal 438, session 031014. Pearson correlation was used for the majority of cells considered. Cell that did not show a normal distribution (in red) were tested using the non-parametric Spearman correlation.

Cell ID	Behavioural Parameter	r/ ρ	p
438_031014_t8_2	Dominant	-0.702	0.01
438_031014_t8_5	Anogenital	-0.585	0.05
438_031014_t1_4	Active Time	-0.74	0.006
	Anogenital	-0.728	0.007
438_031014_t1_11	Dominant	-0.766	0.004
438_031014_t1_18	Active Time	-0.596	0.04
438_031014_t2_3	Active Time	-0.62	0.03
438_031014_t2_4	Active Time	0.789	0.002
	Dominant	0.733	0.007
438_031014_t3_3	Active Time	-0.649	0.02
	Total social contacts	-0.696	0.01
	Anogenital	-0.62	0.03
	Other contacts	-0.613	0.03
438_031014_t3_4	Active Time	-0.575	0.05
438_031014_t3_5	Dominant	-0.617	0.03

11.4.2 Rat 438 session 101014

Session 101014 had three conditions (Base, Fam1 and Fam2), but only the two social conditions (Fam1 and Fam2) were considered in the analysis (n=12, 6 Fam1, 6 Fam2). The implanted rat (438) spent on average the 61% of the social trial time interacting with the social stimulus. About 50% of the trial was spent in proactive exploration of the social stimulus. Overall, the time spent actively engaging with the sister Fam2 was significantly higher than sister Fam1 (active time: $t(5)=-2.6$, $p=0.047$; **figure 11.6 E**). Paired simple t-test revealed no significant difference in ‘total social contact’ ($t(5)=-1.1$, $p=0.3$), ‘anogenital sniffing’ ($t(5)=-1.3$, $p=0.26$), ‘face to face contacts’ (-0.81 , $p=0.46$), and ‘other contacts’ ($t(5)=-0.65$, $p=0.54$)

between the two social conditions Fam1 and Fam2, (**figure 11.6 A,B,C,D**). No dominance behaviour was observed in this session.

Table 11.8 shows the cells whose firing rate exhibited a significant correlation with any of the social behavioural parameters considered in the analysis. Overall, 38% of the cells (15/39) were correlated with at least one of the social behavioural parameters considered in the analysis: total social contacts, anogenital contacts, other contacts and face to face contacts. Eight cells exhibited a positive (n=4) or negative (n=4) correlation with the amount of time spent in anogenital sniffing in each trial, but it is unlikely that the time in anogenital sniffing could predict the firing rate of these cells since the time in a trial spent in ‘anogenital sniffing’ is lower than 6% of the total time in the trial. A similar consideration can be done for the two cells whose firing rate was correlated with the parameter ‘face to face contacts’, since the amount of time spent by the implanted animal in face to face contacts, represent the 2% of the total time in the trial. Four cells (4/15) increased (n=2) or decrease (n=2) the firing rate in relation to the time spent in ‘other contacts’, and 4 (4/15) cells showed a statistically significant correlation with the time spent in social interaction (total social contact). This may suggest that the firing rate of cells positively correlated with these 2 parameters, was influenced by the time spent in interacting with another conspecific. The cells whose firing was negative correlated with the social interaction, may have just showing firing suppression in relation to the close proximity with another social stimulus. In fact, in the model of multiple odorants, in anesthetised animal, the mix of odorants, in this case represented by the social stimulus, suppress the firing rate of cells in the piriform cortex (Stettler and Alex, 2009).

11.4.2.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

In this session, the firing rate of 9 out of 39 cells discriminated between the two sisters (Fam1 and Fam2). The firing-behaviour correlation aimed to understand if changes in firing could have been related to the motivation preference of the implanted animal for sister Fam2. However, none of the 9 cells showed a significant correlation with any behavioural parameters considered in the analysis. This may suggest that changes in firing rate between the two sisters was not reflecting changes in exploration or general social interaction between the two sisters.

Table 11.8: cells that showed significant correlation with some of the behavioural parameters in animal 438, session 101014. Pearson correlation was used for the majority of the cells considered in the analysis.

Cell ID	Behavioural Parameter	r/ ρ	p
438_101014_t5_1	Anogenital	0.597	0.04
	Total social contacts	0.576	0.05
438_101014_t1_6	Other contacts	-0.598	0.04
	Total social contacts	-0.616	0.03
	Active Time	-0.599	0.04
438_101014_t1_7	Total social contacts	-0.623	0.03
	Active Time	-0.627	0.03
438_101014_t1_8	Anogenital	-0.722	0.008
	Active Time	-0.722	0.008
438_101014_t1_10	Other contacts	-0.618	0.03
438_101014_t1_15	Other contacts	0.669	0.02
	Total social contacts	0.61	0.04
438_101014_t1_24	Anogenital	0.738	0.006
438_101014_t4_2	Anogenital	0.695	0.01
	Face to face	0.588	0.04
	Active Time	0.653	0.02
438_101014_t2_1	Face to face	0.626	0.03
438_101014_t2_2	Other contacts	0.592	0.04
438_101014_t2_3	Active Time	-0.577	0.05
438_101014_t2_4	Anogenital	0.677	0.02
438_101014_t2_5	Anogenital	-0.763	0.004
438_101014_t3_c1	Anogenital	-0.605	0.04
438_101014_t3_c5	Anogenital	-0.576	0.05
	Face to face	-0.828	0.001
	Active Time	-0.681	0.02

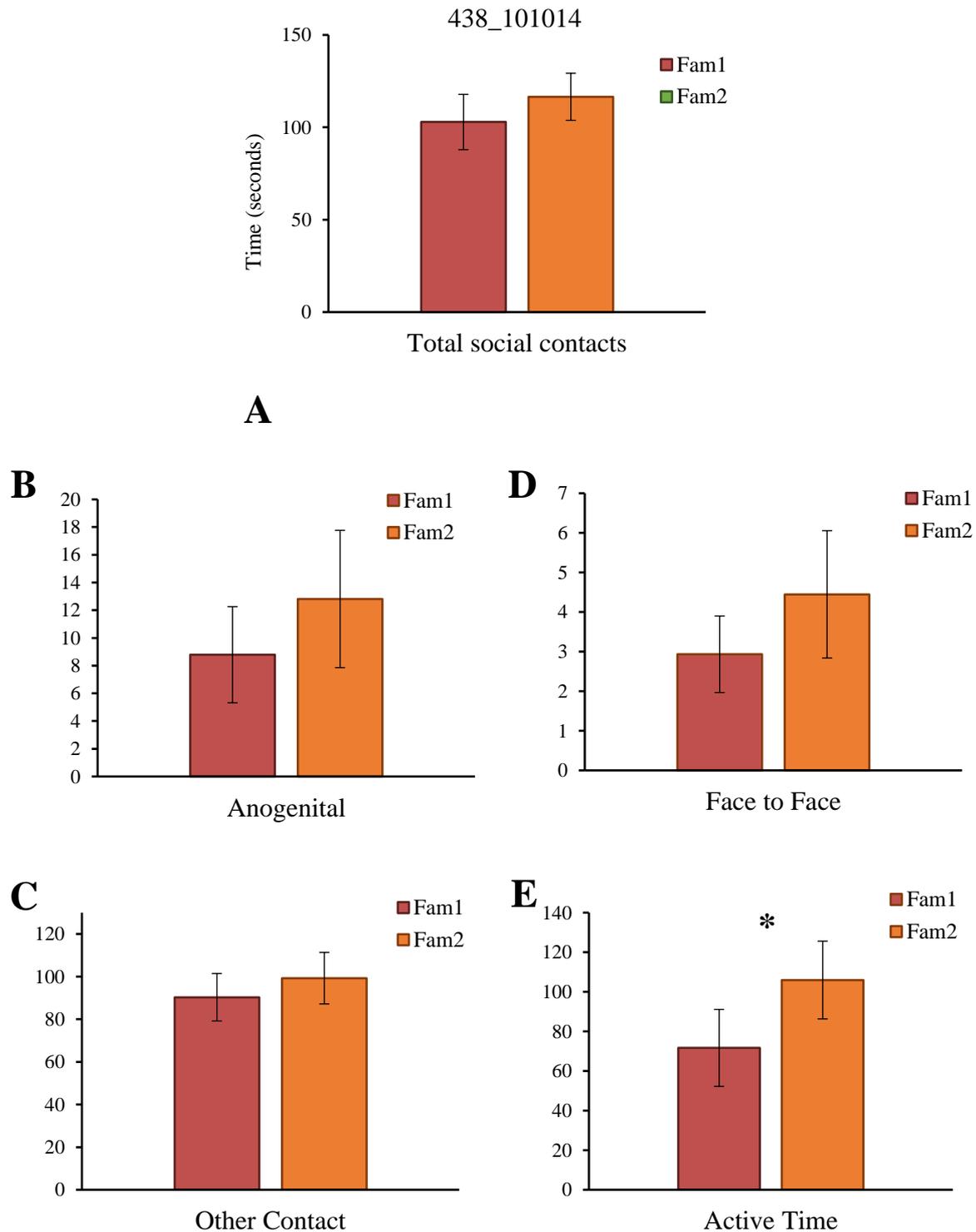


Figure 11.6. Behavioural performance (*mean ± s.e.m.*) of rat 438 during social interaction in session 101014. Paired simple t-test revealed no significant effect of the condition in the following social performance: A) total social contacts ($t(5)=1.1$, $p=0.33$), B) anogenital sniffing ($t(5)=-1.27$, $p=0.26$), and C) other contacts ($t=-0.65$, $p=0.54$). D) face to face contacts ($t(5)=-0.80$, $p=0.46$). E) The graph shows a statistically significant difference in active time between the two conditions ($t(5)=-2.6$, $p=0.047$).

