Distinction between dorsal and ventral hippocampus in processing social cues and the underlying mechanisms essential for social recognition memory in mice

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

genehmigt durch die Fakultät für Naturwissenschaften

der Otto-von-Guericke-Universität Magdeburg

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geb. am 23.12.1987 in Lleida, Spanien

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Eingereicht der Dissertation am: 22.11.2016

Verteidigung der Dissertation am: 30.03.2017

Dedicada a la meva família

Abstract

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The social recognition memory in mice relies primarily on olfactory social cues known as the olfactory signature, which is composed of a volatile and a non-volatile fraction. Although the hippocampus has been described as essential for the intact social recognition memory in mice, the underlying processes induced in this area by a social encounter, as well as the local molecular cascades involved in the formation of social memories, remains to be elucidated. In the present study, the role distinct parts of the hippocampus, dorsal and ventral, play in the processing of information coding for the different fractions of a conspecific' olfactory signature in order to successfully form short-term and/or long-term social recognition memory was investigated. In addition, the function of an adaptor protein potentially able of modulating selected hippocampal molecular cascades thought to be involved in social recognition memory formation was evaluated.

Our results revealed a distinct pattern of hippocampal activation, extracted from the c-Fos *de novo* synthesis, which depends on the defined fractions of the olfactory signature that were presented. When the experimental subjects were directly exposed to the social cues, which allowed access to both fractions of the olfactory signature, a significant increase in the c-Fos synthesis was observed in the ventral but not in the dorsal part of the hippocampus. In contrast, no impact on c-Fos synthesis was detected after an indirect exposure to a conspecific in which the experimental subjects had access to the volatile fraction only. Additional *in vivo* experiments indicated the involvement of both parts of the hippocampus in social recognition memory. The dorsal part of the hippocampus appeared to be responsible for the integration of the information from different modalities, while the ventral part was shown to be causally involved in the initial processing and further transmission of the acquired information. An interesting observation was the presence of intact long-term memory, while short-term memory was impaired, thus suggesting the involvement of independent molecular mechanisms under defined testing conditions. Moreover, the adaptor protein Grb2 was identified to be essential for the consolidation of social memory. Overall, our results provide first evidence for a distinct c-Fos synthesis pattern of the dorsal *versus* the ventral part of the hippocampus induced by social cues, and the involvement of both hippocampal parts in the generation of social recognition memory. Our findings further suggest a contribution of the signalling cascades modulated by Grb2 in the mouse forebrain to consolidate social recognition memory.

Zusammenfassung

Analyse der Bedeutung des dorsalen und ventralen Hippocampus für das soziale Wiedererkennungsgedächtnis und zugrunde liegender zellulärer Mechanismen bei Mäusen

von M. Sc. Judith CAMATS PERNA

Das soziale Wiedererkennungsgedächtnis bei Mäusen beruht hauptsächlich auf olfaktorischen Stimuli, die den individuellen „Geruch" eines Artgenossen ausmachen. Dieser, üblicherweise als „olfaktorischer Fingerabdruck" bezeichnete Geruch besteht aus einer volatilen und einer nicht-volatilen Fraktion. Obwohl die Literatur den Hippocampus als essentiell für ein intaktes soziales Wiedererkennungsgedächtnis bei Mäusen ausweist, sind die in ihm ablaufenden und bei einem Sozialkontakt ausgelösten Mechanismen kaum bekannt, einschließlich der molekularen Kaskaden, die zur Bildung eines „Sozialgedächtnisses" führen. In der vorliegenden Studie wurde deshalb untersucht, welche Bedeutung dem ventralen versus dem dorsalen Hippocampus für die Prozessierung der Information zukommt, die für die unterschiedlichen Fraktionen eines „olfaktorischen Fingerabdruckes" kodiert und letztlich ein intaktes soziales Kurzzeit- und/oder Langzeit-Wiedererkennungsgedächtnis generiert. Zusätzlich wurde die Funktion eines Adaperproteins analysiert, das potentiell in der Lage ist, definierte, intrazelluläre Molekülkaskaden im Hippocampus zu modulieren, die in das soziale Wiedererkennungsvermögen einbezogen sein können.

Unsere Ergebnisse zeigen anhand der c-Fos-de-novo-Proteinsynthese ein distinktes Muster der „Hippocampusaktivierung" in Abhängigkeit davon, welche definierten Fraktionen des „olfaktorischen Fingerabdruckes" präsentiert wurden. Wurden die Versuchstiere direkt einem Stimulustier ausgesetzt, was der Akquisition sowohl der volatilen als auch der nicht-volatilen Fraktion des "olfaktorischen Fingerabdruckes" entspricht, dann konnte eine verstärkte c-Fos-Synthese im ventralen, nicht aber im dorsalen Hippocampus gemessen werden. Die c-Fos-Synthese blieb unverändert, wurde nur der volatile Anteil des „olfaktorischen Fingerabdruckes" präsentiert. Zusätzliche In-vivo-Experimente suggerierten die Beteiligung beider Anteile des Hippocampus an der sozialen Wiedererkennung. Dabei scheint der dorsale Anteil in die Integration von Informationen einbezogen zu sein, die mittels verschiedener sensorischer Modalitäten gewonnen wurden. Demgegenüber legen die Befunde eine Beteiligung des ventralen Hippocampus in die frühe Prozessierung und Weiterleitung der relevanten Information zum dorsalen Anteil nahe. Eine interessante Beobachtung betrifft den Nachweis eines Langzeit-Wiedererkennungsgedächtnisses unter Bedingungen, unter denen kein Kurzzeitgedächtnis nachgewiesen werden konnte. Das impliziert, dass unterschiedliche molekulare Mechanismen

unter den jeweiligen definierten Bedingungen ablaufen, die den definierten Gedächtnisleistungen zugrunde liegen. Das Adapter-Protein Grb2 wurde als ein Kandidat für die Konsolidierung eines intakten Sozialgedächtnisses identifiziert. Zusammen liefern die Ergebnisse dieser Dissertation erste Hinweise für ein distinktes c-Fos-Synthesemuster im dorsalen versus ventralen Hippocampus, ausgelöst durch soziale Stimuli. Außerdem scheinen beide Anteile des Hippocampus in die Generierung eines sozialen Wiedererkennungsgedächtnisses einbezogen zu sein. Schließlich implizieren die Ergebnisse, dass dabei Signalkaskaden, die im Vorderhirn durch Grb2 moduliert werden, an der Konsolidierung des Langzeit-Wiedererkennungsgedächtnisses der Maus beteiligt sind.

List of abbreviations

aCSF	artificial cerebrospinal fluid
Agg	aggressive behaviour
ANOVA	analyses of variance
AOB	accessory olfactory bulb
Aqua dest	*aqua destillata* (distilled water)
BDNF	brain derived neurotrophic factor
Beh.	behaviour
BLA	basolateral amygdala
bp	base pair
CA	*cornus ammonis*
CaMKIIα	calcium/calmodulin-dependent protein kinase II alpha (α-subunit)
c-Fos	cellular homologue of the Finkel-biskis-Jinkis murine osteosarcoma virus protein
c-fos	cellular homologue of the Finkel-biskis-Jinkis murine osteosarcoma virus gene
Ch	choice
cKO	conditional knock-out
Cre	cre-recombinase
CREB	cyclic adenosine monophosphate response element-binging protein
CS	conditioned stimulus (context or cue)
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DAB	3,3'-diaminobenzidin-Tetrahydrochlorid
df	degrees of freedom
DG	dentate gyrus
dHC	dorsal hippocampus
DMSO	dimethylsulfoxide
e.g.	*exempli gratia* (example given)
EC	entorhinal cortex
EI	exposure interval

Erk	extracellular signal-regulated kinase
ES	embryonic stem
Fig.	Figure
flx	floxed
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
h	Hours
HC	hippocampus
HSD	honestly significant difference
IEG	immediate-early genes
Jun	ju-nana
Jv (J)	juvenile
MAPK	mitogen-activated protein kinases
MeA	medial amygdala
min	minutes
MOB	main olfactory bulb
MOE	main olfactory epithelium
mRNA	messenger ribonucleic acid
n	number of subjects per group
NeuN	neuronal nuclei
NMDAR	N-methyl-D-aspartate-receptor
NOR	novel object recognition
opt	*tractus opticus*
P	postnatal day
p	probability of error

PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
Pir	*cortex piriformis*
PKA	protein kinase A
PLCγ1	phosphatidylinositol-specific phospholipase Cγ1
PVT	paraventricular nucleus of the thalamus
RT	room temperature
S	sampling
SA	stimulus animal
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
SDT	social discrimination test
SDT_D	social discrimination test with direct exposure to the juvenile(s)
SDT_I	social discrimination test with indirect exposure to the juvenile(s)
SEM	standard error of the mean
Sex.	sexual behaviour
SH2	src homology-2
SH3	src homology 3
Shc	Src-homology domain-containing transforming protein
Sos	Son of sevenless
Trk	receptor tyrosine kinase
UCS	unconditioned stimulus (foot shock)
VFC	volatile fraction cage
vHC	ventral hippocampus
VNO	vomeronasal organ
vs.	*versus*
WT	wild type

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1 Introduction

At the beginning of the 20th century, Jakob von Uexküll was interested in how living beings subjectively perceived their environments and how this perception determined their behaviour. Focusing on this field, Jakob von Uexküll (1921) was the first to suggest that every species experiences life differently, living as it does in its own "Umwelt", or unique perceptual world, and thus he became one of the founders of behavioural science. Some years later, and largely influenced by von Uexkülls' work, Konrad Lorenz (1935), also considered as one of the founding fathers of the field of ethology, expanded von Uexkülls' idea of a perceptual world to include not only physical surrounding but the social system, suggesting that an animal's perceptual world must include important information about the behaviour of other individuals, and even the group as a whole. The relevance of social perception and the interaction within individuals from the same species in determining an organism's behavioural responses was later confirmed and expanded by numerous studies. Nowadays, it is known that recognition of conspecifics is pivotal for mounting appropriate behavioural responses and thus essential for the maintenance and establishment in social communities. Nonetheless, despite the relevance of this behaviour, for most species little is known about how such social perception occurs or how it leads to the behavioural, physiological, cellular, and molecular changes needed for social learning and memory. During the last years, a high number of behavioural tests have been developed to allow the assessment, in laboratory conditions, of different social behaviours in rodents (van der Kooij and Sandi 2012, Camats Perna and Engelmann 2015). These tests, including the social recognition test, have enabled research into defining the regions of the brain involved in the acquisition and processing of information coded by social cues, which are essential in forming social memory. Similarly, these tests have also allowed the investigation of the molecular pathways involved in these processes, which all together helped to form a better understanding of the functioning of the "social brain".

Currently, there is increasing awareness of the role abnormal social behaviours play, not only in rodent social communities, but in such human disorders such as schizophrenia and autism (Daenen et al. 2002, Hammock and Young 2006, Gras-Vincendon et al. 2008, Marwick and Hall 2008). The knowledge obtained from studies with rodents may help to identify neural substrates of normal social behaviour that might aid in the understanding and treatment of abnormal human social behaviour that are the core symptoms of various psychopathologies.

1.1 Learning and memory

The capability of learning and memory can be seen as fundamental for coping with environmental changes, which are either too short-term or insufficiently predictable, to be coped with by genetic or developmental

mechanisms (Plotkin and Odling-Smee 1979). Hence, learning is defined as the process by which animals acquire knowledge about the environment. This process leads to more or less permanent changes in the animals' behaviour. However, these changes only happen if the learned information is remembered, so if a memory is formed, or in other words, when the knowledge acquired while learning is encoded, stored and can successfully – later- be recalled. Müller and Pilzecker (Müller 1900) first coined the term "consolidation" to describe this post-experience process of memory stabilization. Today, it is understood that consolidation refers to the process during which new memories are transformed from an initially labile state to a more permanent state, gradually increasing their resistance to disruption. Memories are classified dependent on their duration or permanency: short-term memory, usually referred to as working memory (lasting from seconds (s) to hours (h)), intermediate-term memory (lasting from ~ 2 h to ~ 10 h) and long-term memory (lasting from days to months). **Short-term memory** refers to the capacity to maintain through active rehearsal the limited amount that can be held in mind when the information is learnt, usually across a relatively short time interval (Baddeley and Hitch 1974). Although not being as well described as short-term or long-term memory, **intermediate-term memory** addresses those memories which can be recalled several hours after their acquisition, but are still labile, thus susceptible to a variety of influences both facilitating and impairing (McGaugh 1966, Rosenzweig et al. 1993, Perna et al. 2015). Finally, **long-term memory** refers to what can be recalled from the past when the information to be learned no longer occupies the current stream of thought, so needs to be well consolidated making it resistant to disruption (Perna et al. 2015). In animal models of learning and memory, the requirement for *de novo* protein synthesis around the time of training has long been suggested to underlie consolidation and thus, a definitive property of long-term memory that separates it from the other types of memory (DeZazzo and Tully 1995, Davis and Squire 1984). Several studies have demonstrated that the consolidation of memory requires gene expression (Flexner et al. 1965, Davis and Squire 1984, Abel et al. 1997, Silva et al. 1998, Martin et al. 2000, McGaugh 2000) which is mediated by the cyclic adenosine monophosphate response element-binding protein (CREB) family of transcription factors (Lakhina et al. 2015). Indeed, pharmacological studies have shown that inhibition of CREB messenger ribonucleic acid (mRNA) or protein synthesis, disrupted the formation of long-term memory, without affecting short-term memory (Abel et al. 1997, Silva et al. 1998, Kida et al. 2002, Suzuki et al. 2004, Duvarci et al. 2008).

Memories can also be divided independently from their duration, but depending on the conscious awareness of its recall. At the end of the 19th century, Ebbinghaus (1885) distinguished between those memories which need a conscious recall, such as facts or events, and those which does not need a conscious recollection, such as skills or habits (remembered by performance, procedural). More recently, Squire (1992) attributed specific brain areas to

each of these two types of memory. The hippocampus (HC) or medial temporal-lobe dependent declarative memory, which corresponded to the one that needs conscious recollection, and a non-hippocampal dependent (dependent on a composite of other brain structures such as striatum, neocortex, amygdala and cerebellum) non-declarative memory that does not need conscious access (Squire 1992, Squire et al. 2004, Eichenbaum 2004). These types of memory are also referred to as explicit or implicit memory, respectively (Schacter 1987, Squire 1987).

It is important to note that the characteristics introduced for the memory classification based upon its duration or permanency; short-term, intermediate-term and long-term memory, are shared by most types of memory tested in rodents, including declarative and non-declarative, thus independent of the event to be remembered. Nonetheless differences, e.g. activated molecular pathways, pattern of protein synthesis activation or even brain areas involved (as introduced for declarative and non-declarative memories) vary depending on the specific test conditions and on the nature of the memory tested in the study (e.g. depending on the event to be remembered).

1.1.1   c-Fos as an indicator of cellular activity

In the brain, neuronal gene expression is dynamically changed in response to neuronal activity. In particular, the expression of immediate early genes (IEG), such as the cellular homologue of the Finkel-Biskis-Jinkis murine osteosarcoma virus gene (*c-fos*) was described to be rapidly and selectively upregulated in subsets of neurons from specific brain regions associated with learning and memory formation (Greenberg and Ziff 1984, Sagar et al. 1988, Herrera and Robertson 1996, Bullitt 1990). For instance, the protein product c-Fos induced by the transient activation of *c-fos* expression by neuronal excitation, forms heterodimeric complexes with "ju-nana" proteins (more widely known as Jun proteins) to build up the transcription factor activator protein 1, which regulates the expression of a variety of effector genes (Greenberg and Ziff 1984, Kubik et al. 2007). This increase of c-Fos synthesis was associated with the initial steps of long-term memory in different learning tasks (Guzowski 2002, Tischmeyer and Grimm 1999, Kubik et al. 2007). Moreover, deletion of the *c-fos* gene or inhibition of its expression at the time of training impaired memory (Tischmeyer and Grimm 1999, Lamprecht and Dudai 1996). However, the impairment induced by the lack of c-*fos* was dependent on the type of memory under study. In mice, genetic ablation of the *c-fos* gene limited to the central nervous system exhibited impaired long-term potentiation, which could be restored by repetitive stimulation. These mice also displayed impaired contextual but not cued fear conditioning, and impaired memory retrieval in the spatial water maze despite good performance during training

(Fleischmann et al. 2003). Thus, suggesting a specific involvement of c-*fos* in learning and consolidation of HC-dependent memories.

In recent years, optogenetic and pharmacogenetic studies of neurons expressing *c-fos* revealed that during learning IEG-positive neurons encoded and stored information that was required for memory recall, suggesting their involvement in the formation of the memory trace (Ohkawa et al. 2015, Matsuo 2015). Nonetheless, the molecular mechanisms of their cellular functions and their involvement in synaptic plasticity continue to be a matter of intense research. In an independent approach, IEG have also been used as activity reporters, making use of their rapid induction by behavioural experiences (IEG imaging) (Hughes and Dragunow 1995, Dragunow 1996, Brennan and Keverne 1997, Tischmeyer and Grimm 1999, Cammarota et al. 2000, Eagle et al. 2015, Morgan et al. 1987). In its conventional form, IEG imaging uses either immunohistochemistry or *in situ* hybridization to detect IEG protein or mRNA, respectively. In the case of *c-fos*, the most widely IEG studied, its mRNA and protein levels are hardly measurable in most of the brain areas under basal conditions (Hughes et al. 1992). However, in many regions of the brain *c-fos* mRNA levels generally peaked at 30 min post-stimulation, and by 120 min tended to be reduced to basal levels (Cullinan et al. 1995, Richter et al. 2005b). This pattern was different when analysing protein expression, since the highest levels of c-Fos protein in the nucleus were observed between one and three hours after stimulation, which decreased after four to six hours (Sonnenberg et al. 1989, Ikeda et al. 1994, Cullinan et al. 1995). It is important to note that IEG protein levels are subject to both transcriptional and post-transcriptional regulation. In this sense, the detection of mRNA levels is more directly connected to the inducing event, thus providing a more direct readout of neuronal activation. However, the half-life of IEG mRNA is also shorter, yielding higher signal-to-noise ratio (Guzowski 2002). In contrast, steady states of protein levels reflect integration of multiple cellular processes and modulatory signals and, as such may be more closely associated with neuroplastic changes engaged by distinct behaviours. Although IEG protein imaging has much poorer temporal resolution, it provides exceptional anatomical resolution (down to individual neurons). In order to get the peak synthesis of c-Fos, there is often a gap of around 70 - 120 min between the target learning behaviour and the sacrifice of the animal, this gap inevitably means that the source of the signal may become blurred. A further issue is that most IEG imaging studies require a control group that is matched for sensorimotor demands but is expected to show little or no learning when compared with the experimental group. Differential c-Fos levels are then assumed to reflect the learning condition. Thus, the validity of this subtraction method depends on the appropriateness of the control condition.

Numerous studies have demonstrated the utility of this approach to identify which cells are active "simultaneously" in specific brain areas during the formation of a memory, however, as previously exposed with a number of caveats (Hoffman et al. 1993, Kovacs 2008, Guzowski 2002, Bertaina-Anglade et al. 2000). Of particular relevance is to consider the fact that the absence of the IEG induction does not necessarily indicate a lack of neuronal activity. One effort that can be made in this regard is the examination of multiple IEGs. In spite of these limitations, the use of IEGs and other regulatory- or effector- transcription factors as indicators of recent neural activity, have been and still are an extremely powerful technique to locate the cellular basis of the "memory engram" (Ramirez et al. 2013).

1.1.2 Social recognition memory

Mice, as almost all rodents, are animals which live in social communities. Thus, recognition of conspecifics, described as the ability to distinguish and recognize individuals, is crucial for these animals to mount appropriate behavioural responses, built social relationships, and is ultimately vital for reproductive success. Even for the choice of mates, social recognition is essential, since the ability to assess the degree of relatedness of a potential mate is thought to reduce inbreeding and maximize the fitness of the offspring (Meagher et al. 2000, Isles et al. 2001). This ability is also needed to form parent-offspring bonds and is the basis of territorial behaviour (Borelli et al. 2009). These, among many, are some of the behaviours which rely on social recognition memory, highlighting its relevance in everyday life.

Information from a range of senses can be used for discrimination among conspecifics, however in rodents, olfaction is their dominant sense and their behaviour is heavily influenced by the social chemosignals secreted by individual conspecifics (Matochik 1988, Popik et al. 1991). Several investigations described the olfactory nature of the recognition cue, since lesions of the olfactory bulb and chemically induced anosmia, impaired individual recognition in rodents (Noack et al. 2010, Popik et al. 1991). Thus, investigation of conspecifics (e.g. direct sniffing) allows mice to acquire the mixture of social chemosignals, unique for each individual and known as the **olfactory signature**, which is required to form social recognition memory in rats and mice. Recognition memory has been theorized to underlie two latent cognitive processes. One of these processes is described as a vague feeling of familiarity, which is experienced rapidly and varies from a weak intuition to a compelling strong belief. While the second, known as the recollection process, involves the recovery of qualitative associations prompted by a critical cue (Yonelinas and Levy 2002, Eichenbaum et al. 2007). Whether both of these processes rely on the HC remains a subject of debate, however, some studies revealed that the HC contributes to both, familiarity and

recollection, components of recognition memory in humans (Merkow et al. 2015). In contrast, studies with rats claim that recollection is reduced whereas familiarity is increased following HC damage tested on short-term memory (Sauvage et al. 2008, Clark et al. 2001). Nevertheless, the majority of the data obtained from experiments on rodents suggest that the HC is critical for recollection, but not for familiarity (Eichenbaum et al. 2007). When social recognition memory in mice is concerned, the involvement of the HC in immediate social recognition memory (lasting seconds) has been described as minor, since it was intact after HC permanent lesions, while short-term and long-term social recognition memory were impaired (Kogan et al. 2000). Thus, the impairment observed on short-term and long-term social recognition memory after HC lesions supports the idea that while testing social recognition, recollection instead of familiarity is evaluated.

1.1.2.1   Processing of the olfactory signature

How the brain encodes memory as a biological entity is not yet well understood. However, the brain architecture provides some clues. The ability to recognise, use and behave according to socially relevant information requires a neuronal system that not only processes the information of the perceived social cues but also links it to emotion, motivation and adaptive behaviour. The capacity to generate these associations is essential for triggering what we call memory. Present research aimed at analysing the brain areas involved in social memory focused on those areas which can easily be linked to processing of olfactory social cues. As outlined above, in rodents the olfactory system is the most important sensory system to form social memories. Hence, the initial processing of conspecific social cues takes place in the olfactory bulb, a well described structure, ideal to study the involvement of the different neural substrates from the initial sensory detection through to limbic and higher cortical processing areas, which modulate complex social behavioural responses including recognition memory (Camats Perna and Engelmann 2015) (Fig. 1).

The sources for the chemosensory signals used by rodents primarily to identify conspecifics are body fluids as urine or secretions from skin, reproductive tract or specialised scent glands producing pheromones and other semiochemical compounds (Natynczuk and Macdonald 1994, Heiss et al. 2009). Thus, the olfactory signature is composed of **a volatile and a non-volatile fraction** (Popik et al. 1991, Sawyer et al. 1984, Carr et al. 1976, Matochik 1988) which, after being detected in social encounters, are processed by two segregated neuronal pathways, the main and the accessory olfactory system (Noack et al. 2010), respectively. The **main olfactory bulb** (MOB) is specialised in the processing of volatile stimuli. Volatile odours are detected mostly by sensory neurons in the main olfactory epithelium (MOE) that transfer the information to the MOB as the relay station to higher brain areas, including primary olfactory cortex and the HC (Fig. 1). The second pathway comprises the **accessory**

olfactory bulb (AOB) that processes the information linked to non-volatile, predominantly pheromonal signals (Dulac and Torello 2003). Such stimuli are collected by direct contact to the conspecific's body fluids (Luo et al. 2003) and predominantly detected via the vomeronasal organ (VNO). Sensory neurons of the VNO project to interneurons in the AOB, which in turn signal to the hypothalamus and via the amygdala to the HC, and finally elicit neuroendocrine and behavioural effects (Li et al. 1990, Swanson and Petrovich 1998) (Fig. 1).

Fig. 1. Main brain circuit processing olfactory information linked to social recognition memory in the rodent brain. Non-volatile stimuli are processed mainly by the vomeronasal organ (VNO) which projects to the accessory olfactory bulb (AOB) transmitting the information to higher limbic and cortical areas essential to form social recognition memory. Volatile stimuli instead are mainly processed by the main olfactory epithelium (MOE), which projects to the main olfactory bulb (MOB) and sends information to the primary olfactory cortices from where they will be transferred to tertiary projection areas, including the medial amygdala (MeA) and the hippocampus (modified from Camats Perna and Engelmann (2015)).

Inputs originating mainly in the VNO converge in the medial amygdala (MeA), which seems to act as a major site for the integration of accessory and main olfactory pathways. Efferent connections from the MeA signal back to the AOB thereby likely controlling the impact of the non-volatile fraction of the conspecific's olfactory signature on approach-avoidance behaviour (Baum and Bakker 2013, Martel and Baum 2009) (Fig. 1). The MeA had been proven to be essential in processing the non-volatile fraction of the olfactory signature as its blockage immediately before the memory session, but not during the learning session, impaired social recognition memory in mice (Noack et al. 2015). Furthermore, this area is among the sites of action for different steroids and neuropeptides, therefore is sensitive to hormonal states and able to strongly modulate social recognition memory through neuropeptides such as oxytocin and vasopressin (van der Kooij and Sandi 2012, Neumann and Landgraf 2012, Ferguson et al. 2002, Hammock 2015, Wacker and Ludwig 2012, Arakawa et al. 2010). Consequently, the MeA together with the basolateral amygdala (BLA) have been critically implied in the emotional potentiation of memory by facilitating information processing and storage in other structures, like the HC (McGaugh 2004,

Roozendaal et al. 2009, Wang et al. 2014). Thus, the amygdala and the HC constitute major components of the limbic system linked to emotion and memory (Uematsu et al. 2012), however the molecular pathways involved are not yet described.

To sum up, multiple brain areas communicating with the olfactory bulb are involved in the processing of the information from the perceived olfactory cues, including, among others, the HC. The HC is critical to declarative memory such as recognition memory (Eichenbaum 2000, Suzuki and Eichenbaum 2000, Teyler and Rudy 2007, Squire 2009). However, the specific function including the nature of its contribution to recognition memory is not completely understood. Hence, further studies are necessary to reveal which role the HC plays in the complex process of social memory formation.

1.1.2.2   Hippocampus in social recognition memory

Over the course of the 20th century, the progressive tools created to investigate higher cognitive function changed the scientific understanding of the HC formation from a lowly olfactory structure, to the seat of a cognitive map forming the cornerstone of orientation in space and time (O'Keefe and Nadel 1978). Nowadays the HC formation, which encompasses the HC proper (*cornus ammonis* (CA) 1, CA2, and CA3 regions), as well as the dentate gyrus (DG), the subiculum and the entorhinal cortex, is at the centre of the quest to understand the brain's ability to learn, remember and predict.

The HC receives input, either directly or indirectly, from much of the rest of the brain. Highly processed cortical input arrives via the perforant path projection from the entorhinal cortex. The main subcortical input comes to the HC from the medial septum via the fornix and emotional and motivational modulatory input comes from the amygdala (Amaral and Witter 1989, Pikkarainen et al. 1999, Kubik et al. 2007). Thus, it is not surprising that this central area was soon related with social behaviours in rodents. The HC was first implicated in social interaction, specifically the ventral HC (vHC), which shares reciprocal projections with the BLA (O'Donnell and Grace 1995, Pikkarainen et al. 1999). Later, lesion studies provided the first evidence of an involvement of the vHC in social interaction (Cadogan et al. 1994, Deacon et al. 2002, McHugh et al. 2004) and defensive behaviours (Pentkowski et al. 2006). Furthermore, studies using selective lesion methods suggested that the vHC, but not the dorsal HC (dHC), was required for social interaction during a resident-intruder test (McHugh et al. 2004). Thus not surprisingly, the HC was finally described to be involved in social recognition memory. Although lesion studies in rats tend to confirm a lack of HC involvement in short-term social recognition memory (Bannerman et al. 2001, Squires et al. 2006), permanent HC lesion in mice impaired social recognition memory for a conspecific juvenile 30 min after the first exposure without affecting immediate social recognition (Kogan et al. 2000). IEG imaging

was also used to study the involvement of specific brain areas in the formation of social recognition memory after an initial social encounter, mimicking the learning session in a social memory test. Male mice showed increased c-Fos synthesis in the MeA, the medial preoptic area and the piriform cortex, whereas the number of c-Fos-positive cells in the dHC area was not significantly affected (Ferguson et al. 2001, Richter et al. 2005b, Engelmann 2009, Samuelsen and Meredith 2011). The discrepancies between the findings on c-Fos activation in the dHC of mice after social stimulation, and the effect of HC lesions, challenges the interpretation of data from IEG activation in the context of memory formation and illustrates the need to do more accurate quantifications. In this context, it has been demonstrated that distinct parts of the HC are involved in different behaviours. This functional dissociation is supported by its anatomical connectivity and gene expression, and therefore a more detailed look at IEG synthesis by analysing each of these HC parts might help to clarify its involvement. However, instead of dividing the HC by anatomical connectivity according to its septotemporal axis, recent studies analysed the involvement of specific HC areas such as the CA2 in social recognition memory. CA2 projects almost strictly inside the HC itself, to CA1 and CA2 (contralateral) and CA3 (Hitti and Siegelbaum 2014), and back projects to the medial entorhinal cortex layer II (Rowland et al. 2013), from which it also receives olfactory responsive inputs (Gnatkovsky et al. 2004, Boisselier et al. 2014). Recent studies suggested CA2 to be critical for an intact social recognition memory (Hitti and Siegelbaum 2014), since inactivation of CA2 pyramidal cells or lesions in this region impaired social recognition memory without impacting other forms of HC-dependent memory (Stevenson and Caldwell 2014). CA2 receives abundant projections from brain areas known to be relevant for social behaviour including the amygdala (Pikkarainen et al. 1999) and its neurons are modulated by numerous neuropeptides, such as vasopressin, widely known for its critical role in social memory and social aggression in rodents (Pagani et al. 2015). Moreover a recent study reported that targeted activation of CA2 strongly enhanced social memory (Smith et al. 2016), supporting the findings that this area is crucial for the consolidation of socially relevant information into long-term memory (Dudek et al. 2016). Thus, the reported studies support the HC-dependent nature of the social recognition memory, especially of the HC area CA2. This area serves as a link between CA1 and CA3 neighbouring areas (Sekino et al. 1997), thus it would seem likely that also this subareas are necessary for the formation of social memory. Accordingly, a recent study performed in rats suggested the CA1 area from the dHC to be essential for social recognition memory (Garrido Zinn et al. 2016).

Overall, there is increasing evidence that the HC might be critically involved in the generation of social memory. However, the outcome of behavioural and molecular studies is still inconsistent, as no HC activation, in terms of an increased c-Fos synthesis, has been observed after social learning episodes. Moreover, it is important

to note that only the HC as a whole (such as in HC-lesioned studies) or the dHC and CA2 areas only, have been studied, hence the possible different roles that distinct parts of the HC might play in social memory formation remains unclear. In addition, it is likely that specific brain regions are only temporarily involved in acquisition, consolidation and retrieval encoding, as this time-dependent contribution has been demonstrated for the HC (Kogan et al. 2000). Thus, further research investigating the potential different involvement of the distinct parts of the HC, i.e. dHC *versus* (*vs*.) vHC, at several time points during social recognition memory formation, might help to understand and solve the discrepancies present in the literature.

1.1.3 Memory assessment

Behavioural testing has been extensively used to gain a better understanding of learning and memory. Mice models are used to investigate the effects of brain damage, permanent or temporal inactivation of neuronal activity transmission in defined brain areas, as well as novel compounds on cognition, among many others. Moreover, investigators produced many strains of mutant organisms to examine the roles of different genes in cognitive behaviour. However, the focus on this domain of behaviour neglects the other possible altered responses of these animals, some of which may influence performance on cognitive tasks. In all behavioural tests, the responses of the experimental subjects towards a given stimulus or a defined challenging environment are measured. These responses are later interpreted and extrapolated as the presence or absence of a successful learning and/or memory recollection episode. Notwithstanding, those responses do not depend solely on the experimental subjects' learning and memory abilities, but also on its general health, well-being, reflexive and motor capabilities, emotionality, anxiety and many other physiological conditions of the experimental subject. Thus, due to the inherent complexity of behavioural tests, their use requires consideration of several aspects such as sensory-motor function, anxiety-like and depressive-like behaviours, innate fear responses, social interactions, and various forms of cognitive functions. A large variety of rodent behavioural tests are currently being used to evaluate these traits before testing learning and memory abilities, and thus avoid their influence on the performance of cognitive tasks. In this section, some of those tests, referred to as pre-tests will be introduced, together with memory tests which assess different types of memory. Only the tests used in the present study were included in this section.

1.1.3.1 Pre-tests

The following tests were performed to evaluate general traits, such as locomotor activity, exploration of new environments, anxiety related behaviours and olfactory functions, as well as basic cognitive functions of defined groups of experimental subjects:

1.1.3.1.1 Open field

The open field test was originally introduced as a measure of emotional behaviour in rats (Hall 1934), however open field exploration was proved to be equally applicable in mice (Christmas and Maxwell 1970). The procedure consists of exposing an animal to an unknown environment from which escape is prevented by surrounding walls (Walsh and Cummins 1976). Anxiety behaviour in the open field is triggered by two main factors: individual testing, as the animal is separated from its social group, and agoraphobia, created by the brightly lit, unprotected, novel test environment, which is also very large relative to the animal's usual environment. Rodents will typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls (thigmotaxis), than the unprotected centre area. Mice that spend significantly more time exploring the unprotected centre area demonstrate anxiolytic-like baseline behaviour (Prut and Belzung 2003). Hence, this test offers the opportunity to systematically assess novel environment exploration, general locomotor activity and provides an initial screening for anxiety-related behaviour in rodents (Prut and Belzung 2003).

1.1.3.1.2 Odour habituation and dishabituation

Olfactory information is essential for a wide range of mouse behaviours, including navigating, foraging, kin recognition, bond formation and parental behaviours, among others (Kavaliers et al. 2005, Doty 1986, Brennan and Keverne 2004, Brennan 2004). Many behavioural tasks used to test learning and memory abilities depend primarily on olfactory cues. Since olfactory deficits can interfere with performance and led to spurious results, accurate assessment of olfaction is critical for proper interpretation of various mice behaviours, especially within the social domain. The olfactory habituation and dishabituation test relies on the animals' tendency to investigate longer novel odours than familiar ones. This tendency is used as a paradigm to assess whether the animal can detect and distinguish between different odours. The test consists of sequential presentations, within a short inter-trial interval, of the same odour, followed by a last presentation of a mixture of the previously presented odour together with a novel one. Habituation is defined by a progressive decrease in olfactory investigation towards a repeatedly presented odour stimulus, due to the increase of familiarity since it is no longer perceived as being novel and thus the interest to investigate it diminishes. Accordingly, dishabituation is defined by the reinstatement of the olfactory investigation when a novel odour is presented, due to its novelty. In addition to reducing experimental confounds, this test can provide information on the functioning of the olfactory system of new knockout, knock-in, and conditional knockout (cKO) mouse lines.

1.1.3.2   Recognition memory assessment

Recognition memory, a subtype of declarative memory, is defined as the ability to identify a particular item that has been previously encountered. In that sense, recognition memory is fundamental for the ability to separate familiar from novel items to guide prospective behaviour (Aggleton and Brown 2006). This ability can be studied in mice models. Tasks assessing recognition memory exploit the natural tendency of rodents to explore novel items longer compared to familiar ones (Ennaceur 2010). Its application enables the study of various parameters, such as the ability to recognize the items and to later remember them, thus analysing the preference for novelty, for which the recognition of the familiar item is needed. The non-conditioned nature of these tasks, since they require no external motivation, reward, or punishment, allow animals to be tested repeatedly, e.g. under different treatment conditions, which provides a high statistical power in data analysis and allows the detection of side effects that may affect the animals' behaviour.

1.1.3.2.1   Novel object recognition

The spontaneous novel object recognition (NOR) test has emerged as the most popular test for assessing a rodents' ability to recognize previously presented stimuli (Ennaceur and Delacour 1988). The NOR test basically consists of two sessions of 10 min each. During the first session (training), the experimental subject is placed in a rectangular high-walled arena lacking polarizing spatial cues, in which two identical objects are placed and thus the experimental subject can explore freely. After a defined inter-trial interval, which depends on the type of memory under study, the memory session takes place. During the memory session the experimental subject is again placed in the NOR arena, however this time one of the objects has been replaced by a new one. Significant longer exploration of the novel object compared to the exploration duration of the familiar object is interpreted as recognition of the familiar object. Using this test, short-term and long-term object recognition has been observed in mice (Vogel-Ciernia and Wood 2014). Despite its popularity, the underlying neuronal circuitry and mechanisms supporting object recognition are still being studied. In particular, the role of rodent HC in object memory remains highly debated due to conflicting findings across temporary and permanent HC lesions studies and evidence that the perirhinal cortex may support this type of memory. Recently, a great number of studies were reviewed by Cohen and Stackman (2015), who concluded that the HC is necessary for the retention of object recognition memory when a delay greater than 10 min was imposed between the NOR training session and the memory session. In the case of shorter delays, formation of object memory is mainly dependent on information processing in the perirhinal cortex (Cohen and Stackman 2015). Thus, the non-conditioned nature of the NOR task provides

an excellent tool to test HC-dependent memory, if delays longer than 10 min are used between training and memory sessions.

1.1.3.2.2 Social recognition memory

Several non-conditioned paradigms have been widely used in order to measure social recognition, exploiting the spontaneous investigatory behaviour of the animals towards conspecifics, thus showing more interest in investigating a conspecific than an object, as described previously (Perna et al. 2015).

The first experimental model to test social recognition, based on rats' olfactory social capacities, was introduced by Thor and Holloway (Thor and Holloway 1982) and revised by Dantzer et al. (Dantzer et al. 1987). Social recognition was measured as the ratio of the investigation duration in two or more successive exposures, during which significantly reduced investigation duration during the second or following exposures, indicated recognition of the juvenile (Thor and Holloway 1982). The relatively fast extinction of juvenile recognition abilities allows the use of the same experimental subjects repeatedly in different sessions to serve as their own controls. Despite the popularity of this test, it presents some difficulties in data interpretation as repeated testing of the same animal can lead to nonspecific behavioural changes, such as sensitization to the testing procedure (Engelmann et al. 1995). Further, this test has only limited suitability to analyse the duration of the recognition memory performance. As an alternative method, Engelmann et al. (1995) first described the **social discrimination test (SDT)** for the investigation of short-term olfactory memory processes in rats, solving the main limitations present in the social recognition test. The SDT was later adapted and tested for its use with mice (Engelmann et al. 2011). This paradigm is composed of only two sessions, with the first consisting of a given stimulus animal being introduced into the cage of the experimental subject, allowing direct interactions, thus the acquisition of the information coded by the stimulus animal's olfactory signature. This session ends with the removal of the stimulus animal from the experimental subject's cage after four minutes. In the subsequent session, separated by the desired exposure interval (EI), two stimuli animals are introduced at the same time in the experimental subject's cage; the familiar stimulus animal (presented in the first session) together with an unfamiliar one. During this session, which also lasts for four minutes, significantly longer investigation duration towards the unfamiliar stimulus animal compared to the familiar one is interpreted as intact social recognition memory. The main differences between the social recognition test and the SDT is that the latter measures the presence or absence of recognition categorically; the SDT presents a choice to the experimental subject as not only is the previously encountered conspecific presented (= social recognition test) but also, simultaneously, a previously not encountered conspecific. Thus, the experimental subject is allowed to discriminate between both stimuli animals simultaneously in one session. This

provides an internal control under identical experimental conditions and allows the separation of specific from non-specific effects in pharmacological studies and thereby reducing the number of sessions for a given experimental series. By using different EIs, the SDT enables the investigation of the impact of manipulations on the different types of memory. Moreover, the SDT allows the emergence of social memory in animals that appeared to possess no social recognition when tested in the social recognition test, thus showing a higher sensitivity in assessing this type of memory (Montkowski et al. 1995, Choleris et al. 2006).