11.4.3 Rat 464 Session 270716

This session included 12 trials (6, Fam1; 6, Fam2). The implanted rat (464) spent in average the 33% of the time in a trial engaging with the familiar social stimulus. On average, 17% of the trial was spent by the implanted rat in proactive exploration of the social stimulus. The implanted animal spent much more time in contact with Fam1 than Fam2 (Fam1: 96.5 ± 8.6 ; Fam2: 26.1 ± 2.3 ; $t(5) = 6.8$, two tailed $p = 0.001$; **figure 11.7 A**) However, the difference in ‘total social contacts’ was likely mainly due to the higher sociability of sister Fam1 in as much as there was no significant difference in ‘active time’ between the two sisters (Fam1: 44 ± 24.5 ; Fam2: 17 ± 5 ; $t(5) = 1.0$, $p = 0.35$ **figure 11.7 E**). The implanted animal spent more time in ‘other contacts’ with Fam1 than Fam2 (Fam1, 95 ± 8.7 ; Fam1 24 ± 2.6 ; $t = 6.842$, $p = 0.001$; **figure 11.7 C**). No ‘dominant behaviour’ was observed in this session, while, data from ‘face to face contacts’ and ‘anogenital contacts’ were not sufficient to perform any statistical analysis (**figure 11.7 B, D**).

Table 11.9 shows the cells whose firing rate significantly correlated with one or more social parameter considered in the analysis. The parameters anogenital contacts and face to face sniffing, were not included in the analysis because they represented less than 1% of the total time in a trial. Of the 24 cells considered in the analysis, 21 (87.6%) showed a significant positive correlation, and only 3 (12.4%) showed a significant negative correlation, with time spent in social interaction (total social contacts) and ‘other contacts’.

11.4.3.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

In this session, 6 cells (25%) out of 24 showed firing discriminations for one of the two sisters (Fam1 and Fam2). Two cells (2/6), showed the negative significant correlation with the time spent in other contacts and total social contacts (464_270716_t5_c1: 'other contacts' $\rho=-0.63$, $p=0.03$, 'total social contacts' $\rho=-0.66$, $p=0.02$; 464_270716_t8_c6: 'other contacts' $r=-0.64$, $p=0.02$, 'total social contacts' $r=-0.68$, $p=0.02$). This may suggest that at least 4 out of 6 cells were not firing in relation to a specific social behaviour or the total amount of social interaction.

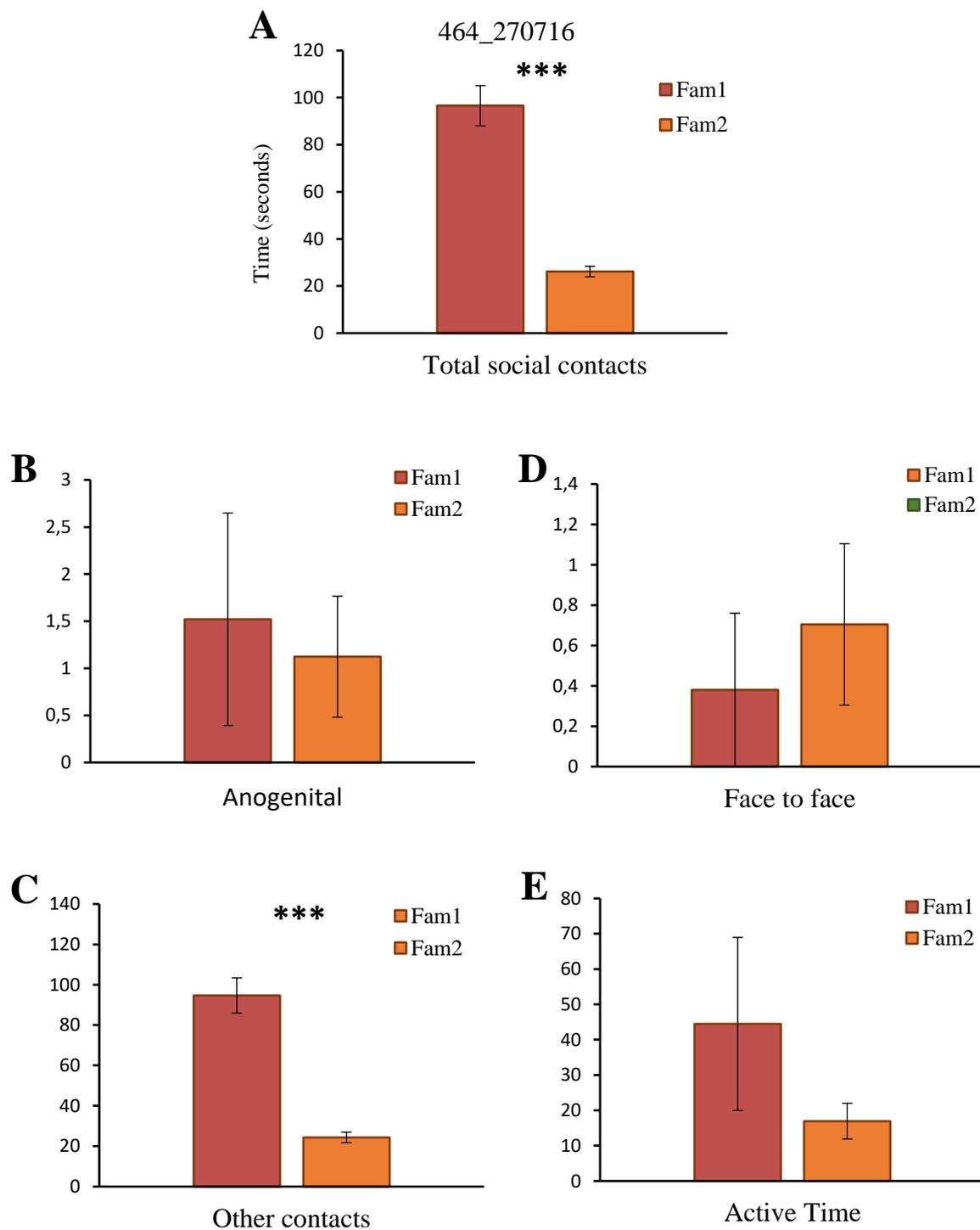


Figure 11.7. Behavioural performance (*mean* \pm *s.e.m.*) of rat 464 during social interaction in session 270716. Graphs show (A) total social contacts ($t(5)=6.8$, $p=0.001$); (B,D) respectively anogenital and face to face contacts (no statistical analysis was performed); (C) other contacts ($t(5)=6.842$, $p=0.001$) and (E) active time ($t(5)=1.0$, $p=0.35$). No dominant behaviour was observed in this session

Table 11.9: cells that showed significant correlation with some of the behavioural parameters in animal 464, session 270716. Pearson correlation was used for the majority of the cells considered. Cell that did not show a normal distribution (in red) were tested using the non-parametric Spearman correlation.

Cell ID	Behavioural Parameter	r/ ρ	p
464_270726_t5_c1	Other contacts	-.634*	0.027
	Total social contacts	-.655*	0.021
464_270726_t8_c1	Other contacts	-.629*	0.028
	Total social contacts	-.643*	0.024
464_270726_t8_c6	Other contacts	.643*	0.024
	Total social contacts	.676*	0.016

11.4.4 Rat 464 Session 280716

Session 280716 had three conditions (Base, Fam1 and Fam2), but only the two social conditions (Fam1 and Fam2) were considered in the analysis (n=12, 6 Fam1, 6 Fam2). The subject rat (464) spent in average the 54% of the time in social trials proactively engaging with another familiar social stimulus (Fam1 or Fam2). The implanted animal spent more time in ‘total social contacts’ with sister Fam1 compared to sister Fam2 (Fam1: 123.5±16; Fam2: 70±9; t(5) =2.7, two tailed p=0.045; **figure 11.8 A**). The implanted animal tended to actively interact more with the sister Fam1, though this did not reach significance (‘active time’: Fam1, 114.6±20.3; Fam2, 83±16, t (5) =2.2, p=0.076 **figure 11.8 E**), The implanted animal spent more time in ‘other contacts’ with sister Fam1 compared to sister Fam2 (t(5)=2.62, p=0.045, **figure 11.8 C**), while there was not significant difference in the time spent in ‘anogenital contacts’ and ‘face to face contacts’ between the two sisters (respectively: (z(n=6))=-1.8, p=0.075; z(n=6)=-0.7, p=0.47; **figure 11.8 B and D**). Data from dominance behaviour were not sufficient to perform any statistical analysis (**no shown**).

Table 11.10 shows the cells whose firing rate was significantly correlated with any of the social parameters considered in the analysis. Of the 27 cells considered in the analysis, 11 (41%) showed significant correlation with at least one of the behavioural parameters. Eight cells (8/11) were correlated with ‘anogenital sniffing’ or ‘face to face contacts’, which have been suggested to be relevant behavioural interaction to acquire odour information. However, it is unlikely that the time in anogenital sniffing and face to face contact could predict the firing rate of these cells since these were not representative behaviour in a trial (anogenital sniffing: 7.4%; face to face contacts: 2% of the total time in the trial). Three cells (3/11) showed statistically correlation with ‘total social contacts’ (positive n=2, negative n=1) and 4 cells (4/11) with ‘active time’(positive n=2, negative n=2). A positive correlation may suggest that changes in firing could reflect differences in social interaction. However, it is unlikely that the negative correlation may reflect changes in social interaction. The negative correlation may suggest that these cells were processing multiple odorant cues like the one representative of a social stimulus, given that the mix of odorant cues can suppress the firing rate of cells in the piriform cortex (Stettler and Alex, 2009).

11.4.4.1 Social discriminatory firing patterns were not better explained by aspects of social behaviour

Notably, cell 464_280716_t8_c1, which showed a significant increase in firing when the subject rat was paired with Fam 1, but not with Fam2, was not correlated with any of the behavioural parameter above mentioned. The differences in firing appeared to be related to the presence of a specific sister inside the arena, independently by the behavioural exploration of the sister.

Table 11.10: cells that showed significant correlation with some of the behavioural parameters in animal 464, session 280716. Pearson correlation was used for the majority of the cells considered. Cell that did not show a normal distribution (in red) were tested using the non-parametric Spearman correlation.