Using the SDT, the performance of mice and rats has been investigated and revealed interesting findings: Mice show a memory performance that lasts at least 24 h, whereas rats form short-term social recognition memory only (Noack et al. 2010). A more detailed analysis in male rats revealed recognition memory to be extinct after ~ 45 min, whereas female rats show a slightly, but significantly longer recognition lasting ~ 2 h (Dantzer et al. 1987, Engelmann et al. 1998). Although a large number of studies confirm the absence of long-term memory in rats, it must be noted that a few published studies suggested that male rats acquire long-term social recognition memory and retained it for at least a week, attributing the discrepancies with the rest of studies to the different housing conditions (Shahar-Gold et al. 2013) (further discussed in (Camats Perna and Engelmann 2015)).

Later, our research group described a variation of the SDT, using the **volatile fraction cage** (VFC) (Engelmann et al. 2011). In this test the juveniles are confined in two tubes separated by two fences from the experimental subjects' cage, preventing direct tactile contact. The tubes are connected to two fan units which provide an air stream towards the experimental subject's cage, facilitating only the access to the volatile fraction of the olfactory signature of the stimuli animals. The VFC is suitable for studies aimed at discriminating the relevance of each fraction of the olfactory signature, the volatile and the non-volatile, in order to establish the social recognition ability (Noack et al. 2010). Using this variation of the SDT, differences between mice and rats in the processing of the different fractions of the olfactory signatures of respective conspecifics were confirmed. Mice recognize juvenile conspecifics on the basis of both, the volatile and the non-volatile components of their olfactory signatures. However, mice are also able to form long-term memory by just having access to the volatile fraction. Rats, in contrast, require access to the non-volatile fraction of the olfactory signature, which is predominantly processed by the AOB and results in short-term recognition memory only (Noack et al. 2010). Due to the widely described differences between rats and mice in social recognition memory and even in the processing of the conspecifics' olfactory signature, for the rest of the manuscript I will focus my attention on studies carried out only with mice (when referring to social recognition memory), if not stated otherwise.

1.1.3.3 Pavlovian fear conditioning

Associative learning is an adaptive process that allows an organism to learn to anticipate events, as Ivan Pavlov first described nearly 100 years ago (Pavlov 1927). Pavlovian fear conditioning represents a form of associative learning that has been widely studied in many species (Kim and Jung 2006). The dependent measure used in fear conditioning tests is the freezing response that takes place following pairing of an unconditioned stimulus (UCS) such as a foot shock, with a conditioned stimulus (CS), such as a particular context or an auditory cue. These tests elicit robust associative learning and is a commonly used paradigm to study emotional learning and memory in rodents (Johansen et al. 2011, Endres and Lessmann 2012, Phillips and LeDoux 1992, Fanselow and Kim 1994, Caldarone et al. 1997b, Caldarone et al. 1997a). Under laboratory conditions, fear conditioning tests trigger fear responses in the experimental subjects with two different types of conditioned stimuli, cued and contextual, each engaging different brain structures (Kim and Fanselow 1992). Thus, animals are trained to associate a foot shock with either a context (contextual fear conditioning) or/and a tone (cued fear conditioning). During **contextual fear conditioning**, in order for context learning to occur, animals must first form a representation of the context. After it has been encoded, context representations can themselves come to be associated with other events, such as the occurrence of an aversive foot shock, UCS. Hence, during the typical contextual fear-conditioning procedure, animals first encode a representation of the context and then associate that representation with the UCS. **Cued fear conditioning** is similar to contextual conditioning, with one notable exception: instead of contextual conditioning, a tone (or another mono-modal stimulus) is used as a cue to induce the conditioning of the experimental subject. In the cued fear conditioning, the memory session is performed in a different context than the one in which the experimental subject was conditioned, thus the fear response is induced by the presented cue (tone) and "independent" of the context. Memory is assessed by comparing the freezing behaviour of the animal under the conditioned condition *vs.* the non-conditioned or habituation session. Since fear conditioning occurs very rapidly (capable with a single CS-UCS pairing) and induces a lasting effect, this task has become a popular behavioural tool for studying the cellular-molecular substrates of fear learning and memory (Davis 1997, Lavond et al. 1993, LeDoux 2000, Maren et al. 1996b).

A large body of evidence, from lesions to pharmacological studies, point to the amygdala as the key neuronal system behind fear conditioning (Davis 1997, Fendt and Fanselow 1999, Lavond et al. 1993, LeDoux 1996). Anatomically, the amygdala receives sensory inputs from diverse areas of the brain (e.g. thalamus, neocortex, olfactory cortex, HC) and sends projections to various autonomic and somatomotor structures thought to mediate specific responses (e.g. bed nucleus of stria terminalis) (LeDoux 1996). It is commonly accepted that

sensory information enters the amygdala through its basal and lateral nuclei (Aggleton et al. 2000) where CS - UCS association (or fear memory trace) formation is believed to take place (Goosens and Maren 2001). Rodents with lesions in the lateral and central nucleus of the amygdala demonstrate a lack of freezing in the presence of cues previously paired with foot shock (Goosens and Maren 2001, Wilensky et al. 2006). Thus, suggesting that contextual and cued fear conditioning responses are mediated by the amygdala, however it is unclear whether the permanent storage for long-term fear memory remains also in the amygdala. However, studies showed that if the amygdala is lesioned or reversibly inactivated shortly (1 day) or long (7 or 28 days) after training effectively abolished conditioned freezing response (Maren et al. 1996a, Wilensky et al. 2006), thus placing the amygdala as the default fear learning structure.

Contrary to the involvement of the amygdala in fear conditioning, the HC seems to be involved in defined procedures of conditioned fear memory. An earlier study suggested that the HC is transiently involved in storing contextual fear memory (Hirsh 1974). Later investigations extended this finding by showing that pre-training HC lesions selectively block the acquisition of context fear memory, but not cued fear memory (Phillips and LeDoux 1992, Maren and Quirk 2004). These results were also supported by several knockout / transgenic mice studies. Specifically, mutant mice with deficient long-term potentiation in the HC exhibit deficits in contextual, but not tone, fear conditioning (Abeliovich et al. 1993, Bourtchuladze et al. 1994). Moreover, strong additional evidence were provided by a study, in which it was demonstrated that contextual fear memory and HC long-term potentiation deficits observed in brain-derived neurotropic factor (BDNF) knockout mice could be rescued by infusing BDNF proteins into the HC (Liu et al. 2004), thereby demonstrating the specific HC involvement in contextual fear memory formation. Similar results were obtained when the Ras signalling cascade was inhibited in these areas, thus impairing cued (Schafe et al. 2000) and contextual (Athos et al. 2002) fear memory when infused in the lateral amygdala and the HC, respectively (Kelleher et al. 2004, Shalin et al. 2004).

As a consequence of the different brain areas involved in the different procedures of the fear conditioning test, this task is often heralded as a tool to index both, HC and amygdala-dependent contextual fear memory and HC-independent but amygdala dependent cued fear memory.

1.2   Growth factor receptor-bound protein 2

The growth factor receptor-bound protein 2 (Grb2) is a ubiquitously expressed adaptor protein, which to date, is the most studied member among the growing family of adapter proteins. Adaptor proteins are made of non-catalytic domains, such as Src homology-2 (SH2), Src homology-3 (SH3), phosphotyrosine-binding, PDK, and

WW domains (Pawson and Scott 1997). These domains allow adapter proteins to bind to a diverse number of proteins so that multiple signal transduction pathways can be activated.

Grb2 is essential for multiple cellular functions, the impairment of which induced defective development processes in various organisms and blocked transformation and proliferation of various cell types (Tari and Lopez-Berestein 2001). Mouse embryonic stem (ES) cells, homozygous for the Grb2 null mutation are defective in their endoderm differentiation and epiblast development, thus homozygous mutations in Grb2 lead to embryonic lethality (Simon et al. 1993, Cheng et al. 1998). Sequencing of the *grb*2 gene showed that it encodes for a protein composed of one SH2 domain flanked by N- and C-terminal SH3 domains (Lowenstein et al. 1992) (Fig. 2). Grb2 uses its SH2 domain to bind to tyrosine-phosphorylated residues in growth factor receptors. At the same time, Grb2 uses its SH3 domains to bind to proline-rich-containing motifs found in various signalling molecules (Chardin et al. 1993, Li et al. 1993, Rozakis-Adcock et al. 1993).

Fig. 2. Schematic illustration of Grb2 adaptor protein domains. Grb2 is a 217 residue protein with an expected molecular mass of 25,206 Da. Grb2 consists of a single Src homology 2 (SH2) domain flanked by N- and C-terminal Src homology 3 (SH3) domains.

1.2.1   Grb2 in neuronal signal transduction

In neurons, the adaptor protein Grb2 is thought to integrate extracellular signals, such as growth factors or neurotrophins, through the activation of receptor tyrosine kinase (Trk), and link them to intracellular cascades and its downstream kinases, for instance extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2) (Borrello et al. 1994, Nimnual et al. 1998, MacDonald et al. 2000).

Binding of neurotrophins to Trk receptors results in their dimerization and the autophosphorylation of the tyrosine residues in their cytoplasmic regions (Fig. 3). The phosphorylation of tyrosine residues recruit several adaptor proteins, including Grb2, which are able to induce intracellular signalling cascades, including; (1) the phosphatidylinositol-3-kinase-akt pathway, involved in promoting neuronal survival (Vaillant et al. 1999); (2) the phosphatidylinositol-specific phospholipase Cγ1 (PCLγ1) pathway, which was described to control expression and / or activity of many proteins, including ion channels and transcription factors, however whether Grb2 is involved in this process is still discussed (Minichiello et al. 1999, Minichiello et al. 2002); or (3) the Ras-ERK

pathway, described to control synapse formation and synaptic plasticity, as well as long-term potentiation in memory formation (Chao 2003, Huang and Reichardt 2003, Huang and Reichardt 2001) (see also Huang and Reichardt (2003) or Reichardt (2006)).

Fig. 3. Representation of the Ras signalling cascade in which Grb2 acts as an adaptor molecule. This pathway is activated by growth factors as neurotrophins mediating the Trk autophosphorylation, triggering a cascade of reactions, modulated by Grb2 (green), which provide a complete link between the cell surface and the nucleus. Activated Erk can translocate into the nucleus to phosphorylate and activate transcription factors, such as CREB or activator protein 1, which controls the expression of several immediate early genes, such as c-fos (red). Modified from *Increasing complexity of the Ras signalling pathway* (Vojtek and Der 1998)

Thus, the best-known cascade regulated by Grb2 and involved with learning and memory processes is the Ras-Erk pathway (also known as mitogen-activated protein kinases (MAPK) cascade). Activated Trk receptors can associate with Grb2 via direct interaction (MacDonald et al. 2000) or via interactions with Src homology domain-containing transforming protein (Shc) (Borrello et al. 1994), fibroblast growth factor receptor substrate 2 (Kouhara et al. 1997, Zhou et al. 2015), SH2-B, and rAPS (Qian et al. 1998). Grb2 recruits son of sevenless (Sos), a guanine nucleotide exchange factor that acts on Ras. On growth factor stimulation, the Grb2-Sos complex is recruited to the plasma membrane, where it uses its SH2 domain to bind to growth factor-stimulated Trk. This binding allows Sos to be in close proximity to Ras, beside the intracellular region of Trks, which is also localized in the plasma

membrane, thereby activating the Ras-Erk signalling pathway (Lowenstein et al. 1992, Buday 1999) (Fig. 3). Ras is a small GTPase protein, which is active when bound to guanosine triphosphate (GTP), and inactive when bound to guanosine diphosphate (GDP) (Downward et al. 1990). Guanine nucleotide exchange factors, such as Sos, are positive regulators of Ras activity that increases the exchange of GDP and GTP (Simon et al. 1991) (Fig. 3). All this data places Grb2 downstream of the growth factor receptor, however upstream of Ras (Alonso et al. 2002) (Fig. 3). The Ras/Raf/MEK/Erk cascade, which is often referred as the MAPK cascade, is the best known Ras signalling pathway. Ras binds to Raf in a GTP-dependent manner; the binding of Ras to Raf leads to Raf activation (Warne et al. 1993) (Fig. 3). Activated Raf stimulates the activity of MEK (Crews et al. 1992), which in turn phosphorylates and induces the activity of Erk1 and Erk2 (Boulton et al. 1991). Activated Erk1 and Erk2, the two best characterized serine / threonine kinases, translocate to the nucleus and activate transcription phosphorylating transcription factors such as CREB, Elk-1, c-Myc and activator protein 1 (Rao and Reddy 1993, Chuang and Ng 1994, Whitmarsh and Davis 1996, Impey et al. 1998) (Fig. 3). These transcription factors are involved in the transcriptional regulation of IEGs, as for example *c-fos*, which upregulates transcription of a diverse range of other genes and thereby inducing long-lasting synaptic changes (Skolnik et al. 1993, West et al. 2001, Mazzucchelli and Brambilla 2000). Despite the relevance of this cascade for memory formation, neither the requirement of Grb2 downstream of Trk receptors in different learning and memory tasks nor the mechanisms of proper distribution of Grb2 in neuronal cells, is well described.

1.2.2   Role Grb2 plays in memory formation

As previously introduced, in response to extracellular signals Grb2 brings various proteins into close proximity, forming molecular complexes that propagate intracellular signals and induce cellular responses (Buday 1999). These complexes have been implicated in cytoskeletal rearrangement, cellular adhesion, and vesicle docking, cellular events that contribute to changes in synaptic efficacy during development (Luo 2000), but also in memory formation during adulthood (Lamprecht et al. 2002, Zhao et al. 1999, Huang and Reichardt 2001, Huang and Reichardt 2003, Chao 2003).

The Ras signalling cascade, induced by Trk activation and described to be modulated by Grb2, is critical for processes such as neuronal plasticity and memory formation (Alonso et al. 2002, Lu et al. 2011, von Bohlen et al. 2007), due to its relevance for induction and maintenance of long-term potentiation (Zhu et al. 2002). Consequently, its alteration had detectable behavioural consequences. Pharmacological inhibition of TrkB during training blocked consolidation, nonetheless intact short-term memory was observed (Lu et al. 2011). Moreover,

downregulation of effectors from the Ras signalling cascade, such as Erk, produced similar consequences as those obtained with the inhibition of different kinases of the Ras signalling cascade (Blum et al. 1999, Selcher et al. 1999, Atkins et al. 1998, Schafe et al. 2000), and also observed in Erk2 cKO mice which exhibited impaired long-term memory (Satoh et al. 2011). Interestingly, the Grb2 complex has also been related to HC-dependent learning events, for instance during spatial learning (Zhao et al. 1999). Moreover, a previous study linked the Ras signalling pathway activated through the Shc adaptor protein molecule (which interacts with Grb2 / Sos complex) to long-term memory formation *in vivo* in area CA1 of the HC, in a time dependent manner via BDNF-induced CREB activation (Alonso et al. 2002). Thus, the Ras signalling pathway seems to be an essential cascade, induced by growth factors and activating gene transcription, to form long-term memories (Brambilla et al. 1997, Darcy et al. 2014, Chen et al. 2006). However, and despite the high amount of research studying the Ras signalling cascade in learning and memory processes, there are still some discrepancies around which effectors from Trk are the relevant for each of those processes, thereby further studies are needed to investigate the modulation of this cascade, for instance by the different adaptor proteins (Minichiello et al. 2002, Gruart et al. 2007, Margolis and Skolnik 1994, Oku et al. 2012).

It is important to note that despite the likely Grb2 modulation of learning and memory processes through the Ras signalling cascade, other relevant kinases and pathways have been proposed to interact with, or being modulated by, this adaptor molecule. For instance, epidermal growth factor receptor activation and subsequent transmission by the Ras-Erk pathway was described to be modulated via-Grb2 mediated recruitment of protein kinase A type I (PKA) (Tortora et al. 1997), which has also been suggested to be involved with CREB activation (Impey et al. 1998). The p190 RhoGAP/ROCK pathway, which regulates the morphology of dendrites and axons during neuronal development, was found to play a central role, through a Grb2-mediated molecular complex, in fear memory formation in the amygdala (Lamprecht et al. 2002). Also related with the modulation of the neuronal cytoskeleton by extracellular signals, microtubule-associated protein 2 was also described to interact with Grb2 (Lim and Halpain 2000). Moreover, 2 out of 319 different gene products in the HC were dysregulated in mice polytransgenic for chromosome 21, one of them being *Grb2*. The mentioned study concluded that decreased Grb2 levels in the HC of 152F7 mice, seemed to contribute to impaired cytoskeleton functions (Shin et al. 2007, McPherson et al. 1994). Grb2 was also described to interact with disrupted-in-Schrizophrenia-1, modulating neurotrophin 3-induced axon elongation through Erk activation in cultured HC neurons (Shinoda et al. 2007). Thus, although not as broadly studied as the interaction of Trk / Grb2 / Sos and further activation of the Ras

signalling cascade, Grb2 was also described to interact with other molecular pathways likely involved in the process of learning and memory.

Overall, it is clear that growth factors have direct effects on synaptic transmission, plasticity and their possible behavioural correlates, however the downstream mechanisms that mediate these effects and the involvement of adaptor proteins, such as Grb2, is not completely understood. Thus, more detailed studies are needed to understand under which conditions Grb2 induces the Ras signalling cascade and whether other molecular pathways are affected by its interaction with other molecules.

1.3   Cre-*loxP* system to create conditional knock-out mice

The generation of mutant mouse harbouring targeted inactivation of desired genes, using homologous recombination in ES cells, is a powerful tool to analyse their role in complex brain functions such as learning and memory, synaptic plasticity as well as neurogenesis and neuronal cell death (Anagnostopoulos et al. 2001, Bolivar et al. 2000, Chen and Tonegawa 1997). However, inactivation in the germline often results in a lethal phenotype that prevents further analysis of the targeted gene in the adult brain.

To bypass early lethality and to analyse functions of a gene particularly in the adult brain, the Cre/*loxP*-recombination system that allows to conditionally excise *loxP*-flanked DNA segments was implemented. In the Cre/*loxP*-recombination system the bacteriophage P1 Cre-recombinase (Cre) which catalyses defined DNA recombination events between specific target sites termed *loxP* (Hamilton and Abremski 1984) is expressed in a cell-type specific manner. In the Cre expressing cells, the recombinase mediate excision of an essential part of the targeted gene that has been flanked by two *loxP* recognition sequences in the same orientation (Nagy 2000, Gu et al. 1994). *LoxP* sites are composed of two 13 base pair (bp) inverted repeats and an asymmetric 8 bp spacer sequence, which endows individual *loxP* elements with a defined orientation (Hoess et al. 1982). Importantly, both activity and specificity of Cre are retained in eukaryotic cells (Sauer and Henderson 1989), permitting the use of Cre as a genetic engineering tool in any cellular context.