Cell ID	Behavioural Parameter	r/ ρ	p
464_280716_t5_c1	Face to face	-0.592	0.043
464_280716_t5_c2	Face to face	-0.61	0.035
464_280716_t5_c3	Face to face	0.616	0.033
464_280716_t5_c4	Face to face	0.788	0.002
	Total social contacts	0.615	0.033
	Active Time	0.825	0.001
464_280716_t5_c6	Face to face	0.667	0.018
464_280716_t6_c3	Other contacts	-0.727	0.007
464_280716_t6_c6	Anogenital	-0.697	0.012
	Active Time	-0.762	0.004
464_280716_t6_c9	Anogenital	-0.611	0.035
464_280716_t8_c2	Face to face	0.725	0.008
	Total social contacts	0.593	0.042
464_280716_t8_c4	Total social contacts	-0.623	0.03
	Active Time	-0.648	0.023
464_280716_t8_c10	Active Time	0.576	0.05

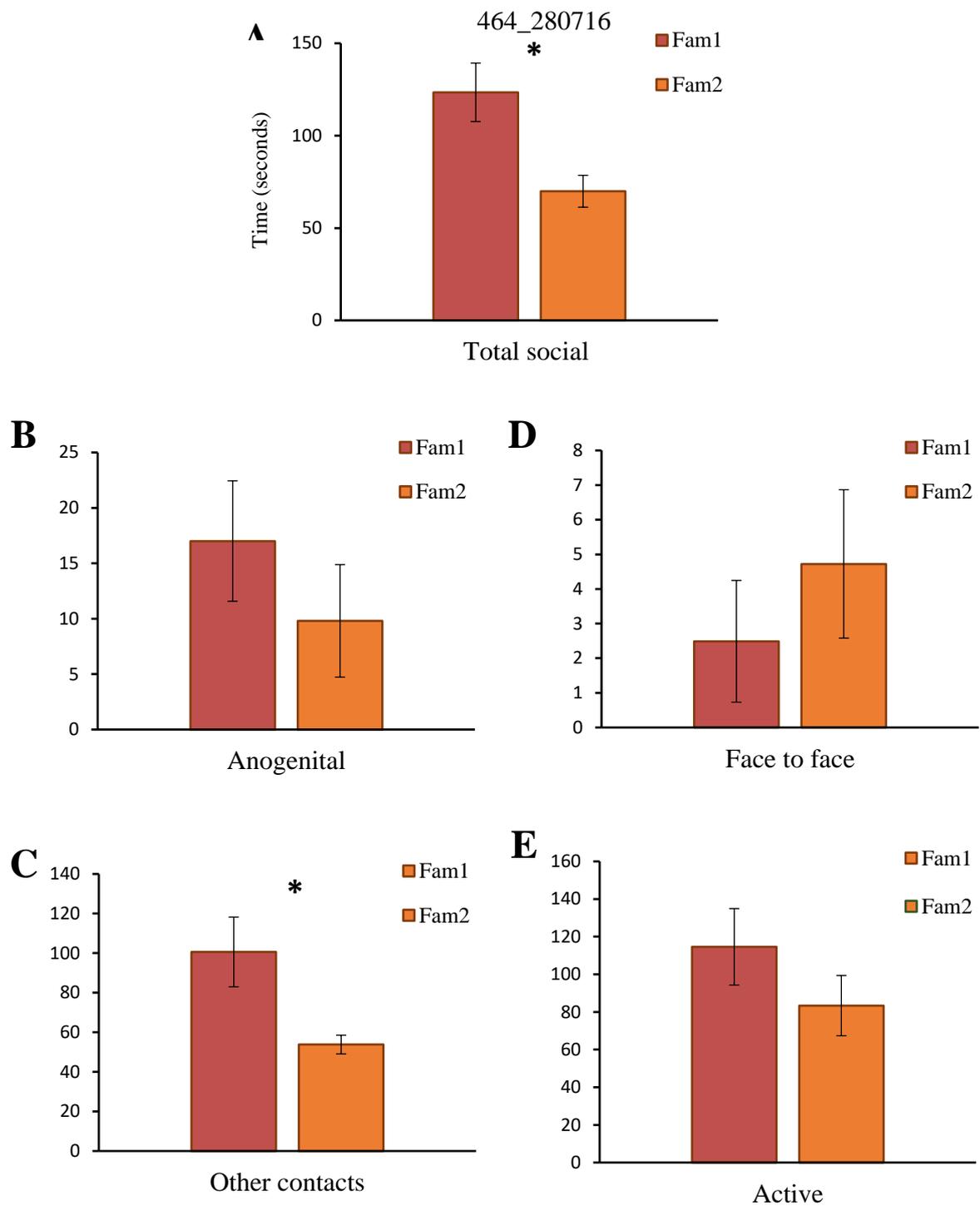


Figure 11.8. Behavioural performance (*mean ± s.e.m.*) of rat 464 during social interaction in session 280716. Graphs show (A) total social contacts ($t(5)=2.7$, $p=0.045$); (B) anogenital contacts ($z(n=6)=-1.8$, $p=0.075$); (C) other contacts ($t(5)=2.6$, $p=0.047$); (D) face to face contacts ($z(n=6)=-0.7$, $p=0.47$), and (E) active time ($t(5)=2.2$, $p=0.076$).

11.5 Summary of behavioural analysis

Eight sessions from three animals (438, 451 and 464) were considered for the behavioural analysis (4 Nov-Fam, and 4 Fam-Fam). In 4 out of 8 sessions, the implanted animal showed behavioural discrimination between the two conspecifics (Nov vs Fam and Fam vs Fam). While it should be expected rats to show behavioural discrimination between a novel and a familiar conspecific, but not between two familiar conspecifics, in the 4 sessions where behavioural discrimination was observed only 2 were Nov-Fam sessions, and unexpectedly, 2 were Fam-Fam sessions.

The implanted animal 464 showed what could be considered a typical social behaviour, where the novel social stimuli were explored more than the familiar stimuli. In the 2 Nov-Fam sessions recorded from animal 464 (010716 and 260716), the implanted animal showed behavioural discrimination between novel and familiar conspecifics (active time 010716, $t(5)=-4.4$, $p=0.01$; 260716, $t(5)=-3.6$, $p=0.02$), while there was no difference in the time spent in the proactive exploration of the sisters in the Fam-Fam sessions (active time: 270716, $t(5)=1.0$, $p=0.35$; 280716, $t(5)=2.2$, $p=0.08$).

Only one session of animal 451 was considered in the analysis. In this Nov-Fam session, the implanted animal appear not to behaviourally discriminate between the novel and familiar social stimuli (active time active time: 270716, $t(4)=-2.0$, $p=0.12$)

The implanted animal 438 showed behavioural discrimination in the two Fam-Fam sessions (active time: 101014, $t(5)=-0.05$, $p=0.35$; 031014, $z(n=6)=-2.0$, $p=0.04$), while it did not appear to discriminate between novel and familiar conspecific (active time, $t(5)=0.2$, $p=0.8$)

In summary, in the 8 sessions considered, 36% of the cells recorded (84/231) showed a significant correlation with at least one of the behavioural parameters considered in the analysis (**table 11.11**). Of the cells recorded in the Nov-Fam sessions, 41% (45/109) showed firing correlation with at least one of the behavioural parameters. Twenty-seven out of 64 (42%) were recorded from the 2 sessions that showed behavioural discrimination between novel and familiar conspecific, while eighteen out of 45 (40%) were recorded from the 2 session that did not show any behavioural discrimination. Of the cells recorded in the Fam-fam sessions 32% (39/122) showed firing correlation with at least one of the behavioural parameter. Twenty-five out of 71 (35%) were recorded from the 2 sessions that showed behavioural discrimination between the two familiar sisters, and 14 out of 51 were recorded from the 2 sessions where no behavioural discrimination between the sisters was shown. **Table 11.11** shows the number of cells correlated with each individual parameter in the eight sessions considered for the behavioural analysis. The firing rate of some cells correlated with more than one parameter.

Table 11.11: Number of cells that showed firing correlation with the behavioural parameters considered in the analysis. Each Row shows the number of cells in the Nov-Fam sessions, Fam-Fam sessions and the overall total number of cells, significant for the specified behavioural parameter.

Behavioural Parameter	Significant cells Nov-Fam	Significant cells Fam-Fam	Total significant cells
Total social contacts	12	11	23
Active time	22	15	37
Anogenital sniffing	21	13	34
Face to face contacts	6	9	15
Other contacts	9	9	18
Dominance	11	4	15
Total number of cells recorded	109	122	231

11.6 General Discussion

The present chapter aimed to further investigate the nature of the firing rate of neurons recorded in the piriform cortex and examined in chapter 9 and 10, through behavioural scoring that was done blind to trial status. Chapter 9 showed evidence of how neurons in the piriform cortex could discriminate between novel and familiar conspecifics. Chapter 10 showed evidence of piriform neurons whose firing rates discriminated between familiar sisters of the implanted rat. However, the open-ended approach used in these experiments leave open to different interpretations that needed these findings to be further investigate with the behavioural analysis. The present chapter provided results from blind behavioural scoring with the aim to narrow down interpretation to findings presented in chapter 9 and 10. In summary, importantly, *the firing rate of 20 cells out of 25 familiar-vs-novel specific cells, and the firing rate of 17 out of 21 sister-specific cells, was not correlated with any behavioural parameters.* Differences in firing appeared to be related to the presence of a certain rat inside the arena (Fam vs Nov; and Fam1 vs Fam2), independently by the social interaction with a social stimulus.

11.6.1 Behavioural correlates of the Familiar-vs-Novel Social distinction

The Fam-Nov sessions were designed to understand if piriform neurons could discriminate between novel and familiar conspecifics. To accomplish that, the Fam-Nov experiments adapted the Crawley's sociability and preference for social novelty protocol, leaving the implanted animal the free choice to interact with the social stimulus. This test, using the natural tendency of rats to explore more novel social stimuli compared to familiar ones, to study social recognition memory. (Dantzer et al, 1988, Crawlwy, 2004). This general increased investigation should be accompanied by higher anogenital investigation, important to acquire

odorant information (Dantzer et al, 1988). Two of the behavioural scored sessions followed these expectations, showing not only a significantly increase in the time spent by the implanted animal in proactively exploring the novel social stimuli (active time), but also an increase in anogenital exploration (anogenital sniffing) of the novel social stimuli compared to the familiar one. However, two sessions showed unexpected results. In session 451_080415, the difference in 'active time' was not significant between the two conditions, even if there was a slight tendency of the implanted animal (451) to interact more with the novel than the familiar rat. In session 438_040914, the implanted animal spent more time in close proximity with the familiar social stimulus, perhaps due to the sociability of the familiar stimulus itself, a sister. Furthermore, in both sessions, we did not observe any difference in anogenital exploration between the novel and the familiar conspecific. Just based on the behavioural results, the findings in these two sessions may inappropriately suggest that the implanted animals (438 and 451) did not have a clear memory of the familiar conspecific. There are three possible explanations for these findings. Firstly, the social recognition memory of the implanted animal might have been affected by the long-term social isolation. On this regard, Kogan and colleagues (2000) showed that both acute and chronic isolation in mice affected the long-term memory in social recognition, but not the short-term memory after 30 minutes from a 2-minute encounter with a conspecific. Since that the familiarization period used in my experimental design required 2 pre-test familiarization session, the last one only 1 hour before the testing phase, and comprising in total 30 min of exposure to the familiar social stimulus, it is very doubtful that the implanted animal could not discriminate familiar from novel conspecifics. Secondly, differences in social interaction may be explained by the oestrus cycles. Previous studies showed that oestrus is the only stage of the cycle that might influence the animals' behaviour with female animals spending less time investigating social stimuli (Engelmann, 1998). However, during the oestrus stage (in sessions 464_010716 and 464_260716) the

implanted animal spent time exploring both the familiar and the novel stimuli, showing as expected increase exploration for the novel animal. The two sessions where the animal did not significantly explore more the novel animal are proestrus and dioestrus. This may suggest that the oestrus stage should not be responsible for the unexpected behaviour observed in sessions 451_080415 and 438_040914. The third and more plausible explanation is that differences in social investigation may depend by the age of the social stimuli used. In session 438_040914 this may explain the non-behavioural discrimination between familiar and novel conspecific. There was age discrepancy between the familiar and the novel social stimuli, where the familiar animal was already in the adult phase (20 weeks old), while the novel social stimuli, even if sexually mature, were juvenile in an adolescent stage (6-8 weeks old) (Sengupta, 2013). In case of session 451_080415 both the familiar and the novel stimuli were 14 weeks old, and there was a clear tendency of the implanted animal to investigate more the novel social stimuli, though this did not reach significance. The different age of the social stimuli used may then explain the differences in the behavioural discrimination between novel and familiar conspecifics, while the implanted animal is still able to recognise familiar from novel social stimuli.

Even if the ethological open-ended approach used in these experiments, leaves open to interpretation, the behavioural analysis aided to interpret the nature of the firing discrimination between novel and familiar conspecifics. To examine whether the excitation of piriform neurons was reflecting behavioural aspect of social interaction, I performed a correlation analysis between the firing activity of piriform neurons and the time spent in anogenital sniffing, face to face contacts, dominant behaviour, total social contacts, and active time (**table 6.3**, chapter 6). The 38% (41/109) of the cells showed firing correlation with at least one of the social behavioural parameters considered in the analysis. Of these cells, 51%(21/41)

showed firing correlation with 'anogenital sniffing', 15% (6/41) with 'face to face contacts' and 5% with 'dominant behaviour'. However, it is unlikely that time spent in 'anogenital sniffing', 'face contacts' and 'dominant behaviour' could influence changes in firing since these behavioural parameters were not representative behaviour in a trial. Of the cells which showed firing correlation with at least one behavioural parameter, 54% (22/41) showed firing correlation with the time spent by the implanted animal proactively exploring the conspecific (active time), 29% (12/41) showed firing correlation with 'total social contacts' and 22% (9/41) with 'other contacts'. A positive correlation with these social parameters may suggest that the firing of these cells could reflect changes in social interaction. However, the firing of 29% of these cells (12/41) were negatively correlated with 'other contacts', 'total social contacts' and 'active time'. It is unlikely that firing suppression may reflect increased in social interaction since social interaction can be associated to increase in the arousal state. It is plausible that these cells were processing olfactory cues since increased social interaction is associated to a close proximity of the implanted animal with the social stimulus. This may explain the firing suppression of certain cells. In fact, a rat is represented by multiple odorant cues, like odours coming from different body parts (mouth, anogenital areas, tail) and on the model of exposure to multiple odorants in anaesthetised animals (Stettler and Alex, 2009), the mix of odorant cues can suppress the firing rate of cells in the piriform cortex.

11.6.2 Only a few Familiar-vs-Novel social discriminatory firing patterns might be explained by correlates of social behaviour

Of the 25 neurons whose firing discriminated familiar from novel conspecifics, only 20% (5/20) showed firing correlation with one of the behavioural parameters. These findings may suggest that in the 80% of these cells (20/25), changes in firing rate was not reflecting changes in social behaviour. This is further supported by data from session 438_040914. In fact, while during the other three testing sessions (010716, 260716 and 080415) the implanted animal showed social motivational preference for the novel conspecifics, in the testing session 438_040914 the implanted animal explored similarly familiar and novel animals (**figure 11.3**). Since there was no difference in the time spent by the implanted animal engaging with novel and familiar conspecific, the firing rate of these novel-vs-familiar discriminative cells could be attributable to the presence of a specific rat inside the arena, independently of the behavioural exploration of the rat itself.

However, it must be taken in consideration that the failure in finding correlation between social behaviours and firing rates is not a clear evidence that the two measures are unrelated. In fact, this null result is limited to the correlation of the firing rate of a whole trial with only the parameters considered in the analysis, while non-social parameters such as grooming, rearing, or motion, were not considered. Furthermore, some behavioural parameters incorporate more than one behavioural aspect. For example, the parameter 'Other contacts' includes body sniffing and body touching, and it can be either active, meaning that the implanted animal is investigating the social stimulus, or passive, meaning that the implanted animal is investigated by the social stimulus. Because of the limitation on the behavioural analysis, these results must be taken cautiously and behavioural influence in the firing rate cannot be completely excluded.

Taken together, the present findings appear to suggest that behavioural differences in social interaction may not play a primary role in generating the novel-familiar discrimination firing of the familiar-novel specific cells in the piriform cortex. This in turn suggests that cell assemblies in the piriform cortex could support social recognition memory. However, future investigation needs to be provided to rule out further interpretation. Future analysis should provide a more cautious approach to investigate the influence of the behaviour in the firing activity of piriform cortex neurons. Perhaps, the investigation may involve a second by second analysis of firing activity and behaviour.

11.6.3 Behavioural correlates of the Familiar-vs-Familiar Social distinction

The Fam-Fam sessions were designed to reveal if piriform neurons could discriminate between two familiar conspecifics. In each of the 4 sessions, the implanted animal (438 and 464) was exposed to two familiar animals. In these experiments, the social stimuli were equally familiar to the implanted animal, and differences observed in social interaction between the two familiar social stimuli, were due to the sociability of the social stimulus in that session or to the motivational preference of the implanted animal for one of the sisters. The implanted animal generally showed a high social exploratory activity, with the exception of session 464_270716 where the implanted animal was less sociable (average 17% of trial time in 'active' mode). The unsociability of the implanted rat in this specific session could be related to the oestrus phase, in fact, accordingly to previous study, this phase is associated to lower levels of social exploration compared to the other phases (Engelmann, 1998).

The aim of this experiment was to understand to what extent piriform neurons could discriminate between two familiar conspecifics. It is important to note the comparative difficulty of this discrimination problem. The familiar stimuli used were sisters and they were caged together. This means that the two sisters were very similar in many dimensions, such as genetics, diet and environment. Nevertheless, 30 neurons (30/167, 18%) in the piriform cortex showed firing discrimination between two very similar familiar conspecifics (Chapter 10). To examine whether changes in firing could reflect changes in social interaction I performed a correlation analysis between the firing activity of piriform neurons and the time of social interaction in different behavioural parameters for 4 sessions (122 cells recorded in these 4 sessions, 21 were sister-specific cells). The firing rate of 32% of the cells (39/122) showed firing correlation with at least one of the social behavioural parameters considered in the analysis.

11.6.4 Only a few sister-specific social discriminatory firing patterns might be explained by correlates of social behaviour

Only 19% (4/21) of the sister-specific cells showed firing rate correlation with one of the behavioural parameters. This means that the firing of 81% of the Sister-Specific cells (17/21) appeared not reflecting changes in behavioural social interaction, instead it could be speculated that the firing was driven by the presence of the sister itself. This interpretation is further supported by data from session 464_270716. In fact, even in a session when there was no difference in time spent by the implanted animal engaging with the two familiar conspecifics, four cells still showed discriminative firing between the two sisters.

The naturalistic, open-ended social interaction leaves open different potential interpretations. For instance, it may be plausible that at least some sister specific cells may fire motivationally, in line with changes in levels of ‘sociability’. For example, in session 464_270716, sister Fam1 spent a significantly longer amount of time trying to engage in social interaction with the implanted rat (total social contacts). If this was the case, I should expect firing excitation in presence of sister Fam1, due to the higher arousal state that social interaction could induce. However, *there were sister-specific cells simultaneously recorded in session 464_270716 which showed higher firing in presence of sister Fam1 (2/6), while others showed higher firing in presence of sister Fam2 (4/6)*, suggesting that the increased sociability of the social stimulus may not be primarily involved in generating the firing discrimination, at least not for all the cells (6 in this session). This is further supported by data recorded from the two sessions 438_031014 and 438_101014 where there were no differences observed in total social contacts between the two familiar sisters, but still 20% (14/71) of cells recorded in these sessions showed firing rate discrimination between the two familiar conspecifics. Indeed, in these two sessions, the proportion of sister-specific cells (20%) was similar to (if anything higher than) the proportion observed in sessions where some behavioural differences emerged (7/51, 14%).