In most cases, Cre and *loxP* containing strains of mice are developed independently and then crossed to generate offspring with the tissue-specific gene knockout (Fig. 4). The first mouse strain contains a targeted gene flanked by two *loxP* sites (floxed gene). The location of *loxP* sites has to be appropriately chosen so that the function of the gene is not affected and that deletion of the floxed gene segment will lead to the inhibition of transcription and / or translation of the gene of interest, or to the synthesis of a non-functional protein (Misra and Duncan 2002). The mouse strain harbouring the floxed gene can be crossed to any other strain of mice expressing

Cre in a specific tissue, cell type, or in a developmentally regulated manner. Thus, the second mouse strain is a conventional transgenic mouse line expressing the Cre under the control of a promoter that is specific for a particular cell or tissue type. When the mouse strain containing the floxed gene and the Cre-expressing mouse are crossed, a proportion of the offspring will inherit both the floxed gene and the Cre-expressing transgene. In the tissue where the Cre is expressed, the DNA segment flanked by the *loxP* sited will be excised, and consequently inactivated. The targeted gene flanked by *loxP* sites remains active in the cells and tissues that do not express Cre (Fig. 4).

Fig. 4. Generation of tissue / cell-specific knockout mice using the Cre-*loxP* system. In the mouse line containing the Cre-recombinase (CRE), the expression of CRE is under control of a tissue/cell-specific promoter, such as calcium/calmodulin-dependent protein kinase type II subunit α (CaMKIIα). The floxed target gene mouse line contains *loxP* sites flanking the region of the target gene to be deleted. When the two mouse lines are bred together, the CRE enzyme recognizes the *loxP* sites and deletes the intervening DNA sequence only in tissue/cells where the CRE is expressed. When CRE expression is controlled by CaMKIIα promoter, the floxed gene will be excise in excitatory neurons from the forebrain. The target gene remains floxed and functional in all the other tissues. pA: poly (A) tail.

Conditional deletion of floxed genomic DNA sequences has become a rather standard approach for assessing tissue, cell type or developmental stage specific gene functions (Nagy 2000, Branda and Dymecki 2004).

1.3.1   Cre-recombinase expression controlled by CaMKIIα-promoter

The Cre/*LoxP* system is a well-established approach to spatially and temporally control genetic inactivation. The promoter of the α subunit of the calcium/calmodulin-dependent protein kinase II gene

(CaMKIIα) limits expression to specific regions of the forebrain and thus can be used to induce brain-specific disruption of a floxed gene using the Cre/*LoxP* system.

An 8.5 kilobase 5' flanking genomic fragment of the gene CaMKIIα has been shown to confer brain specific expression on a variety of transgenes in mice, including Cre (Mansuy et al. 1998, Mayford et al. 1995, Tsien et al. 1996). The CaMKIIα gene is expressed with tissue-specificity predominantly in the adult forebrain (Benson et al. 1992, Jacobs et al. 1993). This gene encodes the α subunit of a serine-threonine protein kinase that is involved in the regulation of a diverse set of cellular processes, including synaptic plasticity (Mayford et al. 1996b, Stevens et al. 1994). The expression of the endogenous gene is developmentally regulated, since CaMKIIα is not expressed during embryogenesis and is barely detected in the first few postnatal days (P). Expression increases ten-fold between P1 and P21, with a further 2.5-fold increase by P90 (Zou et al. 2002, Sugiura and Yamauchi 1992, Colbran 1992). This significant increase during the second and third postnatal weeks coincides with the most active period of synaptogenesis in the forebrain. A CaMKIIα - green fluorescent protein (GFP) transgenic mouse line was used for the neuroanatomical characterization of GFP expression in the central nervous system, providing detailed information regarding the expression of CaMKIIα in the mouse brain (Wang et al. 2013). The granular layer of the DG of the HC was where CaMKIIα -GFP was expressed the most. Within the HC a strong expression was also observed in pyramidal cells layer within field CA1 and CA3, being lower in CA2 area. Overall, in the HC, around 70 % of granule and pyramidal neurons expressed strong GFP. In the neocortex, presumed pyramidal neurons were GFP positive: around 32 % of layer II/IIII and 35 % of layer VI neurons, and a lower expression rate was found in other layers. In the thalamus and hypothalamus, strong GFP signals were detected in the neurophil. GFP-positive cells were also found in many other regions such as spinal trigeminal nucleus, cerebellum and basal ganglia, however in very low amounts (Wang et al. 2013). High levels of CaMKIIα were also detected in the olfactory bulb (Zou et al. 2002). In the piriform cortex and the BLA, as in the majority of cortical regions CaMKIIα was expressed in the glutamatergic neurons but not in the GABAergic neurons (Zou et al. 2002, McDonald et al. 2002, Benson et al. 1992, Jones et al. 1994, Benson et al. 1991).

Thus, the control of Cre expression under the promoter of CaMKIIα using the Cre/*LoxP* system is a convenient tool to study the relevance *in vivo* of a target gene and encoded protein in specific brain processes, such as memory acquisition, consolidation or recall (Minichiello et al. 1999). Behavioural phenotypes identified by transgenic mice may lead to important medical applications essential to further understand the genetic basis of behaviour.

2   Aims and experimental approach

As explained above, learning and memory are processes which constantly adjust our behavioural responses to everyday life situations. For animals living in communities, social memory is one of the most relevant abilities to recognize conspecifics and thus built proper social interactions. It is known, that social memory formation consists of a complex cooperation between different brain areas activating multiple cascades, which induce defined molecular reactions. Nonetheless, this work is focused on the analyses of the interplay of inputs to and circuits within the HC. The HC has been widely studied for its role in learning and memory, and was described to be essential for social memory formation, consolidation and, although less studied, retrieval. However, despite the relevance of this type of memory, there is little information available about the underlying mechanisms involved in these processes. Moreover, there are salient controversies between behavioural and molecular studies regarding the involvement of the HC in the acquisition of social memories which need to be addressed in more detail. Thus, in order to study the HC involvement and the underlying mechanisms implicated in social memory formation, two main aims were set in the present work:

- o   To better understand the HC involvement during the acquisition of social cues essential for social memory formation (short-term and long-term social recognition memory) distinguishing between the dHC and the vHC.

- o   To study whether HC-molecular pathways modulated by Grb2 are involved in social recognition memory formation.

In order to achieve the reported aims the following experimental approach was followed:

Firstly, the neuronal HC activation induced by a social encounter should be assessed, followed by the study of the functional involvement of the activation observed to form social recognition memory. As it has been previously described, mice can form short-term and long-term social recognition memory either when they have direct access to the stimulus animal's social cues, including both fractions of the olfactory signature (direct exposure), or when only the volatile fraction of the olfactory signature is presented (indirect exposure) (Noack et al. 2010). In order to study whether the different fractions of the olfactory signature, volatile and non-volatile, are processed differently in the HC, both types of exposures, direct and indirect, were included in the experimental design. Neuronal activation induced by direct and indirect social exposures was studied measuring the increase of c-Fos synthesis in the HC and other defined brain areas. For the quantification and analyses of c-Fos positive cells,

the HC was divided in its septotemporal axis thus distinguishing between its dorsal and ventral parts. Once revealed the HC-activation pattern induced by the direct and the indirect social stimulation, the functionality of each HC part during the acquisition of the information coded by the distinct fractions of the olfactory signature was tested *in vivo*. Temporal bilateral dHC- or vHC- inactivation was used to block the neuronal activity only during the acquisition of the information coded by a conspecific's social cues, under direct and indirect social exposure. The inactivation lasted approximately 20 min, thus the HC function was again intact shortly after the acquisition session. The ability to recognize the familiar conspecific one hour and 24 hours after the acquisition session were tested on those mice. Thus, dHC- and vHC-temporal inactivation allowed us to determine whether the observed neuronal activation induced by a social encounter in these HC parts was essential to form either short-term or long-term social recognition memory, if any. With this approach we aim to reveal whether distinct parts of the HC are involved differently during the acquisition of the information coded by a conspecific's social cues in order to form social recognition memory, and to better understand how the olfactory signature, distinguishing between the volatile and the non-volatile fraction, is processed by these distinct parts of the HC.

Secondly, the underlying molecular pathways necessary to form social recognition memory were investigated. Ras-MAPK cascade, which has been described as being essential for learning and memory processes, is one of the most studied pathways regulated by Grb2 adaptor protein after being induced by Trk activation. To achieve the second aim, Grb2 conditional knock out (cKO) mice were created, in which expression of Grb2 adaptor protein was downregulated postnatally only in the anterior forebrain, using the Cre/*loxP* technology. This mouse line was generated in order to study whether the downregulation of Grb2-dependent signalling cascades are involved in social recognition memory formation. However, since it is a new transgenic mouse line, previous studies had to be performed before social recognition abilities were tested. Specific downregulation of Grb2 protein expression in the forebrain and the absence of any developmental disorder had to be confirmed. A battery of behavioural tests was also performed in order to discard any sensory or motor deficit. An open field test was used to assess a novel environment exploration, general locomotor activity, and to provide an initial screen for anxiety-related behaviour (Walsh and Cummins 1976, Archer 1973). To ensure that transgenic mice had intact olfactory functions, the habituation and dishabituation test was also performed, thus assessing the ability to habituate to an odour, and to discriminate between different odours (dishabituation). Once confirmed that the mutant mice did not show any altered phenotype which could affect the outcome of further behavioural tests, social recognition memory was assessed. However, the study of the memory abilities of the mutant mice did not end with the social recognition test, as novel object recognition and fear conditioning memory were also performed

in order to more accurately predict the function of Grb2 in different types of memories, or in different brain areas. Thus, the study of Grb2 cKO mice phenotype could provide direct evidence of Grb2 involvement in the modulation of processes essential for social memory formation.

3 Material and Methods

3.1 Animals

Mice were housed in groups from two to five per cage in transparent polycarbonate type II cages (size: 22 x 16 x 14 cm) under standard laboratory conditions: 22 ± 1 °C, 60 ± 5 % humidity, 12 - h light: 12 - h dark cycle, with lights on at 07:00 a.m., and food and water *ad libitum*. Animals were transferred weekly into fresh cages. Adult mice of different lines including mutant mouse lines (see below) are subsequently termed "**experimental subjects**". All experimental protocols were approved by the local governmental body (Regierungspräsidium, Halle) and all efforts were made to minimise animal suffering during the experiments and housing. All experimental manipulations were performed in strict compliance with the European Commission recommendations for the care and the use of laboratory animals for scientific purposes (2010/63/EU).

3.1.1 C57Bl/6JOlaHsd Inbred mice

Adult male C57BL/6JOlaHsd mice (Harlan-Winkelmann, Borchen, Germany) were used for defined experiments at an age of 9 to 30 weeks.

Juvenile (25 to 35 days old) C57BL/6JOlaHsd mice of both sexes were used as **stimuli animals** in the experiments employing the SDT (see 3.2.5) throughout the study (including experiments with Grb2 cKO mice and their littermate controls). Previous extensive studies in our laboratory demonstrated that neither the sex nor the age within the defined range of the stimuli animals, affected recognition performance of adult male mice of different genotypes significantly *per se* (Engelmann et al. 2011).

3.1.2 Mutant mice

Adults from both sexes of the Grb2 cKO mice (see 3.1.2.3) were used for the behavioural screening or / and biochemical analyses at an age of 9 to 30 weeks old. Only adult males were used as experimental subjects for the novel object recognition test and fear conditioning experiments.

3.1.2.1 Floxed Grb2 mice (Grb2 $^{flx/flx}$)

The preparation of the Grb2 $^{flx/flx}$ mice is described in detail elsewhere (Ackermann et al. 2011). Briefly, the targeting vector was generated containing a floxed second exon of the *grb2* gene and flanking arms by polymerase chain reaction (PCR) cloning. "The short arm ($\sim$ 1 kb) was cloned into the pRAPIDflirt vector

(Hovelmeyer et al. 2007) by introducing *Not*I and *Bam*HI sites. It was amplified with the following primers: Grb-in2S-Not, 5′-AGT GAT <u>GCG GCC GC</u>C ACC ACG CCT GAT GCT TCT A-3′ and Grb-in2AS-Bam, 5′-AGT GA<u>G GAT CCT</u> GGG CTT GTG GAA GGC TTA-3′ (restriction sites underlined). The long arm (~ 5.3 kb) was cloned by one introduced *Fse*I and one already present *Xho*I site and was amplified with the following primers: Grb-in3S-Fse, 5′-AGT GAC <u>GGC CGG CC</u>T TAG CCC ATA GAC ATT A-3′ and Grb-in4AS 5′-GTC ACC GTA AGT GCA GCA GA-3′. The central part with the Grb2 floxed second exon (~ 0.5 kb) was cloned by introducing *Sda*I and *Sal*I sites and amplified with the following primers: Grb-in2S-Sda, 5′-AGT GAC <u>CCT GCA GG</u>C CCA GAC AAC ATA ATT GTC T-3′ and Grb-in3AS-Sal, 5′-AGT GAC GTC GAC CGC CAG AGC ACT AGA ATT A-3′. BALB I ES cells (Noben-Trauth et al. 1996) (derived from BALB/c mice) were transfected with the target vector by electroporation. Clones were screened for homologous integration by PCR and verified by Southern blot with an external probe (generated with primers 5′-ACC CAT AGC CAC TGC GTC AT-3′ and 5′-GAC ATC CAC GGT ATG CCA AG-3′). Positive clones were identified, injected into blastocysts which were transferred into pseudo-pregnant females to produce germline transmission. Mice carrying the correct mutation in their germline were identified with PCR and bred to homozygosity" (Ackermann et al. 2011). Grb2$^{flx/flx}$ mice were obtained from Prof. Dr. Lars Nitschke, Department of Genetics from the University of Erlangen, Germany.

3.1.2.2   CaMKIIα-Cre recombinase

These mice expressed a transgene encoding Cre under the control of the promoter for the CaMKIIα. For a detailed description of the generation of this mutant mice line refer to (Mayford et al. 1996a). The expression vector for CaMKIIα-CRE transgenic mice consists of the 8.5 Kb CaMKIIα promoter followed by a hybrid intron in front of the Cre coding region with nuclear localization sequence and a SV40 polyadenylation signal (Mayford et al. 1996a). The transgenic line CaMKIIα–CRE-159 was generated as described elsewhere (Hogan 1994). Southern blot analysis for the recombination of the *loxP*-flanked betaT14 allele (Gu et al. 1994) revealed CaMKIIα–CRE-159 50 % recombination, at an age of eight weeks, in the HC, cortex, and olfactory bulb, but no deletion in the cerebellum, liver, kidney, spleen, or tail (Minichiello et al. 1999). The CaMKIIα-CRE mice were kindly provided by Prof. Dr. Oliver Stork, Institute of Biology from the Otto von Guericke University of Magdeburg, Germany.

3.1.2.3   Generation of Grb2 $^{flx/flx}$ CamCre $^{+/-}$

In mice with homozygous floxed Grb2 allele (designated Grb2 $^{flx/flx}$), the allele sequence was conditionally inactivated via Cre/*loxP*-mediated DNA recombination (*loxP*-mediated excision of exon 2 of *Grb2* gene). Deletion of the floxed *Grb2* gene fragment lead to the deletion of part of the first SH3 domain of the Grb2 protein, as well as to a translational frame shift which produced an aberrant splicing, thereby generating a functionally-null allele (Ackermann et al. 2011). To generate the cKO mice, Grb2 $^{flx/flx}$ mice (see 3.1.2.1) were crossed with mice in which the Cre was selectively expressed under control of the CaMKIIα gene promoter (see 3.1.2.2). Grb2 $^{flx/flx}$ and CaMKIIα-CRE mice were backcrossed to C57BL/6JOlaHsd for at least ten generations before using them for experimentation. The obtained Grb2 $^{flx/flx}$ CaMKIIαCre $^{-/-}$ were used as control littermates (WT) *vs.* the Grb2 $^{flx/flx}$ CaMKIIαCre $^{+/-}$, referred as Grb2 cKO mice.

3.2   Behavioural tests

All behavioural tests were performed during the light phase between 07:00 and 19:00 h. The animals were kept in their original groups between tests.

3.2.1   Open field test

The open field test apparatus was made out of white polyacrylics and consisted of a white square floor (of 100×100 cm^2) and had 50 cm high walls. The floor was divided in 25 different areas (of 20×20 cm^2) with black lines drawn on the floor surface. The outer area or periphery included all the subareas of the field in contact with the walls (64 % of the total area); the rest was defined as centre or inner area (36 % of the total area). Testing was done under uniform bright illumination (170 ± 10 lux) and a background white noise of 50 dB. Experimental subjects were carried to the testing room in their home cages and placed into the centre of the open field and allowed to freely explore it for 10 min. The behaviour of the experimental subjects during the test was recorded using a videorecorder connected to a video camera located above the centre of the arena. The tapes were later analysed by a trained observer, unaware of the animals' genotype, by pressing pre-defined keys on a PC using the Eventlog Event recorder version 1.0. software (Robert Hendersen, 1986). The following behavioural parameters were scored: time spent investigating the open field (sniffing on the field's surface), time spent in the inner area (60×60 cm^2 in the centre of the arena) and in the outer area (periphery), number of times the subject crossed the black lines (discriminating between inner and outer area), the number of rearing (erected posture sustained by the hind paws on the floor) and grooming (including washing or mouthing of forelimbs, hind paws, face, body and

genital) episodes the mice performed during the test. The number of defecations (number of fecal boli produced) and the presence of urination (number of puddles or streaks of urine) were also registered after the end of each test. Before the start of each session, the open field test apparatus was carefully wiped with a 30 % alcohol solution. The open field test procedure was well established in the laboratory and widely used in other studies (e.g. see supporting information from (Hadicke and Engelmann 2013)).

3.2.2   Odour habituation and dishabituation test

Experimental subjects were separated by transferring them to transparent polycarbonate type II cages with fresh bedding 2 h before commencing the test. The test is based on the presentation of two different odours. For the odour habituation 5 µl of diluted (R)-(-)-Carvone (1 / 200) (Sigma-Aldrich, further referred as Carvone) were dropped onto filter paper (1 cm x 1 cm^2), which was placed inside a stainless steel tea egg. The scented tee egg was then placed in the home cage of the experimental subject for 1 min (first trial). Four trials were performed with 10 min intervals between each (habituation). Ten minutes later in a fifth, so-called dishabituation trial, a mixture of Carvone (1 / 200) and Isoamyl acetate (1 / 2000), 1:1, was applied to the filter paper, placed inside the tea egg and presented for one minute to the expeirmetnal subject. During the inter-trials intervals the experimental subject remained in the test cage without the presence of the tee egg. During each trial the duration of investigatory behaviour (sniffing directed to the tea egg with a maximal distance of 1 cm) was measured by a trained observer blind to the treatment / genetic background of the experimental subject, using the Eventlog Event recorder version 1.0. software. After the dishabituation trial, the experimental subject was returned to its original cage. Significantly decreased investigation duration in the fourth trial *vs.* the first habituation trial was taken as intact habituation. An increased investigatory duration in the dishabituation trial *vs.* the fourth habituation trial was taken as an evidence for an intact dishabituation. In addition to measuring habituation and / or dishabituation this procedure may also provide an indication for the presence of an intact short-term olfactory memory. Extensive studies using different mouse lines were performed to confirm the suitability and robustness of this protocol in the laboratory (A Fiedler, unpublished; A Fatima, unpublished).