Since the piriform cortex is predominantly involved in processing olfactory cues (Chapuis et al, 2013; Wilson and Sullivan, 2011), I analysed the time spent in anogenital sniffing and face to face contact. The direct investigation toward the anogenital region and the face are important to acquire volatile odour that should involve the activation of neurons in the piriform cortex. (Kippin et al., 2003). However, in the four sessions analysed, the amount of time spent by the implanted animal in these two type of social interaction was less than 8% of the trial time, meaning that it is unlikely that the firing rate of these cells could be influenced by social behaviours which were not representative in the trials. Furthermore, of the 21 sister-specific

cells, none showed firing rate correlations with anogenital investigation, and again in three of the sessions considered for the behavioural analysis, the levels of anogenital investigation and face to face contacts were very similar for both the two sisters.

The present findings suggest that differences in social interaction cannot fully justify the sister-sister discriminative firing of cells in the piriform cortex, and it could be speculated that the firing rate of cells in the piriform cortex support social recognition memory. However, as already discussed in section 11.6.2, must be considered that the lack in correlation between social behaviours and firing rate cannot exclude a relation between this 2 variables. Further investigation may include a more cautious behavioural analysis perhaps including more behavioural parameters such as active body touching and active body sniffing to rule out alternative interpretations.

Chapter 12: Exploring the Bases of Discriminatory

Firing Patterns

In this brief chapter, I present two kinds of analysis aimed at trying to understand further the nature of the discriminatory firing patterns set out in Chapters 9 and 10. Broadly speaking, I ask the two following questions:

- 1) Are discriminatory firing patterns present early on in the 3-minute social interaction trial, as would be expected from ethologically relevant signals?
- 2) Can we begin to specify the kinds of sensory cues that do, and do not, elicit the discriminatory firing patterns?

The second part of this chapter instead aimed to understand if the firing rate was influenced by the progression of trials during the testing session. I asked the following question:

Did the firing rates of the neurons in the amygdala and/or in the piriform cortex decrease monotonically over the session? or did the firing rates increase?

12.1 Temporal analysis: Are discriminatory firing patterns present early on in the 3-minute social interaction trial?

There was no experimenter control of behaviour, but it would be expected and was observed that bouts of behaviour were more likely to be overtly related to social investigation early on in a trial. Accordingly, one would expect that a discriminatory firing pattern would be present early on in a social interaction trial. Later on in the trial, even with a relatively short trial of 3

minutes, socially-directed motivation may well be decreased. In terms of social identity more specifically, most researchers would hypothesise that if neural mechanisms for detecting and remembering social identity are to be ethologically useful, they should be active in the first moments of a trial. For instance, one might expect that rodents engage in social identity recognition processes early on in an interaction, and that this initial recognition might then determine the behaviour that is subsequently seen in the interaction (e.g. affectionate, reciprocally altruistic, anxious, hierarchical, playful).

Two specific versions of this hypothesis were tested.

Hypothesis 1: Discriminatory firing in the first 30 seconds should mirror the pattern seen over the whole trial.

Hypothesis 2: Discriminatory firing should be stronger in the first 30 seconds than the last 30 seconds of the trial.

The sample size of discriminating cells was not sufficient to evaluate these hypotheses separately for Fam-vs-Fam and Fam-vs-Novel sessions. *A reasonable merged dataset was that which comprised all the neurons that fired higher for a familiar sister rat.* This comprised all neurons that fired higher for one familiar sister either in sessions when two familiar sisters were presented, or when a familiar sister and novel females were presented.

12.2 Testing the temporal hypotheses

Testing Hypothesis 1: Discriminatory firing in the first 30 seconds should mirror the pattern seen over the whole trial. This was tested by correlating signed discrimination ratios obtained from whole-trial epochs and first-30-seconds epochs. The discrimination ratio was defined as:

$$(\text{Mean Firing for A}) - (\text{Mean Firing for B}) / (\text{Mean Firing for A}) + (\text{Mean Firing for B})$$

where A was ‘Familiar 1’ or ‘Familiar’, and B was ‘Familiar 2’ or ‘Novel’.

Thus in this dataset, negative values meant that the cell showed higher firing for Familiar 2.

As predicted, discrimination ratios for the first-30-seconds epochs strongly correlated with discrimination ratios for the whole-trial epochs ($n = 41$, $r = +0.84$, $p \ll 0.0001$).

Hypothesis 2: Discriminatory firing should be stronger in the first 30 seconds than the last 30 seconds of the trial. This more-restricted and directional hypothesis was tested by comparing first-30-second epoch discrimination ratios to last-30-second epoch discrimination ratios.

For this analysis, all negative discrimination ratios were converted to positive discrimination ratios, since it was the *strength* of the discrimination ratio that was important. However, in a few cases, it was necessary to then correct these positive values using the whole-trial epoch values as the gold standard, *where the sign of the 30-second epoch was opposite to the sign of the whole-trial epoch*. For instance, in the corrected cases, a cell might have a whole-trial epoch discrimination ratio of 0.25, but with a first-30-second epoch value of -0.02. In all, 3 out of 41 first-30-second epoch values, and 5 out of 41 first-30-second epoch values, were corrected. The most negative corrected discrimination ratio value was -0.0469.

As predicted, discrimination ratios were higher in first-30-second epochs than last-30-second epochs (1st-30s DRs: $+0.26 \pm 0.04$; Last-30s DRs: $+0.17 \pm 0.02$; paired $t_{40} = 1.99$, $p = 0.05$). Interestingly, the mean discrimination ratio in the first-30-second epochs was comparable to (if anything slightly higher than) the mean discrimination ratio in the whole-trial 180-second epochs (Whole-trial 180s DRs: $+0.24 \pm 0.02$; 1st-30s: $+0.26 \pm 0.04$, paired $t_{40} = 0.595$, $p =$

0.56). This was despite considering the 180-second trials as the gold standard, asymmetrically ‘penalising’ only the first-30-second epochs with negative-value corrections.

Taken together, these results show that, as predicted, robust discriminatory firing patterns are already present in the early portion of the trial, and firing patterns are more discriminatory in these early-epochs than the late-epochs. Perhaps surprisingly, any assumptions that there might be too much ‘noise’ in this early epoch, representing just 16.7% of the data, appear to be unfounded. In retrospect, one might ask if future analysis could focus on even smaller-duration early epochs.

The interpretation of these data seems relatively straightforward. The data support the idea that rodents engage in social identity recognition processes early on in an interaction, and that this initial recognition might then determine the behaviour that is subsequently seen in the interaction. Neuronal mechanisms underlying social identity recognition processes are accordingly likely to be biased towards early than late epochs of social interaction. Although clearly still discriminatory, firing towards the end of the trial may be more noisy due to other types of neuronal processing than involved in social identity recognition, such as subserving exploration, foraging, and so on.

12.3 Probe trial analysis: Can we begin to specify the kinds of sensory cues that do, and do not, elicit the discriminatory firing patterns?

Here I do some overview analysis to ask if we can begin to specify the kinds of sensory cues that do, and do not, elicit the discriminatory firing patterns.

12.3.1 Overview of bedding probes

To remind the reader, some probe trials consisted of bedding taken from the cages of the novel or familiar rats. Bedding probes have been described in more detail in the Materials and Methods Chapter 6.

The key question to address, is how effective are the bedding probes at triggering the social-specific responses seen in the whole-animal session data. For instance, if a cell fired significantly higher for the familiar rat than to novel rats, did that cell also fire higher to the bedding of the familiar rat than to the bedding of the novel rats? If so, this could indicate that volatile odours could be sufficient to drive a social discriminatory response.

I selected all neurons that were shown to be statistically discriminatory and where probe trials were done at the end of the recording session, and divided these neurons into two categories: *Novel-social higher*, or *Familiar-social higher* neurons. Ten *Novel-social higher* neurons were taken from two Novel-Vs-Familiar Social sessions from rat 464. Nine *Familiar-social higher* neurons were taken from the same two Novel-Vs-Familiar Social sessions just mentioned and one Familiar-Vs-Familiar social session (Rat 464).

Although the sample size is small, a trend emerged whereby it was more common for the bedding odorants to mimic the whole-animal session response of *Novel-social higher* neurons than for bedding odorants to mimic the whole-animal session response of *Familiar-social higher* neurons. Eight out of ten *Novel-social higher* neurons (80%) fired higher to the novel-bedding than to the familiar-bedding. In contrast, only three out of nine *Familiar-social higher* neurons (33%) fired higher to the familiar-bedding than the other bedding. This difference was statistically significant in a Chi-squared test ($n = 19$, $\chi^2 = 4.23$, $p = 0.04$).

12.3.2 Novel-social higher neurons

In bedding-probe trials, eight out of 10 *Novel-social higher* neurons replicated the pattern observed in whole-animal session. That is, they fired higher to the bedding of the novel rats than to the bedding of the familiar rat. Six of these eight neurons exhibited firing rates that were *higher than the mean* of the novel-social trials in the whole-animal session, or were *close to this mean* (defined as “[Novel bedding rate] \geq [Novel/Social mean – 0.5 Standard Deviation]).

This would suggest that volatile odorants present in the urine/faeces of bedding were generally sufficient to detect the presence of novel conspecifics. For the two (of ten) *Novel-social higher* neurons where the bedding did *not* mimic the whole-animal session response, we can be sure that this was not due to inadequate bedding stimuli (e.g. staleness) because these two neurons were simultaneously recorded with four *Novel-social higher* neurons.

12.3.3 Familiar-social higher neurons

In bedding-probe trials, only three out of nine *Familiar-social higher* neurons exhibited similarly-higher firing rates to bedding containing odorants of familiar animal. Two of these three neurons exhibited firing rates to the familiar-bedding that were *higher than the mean* of the familiar-social trials in the whole-animal session; the other did not and was not *close to this mean* (defined as [Familiar bedding rate] \geq [Familiar/Social mean – 0.5 Standard Deviation]).

This would suggest that, for the majority of *Familiar-social higher* neurons, volatile odorants present in the urine/faeces of bedding were generally *not* sufficient to detect the presence of familiar conspecifics, even though these conspecifics were highly familiar.

What are the implications of the differences between *Familiar-social higher* and *Novel-social higher* neurons? One interpretation is that neuronal firing patterns in *Familiar-social higher* and *Novel-social higher* neurons are qualitatively different. Within this idea, *Familiar-social higher* firing patterns may largely reflect *individual-specific* conspecific patterns that have a higher requirement for pattern completion like processes to *reactivate* an earlier firing cell assembly. Yes, partial cues may eventually trigger the unique individual-specific cell assembly for a given conspecific, but there needs to be a good overlap between the partial triggering cues and those available with full bodily presence. The overlap may require a *higher number of cues* than contained in bedding odorants, or a set of cues that are more *body-based* than *excretory*. (In anxiety research on predator odours, cloth containing odours derived from rubbing the cat's neck are generally much more anxiogenic than cat urine.)

12.4 Did the firing rates of the neurons in the amygdala and/or in the piriform cortex decrease monotonically over the session? or did the firing rates increase?

12.4.1 Amygdala

To answer the question, the mean firing rate of the population of cells recorded in the amygdala (63 cells), were linearly correlated with the trial number (1-12 or 1-18). The analysis showed that the neuronal population in the amygdala appear not to be influenced by the progression of trials during the testing session ($r=-0.36$, $p=0.26$).

12.4.2 Piriform cortex

The analysis run on 562 cells, where the firing rate mean of the cells taken together as a population was correlated with the trial number (1-12) showed a significant reverse correlation between the two variables. This means that the population of neurons in the piriform cortex decrease the firing activity along the 12 trial session ($r=-0.67$, $p=0.017$). However, the firing rate of cells in the 18 trial session was unchanged throughout the session ($r=-0.32$, $p=0.2$). This insignificant result may be attributed to the variance of the firing rates between the groups (i.e Base/Fam/Nov/Sisters). This would suggest that probably the neuronal population in the piriform cortex is influenced by the progression of trials during the testing session.

A Spearman's rank-order correlation was used to determine if each cell singularly taken increase/decrease the firing rate monotonically within session. This was tested by correlating the trial number (1-12 or 1-18), depending on the length of the session) and the mean firing rate of each cell in each trial. In the piriform cortex, the 8.4% of the cell showed an increased activity during the session, while 10.5% of the cells showed decreased the activity during the session. Among the 4 rats, the proportion of cells whose firing rate changed monotonically within session, was similar (**table 12.1**). The main variance can be observed in the case of rat 464, where the proportion of cells which increased the firing monotonically within session was higher compared to the other rats.

were the Novel-vs-familiar and the sisters-specific cells influenced by this general decrease in firing in the piriform cortex?

Only 4 cells out of 30 showed a significant decrease in firing during the session and only 1 out of 27 cells of the Fam-Nov cells showed a firing decreasing during the session.

Table 12.1. Summary of cells recorded in each animals and the percentage of cells that decrease or increase the firing rate monotonically within session on animal by animal bases.

Animal code	Total cells considered (N)	Firing decreasing cells (%)	Firing Increasing cells (%)
426	26	7.7	7.7
438	206	6.7	10.2
451	81	2.5	8.6
464	217	16.6	6.5
Total	562	10.5	8.4

Chapter 13: Discussion

This present study represents a further step in understanding the neurobiological mechanisms of social behaviour. Although the findings are just a first step, they form ‘proof of concept’ foundations for understanding social motivation and social recognition memory. Furthermore, this thesis is the first attempt to examine the role of neurons in the piriform cortex in rats free to engage in positive social interaction, and one of the very first for amygdalar neurons. In order to further explain my current findings, I will discuss them considering previous studies which, though different in nature, share some similarities.

13.1 Amygdala’ s involvement in social behaviour.

13.1.1 Summary of the main results

The present study, in line with previous findings, showed that the amygdala is involved in social interaction. The presence of a conspecific inside the arena represents an emotional relevant stimulus which altered the firing of most of the neurons recorded in the amygdala (56%). Of these cells, 65% of the cells showed firing excitation (Social-higher neurons) and a lower 35% (Non-social higher neurons) showed firing suppression in social trials compared to the baseline trials where the implanted animal was alone inside the apparatus. The most interesting result is that 1 cells recorded from the central amygdala showed firing excitation in novel social compared to social familiar trials, and firing suppression in familiar trials compared to baseline trials and novel trials.

Even if social trials are likely to increase the general arousal state of the implanted animal, in about 35% of the social/non-social discriminative neurons, the firing rate was related to the presence of the animal in the arena but not by the general arousal state, expressed as average trial speed. The firing rate of 14 % of the social/non-social discriminative neurons was predicted by both the speed and the social condition, while the firing rate of about 23% of was predicted only by the running speed.

Considering the social/non-social discriminative neurons it is notable that about 14% of the cells responded broadly similarly to the presence of a familiar object, but 86%. In few words, the majority of the social/non-social cells did not reproduce the pattern of the social trials in presence of a still object in the arena.

The analysis of trials speed and probe trials may support the primary global theory that changes in firing may be related to change in motivation and cognition. The motivation of interacting with a novel animal instead of a familiar one, may also explain the strong increase in firing observed in one cell when the implanted animal was exposed to social familiar versus social novel trials.

13.1.2 Amygdala's role in social interaction

The amygdala has been previously implicated in a processing negative emotions, like anxiety and fear (Adhikari, 2015). Adhikari and colleagues, for example, showed that the amygdala is target of top-down pathways from cognitive control area locates in the prefrontal cortex. They showed how neurons in the basolateral amygdala increase or decrease the activity in relation

to a safe or aversive environment. On the other side, the amygdala has been widely implicated in positive emotions (Baxter and Murray, 2002), and social interaction can be considered a positive reward. Clearly, here it is important to make the point that certain types of social interaction can create anxiety, including those that involve the introduction of a novel conspecific inside the animal home-cage. For this reason, most of the studies which take as their starting point rodent social interaction as neutral or as positively rewarding use younger animals as social stimuli, so that any such 'intruders' do not elicit undue anxiety in the subject animal (Thor and Holloway, 1981). In a similar way, my testing set up was organised in a way to reduce anxiety and minimise the common aggressive social interaction in favour of a more positive range of behaviours which includes more investigation of the conspecific. Firstly, I used female animals, which are less aggressive compared to males. Female animals tend to be aggressive only during maternal care (Mayer and Rosenblatt, 1987). Secondly, to reduce the social anxiety component in the social interaction, only familiar animals were used, as social stimuli for the social condition. Finally, in informal experiments, I collected sporadic ultrasonic evidence in a couple of rats that no distress calls were made during social interaction. This was not due to any insensitivity of the recording apparatus, because distress calls were picked up from a rat hooked up for the first time in the adjoining lab. (Future electrophysiological recording studies should incorporate ultrasonic recordings.) In few words, it is reasonable to think that the neuronal response I observed in amygdala neurons was hardly related to anxiety and fear, there are instead better theories which could help to interpret better my findings

While my discussion focuses on the idea that the amygdala is involved in social interaction, it is important to realise that the amygdala has been linked with a widely amount of behaviour and social aspects and from a certain point of view the interesting percentage of cells that were related to social behaviour may just be influenced by general arousal. It would not be the first

study which correlates the firing rate of cells with increase in the arousal state. This effect has been observed in cortical areas as well as in the hippocampus. For example, the electrophysiological activity in the cortex differs across behavioural states such as different sleeping phases, walking or even anaesthesia (Haider et al, 2012; Steriade et al, 2001). In mice, primary neocortices are strongly influenced by the animal's locomotion, showing altered firing activity, with a prominently increased activity in inhibitory interneurons, and an increase of LFP gamma-band oscillations (Keller et al, 2012; Niell and Stryker, 2010; Polack et al, 2013). In a similar way, hippocampal place cells fire at higher rates when the animal increases the locomotion inside the place field (McNaughton et al., 1996; O'Keefe et al, 1998). Parts of the amygdala are activated by emotional arousal per se (McGaugh, 2004), and certainly a rat-rat trial is more emotionally relevant than alone time in the arena. From a certain point of view, it may be plausible that amygdala neurons are partially influenced by arousal since social interaction was also accompanied by increased locomotion. However, together with cells whose firing rate correlated with average speed, some cells appeared to be influenced only by the rat-rat interaction.

One potential comparison is to the primate literature, where social stimuli are generally always visual. A study by Gothard and colleagues (2007), on primates, showed that a portion of neurons in the amygdala can respond selectively to faces or only to objects. Furthermore, Gothard and colleagues showed that the 64% of neurons were responding to facial expression and identity, but there were neurons in the amygdala which were only identity-selective. Even if in the present study, the amygdala neurons were recorded in rodents, both Fried et al (1997) and Gothard and colleagues' findings reported observations that are in some respects similar to my observations; that there are neurons in the amygdala which showed a firing response similarly to different familiar stimuli, like a familiar object and a familiar conspecific, but at

the same time there are neurons which selectively responds to familiar objects or familiar conspecifics.

Even if the present result represents a closer step to our understanding of the amygdala, future work needs to be done to rule out other possible interpretation, such as arousal. However, my findings, together with previous studies support the speculative theory that some neuronal activation recorded in the amygdala during emotional relevant event, may not be mainly due to neuronal arousal, but could reflect a memory trace, and in case of social interaction, the coding for the identity of a conspecific. In the present thesis, this kind of coding was examined in the piriform cortex.

13.2 The piriform cortex

13.2.1 Summary of the main results

The present study represents the first attempt to examine the neuronal activity in the piriform cortex in relation to an important aspect in rodent life: social interaction. The experiments discussed in chapter 8 showed, for the first time, that neurons in the piriform cortex were responsive during unconstrained social interaction with another conspecific, and while some cells were responsive both to the conspecific and to the conspecific's volatile scent in urine, other cells were responsive only in the presence of the conspecific itself. Two thirds of the cells with socially-responsive firing showed a firing excitation following the exposure to a conspecific, while one third showed firing suppression. On the model of exposure to multiple odorants in anaesthetised animals (Stettler and Alex, 2009), the suppression could be due to

the fact that another rat presents many odorants during a given trial exposure, including from different parts of the body.