3.2.3   Novel object recognition

Using the NOR test, the experimental subjects' ability to recognize a familiar object was assessed. The task was performed in a rectangular arena measuring 30 x 45 cm^2 surrounded by 35 cm high walls and made out of stainless steel. The arena was virtually divided into a peripheral and a central zone. The peripheral zone consisted of the 10 cm wide edge next to the arena walls and thus about 80 % of the arena area. Two objects

were placed in the peripheral area, both of them in the same side of the arena and ~ 7 cm from the walls. The positions of the objects were pseudorandomized between different experimental subjects; however the side in which the objects were placed was the same within the first trial (sampling) and the second trial (memory session) for each experimental subject. During the first trial, experimental subjects were placed for 10 min in the NOR arena where two identical objects (children bricks made out of wood, with either triangular or square shape (~ 4 cm x 4 cm) and of either red or blue colour) were presented. Experimental subjects were allowed to freely explore the arena and the objects and then placed back to their home cage in the original animal group. After a given inter-trial interval (1 h or 24 h), during the second trial, one of the two objects previously presented in the first trial was replaced by a novel object and the experimental subject was again allowed to explore the NOR arena with the two objects (novel and familiar) for 10 min. The pairs of objects used had different shapes (triangular *vs.* square) and colours (red *vs.* blue). The novel object always had both features distinct from the familiar object. The objects and arena were thoroughly cleaned with Descosept AF (Dr. Schumacher GmbH, Melsungen, Germany) before the beginning of each session. The test was conducted under dim light conditions (8 lux) and recorded by a ceiling mounted camera which enabled a manually score of the exploration duration towards each object (defined as sniffing behaviour towards the object, within ~ 1 cm from the object and / or physically exploring it, e.g. climbing on it) using the software ANY-maze (Stoelting Co., Wood Dale, IL), by an observer blind to the subjects' genotype. These scores were later used to analyse whether the experimental subject was able to discriminate between the familiar and non-familiar object during the second trial or memory session. The preference index was calculated as the ratio of the amount of time spent exploring the new object over the total time spent exploring both objects during the memory session. Furthermore, exploratory behaviour (total distance travelled, time in the centre or in the periphery) was automatically recorded and analysed using video tracking software ANY-maze. This behavioural procedure was well established and routinely used in the laboratory of Dr. Thomas Endres (Psotta et al. 2015), Institute of Physiology from the Otto von Guericke University of Magdeburg, Germany, where the experiments were performed.

3.2.4 Fear Conditioning

The experimental subjects' ability to learn associative fear was tested using the TSE Fear Conditioning System (FCS, TSE Systems GmbH, Bad Homburg, Germany). The fear-conditioning device consisted of a quadratic box (23 x 23 cm^2) located in a sound-attenuating chamber. A loudspeaker was installed on the ceiling of the chamber to present the CS (tone: 8-kHz single frequency with an intensity of 70 dB for 30 s). The chamber and

box were illuminated with a house light (100 - 200 lux). The floor of the fear conditioning box consisted of a grid floor that allowed the delivery of an electric foot shock (UCS; 0.7 mA, 1 sec) to the experimental subjects. Two levels of infra-red light photo sensor system allowed analysing of the different movements of the experimental subjects within the box during the sessions. Background noise (65 dB, A-scale) was supplied by ventilation fans in each chamber. In order to change the contextual setting between fear conditioning and fear memory retrieval sessions, the walls of the fear conditioning box were made of either transparent (context A) or black Plexiglas (context B). When using context B also the grid floor was covered with a black Plexiglas plate. Furthermore, the boxes were cleaned before the learning session with 70 % alcohol and before the memory session with Descosept AF, in order to provide two different odour stimuli. Memory was assessed in the memory session as the percentage of time mice spent freezing when re-exposed to the conditioned stimulus (either tone or context, see below). The different behaviours such as, travelled distance, rearing episodes, number of jumps or freezing behaviour were scored automatically by the TSE FCS Software. In general, a significant increase in the percentage of freezing behaviour (defined as complete lack of movement, except for respiration) observed in the conditioned condition *vs.* the neutral condition was interpreted as intact associative fear memory. This behavioural procedure, including the modifications detailed below, was routinely used in the laboratory of Dr. T. Endres (Endres and Lessmann 2012), Institute of Physiology from the Otto von Guericke University of Magdeburg, Germany, where the experiments were performed.

3.2.4.1 Cued Fear Conditioning

During the cued fear conditioning test an 8 kHz pure tone was used as CS. The cued fear conditioning consisted of two sessions: the fear conditioning and the fear memory retrieval (or memory session). The fear conditioning session lasted 12 min, during which the CS paired with a co-terminating UCS was presented three times during the last 10 min of this session (after the first 2 min habituation), always separated by randomly chosen intervals in order to avoid periodicity. This first session took place in context A. Twenty-four hours after the fear conditioning session, the associative fear memory of the experimental subjects was assessed in the fear memory retrieval session by exposing them five times to the CS (30 s each) after a 120 s habituation period (without UCS or CS). The fear memory retrieval session lasted 14 min and took place in context B in order to minimize the impact of contextual memory.

To analyse the behaviour of the experimental subjects during each session, they were divided in four different intervals: **habituation** (first 90 s in the new context), **pre-CS** (30 s before receiving each UCS), **CS** (while the CS was presented) and **pause** (time between CS presentations). The behavioural values represented in

the graphs are the mean of each 30 s measurement contained in each interval. During both the conditioning and fear memory retrieval sessions, the **60 s after each CS** presentation were analysed independently, in order to observe the specific response to the CS *vs.* the time of presentation.

3.2.4.2 Contextual Fear Conditioning

Contextual fear conditioning occurred in context A. In the fear conditioning session (10 min), mice were placed in the fear conditioning box during 2 min before they received the first UCS. Two additional UCSs were later presented: the second 2 min later and the last at minute nine, in order to avoid periodicity between the UCSs. In this test the context A was the CS. At the end of the conditioning session, mice were returned to their home cage with the original group. The fear memory retrieval session consisted in two trials: the first took place in a neutral context (context B) while the second took place in the same context as the conditioning session (conditioned context, context A). The first lasted 5 min and took place 24 h after the conditioning session. The second trial took place 1 h later than the first trial and lasted 10 min. The UCS was not presented in any of the trials from the fear memory retrieval session.

To provide a more detailed analysis of the experimental subjects' behaviour during the conditioning session, the behavioural parameters measured were separated in different intervals depending on their timing of presentation *vs.* the UCS presentation: **Habituation** (first two minutes, habituation to the context, before any contact with the UCS), **UCS** (when the UCS was presented) and **Pause** (intervals between UCS presentations). During these session the **60 s after each UCS** presentation were analysed independently, in order to observe the specific response to the UCS *vs.* time of presentation.

To analyse the behaviour during the fear memory retrieval session, the second trial was divided in two parts of 5 min each**; Cond (1 - 5 min)** and **Cond (5 – 10 min),** in order to provide a better comparison of the behavioural parameters measured during these intervals with the ones measured during the 5 min of the first trial (**Neutral**).

3.2.5 Social discrimination test

During the first session of the SDT, called sampling (4 min), a given stimulus animal (SA1) was presented to the experimental subject to allow the acquisition of the information coded by the stimulus animal's olfactory signature (Fig. 5). The SA1 was then removed and kept individually in its cage with food and water *ad libitum,* which initiated the EI. Memory was tested in a second session, called choice (4 min). During choice SA1 was exposed to the experimental subject simultaneously with a second, novel stimuli animal (SA2) (Fig. 5). Different

EIs were used to test for short-term memory (EI = 1 h), intermediate-term (EI = 6 h) and long-term (EI = 24 h) memory.

The computer software Eventlog Event recorder version 1.0 software was used to register the different behavioural parameters shown by the experimental subjects during the test. The following measures during each sampling and choice session were taken by pressing pre-set keys on a computer keyboard by a trained observer unaware of the treatment or genotype of the experimental subject: (1) the total duration of a defined behaviour, (2) the number of bouts and (3) the latency after starting the session until the first bout of the defined behaviour was observed. The selection of the parameters which were used for further analysis depended upon the defined experimental protocol, detailed below. At the end of each choice session, the experimental subjects and the stimuli animals were returned to their original groups in their home cages.

Fig. 5. Schematic drawing illustrating the principle of the SDT. **A.** Scheme of the experimental sessions for testing social recognition memory; sampling (learning session) and choice (recall session). Each session was separated by an exposure interval that defined the type of memory that was being tested (from short-term to long-term memory). **B.** During the sampling session, a stimulus animal (SA1) was presented to the experimental subject (ES), which during this session will acquire the information contained in the olfactory signature of the stimulus animal. After a defined exposure interval, the choice session took place, during which the SA1 was re-exposed to the experimental subject together with a previously not encountered stimulus animal, SA2. In this session the experimental subject will have the chance to recall the information learned during the sampling session recognizing SA1, and thus longer investigating SA2. (Image modified from (Engelmann et al. 2011)).

The SDT was performed in two modifications:

3.2.5.1 The social discrimination test with direct exposure

Two hours before the start of the SDT with direct exposure (SDT_D), the experimental subjects and stimuli animals were transported in their home cages to the testing room and were individually separated by transferring each of them to a transparent polycarbonate type II cage with fresh bedding and food and water *ab libitum*. This procedure allowed the habituation of the experimental subjects to the new cage. Each SDT_D consisted of two 4 min exposures to stimulus animal/s directly in the experimental subjects' cage (Fig. 5). The following EIs were used:

1 h, 6 h and 24 h. During sampling and choice, the duration of the investigatory behaviour of the experimental subject towards SA1 (and SA2; = investigation duration; direct sniffing on the stimulus animal's surface) was monitored. A significantly longer investigation duration of SA2 *vs.* SA1 during choice was taken as evidence for an intact recognition memory (Engelmann et al. 1995). In addition to the investigatory behaviour, aggressive/sexual behaviour (chasing the stimuli animals and (attempting to) bite and mount them, respectively) of the experimental subjects towards SA1 (and SA2) was also measured. The described procedure was established and widely used in the laboratory (Noack et al. 2015, Engelmann et al. 2011).

3.2.5.2 The social discrimination test with indirect exposure

As described in detail by Noack et al. (2010) for investigating SDT with indirect exposure (SDT_I) the VFC was used. The VFC consisted of a transparent polycarbonate type III cage (37 x 21 x 15 cm) with two holes (ø 7 cm) in one side wall where two tubes were entered, each connected to a fan unit. During the sessions the stimuli animals were placed in these tubes. The inner parts of the tubes were separated from the inner part of the cage by a double metallic fence (approximately 2 cm far from each other), hence direct contact between experimental subjects and stimuli animals was blocked. The fans provided an air stream with a flow rate of approximately 1 m/s in the centre of the tube. This air flow crossed the tube were the stimulus animal was placed and transported the volatile fraction of the olfactory signature of the stimulus animal into the type III cage where the experimental subject was kept.

The experimental subjects were transferred to the VFC containing fresh bedding 10 min before starting the session. Animals were tested with EIs of 1 h or 24 h. The stimuli animals were placed in random order within either of the two tubes connected to the fan units to avoid a non-specific preference of the experimental subjects towards a specific location. The VFC, including the tubes, were thoroughly cleaned before the start of each session to make sure that no residual odours remained between the trials. The duration of the investigatory behaviour towards the stimuli animals' odour (active sniffing behaviour towards the air stream produced by -the fan unit within a 5 cm distance from the fence, but no chewing at the fence) was measured. This behavioural procedure was established and extensively used in the laboratory (Noack et al. 2010, Engelmann et al. 2011).

3.2.6 Sequence of behavioural tests

Fig. 6 shows the sequence of tests which the Grb2 cKO mice and their wildtypes littermates underwent in order to characterise their behavioural performance. This sequence was carefully designed to minimize the possible

impact of the more aversive behavioural test on the performance of the experimental subjects in the subsequent behavioural tests e.g. the open field test first and the fear conditioning tests last in this sequence.

Fig. 6. Time line for the behavioural screening of the Grb2 cKO mice and their control littermates. Exploratory behaviour and general activity were tested first in the open field test (OF), when the mice were approximately 10 weeks old (week 1). Following, their basic olfactory functions were tested using the olfactory habituation and dishabituation test (week 2). Once intact olfaction was confirmed, social recognition memory was tested using the social discrimination test with direct exposure (SDT$_D$). Due to the distinct location of the required equipment to perform the novel object recognition test (NOR) and fear conditioning tests, the mice had to be transported to another animal facility from the same campus (Haus 13, Institute of Physiology, OvGU). There, after two weeks of habituation (week 4 – 5), NOR and fear conditioning test were performed in order to study other types of memory apart from social recognition memory (week 6 – 8).

3.3   Invasive manipulations

3.3.1   Stereotaxic surgery

Experimental subjects of the C57BL/6JOlaHsd strain were anaesthetised with isofluorane (2 % Baxter, Unterschleißheim, Germany) applied via an anaesthesia system (MLW, Leipzig, Germany) by a constant flow of 1.2 l / min and then injected intraperitoneally (intraperitoneally; 0,1 ml / 10 g) with a mixture of ketamine and xylazine (Ketavet®, "20 %; Pfizer Pharmacia, Berlin, Germany", and Rompun®, "5 %; Bayer Vital GmbH, Leverkusern, Germany" in 0,9 % sodium chloride solution "Braun Melsungen AG, Melsungen, Germany") and subcutaneously with the analgesic Meloxicam (0.05 mg / Kg body weigth, Metacam® Boehringer Ingelheim Vetmedica, Ingelheim, Germany) to temper post-operative pain. The experimental subjects were fixed into a TSE stereotaxic frame with the help of the TSE mouse adapter and the TSE ear bar set (TSE Stereotaxic Instruments, TSE Systems GmbH, Bad Homburg, Germany). The eyes of the experimental subject were protected from drying out by applying Vidisic (Bausch & Lomb; Dr. Gerhard Mann GmbH, Berlin, Germany) to them before the start of the surgery. The fur from the upper part of the head was shortened with scissors and the area was disinfected with Descosept AF before accessing the skull through a small rostro-caudal incision (approximately 7 mm) in the scalp. With the help of a wet swab soaked with hydrogen peroxide (3 % H_2O_2), the delimitating areas from the skull were made visible, which allowed the Bregma and Lambda to be align in the same horizontal plane, and to calculate the coordinates for the cannula placement aiming at the dHC or the vHC. The coordinates were selected according to a

stereotaxic atlas (Franklin 1997) and the cannulae positioned with the aid of micromanipulators (TSE Stereotaxic Instruments, TSE Systems GmbH, Bad Homburg, Germany) fixed to the stereotaxic frame (for coordinates see Table 1). Small bilateral holes were drilled into the skull with a dental drill at the calculated x-y-coordinates for the defined brain areas. Two guide cannulae (0.55 x 0.08 x 9.00 mm; Injecta, Klingenthal, Germany) were slowly introduced via the holes into the brain tissue and placed bilaterally into either the dHC or the vHC. Additionally, two anchoring screws (1.0 x 2.0 mm; Paul Korth, Lüdenscheidt, Germany) were inserted into the skull next to each guide cannula. The wound was dried with a swab and a drop of tissue adhesive (Histoacryl®, B. Braun Surgical, S.A. Rubí, Spain) was applied to the base of the implanted screws and cannulae and also on the surface of the skull. Once dried, the implant was fixed to the skull and the screws with a light-curing dental cement (Ivoclar vivadent, Schaan, Liechtenstein). This procedure was also used to close the wound. To prevent clogging, a stylet (insect pins shortened to the length of the guide cannulaes; Ento Sphinx, Pardubice, Czech Republic) was inserted into each guide cannula. Experimental subjects were allowed to recover from surgery individually in type II cages for 24 h and then put back to their original groups for approximately two weeks before the start of the experiment. Pilot studies revealed that at this time point both body weight and social behaviour reached pre-surgery levels. Experimental subjects' weight was always checked the days after surgery to ensure all animals were recovering successfully.

Table 1. Reference coordinates for the implantation of the guide cannulae according to the stereotaxic atlas of Franklin (1997). Minor adjustments were made from the original coordinates in order to compensate instrumental errors

mm from Bregma	Posterior	Lateral	Ventral
dorsal hippocampus	- 2.0 mm	± 1.3 mm	- 1.0 mm
ventral hippocampus	- 3.0 mm	± 2.8 mm	- 2.5 mm

3.3.2   Substances injected

The following substances were used in the present study:

3.3.2.1   aCSF

The internal fluid environment of the skull is critical to brain function. Anatomy texts depict the brain as "floating" in a clear fluid excreted mainly by the choroid plexus. This fluid, termed cerebrospinal fluid (CSF), baths the brain and spinal axis, and serves as a third circulation buoying the brain, while providing a conduit for

clearing brain metabolic bioproducts (Sakka et al. 2011). In the present study, artificial CSF (aCSF) was used as the control solution (vehicle), in order to ensure that any of the behavioural differences observed after the injection procedure were not due to the procedure *per se* (Vetrivelan et al. 2004). Thus, aCSF was injected in the same experimental subjects used for drug injection, minimizing the number of animals needed for the study. Moreover it also helped to reduce the animal variability since the drug and vehicle injections were performed in the same experimental subjects. aCSF closely matched the electrolyte concentration of CSF. It was prepared from high purity water and analytical grade reagents. It was microfiltered and sterile, with a final ion concentration (in mM): Na 150; K 3,0; Ca 1,4; Mg 0,8; P 1,0; Cl 155, supplied by Harvard Apparatus.

3.3.2.2 Lidocaine

The principle mode of action of lidocaine (2-Diethylamino-N-(2,6-dimethylphenyl) acetamide) is the blockade of the voltage-gated sodium channels (Hille 1966), which consequently inhibits the induction of action potentials in neuronal cell membranes, and therefore their transmission or propagation. This mechanism leads to the local anaesthetic effects of lidocaine, since lidocaine injections inhibit action potentials in both the cell body and the axons of the neurons (Malpeli 1999, Martin and Ghez 1999). Low lidocaine concentrations (10 - 40 μg) in small injection volumes (0,5 to 4 μL) inactivate an area with an average distance of one to two millimetres from the injection site (Albert and Madryga 1980, Sandkuhler et al. 1987, Fenton and Bures 1994, Boehnke and Rasmusson 2001). Its effect start within two to three minutes, reaching its maximum after 10 to 20 min and decays, depending on the injected concentration after 20 to 60 min (Malpeli 1999, Martin 1991, Tehovnik and Sommer 1997, Boehnke and Rasmusson 2001). The injection of 10 μg of lidocaine caused a ten- to 20- percent functional inhibition (Pereira de Vasconcelos et al. 2006).

A major advantage of lidocaine-induced neural inactivation is that the effects are temporary. Since, traditional permanent lesions (e.g. electrolytic, excitotoxic) of the HC may result in compensatory changes in the surrounding neural circuitry, making it difficult to attribute behavioural effects to the loss of the missing structure (Cassel et al. 1997, Clusmann et al. 1994).

3.3.3 Injection procedure and experimental design of injection experiments

Simultaneous bilateral microinjections were done under light isofluorane (2 %) inhalation anaesthesia. Stylets were removed from the guide cannulae and injection cannulaes (0.36 x 0.08 x 20.00 mm; Injecta, Klingenthal, Germany) were inserted. The injection cannulae extended the ventral tip of the guide cannulae by 1 mm. The dorsal tip of the injection cannula was connected via a piece of tubing (Tygon® R3607, wall thickness:

0.91 mm, inner diameter: 0.19 mm) to a constant-rate microinfusion pump (CMA Microdialysis, Stockholm, Sweden). Injections were conducted over a 1 min period. In all cases, correct injection flow rates were visually controlled. The distal part of the tubing was filled with distilled water and the proximal part to the injection cannula with the defined substance (lidocaine or aCSF, see 3.3.2); both parts were separated by a small air bubble. The movement of the air bubble in the tubing during the injection controlled the amount of fluid/substance that was administered. aCSF (Harvard Apparatus, Catalog n°59.7316) or lidocaine at a concentration of 20 µg / µl (dissolved in aCSF) were delivered bilaterally into the distinct parts of the HC, dHC or vHC; ~ 8 min prior to the sampling session. The substances were delivered in 1 min at a total volume of 0.5 µl per hemisphere, thus experimental subjects which underwent lidocaine injections received 10 µg of lidocaine in each HC hemisphere. The injection cannulae were left in place for a further minute before removal, to enable the complete diffusion of the substances in the surrounding tissue.