The main findings presented in this thesis, due to the sacrificing controlled cue in favour of a naturalistic social interaction, leave open more than one interpretation. Firstly, the firing changes could have just been reflecting changes in the arousal state. Secondly, the piriform cortex may respond to multi-sensory inputs that only the unconstrained social interaction may provide (i.e. the exposure to the bedding of a conspecific may not provide the same firing response observed for the conspecific trial). Thirdly, piriform neurons may contribute to social identity coding of individual conspecific.

The experiments discussed in chapter 9 and 10 aimed to constrain interpretation and provide evidence that neurons in the piriform cortex not only change the firing activity during social interaction compared to baseline, but also there are neurons that can discriminate between novel and familiar conspecifics. Even more interestingly, the piriform neurons could discriminate between two different sisters.

The bedding manipulation aimed to simply provide a control for odour cues, however, since the sense of smell has been shown to be involved in memory recall and social recognition (Porter et al, 1986; Holand and Schleidt, 1997), the data came from the analysis of the bedding probe trials, allowed to speculate whether the odour cues itself can lead to the memory retrieval of a conspecifics. Considering those neurons which discriminated novel vs familiar conspecifics or familiar sisters, it was notable that there was a significant higher proportion of 'Novel-Social-higher' neurons (80%) compared to 'Familiar-Social-higher' neurons (33%) that similarly increased their firing in response to the appropriate social bedding cues

(respectively novel or familiar). Taken together, about 58% of the cells responded broadly similarly to the presence of just the relevant conspecific's bedding. In this case, it shows that the activation of volatile odour associated with the conspecific was sufficient to drive the neuron in the socially-specific way (e.g. higher to novel rats, higher to sister fam1). What is not clear, however, is whether such activation reflects pattern completion. In other words, one might theorise that an individual-specific cell assembly is created by various odorants (and possibly non-olfactory stimuli), and that a few odorants are sufficient to cue a mnemonic, pattern completion-like process, as occurs in the hippocampus with place cells (Nakazawa et al, 2002, Science; Wills et al, Science, 2005). However, it cannot be ruled out that during social interaction, urine, for instance, is being sampled from the anogenital or other bodily region of a stimulus rat, and that the firing during both presence and absence reflects perception, without any memory. This means that it may be possible that no memory trace was involved and cells fired specifically to the odour cues irrespective of whether the conspecific itself was present or not.

Considering those neurons which discriminated novel vs familiar conspecifics or familiar sisters, it was also notable that some (about 42%) did not respond to bedding in a manner similar to the social trials. Importantly, excluding the single sister specific cell recorded in session 280716, it was possible to show that this was a neuron-specific response, not a general one, since bedding-non-replicating neurons were sometimes simultaneously recorded with bedding-replicating neurons. Accordingly, we can be sure at least some bedding-non-replicating neurons were not just attributable to some failure of the bedding as a sensory triggering cue (e.g urine too stale). This suggests that at least for this type of cell, or this type of context-specific response, that volatile odours were not a major contributor to the firing conspecific pattern.

The firing rate of familiar-vs-novel and sister-specific cells appeared not to be reflecting changes in running speed. Examining the average trial running speed is one approach to considering the effect of arousal, and from a certain point of view it can be considered an index of general arousal; and more strictly as a locomotion variable well-known to positively correlate with neurons throughout the hippocampal formation (O'Keefe et al, 1998; Lever et al, 2003; Kropff et al, Nature, 2015). However, my findings, showed that the firing rate was never related to running speed alone, while the firing of 45% of the socially discriminating cells appeared to predict the presence of Familiar vs Novel and Familiar-1 vs Familiar2.

The behavioural analysis in chapter 11, offered the best avenue to aid the understanding of the change in firing of piriform neurons. In fact, the firing of the 80% of the *familiar-vs-novel* and the *sister-specific cells* was not correlated with any social behavioural parameter considered in the analysis, even given six such parameters. Furthermore, the behavioural scoring showed that even when there was not difference in time spent exploring two conspecifics (i.e. novel vs familiar), there were cells that still showed a distinctive conspecific pattern. The present findings suggest that behavioural differences in social interaction are unlikely to play a primary role in generating the novel-familiar discrimination firing of the familiar-novel specific cells in the piriform cortex

Another interpretation of my data comes from the oestrus characterization for the social stimuli. It is unlikely that the firing discrimination between the two sisters may be caused by differences in the oestrus stage, in fact when two conspecifics were both at the same oestrus stage (i.e. dioestrus, proestrus, oestrus), cells could still discriminate between the two conspecifics (familiar vs novel and familiar-1 vs familiar-2).

In summary, these findings even if represent a step forward in our understanding of the piriform cortex, need further work to narrow down interpretation. These results may be explained by different factors, and even if supplementary probe trials, behavioural scoring, speed analysis and oestrus characterization, helped to narrow down interpretation, by themselves, these data do not provide sufficient information to understand if the change in firing was purely sensory or if involved a pattern completion type of response.

13.2.2 Piriform cortex as associative cortex

The present study represents the first attempt to examine piriform cortex neurons in the context of social interaction and social recognition memory. Previous studies focused the attention on the role of the piriform cortex in olfaction, and even if limiting in terms of neuroethological approach, reported very detailed cellular response which have been useful to interpret the findings of the present study. What is known is that the piriform cortex receives strong inputs from the main olfactory bulb which project broadly to the cortex without an apparent topographic organisation (Gosh et al, 2011, and Miura et al, 2012). Surely, olfaction is an important aspect of my findings since a social stimulus has a smell, However, the idea of ‘just olfaction’, cannot explain all the findings in these experiments. ‘Just olfaction’ may explain why cells are firing in presence of a conspecific instead of an empty box, but it cannot explain why cells simultaneously recorded were firing differently between novel and familiar conspecifics and even between two sisters.

The interpretation of my findings rely more on the poorly understood and usually underestimated reciprocal strong connections of the piriform cortex with other brain areas, such

as the amygdala, the orbitofrontal cortex and the entorhinal cortex (Linster and Cleland, 2003); Easy to understand is why the piriform cortex send efferents to these higher cortical regions, since smell is a strong sensory stimulus which alone is sufficient to induce memory recall, or the recognition of a conspecific (Porter et al, 1986; Holand and Schleidt, 1997). Then, what is the meaning of these descending inputs and what may be the contribution of the piriform cortex in social recognition memory?

The reciprocal connections that the piriform cortex shares with entorhinal cortex, amygdala and orbitofrontal cortex and other brain areas, may suggest that the piriform cortex has a role in associative connectivity, on which social identity coding may rely. The firing of neurons in the piriform cortex appear to be shaped by previous experiences, but also expectations as well as current behavioural state (Sadriani and Wilson, 2015). Karunanayaka et al, 2015, showed for example that odour perception in humans, is strongly associated with visual cues. Using functional magnetic resonance imaging, they provided evidence that the piriform cortex may be involved in learning response, since pairing an odour with a visual cue, increased the activity of the piriform cortex when only the visual cue was presented. Clearly, it may be claimed that the piriform cortex like other areas in the brain is simply responding to different sensory cues, like the olfactory tubercle responds not only to olfactory stimuli, but also to auditory stimuli (Karunanayaka et al, 2015). However, the exposure to visual cues previously not paired with odours do not elicit any activity in the piriform cortex (Karunanayaka et al, 2015; Gottfried et al, 2004). The theory that the piriform cortex is involved in learning, can be confirmed by studies in rodents. For example, Ross and Eichenbaum (2006) showed that the hippocampus play a critical time-limited role in the consolidation of memories. In fact, they showed that the long-term memory of food transferring preference is not supported by the hippocampus, but by olfactory involved regions which include the piriform cortex and the orbitofrontal cortex.

In a similar way, the firing rate of piriform neurons following the exposition of a familiar conspecific, may be the coding learned after multiple presentation of the same conspecific and could be facilitated by the experience of the conspecific multi-sensory information. After all, social interaction comprises more than the two sensory cues presented by Karunanayaka and Gottfried (Karunanayaka et al, 2015; Gottfried et al, 2004). It is a complete experience which includes the 5-sensory information (olfaction, vision, audition, gustation and touch), but also the episodic experience of the animal, and it represents a more salient relevant stimulus compared to a visual cue or an object. Furthermore, like the Karunanayaka study, the presentation of a single sensory stimulus, in my case olfaction, could recall the firing suppression or excitation of the social trial. At the same time, not all simultaneously recorded neurons in the piriform cortex showed a reactivation of the individual conspecific pattern. The nature of this finding was only informative, for this reason it is not possible to understand if the pattern reactivation is purely sensory or if it may involve the pattern completion response.

Indeed, it is likely that social recognition memory relied on circuits which support pattern separation and pattern completion memory and the structural organization of the piriform cortex may support pattern completion. Haberly (2001) described the many similarities of the piriform cortex with hippocampal connectivity, notably the recurrent connectivity of CA3, and how this might support pattern completion. In fact, the piriform cortex, as well as the CA3 is predominantly connected to itself with a broadly overlapping spatial organization (Haberly, 2001; Amaral et al, 1990). Each pyramidal cell in the piriform cortex takes connection with at least 2000 other pyramidal cells and also activates interneurons for the local-feedback inhibition (Franks et al, 2011). Finally, they show similarities in laminar patterns of connectivity organised in parallel (Haberly, 2001). The hippocampal-CA3 like organisation

may then support my findings where a partial cue, like the smell of a familiar conspecific, was able to provide the whole memory retrieval of the conspecific itself.

13.3 Strengths and limitations of the present study and future prospects

13.3.1 In vivo naturalistic approach

My study is not the first to use an electrophysiological approach to understand the role of the piriform cortex. To my knowledge, most of the studies which involve the recordings from cells in the piriform cortex are carried out on anaesthetised animals. Such an approach has been preferred to freely moving animals to simplify the technical problems during electrical recording and to give a better control of physiological variables (Vanderwolf and Leung, 1997). In fact, recording from anaesthetised animals permitted to study the piriform neurons restricting the paradigm to a single variable, olfaction. This very conservative approach, offered detailed neuronal response of how piriform neurons process olfactory information. For examples, these studies provided informative knowledge on how the odorant cues, perceived by the olfactory epithelium, activated broadly distributes, moderately sparse ensemble of neurons (Miura et al, 2012; Franks et al, 2011; Wilson 2001); how mix of odours induced firing suppression (Stettler and Alex, 2009); or even how olfaction in piriform neurons is modulated by other brain areas (Chapuis et al, 2013; Sadrian and Wilson, 2015).

Also in anaesthetised animals, Wesson and Wilson (2010) were able to show that certain areas are not just responding to a single sensory cue. In fact, they found that the 20% of neurons in the olfactory tubercle responds also to auditory tone. These studies on anaesthetised animals

clearly show the advantage of ruling out different variables, like general arousal or other sensory stimuli.

However, anaesthetics can induce a general disturbance of the overall brain functions and provide incomplete or even misleading information. For example, the mix of aesthetic with certain type of drugs, such as mianserin, abolishes the ability of serotonin to activate the neocortex (Vanderwolf and Leung, 1997, Neuman and Zebrowska, 1992).

Furthermore, in anaesthetised animals, awake behaviour is completely abolished, making impossible to relate brain activity to behaviour (Vanderwolf and Leung, 1997). In few words, studying the brain activity during social behaviour and social identity coding, requires a more spontaneous approach that only in vivo electrophysiology in freely moving animal can give.

13.3.2 Unconstrained social interaction

The present study provided a brave step forward to the nowadays knowledge of the amygdala and piriform cortex in the context of social interaction and social recognition memory. The approach adopted in my study provided a very naturalistic approach since the implanted animal was free to socially engage with a conspecific. The only restraint was presented by the testing arena, whose small dimensions had the aim to increase the proximity of the implanted animal with the social stimulus, even during the non-social contact period in a social trial. Furthermore, the length of the trial was limited to 3 minutes to increase the motivation of the animals to interact with each other's.

It is disputable that the unconstrained interaction adopted in this experiment may give space to more than one interpretation. The unconstrained interaction from a certain point of view, offered a too open approach that did not leave the possibility to control cue delivery. For example, each trial was different, and as I observed during my experiments the implanted animal tended to interact more with a familiar sister compare to another. Furthermore, this naturalistic approach did not offer the possibility to control which sensory stimulation was driving the firing rate of neurons, leaving the door open to different interpretation.

However, restrictive paradigms offer a certain advantage. Some studies, as in the case of Petrusis and colleagues (2005), report very detailed neuronal response but they offer informative knowledge of how social recognition memory can be based on a specific sensory stimulus, missing the fact that social recognition may not be based on a single sensory cue. In some case, a too restrictive paradigm could be even one of the several explanations that could bring to negative results (Heimendahl et al, 2012).

Furthermore, thanks to this naturalistic approach rats are free to engage with the social stimulus using all sensory modalities, olfactory, gustatory, auditory and visual which may all play a significant role in different aspects of social behaviour, including social recognition memory. For example, olfactory cues are important in any aspect of social behavioural interaction, from food preference to social recognition. In fact, positive social interaction includes the sniffing of the anogenital area and/or the body of the social stimulus, to acquire information. The gustatory sensory information is important for example in the food preference transmission (Van Der Kooij, Sandi, 2012). Ultrasonic vocalizations are important in social communication but also give information of the emotional state of a conspecific (McGinnis and Vakulenko, 2003; Panksepp and Burgdorf, 2003; Woehr and Schwarting, 2007); their role as distinguishing

conspecifics is unclear, but should be explored. Visual cues appear to be important for example to interact with rat pups in food location (Galef and Clark, 1971). Finally, the somatosensory stimuli have been suggested to be one of the most rewarding components in social interaction, since the full restriction of touch, even in presence of visual and olfactory cues, induces place aversion in rats (Kummer et al, 2011). Here I described just few examples of how rodents use all the sensory stimuli to interact with another conspecific, but these examples make the point on the importance of the freely behaving paradigm as a first step approach to study brain areas that like the piriform cortex may be related in aspects of social behaviour.

This is relatively important considering that even if the piriform cortex predominantly receives olfactory inputs, it also connects to another sensory primary cortex. On this point, in 2004, Gottfried and colleagues, showed that the retrieval cue of one sensory modality, was sufficient to elicit sensory-specificity neuronal activity of the piriform cortex, following the explicit encoding of cross-modal association (Gottfried et al., 2004). In fact, following a previous familiarization where the visual presentation of an object was paired to an odorant cue, during the retrieval phase the visual presentation of the same object this time not paired with olfactory cues, was sufficient to activate the piriform cortex in humans. Vice-versa, this activation was not observed following the visual stimulation of novel objects. These findings support the theory that social recognition is a multisensory modality and non-olfactory cues may sometimes be sufficient to provide the information for retrieval of a conspecific identity.

In summary, even if the naturalistic approach presents certain limitations, the freely-behaving paradigm gave the advantage to link the piriform cortex to social interaction and potentially social identity coding, and was the necessary first step for revealing a phenomenon that clearly will need future work to be explained.

13.3.3 Behavioural analysis

The blind behavioural scoring paired with the electrophysiological recording is one of the main strengths of this thesis. To my knowledge, this study is the first study to pair the electrophysiological results from neurons in the piriform cortex with the scoring of behavioural social interaction with novel or familiar conspecific. The behavioural scoring permitted to identify possible differences in the social behaviour of the implanted rat with two different conspecifics, for example, familiar and novel conspecifics and aided to constrain interpretation. The blind approach provided a more reliable analysis avoiding possible result bias.

This approach allowed to correlate the firing rate of cells in each trial with the behavioural scoring of each trial, but correlation does not mean causation. Furthermore, it can be said that the correlation approach was relatively crude, and made most sense for behaviours that took up large proportions of the trial (e.g. ‘total contact’ time, ‘active’ time). A more careful approach is likely needed in future, especially for behaviours of short duration like anogenital sniffing, whose total duration was typically less than 10% of the whole trial, even with novel rats. Assumption-free, moment-to-moment micro-behavioural analyses may lead to better interpretation, and show that at least some cells do have sniffing-related correlates. Arguably, overall, the strength of the behavioural scoring was largely in showing that the social-specificity of a clear majority (80%) of socially-discriminating cells could not be primarily attributed to gross behavioural differences.

13.3.4 Female rats

A general limitation of most of animal studies is the prevalent use of male rodents, with much less focus on females. Underrepresentation of females in animal models often cause a misleading understanding of female biology (Beery and Zucker, 2011). My study offered instead a completely opposite scenario, where females have been preferred to males for their behavioural and neurophysiological characteristics. From a behavioural point of view, female rats show better social cognition (Markham, 2007, Engelmann, 1998). The piriform cortex of female animals shows a high density of oxytocin receptors, which is interesting from a neurophysiological point of view since oxytocin is a prosocial neuropeptide (Mitre et al, 2016, Gabor et al, 2012). However, the use of female animals adds further variables to this study due to the hormonal fluctuation of the oestrus cycle. In fact, hormones like oestrogen influence social behaviour interacting with receptors in areas like the amygdala (Spiteri et al, 2010) and females in oestrus are normally less sociable (Markham, 2007). Nevertheless, females at any stage of the oestrus cycle still maintain a better social memory compared to males (Markham, 2007) which made female animals the right subject for my study.

13.3.5 Social Isolation

The chronic isolation of the implanted animals is a clear limitation of my study. In fact, after the surgery, the subjects were kept under isolation to avoid technical problems with the implants. Even if this type of social isolation cannot be compared to the social isolation model of long-term stress and depression because the isolation started not earlier than 3 months after the birth of the animal and not immediately after weaning, social isolation still represents a stress and there are studies which shows social memory impairment following chronicle social

isolation in rodents which needs to be taken in consideration. However, most of the study use as model of social isolation, the social isolation from weaning, while the acute/chronicle social isolation after the adult age, is not well study. Indeed, a study in mice (Kogan et al 2000) showed that not only chronic, but also acute social isolation, can cause social recognition deficits after 24h a single 2 minutes' encounter with a juvenile conspecific. Clearly, the 2 minutes single encounter presented in the Kogan's study, cannot be compared to the 2 familiarization phases that precede my experiments, and the inter-period of time elapsed between the first encounter and the second encounter cannot be compared with the second familiarization phase that precede the testing phase of my experiments of only 1 hour.

13.3.6 Future work

Despite the significance of these results, the present study bears undeniable limitations, which also direct future work. Firstly, the most critical analysis, which undoubtedly will allow a better explanation of my results, is understanding if ensemble of neurons represent and transmit social identity information in the patterns of their joint firing activity. In fact, the use of specific decoding algorithms may help to understand if the simultaneously multiple single neurons activity during social encounters, can predict if the conspecific is familiar or novel, or even if the implanted animal is interacting with sister Mary or sister Jane.

Secondly, since the results in this thesis are limited to the correlation of the firing rate of each trial with the total amount of time in each trial that the implanted animal spent in a certain behavioural parameter, for future work it would be expected a more careful micro-behavioural analysis. This would allow to associate burst of spikes with the exact behaviour in that specific second, or fraction of a second.

Thirdly, future work is needed to limit the parameter space of variables. A first option could be the inclusion of more probe trials and controls in the fam-fam session, for example, using a moving object like Sphero which presents the smell of a conspecific. A second useful approach would be the disruption of the olfactory bulb and/or vomeronasal organ during the retrieval phase of a familiar conspecific, and compare if the suppose social identity cells can still be excited or suppressed following the lack olfactory stimuli. A third option could be the use of more restrictive paradigms to have a better control of the variables. For example, limiting the sensory information to only vision and auditory and olfaction, and excluding somatosensory information that may be an important component in social behaviour.

Fourthly, this study did not distinguish pyramidal cells from interneurons. The piriform cortex is organised in three layers and pyramidal cells are mostly localised in layer 2. The use of neurobiotin would allow neuron labelling and a better understanding of the process.

Finally, only female rats have been included in the present study. To avoid possible sex bias, next studies should include male rodents.

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