Initially, a given set of experimental subjects was tested in the SDT_D, in order to ensure that the experimental subjects had an intact long-term social recognition memory and showed low levels of aggressive/sexual behaviour towards the stimuli animals. Only these animals were included in the subsequent testing. The selected experimental subjects then underwent the guide cannula surgery (see 3.3.1). Two weeks after the surgery (Noack et al. 2015), the long-term memory was tested to control for possible effects caused by the surgery *per se* that might have corrupted the outcome in subsequent behavioural testing (Fig. 7). Either aCSF or lidocaine was infused and either short-term or long-term memory was tested in the SDT. dHC- and vHC-inactivation effects were tested in both the SDT_D and the SDT_I. A maximum of four injections per animal was performed, with at least seven days between injections. The injections were performed following a cross-over design with aCSF and lidocaine in order to yield a more efficient comparison of the treatments. An advantage of the SDT is its non-conditioned nature, which allowed us to apply this experimental design testing social recognition memory in the same experimental subject under different treatments. One week after the last injection, long-term social recognition memory was again tested (without injections) in order to assess whether experimental subjects showed intact long-term social recognition memory after the whole procedure. Finally, mice were killed and the brains processed for histological analysis.

Previous studies form our laboratory investigated a possible impact of the transportation after the injections, as well as the time needed by the experimental subjects to fully recover from the surgery to still allow the measurement of an intact long-term social recognition memory (Engelmann et al. 2011, Noack 2012, Noack et al. 2015). All social memory tests performed in the present work were designed in accordance with the outcome from previous studies.

3.3.4 Histological analysis

Mice were killed with an overdose of isofluorane. After decapitation the brain was removed from the skull, submerged for two minutes in Isopentane at ~ 50°C and stored at - 80°C until cryo-sectioning (Leica Figocut 2800E, Wetzlar, Germany). The brains were coronally sectioned at 25 μm and every 4th section of the hippocampal formation was mounted on slides (SuperFrost® Plus, Thermo Scientific, Darmstadt). Once the slices dried out, Nissl staining was performed and analysed (see 3.3.4). The placements of the cannulae were reconstructed by the tracks left on the identified slices. Animals in which one cannula or both cannulae were found outside the defined HC part were excluded from further analysis.

3.3.5 Sequence of behavioural test and invasive manipulations

Fig. 7 shows the procedural sequence followed by the mice which underwent dHC or vHC bilateral injections and the respective behavioural tests. As previously introduced, experimental subjects' intact long-term memory was tested before implanting the cannulae and two weeks after recovery. One week after, the experimental subject underwent one injection per week, for a maximum of four weeks, followed by the social discrimination test, which tested either long-term or short-term recognition memory. After receiving a maximum of four injections, the long-term social recognition memory was again tested. During the following week the mice were killed and the HC-coordinates from the injection sites verified (Fig. 7).

Fig. 7. Time line of the behavioural tests and invasive manipulations for the temporal dHC and vHC inactivation experiments. Long-term social recognition memory was tested using the social discrimination paradigm in mice approximately 10 weeks old (week 1 of the experiment). One week later, the selected mice underwent bilateral cannulae implantation either leading to the dHC or to the vHC, which allowed the temporal inactivation of these areas following the injection of lidocaine. After a maximum of 4 injections (1 injection/week), long-term social recognition memory was again tested. Ten weeks after the beginning of the experiment the mice were killed and the coordinates verified using Nissl staining (Abbreviations; SDT: Social discrimination test, aCSF: artificial cerebrospinal fluid).

3.4 Molecular biology techniques

3.4.1 DNA isolation

Isolation of genomic DNA was carried out from tail biopsies. A small piece (≤ 0.5 cm) of mouse tail was incubated overnight in a thermomixer at 55°C, with 500 µl of lysis buffer (50 mM Tris, pH 8; 100 mM Ethylenediaminetetraacetic acid (EDTA); 100 mM NaCl; 1% sodium dodecyl sulfate (SDS)) with 10 µl of Proteinase K (20 mg/mL; GeneON, Ludwigshafen am Rhein, Germany). The following day the sample was allowed to cool down at room temperature (RT) and 200 µl from sterile 6 M NaCl was added to the reaction vials (Eppendorf, Hamburg, Germany). After gently mixing the solution with the help of a vortex, it rested in the tube for 15 min. The samples were centrifuged during 7 min at 14000 rpm. The supernatant was taken (approximately 500 µl) and mixed with 500 µl of cold Isopropanol (2-Propanol; ROTH, Karlsruhe, Germany) in order to precipitate the DNA. The reaction vials containing the samples were manually mixed until the DNA threads were visible. The solution was again centrifuged during 10 min at 14000 rpm. The supernatant was then discarded and 500 µl of cold 70 % Ethanol (Zentral apotheke, Otto von Guericke University, Magdeburg, Germany) were used to re-suspend the pellet following 5 min of centrifugation at 14000 rpm. The supernatant was discarded and the pellet passively dried at RT (~ 5 min). The final pellet was re-suspended with 200 µl of H_2O, 1-2 µl of this solution was used for the PCR reaction of the genotyping process (9.1).

3.4.2 Amplification of DNA fragments with polymerase chain reaction

The genotype of each mouse was verified by performing PCR, a method used to selectively multiply a defined DNA sequence from an obtained complex template DNA. The flanked sequences of the target DNA are used to generate a sense and an anti-sense oligonucleotide primer (usually 18-30 bp; Table 2), thus surrounding the target sequence. The primers were used as the starting point by the polymerase for amplification. Chain elongation in 5' - to 3' - direction was yielded by addition of desoxyribonucleotide triphosphate (dNTPs) (Genecraft, Köln, Germany). The reaction mixture was prepared on ice. Each reaction contained 200-250 µM of each dNTP (Genecraft, Köln, Germany), 2 - 2,5 µl reaction buffer (750 mM Tris-HCl 200 mM $(NH_4)_2SO_4$, 0.1 % (v/v) Tween 20) (Fermentas;ThermoFisher, Darmstadt, Germany), 0.8 - 1 µl of 25 mM $MgCl_2$ (Fermentas;ThermoFisher, Darmstadt, Germany), 3 - 4 µl 5 M Betaine (Sigma-Aldrich, Munich, Germany), 1 µl of each primer (10 mM), H_2O, 1-2 µl template DNA (obtained from DNA isolation from the tail, approximately 200 ng genomic DNA, 3.4.1) and 1 µl of ThermoPrime Taq DNA Polymerase (Fermentas;ThermoFisher, Darmstadt, Germany), which

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was added in a final step. The total reaction volume was between 20-25 µl (the master mix and the cycler program used for each PCR is included in 9.1).

The steps of PCR amplification were as follows:

- Heat denaturation of the double-stranded template DNA at 94 - 95°C.

- Primer annealing to the complementary sequences of the single stranded target (60-63°C).

- Extension by the action of DNA polymerase at 72°C.

After primer extension the mixture was heated again to separate the DNA strands. Cooling down the mixture allowed the primers to hybridize with the complementary regions of newly synthesized DNA. Each cycle doubled the content of the original target DNA. In general, 30 - 35 cycles were run yielding a 10^6- to 10^9- fold increase of the target DNA. Annealing temperature and also elongation time depend on the primer composition and the length of the amplified sequence.

Table 2. Primers used to reveal the genotype of the Grb2 cKO mice and their WT littermates by PCR according to the protocol provided by Prof. Stork and Prof. Nitschke.

Primer	Oligoname	Sequence (5'→ 3')
Cam Cre	Cre-tot 1	ACG ACC AAG TGA CAG CAA TG (20) (forward)
	Cre-tot 2	CTC GAC CAG TTT AGT TAC CC (20) (reverse)
	TR1B	GGC ACA GCT CTC CCT TCT GTT TGC (24) (forward)
	TR3	GCT CTC CTT TCG CGT TCC GAC AG (23) (reverse)
Grb 2	LoxP in 2S	CCA GCA CAC ATG TCC TGC CTT C (22) (forward)
	LoxP in 2 AS	GGT GGC TCA CAA CCA CCT ATA AC (23) (reverse)

3.4.3 Agarose gel electrophoresis

The DNA molecules obtained from the PCR were separated according to their size by agarose gel electrophoresis. Gels containing 1 - 2.5 % (w / v) agarose (Universal-Agarose, peqGOLD, Peqlab, Erlangen, Germany) in 1x Tris-acetate-EDTA (TAE) were used for separation of DNA fragments. Gel percentage was determined depending on the size range of the fragments. Ethidium bromide (Carl Roth, Karlsruhe, Germany) was added to the gels to obtain the final concentration of 1 mg/ml. To prepare the sample, 1 to 2 µl of DNA was mixed with 1/10 volume 10x bromophenol blue loading dye (Carl Roth, Karlsruhe, Germany) and loaded into the gels. A 100 bp DNA ladder (Thermo Scientific GeneRuler 100 bp Plus DNA ladder, Darmstadt, Germany) was also

loaded in order to determine the size of the DNA fragments. The electrophoresis was performed in 2x TAE buffer at 5-10 V / cm (distance between the electrodes), until the bands were separated. An ultraviolet transilluminator (GeneGenius, Syngene International Ltd, Cambridge, UK) was used to visualize the ethidium bromide incorporated into the DNA fragments in the gels (representative pictures of PCR products separated by agarose gel electrophoresis are included in 9.1).

3.4.4 Cell brain lysis and protein quantification

Three to six months-old mice were sacrificed by cervical dislocation. Their brains were removed and dissected on ice; olfactory bulb, frontal cortices, striatum, HC (all from both hemispheres) and cerebellum were separated and later homogenized with a lysis buffer containing 50 mM Tris pH 7.4. 150 mM NaCl, 2.5 mM EDTA, 1 % NP40, 0.5 % sodium deoxycholate (DOC), 0.1 % SDS and 1 mM Na_3VO_4 containing proteases and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The tubes were kept for 30 min in ice and finally centrifuged at 4°C and 208000 rpm for 15 min. Only the supernatant (soluble proteins) was used for Western blot. Protein concentration was determined using the Thermo Scientific Pierce BCA Protein Assay (BCA kit, Pierce, Rockford, IL).The homogenates were stored at −80°C until further use.

3.4.5 SDS-polyacrylamide gel electrophoresis

Electrophoretic separation of proteins was carried out under denaturing conditions in a discontinuous gel system, technique known as SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels were prepared by the protocol of Laemmli (1970). A separating gel with a concentration gradient from 5 % to 20 %, and a 5 % stacking gel were used in all experiments (SDS-polyacrylamide gel). Samples were thawed and an equal amount of protein (25 µg) was diluted 1:4 with Rotiload 4x (Roth, Karlsruhe, Germany), which contained 2-mercaptoethanol and SDS to unfold and negatively charge the proteins. The samples were then boiled at 96°C for 5 min. The denaturised proteins were electrophoresed on the SDS-polyacrylamide gel (for ~ 2 h at 8 - 10 mA/gel).

3.4.6 Western blotting

Using western blotting technique, the proteins separated in SDS-polyacrylamide gel were transferred to a membrane in order to enable the immunodetection of the proteins of interest. Polyacrylamide gels were washed briefly in transfer buffer and transferred onto nitrocellulose membranes (Amersham Biotech, Little Chalfont, UK) by blotting for 1 h at 200 mA using (SE250 Mighty Small II vertical electrophoresis, Hoefer, Inc. USA). The correct protein transfer from the gels to the nitrocellulose membranes and the comparable loading were verified by

incubating 5 - 10 min the membranes in Ponceau (Sigma-Aldrich, Munich, Germany) 0.2 % solution at RT. After washing three times with distilled water, the membranes were incubated with gentle agitation throughout all the following steps. Membranes were bathed with the blocking buffer (5 % non-fat dry milk (Blotting grade, Carl Roth GmbH + Co. KG, Karlsruhe)) in 1x Tris buffered saline with 0.1 % Tween®-20 (TBS-T)) for 2 h at RT to reduce unspecific binding, then washed three times for 10 min with TBS-T and incubated with the primary antibody (Table 3), diluted in 0.5 % non-fat dry milk in TBS-T buffer or 5 % bovine serum albumin (fatty acid free, PAA laboratories GmbH, Pasching, Austria) in TBS-T buffer, overnight at 4°C. Next, the membranes were washed three times with TBS-T, 10 min each, and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (1:5000, Jackson ImmunoResearch Lab., West Grove, PA) (Table 3) diluted in 0.5 % non-fat dry milk in TBS-T, for 2 h at RT. The membranes were washed again three times 10 min each, and the bands corresponding to the proteins of interest were visualised using chemiluminescent substrate ECL (ECL assay kit, Amersham Biosciences, Little Chalfont, UK) and visualized on Amersham hyperfilm ECL autoradiographic film (Amersham Biosciences, Little Chalfont, UK) after automatic development in Curix 60 film processor. Blots were scanned and quantify using Fiji/ImageJ software (ImageJ 1,50b, Wayne Rasband, National Institute of Health, USA).

To normalize the signal of the protein of interest to the total protein content, the membranes were washed three times with distilled water for 10 min each, and re-incubated with mouse anti-alpha-tubulin monoclonal antibody or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls (following the same procedure described above from the incubation of the primary antibody). For graphic representation, the results of the densitometry analysis were normalised to the referent values with the internal control used (α-tubulin or GADPH).

Table 3. Primary antibodies used for Western blot analyses.

Antibody	Host Species	Molecular weight	Dilution WB	Source
α-tubulin	mouse monoclonal	52 KDa	1/5000	Sigma, Steinheim, Germany
GAPDH	mouse monoclonal	38 KDa	1/1000	Chemicon, Merck Millipore, Darmstadt, Germany
Grb2	mouse monoclonal	24 KDa	1/5000	BD Transduction Laboratories, Heidelberg, Germany
pMAPK 42/44	Rabbit monoclonal	42 & 44 KDa	1/1000	Cell Signaling
MAPK 42/44	Mouse monoclonal	42 & 44 KDa	1/2000	Cell Signaling

3.5 Imaging and staining techniques

3.5.1 Nissl staining

Frozen mice brains (-80°C) were later sectioned at a thickness of 25 µm and directly attached to Superfrost™ Plus Microscope Slides (Thermo Scientific, Braunschweig, Germany). Sections were stained with cresyl violet acetate (Merck KGaA, Darmstadt) according to the following protocol: the slides were exposed to 1 min cresyl violet acetate, followed by 30 s to 70 % ethanol (Th. Geyer GmbH & Co. KG, Hamburg, Germany), 20 s to 96 % ethanol, 5 min to isopropanol ("2-propanol", Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 5 min to xylol ("Isomere", Carl Roth GmbH + Co. KG, Karlsruhe, Germany). After treatment with xylol, the sections were dried slightly to get rid of the Xylol surplus and covered using a glass coverslip (Cover glasses, Menzel-Gläser, Thermo Scientific, Braunschweig, Germany) by adding DePeX medium.

3.5.2 Immunostainning

3.5.2.1 Stimulation, perfusion and tissue processing

If not stated otherwise, mice were separated 24 h prior to the exposure of the defined stimulus in type II cages. The stimulus exposure was thought to imitate sampling in the SDT and therefore lasted 4 min and consisted of either a direct exposure to a stimulus animal (SDT_D sampling-like juvenile exposure) or indirect exposure to a stimulus animal (SDT_I sampling-like juvenile exposure, with the VFC). The control experimental subjects underwent similar manipulations, however without the presence of the stimulus animal. Control experimental subjects were isolated in a novel cage 24 hours prior to the start of the experiment. For those which underwent indirect exposure, they were additionally placed 10 min in the VFC before the presentation during four min of the fan-driven stream of room air. Two hours later, the experimental subjects were deeply anaesthetized by a subcutaneous injection of a mixture of 5:3 Ketavet® (Pfizer Pharmacia, Berlin) and Dorbene® (Merlin Vet Export, United Kingdom) and transcardially perfused with physiological saline containing 0.1 % Heparine (Heparin-Natrium 25000 ratiopharm®, Merckle GmbH, Blaubeuren, Germany) followed by 4 % formaldehyde diluted in 0.1 M phosphate buffer (PBS, pH 7.4) for 12 min. Brains were removed, post-fixed for 24 h in 4 % formaldehyde-PBS at 4°C with gentle shaking. The brains were then transferred to a sucrose solution (1 M in 0.1 M NaH_2PO_4 buffer) where they underwent gentle shaking at 4°C until the solution had infiltrated into the whole brain (for two to three days) in order to cryoprotect the tissue. Finally, the brains were embedded in a tissue freezing medium for

cryo-sectioning (Leica Biosystems, Nussloch, Germany) and shock-frozen in isopentane at - 40°C - - 60°C and stored at - 80°C until cryo-sectioning (Leica CM3050 S Reseach Cryostat, Leica Biosystems, Nussloch, Germany).

3.5.2.2   Immunofluorescence

All antibodies were tested in pilot experiments to check their specificity and to establish the best dilution before being used in the routine. Brain coronal sections (25 µm) were processed as free-floating slices. After three initial washing steps in PBS, a pre-incubation of 1 h, at RT, with PBS containing 10 % normal donkey serum (DNS; PAN Biotech GmbH, Aidenbach, Germany) and 0,3 % Triton X-100 (Serva, Heidelberg, Germany) was performed to block unspecific binding sites and permeabilisation, respectively. The sections were then incubated with primary antibodies (Table 4) in PBS with 10 % DNS, 0.3 % Triton X-100 and 0.1 % sodium azide (Serva, Heidelberg, Germany) for ~ 70 h at 4°C on a shaker. After three washing steps and a pre-incubation with PBS in 10 % bovine serum albumin (BSA, PAA Laboratories, Cölbe, Germany) of 1 h, at RT, the slices were incubated for 4 h, at RT, with a secondary antibody donkey anti-rabbit Cy3 or donkey-anti-goat/mouse-Alexa 488 or *vice versa* in 0.1 % Triton, 2 mg/ml BSA. Finally, the sections were washed in PBS, mounted on gelatine coated microscope glass slides (Menzel-Gläser, Thermo Scientific, Braunschweig, Germany), air-dried and covered with Immunomount (Thermo Shandon, Pittsburg, USA) to prevent fading. Slides from immunostained sections were stored at 4°C in darkness. Appropriate negative controls were performed by omission of the primary or/and the secondary antibodies.

Table 4: Primary antibodies used for Immunofluorescence.

Antibody	Host Species	Dilution	Manufacturer
c-Fos	rabbit polyclonal	1 / 500	(sc-52) Santa Cruz Biotehcnology, Inc, USA
Neu N	mouse monoclonal	1 / 100	Chemicom, Merck Millipore, Darmstadt, Germany
GFAP	goat polyclonal	1 / 500	(c-19;sc-6170) Santa Cruz Biotechnology, Inc, USA

3.5.2.3   c-Fos immunohistochemistry

3.5.2.3.1   Processing of the sections

Each of the following steps were performed under gentle shaking at RT if not stated otherwise. The washing steps were conducted with PBS buffer, three times and for 10 min each. To eliminate endogenous peroxidase, floating coronal sections (25 µm) were placed in 24 well-plates and incubated for 20 min with 1:1 Methanol:PBS 1 % H_2O_2 solution. Then the brain sections were washed. After blocking the non-specific binding sites with 10 %

normal goat serum solution (in PBS, 0.3 % triton X-100 and 0.1 % sodium azide) for 30 min, the brain sections were incubated with a rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of human c-Fos p62 (identical to the corresponding mouse sequence; c-Fos sc-52; Santa Cruz Biotechnology, USA) diluted at 1:5000 in normal goat serum solution for ~70 h at 4°C. The sections were washed and incubated for one hour with 0.2 % bovine serum albumin (in PBS, 0,2 % triton X-100 and 0,1 % sodium azide) in order to block unspecific binding sites. Next, the brain slices were incubated with the secondary goat biotinylated antibody (goat anti-rabbit IgG, Vector Laboratories, Inc., Burlingame, USA) which was diluted at 1:2000 in bovine serum albumin solution overnight at RT. The sections were washed and incubated for one hour in 0.2 % bovine serum albumin (in PBS only), and treated over four hours with the avidin-biotin peroxidase complex solution before being washed again. A change of pH was achieved by a 10 min washing step with 50 mM Tris solution. The visualization of the antibody binding sites involved the reaction of the avidin-biotin complex (Vector Laboratories, Inc., Burlingame) and the development by 3, 3'-diaminobenzidine (DAB, Sigma-Aldrich, Steinheim, Germany) (0.05 % in 50 mM Tris solution, 0.5 ml / well) and its reaction with H_2O_2 (0.3 %, 25 μl / well, for approximately four min) to produce a brown colorimetric end product which allows the labelling of c-Fos positive cells (ABC-method described elsewhere (Laube et al. 2002)). This reaction was stopped via washing. The brain slices were transferred to tap water and placed on gelatinized slides and dried with room air for at least one hour. An ascending alcohol series (in this order each for five min: 70 %, 85 %, 96 %, 100 % ethanol twice, and then twice ten min incubations with xylene) was followed by the final step to place glass coverslips onto the slides with the polystyrene mountant for coversliping DePex. The number of c-Fos immunoreactive cells was determined using a bright field microscope and a computer software program (see 3.5.2.3.3). A trained observer, who was unaware of the animals' treatment, counted the immunoreactive cells (c-Fos positive cells) in each area.

3.5.2.3.2 Analysed areas

From three to five representative sections were selected from approximately the same rostro-caudal level (Franklin 1997) to analyse the following brain areas from each experimental subject: the suprachiasmatic nucleus (SCN), the paraventricular nucleus of the thalamus (PVT), the MeA, the dHC and vHC (Fig. 8). For the MeA, a representative area of 0.1 mm^2 was chosen in each section and further analysed. The analysis of the c-Fos synthesis in the SCN and PVT was included to provide an insight into the status of the experimental subject at the moment of sacrifice, concerning their subjective stage of the day-night cycle and the motor activity, respectively. In addition, this approach would theoretically also allow to "normalize" the synthesis of c-Fos in the other brain areas under study.

Fig. 8. Brain areas selected for analysing c-Fos immunoreactivity after a social encounter. A. Paraventricular nucleus from the thalamus (PVT) and suprachiasmatic nucleus of the hypothalamus (SCN) (~ - 0.46 from Bregma) **B.** Medial amygdala (~ - 1.22 from Bregma), **C.** Dorsal hippocampus (~ - 1.94 from Bregma), **D.** Ventral hippocampus (~ - 3.28 from Bregma). Pictures are taken from "The mouse brain in stereotaxic coordinates", from Franklin (1997).

3.5.2.3.3   Image processing

Brain slices were inspected using an epifluorescence and brightfield microscope (Axio Imager M1 microscope, Carl Zeiss AG, Jena, Germany). Digital pictures were taken with the help of the AxioVision software (AxioVision Rel. 4.8.2 sP3, 08-2013, Carl Zeiss AG, Jena, Germany) by an AxioCAM camera (AxioCAM MRc camera and its adapter 60-C 1'' 1.0 X, Carl Zeiss AG, Jena, Germany)). AxioVision software was also used to quantify the c-Fos positive cells from the c-Fos immunohistochemistry (3.5.2.3).

3.6   Statistical analyses

GraphPad Prism 5.04 (GraphPad Software Incorporated, La Jolla, USA) was used to conduct all statistical analyses and produce the graphs of the obtained results. The statistical methods used to analyse the current research results are as follows:

Data normality was assessed using three different statistical tests including, **Kolmogorow-Smirnov, D'Agostino-Pearson** and **Shapiro-Wilk**. Normally distributed data which was confirmed by at least one of the aforementioned tests was further analysed using parametric tests where a confidence interval of 95 % was used.

The **Kruskal-Wallis test**, also called Kruskal-Wallis one-way analyses of variance (ANOVA) by ranks, is a non-parametric test that was used to analyse non-normality distributed data as well as to compare three or more unpaired groups. Using this test the number of quantified c-Fos positive cells in the SCN and PVT were compared between the distinct stimulation and control conditions. No *post hoc* tests were performed as no significant differences were observed. In contrast, quantification of c-Fos positive cells in the MeA, dHC, and vHC were confirmed to show a Gaussian distribution and thus a **one-way ANOVA** parametric test was selected and followed by the *post hoc* **Tukey Honestly Significant Difference (HSD) multiple comparison** test, which allowed for further analyses of the differences between groups.

A **non-parametric correlation analyses (Spearman)** two-tailed p value was used to study the correlation between the investigation duration during the SDT_D sampling-like juvenile exposure towards the juvenile to the later *post mortem* quantification of c-Fos positive cells in the different brain areas analysed. Both parameters belonged to the same experimental subjects.

The **One-way repeated measures ANOVA** test was used to compare the investigation duration of the same experimental subjects before surgery, after surgery and after injections in all trials, during the sampling and choice session, with discrimination between investigation duration towards SA1 and SA2 in the latter session. This statistical test was also used to analyse the length of the investigation time and the number of bouts towards the odour/s presented during the distinct trials of the olfactory habituation and dishabituation test. In case of a significant effect, a *post hoc* **Tukey HSD multiple comparison** test was performed to determine where significant differences were.

A **paired *t*-test** was used to analyse the outcome from both variants of the social discrimination test (SDT_D and SDT_I) with comparisons between the investigation duration that an experimental subject spent towards SA1 *vs*. SA2 during the choice session. Similarly, the same test was applied in the novel object recognition, in order to compare the exploration duration towards each object, novel *vs*. familiar. This statistical tool was additionally used in order to study possible differences on the latency to approach SA1 *vs*. SA2, as well as the duration and number of bouts of the aggressive/sexual behaviour towards each stimulus animal in WT and Grb2 cKO mice during the choice session of the SDT_D.

A **two-way repeated measures ANOVA** analyses was applied to study all the behavioural parameters (travelled distance, rearing episodes, number of jumps and the percentage of time freezing) measured during the conditioning and the fear memory sessions of both fear conditioning tests using as main factors "interval" and "genotype". This statistical tool was further used to study the effect of the UCS-CS presentation on each of these

behaviours and compare it between genotypes, thus in this case a two-way repeated measures ANOVA using as main factors "post-CS /post-UCS" and "genotype" was applied, followed by the **Bonferroni *post hoc*** test which provided more detailed information about the differences between the groups.

Since the experimental subjects were not necessarily the same in all compared conditions, the **standard Two-way ANOVA** with main factors "injected substance" and "session", was used to analyse the duration and the number of bouts of the aggressive/sexual behaviour during each session of the SDT_D tested after either dHC or vHC injections. This analysis was followed by the **Bonferroni *post hoc* test.** These statistical tests were also used to analyse the behavioural responses of the Grb2cKO mice, such as the latency to approach SA1 *vs*. SA2 during the SDT_D, as well as the duration and number of bouts of the aggressive/sexual behaviour, with main factors "exposure interval" and "genotype". The exploration duration during the NOR tests, with main factors "trial" and "genotype", were also analysed with a standard two-way ANOVA.

An **unpaired *t*-test** was used to compare the intensity values of the normalized bands obtained by western blot between the samples from the WT and the Grb2 cKO mice. This statistical test was also used to compare the distinct behavioural parameters registered during the open field and to compare the outcome between WT and Grb2 cKO mice. In addition an upaired *t*-test was also applied in order to compare between genotypes the registered overall behavioural parameters measured during the distinct sessions of the fear conditioning test (travelled distance, rearing episodes, number of jumps and the percentage of time freezing).

The **preference index** was calculated as follows: (exploration duration towards SA2/(total exploration duration (towards SA2 + SA1)). A **one sample *t*-test** was used to compare the observed preference index against a chance value 0.5 (null hypothesis).

The **Mann-Whitney U test** was used to statistically analyse two unpaired groups of not normally distributed data. This test was used for instance to compare the quantification of c-Fos positive cells in the CA2 area of the dHC and the vHC under stimulated (SDT_D) and control conditions.

If not stated otherwise, all data are presented with the mean + the standard error of the mean (SEM). If the probability of error (p) was less than 0.05, the differences between the groups were considered statistically significant. Different levels of significance depending on the probability of error were indicated as follows: *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$ if not stated otherwise.

4   Results

4.1   Role of the hippocampus in social memory

4.1.1   Cellular c-Fos expression

Double immunofluorescence staining combining c-Fos-labelling with either glial fibrillary acidic protein (GFAP)- or Neuronal Nuclei (NeuN)-labelling were performed in mouse brain coronal sections. The brains were obtained from experimental subjects that were stimulated using SDT_D sampling-like juvenile exposure 120 min before being perfused. c-Fos positive cells did not co-localise with GFAP labelling (Fig. 9), however it did co-localize with NeuN-labelled cells (Fig. 10).

Fig. 9. Fluorescent photomicrographs of the dHC from mouse brain coronal sections used to study the co-localisation of c-Fos and GFAP expression. Photomicrographs of the double immunofluorescent staining of the dHC CA3 and DG. c-Fos (red) and GFAP (green) positive cells were labelled together with the nuclear marker DAPI (blue). In the merged images the lack of co-localisation between c-Fos and GFAP can be observed. Scale bar: 100 μm.

Fig. 10. Fluorescent photomicrographs of the dHC from mouse brain coronal sections used to study the co-localisation of c-Fos and NeuN expression. Photomicrographs of the double immunofluorescent staining of the dHC CA3 and DG. c-Fos (red) and neuron-specific nuclear protein named Neuronal Nuclei (NeuN) were labelled, together with the nuclear marker DAPI (blue). In the merged images the co-localisation between c-Fos and NeuN can be observed in orange. Scale bar: 100 µm.

4.1.2   c-Fos expression induced by social stimulation

Statistical analysis failed to provide significant differences in the expression of c-Fos in the SCN (p = 0.8555; Kruskal-Wallis statistics: 0.7749) (Fig. 11) or PVT (p = 0.4074; Kruskal-Wallis statistics: 2.899) (Fig. 12), in experimental subjects after SDT_D or SDT_I sampling-like juvenile exposure compared to their controls (Fig. 11 and Fig. 12).

Fig. 11. Quantification of c-Fos immunoreactivity in the SCN with representative microphotographs. **A.** Quantification of the c-Fos positive cells in experimental subjects' SCN under control conditions (white squares and circles) and/or stimulated conditions (black squares and circles). Figures **B – E** show representative microphotographs from the SCN slices used for the quantification of c-Fos immunoreactivity under different conditions. **Direct** refers to the usage of the SDT_D sampling-like juvenile exposure in controls and stimulated experimental subjects, while **indirect** refers to the usage of the SDT_I sampling-like juvenile exposure instead. The black lines are delimitating the area of the SCN within which c-Fos positive cells were quantified. Scale bar: 200 μm. Kruskal-Wallis test was used for statistical analysis; p = 0.8555. n = 5-6. Mean and individual values are represented.

Fig. 12. Quantification of c-Fos immunoreactivity in the PVT with representative microphotographs. A. Quantification of the c-Fos positive cells in different experimental subjects' PVT under control conditions (white squares and circles) and/or stimulated conditions (black squares and circles). Figures **B – E** show representative microphotographs from the PVT slices used for the quantification of c-Fos immunoreactivity under different conditions. **Direct** refers to the usage of the SDT_D sampling-like juvenile exposure in controls and stimulated experimental subjects, while **indirect** refers to the usage of the SDT_I sampling-like juvenile exposure instead. Scale: 200 μm. Kruskall-Wallis test was used for statistical analysis; p = 0.4074; n = 5-6. Mean and individual values are represented.

The number of c-Fos positive cells detected in the MeA was found to be significantly increased in experimental subjects which underwent SDT_D sampling-like juvenile exposure compared to the respective control

condition, or any other condition ($F_{(3,18)} = 15.81$; $p < 0.0001$). However, no significant differences were found in experimental subjects that underwent SDT$_I$ sampling-like juvenile exposure compared to their control condition (Fig. 13) (for *post hoc* analyses see 9.4.1.1).

Fig. 13: Quantification of c-Fos immunoreactivity in the MeA with representative microphotographs. A. Quantification of the c-Fos positive cells in different experimental subjects' MeA under control conditions (white squares and circles) and/or stimulated conditions (black squares and circles). Figures **B – E** show representative microphotographs from the MeA slices used for the quantification of c-Fos immunoreactivity under different conditions. **Direct** refers to the usage of the SDT$_D$ sampling-like juvenile exposure in controls and stimulated experimental subjects, while **indirect** refers to the usage of the SDT$_I$ sampling-like juvenile exposure instead. Scale bar: 200 µm. An increase of c-Fos positive cells was observed in the MeA when the subjects were directly stimulated. A standard one-way ANOVA followed by Tukey's HSD *post hoc* test was used for statistical analyses. a *** $p < 0.001$ versus b; n = 5 - 6 / group. Mean and individual values are represented.

The standard one-way ANOVA analyses of the c-Fos positive cells in the dHC showed significant differences between the groups ($F_{(3,18)} = 3.698$; $p = 0.0311$) however, the *post hoc* analyses revealed differences between both stimulated conditions, but not with the respective controls (Fig. 14) (for *post hoc* analyses see 9.4.1.2). Thus sampling-like juvenile exposure under both, SDT$_D$ and SDT$_I$ conditions failed to cause significant differences in the number of c-Fos positive cells in the dHC *vs.* their control condition. In contrast, a significant increase of c-Fos positive cells in response to SDT$_D$, but not SDT$_I$ sampling-like juvenile exposure was detected in the vHC using the one-way ANOVA followed by Tukey HSD *post hoc* test ($F_{(3,18)} = 6.806$; $p = 0.0029$) (Fig. 15) (for *post hoc* analyses see 9.4.1.2). A rough estimation suggested that the ventral CA1 and the ventral subiculum were those HC areas in which the increase of c-Fos expression was particularly prominent (Fig. 15D).

Fig. 14. Quantification of c-Fos immunoreactivity cells in the dHC with representative microphotographs. A. Quantification of the c-Fos positive cells in different experimental subjects' dHC under control conditions (white squares and circles) and/or stimulated conditions (black squares and circles). Figures **B – E** show representative microphotographs from the dHC slices used for the quantification of c-Fos immunoreactivity under different conditions. **Direct** refers to the usage of the SDT_D sampling-like juvenile exposure in controls and stimulated experimental subjects, while **indirect** refers to the usage of the SDT_I sampling-like juvenile exposure instead. Scale bar: 1000 μm. A standard one-way ANOVA followed by Tukey HSD *post hoc* test was used for statistical analyses. a' * $p < 0.05$ *versus* b, no significant differences a *vs* b. n = 5-6. Mean and individual values are represented.

Fig. 15: Quantification of c-Fos immunoreactivity cells in the vHC with representative microphotographs. A. Quantification of the c-Fos positive cells in different experimental subjects' vHC under control conditions (white squares and circles) and/or stimulated conditions (black squares and circles). Figures **B – E** show representative microphotographs from the vHC slices that were used for the quantification of c-Fos immunoreactivity for each condition. The figures **B - E** specifically show different areas from the vHC (i.e. CA1, CA3, Dentate gyrus (DG) and the Subiculum (Sub)). **Direct** refers to the usage of the SDT_D sampling-like juvenile exposure in controls and stimulated experimental subjects, while **indirect** refers to the usage of the SDT_I sampling-like juvenile exposure instead. Although in **B - E** microphotographs of distinct areas form the vHC are shown, the quantification was made from the whole vHC part. A standard one-way ANOVA followed by Tukey HSD *post hoc* test was used for statistical analyses. a * $p < 0.05$ *versus* b; b ** $p < 0.01$ *versus* a'. n = 5-6. Scale bar: 100 μm. Mean and individual values are represented.

No correlation was found between the investigation duration under SDT_D sampling-like conditions and the c-Fos synthesis in any of the analysed areas (PVA, r = 0.058, p = 0.919; SCh, r = 0.657, p = 0.175, MeA, r = - 0.371, p = 0.497; dHC, r = - 0.771, p = 0.103; vHC, r = - 0.657, p = 0.175).

4.1.3 Hippocampal temporal inactivation by lidocaine

4.1.3.1 Histology

The cannulae implantation and injection procedures caused minor damage in the brain tissue as revealed in the histological reconstruction of the injection site (Fig. 16). Behavioural performance measured in the untreated sessions at the end of the experiment confirmed the absence of deteriorating effect on social recognition memory performance (see 4.1.3.2).

Fig. 16: Representative examples of the coronal sections used for the reconstruction of the HC injection sites in the mouse brain. A. Photomicrograph from the cannulae placement/injection site in the dHC. **B.** Photomicrograph of the cannulae placement/injection site in the vHC. These are two representative coronal brain slices stained by Nissl staining and used to reconstruct the cannula placement and the injection site from both hemispheres of the HC. Scale bar: 1000 μm

Table 5 shows the total number of experimental subjects that underwent cannulae implantation for both the dHC and vHC, and the drop out caused by defined reasons.

Table 5. Evolution of the experimental subjects which underwent cannulae implantation.

	dHC	vHC
Experimental subjects which underwent cannulae implantation	~60	~50
Discarded before the end of the experiment	8	3
Discarded due to displacement of the cannuale	8	4
Final n° of animals included in the study	44	43

4.1.3.2 Social recognition memory after surgery and injections

The experimental subjects' long-term social recognition memory was tested using the SDT_D before the start of the experiment, after recovering from the cannuale implantation and after the end of all injection procedures. Significant longer investigation duration towards SA2 *vs.* SA1 was observed during the choice session in the experimental subjects tested before starting any invasive procedure (see "before surgery" Fig. 17 and Fig. 18; dHC: t = 7.318, df = 42, p < 0.0001; vHC: t = 5.197, df = 25, p < 0.0001). All experimental subjects with implanted cannulae showed a robust investigation duration towards SA1 during the sampling session, and a significantly longer investigation duration towards SA2 *vs.* SA1 during the choice session (see "after surgery" Fig. 17 and Fig. 18, dHC: t = 5.788, df = 42, p < 0.0001; vHC: t = 6.141, df = 41, p < 0.0001). Similar results were obtained when the experimental subjects were tested using the SDT_D after being injected between two to four times with aCSF and/or lidocaine either on the dHC or the vHC (see "after injections" Fig. 17 and Fig. 18; dHC: t = 3.587, df = 34, p = 0.0010; vHC: t = 4.596, df = 28, p < 0.0001) (to see further detailed statistical analyses see 9.4.2)

Fig. 17. Long-term social recognition memory of the experimental subjects used for the bilateral dHC injection experiments. SDT_D testing long-term memory was performed in all mice before the surgery, after the surgery and after the injections. The investigation duration from the experimental subjects towards each stimulus animal (SA) during each session of the SDT_D, sampling and choice, is represented. During choice: SA1, stimulus animal 1 (familiar); SA2, stimulus animal 2 (unfamiliar). A paired *t*-test was used for the statistical analyses. Before surgery n = 43, after surgery n = 43 and after injections n = 35.

Fig. 18: Long-term social recognition memory of the animals used for the bilateral vHC injection experiments. SDT_D testing long-term memory was performed in all mice before the surgery, after the surgery and after the injections. The investigation duration from the experimental subjects towards each stimulus animal (SA) during each session of the SDT_D, sampling and choice, is represented. During choice: SA1, stimulus animal 1 (familiar); SA2, stimulus animal 2 (unfamiliar). A paired *t*-test was used for the statistical analysis. Before surgery n = 26, after surgery n = 42 and after injections n = 29.

4.1.3.3 Behavioural consequences of intra-hippocampal injections in the SDT

4.1.3.3.1 Bilateral injections in dorsal hippocampus

4.1.3.3.1.1 Sexual and aggressive behaviour

The duration of the experimental subjects' aggressive/sexual behaviour scored during each session of the SDT_D tested after receiving intra-HC injections was analysed with the standard two-way ANOVA test, defining the main factors as "injected substance" and "session". Despite no effect of the "injected substance" was observed on the duration of the aggressive/sexual behaviour of the experimental subjects towards the stimuli animals, a significant effect of the "session" was detected ("injected substance", $F_{(1,154)} = 0.2456$, p = 0.6209; "trial", $F_{(2,154)} = 33.92$, p < 0.0001). No interaction was observed between the two main factors ($F_{(2,154)} = 1.689$, p = 0.1880) (Fig. 19A) (to see *post hoc* analyses see 9.4.3.1).

Because the duration of the aggressive/sexual behaviour was in some instances shorter than one second (which made the reliability of those measurements that were only a few millisecons / bout in duration questionable), the number of bouts showing aggressive/sexual behaviour towards the distinct stimuli animals was also analysed. A standard two-way ANOVA test with main factors "injected substance" and "session" (Fig. 19B) failed to detect a significant effect of the "injected substance" (Fig. 19B; $F_{(1,154)} = 0.5243$, p = 0.4701), but did detect a significant "session" effect (Fig. 19B; $F_{(2,154)} = 28.31$, p < 0.0001). No interaction between the main factors was revealed ($F_{(2,154)} = 2.501$, p = 0.0853).

Fig. 19. Aggressive/sexual behaviour registered during the distinct sessions of the SDT$_D$ tested after bilateral dHC injections of either aCSF or lidocaine. A. Representation of the total aggressive/sexual behaviour duration in each session of the SDT$_D$: Sampling, Choice using an exposure interval of 1 h (Choice, 1 h), and choice using an exposure interval of 24 h (Choice, 24 h). **B.** Representation of the total number of bouts in which the experimental subjects showed aggressive/sexual behaviour towards the stimuli animals during each session. Each bar represents the behaviour of the experimental subjects during each session of the SDT$_D$ tested after receiving injections of either aCSF (white bars) or of lidocaine (black bars). A standard two-way ANOVA followed by Bonferroni *post hoc* test was used for the statistical analyses. Sampling, n = 40; Choice, 1 h, n = 20; Choice, 24 h, n = 20.

4.1.3.3.1.2 Investigatory behaviour

All tested experimental subjects showed vigorous investigation towards SA1 during the sampling session after receiving dHC injections. No significant differences were observed with regards to the investigation duration between the injected substances (SDT$_D$: t = 1.329, df = 78, p = 0.1877; SDT$_I$: t = 0.1288, df = 70, p = 0.8979) (for results and statistical analyses see 9.3). Their behaviour during the choice session, using exposure intervals of 1 h to test short-term memory and 24 h to test long-term social recognition memory, is described below:

SDT$_D$

As shown in Fig. 20A, experimental subjects bilaterally injected in the dHC with either aCSF or lidocaine failed to differ in their investigation duration towards the SA1 *vs.* SA2 when testing short-term memory (Fig. 20A, aCSF: t = 1.381, df = 19, p = 0.1834; Lidocaine: t = 1.855, df = 19, p = 0.0792). In contrast, when testing long-term memory experimental subjects injected with aCSF investigated significantly longer SA2 than SA1 during the choice session (Fig. 20B, t = 2.138, df = 19, p = 0.0457). This difference was abolished under the lidocaine treatment when testing long-term social recognition memory (Fig. 20B, t = 0.1060, df = 1, p = 0.9167).

SDT$_I$

SDT$_I$ was additionally used to test the consequences of aCSF and lidocaine injections in the dHC. Under short-term memory testing conditions, experimental subjects investigated longer SA2 vs. SA1 after aCSF injections, but not after lidocaine injections (Fig. 20C, aCSF: t = 2.191, df = 18, p = 0.0419; Lidocaine: t = 1.456, df = 17, p = 0.1636). In contrast, the experimental subjects that were tested under long-term memory conditions showed significantly longer investigation durations towards SA2 *vs.* SA1 after both aCSF and lidocaine injections (Fig. 20D, aCSF: t = 3.141, df = 18, p = 0.0056; Lidocaine: t = 2.247, df = 18, p = 0.0374).

Fig. 20. Effects of the temporary dHC inactivation by lidocaine injections before sampling on short- and long-term social recognition memory tested in the SDT$_D$ and the SDT$_I$. A-D. Investigation durations towards each stimulus animal (SA1; familiar and SA2; non-familiar) during the choice session are shown. Short-term social memory (**A and C**) and long-term social memory (**B and D**) were tested using the SDT$_D$ (**A and B**) and SDT$_I$ (**C and D**) after dorsal intra-hippocampal injections of either aCSF or lidocaine before the sampling session. A paired *t*-test was used for the statistical analyses. n = 20.

4.1.3.3.2   Bilateral injections in ventral hippocampus

4.1.3.3.2.1   Sexual and aggressive behaviour

A standard two way-ANOVA detected a significant effect of the "session", but not of the "injected substance" on the duration of the aggressive/sexual behaviour during the SDT$_D$ (Fig. 21A; "session":

$F_{(2,154)} = 14.19$, p < 0.0001; "injected substance": $F_{(1,154)} = 0.3953$, p = 0.5305). No interaction was identified between the two main factors ($F_{(2,154)} = 2.208$, p = 0.1134).

For the reasons explained above (see 4.1.3.3.1.1), the number of bouts of aggressive/sexual behaviour towards the stimuli animals was also analysed using a standard two-way ANOVA (Fig. 21B). The results indicated no effect of the "injected substance", although a strong effect of the "session" was detected ("injected substance": $F_{(1,154)} = 0.9805$, p = 0.3236; "trial": $F_{(2,154)} = 28.79$, p < 0.0001) (to see *post hoc* analyses see 9.4.3.2). No interaction of the main factors was identified ($F_{(2,154)} = 0.5088$, p = 0.6022).

Fig. 21. Aggressive/sexual behaviour registered during the distinct sessions of the SDT$_D$ tested after bilateral vHC injections of either aCSF or lidocaine. A. Representation of the total aggressive/sexual behaviour duration in each session of the SDT$_D$: Sampling, Choice using an exposure interval of 1 h (Choice, 1 h), and choice using an exposure interval of 24 h (Choice, 24 h). **B.** Representation of the total number of bouts in which the experimental subjects showed aggressive/sexual behaviour towards the stimuli animals during each session. Each bar represents the behaviour of the experimental subjects during each session of the SDT$_D$ tested after receiving injections of either aCSF (white bars) or of lidocaine (black bars). A standard two-way ANOVA followed by Bonferroni *post hoc* test was used for the statistical analyses. Sampling, n = 40; Choice, 1 h, n = 20; Choice, 24 h, n = 20.

4.1.3.3.2.2   Investigatory behaviour

All experimental subjects tested after receiving vHC injections showed robust investigation towards SA1 during the sampling session showing no differences between substances (SDT$_D$: t = 0.3642, df = 78, p = 0.7167; SDT$_I$: t = 0.3519, df = 73, p = 0.7259) (for results and statistical analyses see 9.3). Their behaviour during the choice session using exposure intervals of 1 h to test short-term memory and 24 h to test long-term social recognition memory is described below:

SDT$_D$

As shown in Fig. 22A, experimental subjects bilaterally injected in the vHC with aCSF failed to differ in their investigation duration towards the SA2 *vs.* SA1, if tested for short-term memory (Fig. 22A; aCSF: t = 1.233, df = 19, p = 0.2328). In contrast, when the short-term memory was tested following lidocaine injections significant differences in the investigation duration towards the SA2 *vs.* SA1 were revealed (Fig. 22A; lidocaine: t = 3.544, df = 19, p = 0.0022). Contrary to the results obtained from the short-term memory test, the experimental subjects that were tested for the long-term memory injected with aCSF, but not with lidocaine, investigated SA2 significantly longer than SA1 during the choice session (Fig. 22B, aCSF: t = 2.239, df = 19, p = 0.0373; lidocaine: t = 0.8005, df = 1, p = 0.4333).

SDT$_I$

SDT$_I$ was also used to test the consequences of aCSF and lidocaine injections in the vHC. Using the indirect exposure of the stimuli animals under short-term testing conditions, aCSF injections had no effect on the experimental subjects' preference to investigate SA2 *vs.* SA1 (Fig. 22C, t = 2.852, df = 16, p = 0.0115). This preference was eliminated after lidocaine injections (Fig. 22C, t = 0.2906, df = 17, p = 0.7748). As shown in Fig. 22D experimental subjects injected with either aCSF or lidocaine failed to differ in their investigation duration towards SA2 *vs.* SA1 if tested for long-term memory (Fig. 22D, aCSF: t = 1.917, df = 19, p = 0.0704, lidocaine: t = 1.773, df = 19, p = 0.0922).

Fig. 22: Effects of the temporary vHC inactivation by lidocaine injections before sampling on short- and long-term social recognition memory tested in the SDT$_D$ and the SDT$_I$. A-D. Investigation durations towards each stimulus animal (SA1; familiar and SA2; non-familiar) during the choice session are shown. Short-term social memory (**A and C**) and long-term social memory (**B and D**) were tested using the SDT$_D$ (**A and B**) and SDT$_I$ (**C and D**) after dorsal intra-hippocampal injections of either aCSF or lidocaine before the sampling session. A paired *t*-test was used for statistical analyses, n = 20.

4.2 Results obtained from Grb2 cKO mice

4.2.1 Brain morphology

A gross anatomical analysis revealed no obvious differences between Grb2 cKO mice and their control littermates, WT (Fig. 23).

Fig. 23. Gross hippocampus anatomy from WT and Grb2 cKO mice. Representative photomicrographs of hippocampal coronal sections stained by the Nissl taining method. **A.** Dorsal hippocampus, **B.** Ventral hippocampus. Scale: 1000 μm.

4.2.2 Grb2 protein levels in the adult mice brain

Significant lower levels of Grb2 were observed in the HC from 10 - 24 weeks old Grb2 cKO mice *vs.* their control littermates (Fig. 24A; t = 6.833, df = 15, p < 0.0001). However, no significant differences between genotypes were observed when the phosphorylation state of Erk1 and Erk2 (also known as p44MAPK and p42MAPK, respectively) in the HC under basal conditions were studied (Fig. 24B; t = 1.266, df = 15, p = 0.2247). Higher amounts of Erk2 and also phoshpo-Erk2 (pErk2) immunoreactivity in comparison to Erk1 and phospho-Erk (pErk1) were observed in all experimental subjects (Fig. 24B).

A significant reduction of Grb2 levels in Grb2 cKO mice compared to WT mice was also observed in the olfactory bulb, frontal cortex and striatum (Fig. 25A, Olfactory bulb: t = 5.647, df = 14, p < 0.001; Fig. 25B, Frontal cortex: t = 5.568, df = 15, p < 0.001 and Fig. 25C, Striatum: t = 4.884, df = 14, p = 0.0002, respectively), but not in the cerebellum (Fig. 25D, t = 1.115, df = 15, p = 0.2823).

Fig. 24. Hippocampal endogenous protein levels of Grb2 and phosphorylated Erk (pErk1 and pErk2) in WT and Grb2 cKO mice. A-B. Representative Western blots are shown for illustration **A**. As shown in the western blot image, hippocampal Grb2 protein levels were significantly reduced in Grb2 cKO *vs.* their wildtype littermates (WT). GAPDH was used as a loading control. **B**. As shown by the western blot illustration, levels of phospho-Erk1 (pErk1) and phosphor-Erk2 (pErk2) in basal conditions were not significantly different between genotpyes. Total levels of Erk (totalErk1 and totalErk2) were used to normalize the levels of pErk1 and pErk2 and α-tubulin was used as a loading control. Mice from 10 to 24 weeks old were used for the experiment. An unpaired *t*-test was used for the statistical analyses.WT, n = 8; Grb2 cKO, n = 9.

Fig. 25. Levels of Grb2 protein expression in different brain areas of WT and Grb2 cKO mice. Representative images of Western blots are shown for illustration **A.** Immunoblot of Grb2 levels in the olfactory bulb, **B**. frontal cortex and **C**. striatum from WT and Grb2 cKO mice and its quantification showing a significant decrease of Grb2 protein levels. **D**. Immunoblot of

Grb2 levels in the cerebellum from WT and Grb2 cKO mice and its quantification showing no differences between genotypes. An unpaired *t*-test was used for statistical analyses.WT, n = 8 *vs*. Grb2 cKO, n = 8 - 9.

4.2.3 Behavioural analysis

4.2.3.1 Open field

Grb2 cKO mice and their control littermates actively inspected the open field arena for a comparable length of time (Fig. 26A; investigation duration, t = 0.9511, df = 38, p = 0.3476), spending similar time in the centre and the periphery of the arena (Fig. 26B; total time 600 s: ~ 80 % of time in the periphery and ~ 20 % in the centre). Significant differences were observed in the total number of times the experimental subject crossed the dividing lines (Fig. 26D; t = 3.009, df = 19, p = 0.0072). When analysed separately, in the centre and in the periphery, significant differences were observed only in the periphery, but not in the centre (Fig. 26E; periphery: t = 2.959, df = 38, p = 0.0053; centre: t = 1.877, df = 38, p = 0.0682). No significant differences between the genotypes were observed in the number of grooming episodes (Fig. 26C; t = 1.622, df = 38, p = 0.1131). In contrast, the number of rearing episodes was increased significantly in Grb2 cKO mice *vs*. WT littermates (Fig. 26F; t = 2.053, df = 38, p = 0.0470). Grb2 cKO mice and control littermates showed similar urination and defecation habits during the open field test (Fig. 27; streaks of urine: t = 0.4049, df = 40, p = 0.6877; puddle: t = 1.763, df = 40, p = 0.0856).

Fig. 26. Behavioural parameters measured during the open field test performed by WT and Grb2 cKO mice. A. Time spent by the experimental subjects inspecting the open field arena. **B**. Time spent in each area of the open field, in the centre or in the periphery of the arena. **C.** Number of grooming episodes. **D.** Number of times the experimental subjects crossed the lines, which divided the field in equal areas. **E.** Number of lines crossed in each of the areas of the open field. **D.** Number of rearing episodes during the open field test. An unpaired *t*-test was used for thevstatistical analyses. WT: n = 20; Grb2 cKO: n = 20.

Fig. 27. Urination and defeacation habits during the open field registered from WT and Grb2 cKO mice. A. Number of puddles or streaks of urine the mice produced during the open field test. **B.** Number of faecal boli produced during the open field test. An unpaired *t*-test was used for the statistical analyses. WT: n = 20; Grb2 cKO: n = 20.

4.2.3.2 Olfactory habituation and dishabituation test

A repeated measures one-way ANOVA was used to analyse the experimental subjects' investigation time and the number of bouts towards the odours presented in each trial of the olfactory habituation and dishabituation test, and revealed significant differences between trials in the investigation time and number of bouts for both, WT and Grb2 cKO mice (Fig. 28A: WT: investigation time, $F_{(4,19)} = 17.66$, $p < 0.0001$, $R^2 = 0.4817$; Fig. 28C: number of bouts: $F_{(4,19)} = 12.25$, $p < 0.0001$, $R^2 = 0.3921$; Fig. 28B: Grb2 cKO, investigation time: $F_{(4,19)} = 9.803$, $p < 0.0001$, $R^2 = 0.3404$; Fig. 28D: number of bouts: $F_{(4,19)} = 4.113$, $p = 0.0045$, $R^2 = 0.1779$).

WT and Grb2 cKO mice showed a significant decrease in the investigation duration between the first and the second habituation trials compared to the fourth habituation trial (Fig. 28A: WT, H1 - H4: q = 9.805, $p < 0.0001$; H2 - H4: q = 7.207, $p < 0.0001$; Fig. 28B: Grb2 cKO: H1 - H4: q = 7.223, $p < 0.0001$; H2 - H4: q = 4.978, $p < 0.01$). The presentation of the odour mixture in the dishabituation trial resulted in a significantly increased investigation of the odour source in both genotypes (Fig. 28A: WT, H4 - D: q = 6.864, $p < 0.0001$; Fig. 28B, Grb2 cKO: H4 - D: q = 7.198, $p < 0.0001$) (for further details from the *post hoc* analyses see 9.4.4). As the investigation durations were found to be relatively low (see Fig. 28A, less than 1 s in control littermates for the third and fourth habituation trials), the number of investigatory bouts was also analysed. This parameter proved the significant reduction between the first and second habituation trials, compared to the fourth habituation trial in both genotypes (Fig. 28C: WT, H1 - H4: q = 7.419, $p < 0.0001$; H2 - H4: q = 7.557, $p < 0.0001$; Fig. 28D, Grb2 cKO: H1 - H4: q = 5.224, $p < 0.01$; H2 - H4: q = 4.460, $p < 0.05$). However a significant increase from the fourth habituation trial to the dishabituation trial was only observed in the WT mice (Fig. 28C: WT, H4 - D: q = 5.907, $p < 0.0001$; Fig. 28D Grb2 cKO: H4 - D: q = 2.421, $p > 0.05$) (for further details from the *post hoc* analyses see 9.4.4).

Fig. 28. Results from the olfactory habituation and dishabituation test performed by WT and Grb2 cKO mice. The two top graphs show the investigation time that the experimental subjects invested in sniffing the presented odour through the different trials of the habituation-dishabituation test, **A**. WT littermates, **B**. Grb2 cKO mice. The two bottom graphs display the number of bouts that the experimental subjects performed while investigating the presented odour, **C**. WT littermates and **D**. Grb2 cKO mice. H1: first habituation trial; H2: second habituation trial; H3: third habituation trial, H4; fourth habituation trial, D; dishabituation trial (fifth trial). The first odour (Odour A, carvone) was presented in the first four habituation trials (H1 - H4, white bars), and change to a new mixed one (Odour B; 1:1, carvone : isoamyl acetate) in the dishabituation trial (black bars). Repeated-measures one-way ANOVA followed by Tukey HSD *post hoc* multiple comparisons test was performed for the statistical analyses. WT; n = 20; Grb2 cKO; n = 20

4.2.3.3 Social discrimination test (SDT$_D$)

4.2.3.3.1 Social recognition memory

WT mice showed significantly longer investigation duration towards SA2 *vs.* SA1 during choice when tested under short-term, intermediate-term and long-term memory testing conditions (Fig. 29A; EI = 1 h: t = 3.6120, df = 19, p = 0.0019; EI = 6 h: t = 3.2014, df = 19, p = 0.0046; EI = 24 h: t = 3.447, df = 19, p = 0.0027).

Grb2 cKO mice also investigated significantly longer SA2 *vs.* SA1 during choice when short-term and intermediate-term memory testing conditions were used (Fig. 29B; EI = 1 h: t = 4.233, df = 19, p = 0.0005; EI = 6 h: t = 3.662, df = 19, p = 0.0067), but showed similar investigation duration towards both SA1 and SA2 under long-term memory testing conditions (Fig. 29B; EI = 24 h: t =0.1402, df = 19, p = 0.8900). Although Grb2 cKO experimental subjects showed a robust investigation towards SA1 during sampling, neither Grb2 cKO males nor females, showed a preference to investigate SA2 over SA1 during choice when tested with an exposure

interval of 24 h (Fig. 29C; males, EI = 24 h: t = 0.4865, df = 12, p = 0.6354; females, EI = 24 h: t = 1.013, df = 14, p = 0.3284).

Fig. 29. Social recognition memory from WT and Grb2 cKO experimental subjects tested with the SDT$_D$. A – B. Representation of the investigation durations spent by the experimental subjects, either **A.** WT or **B.** Grb2 cKO, towards each stimulus animal (SA1 "familiar" and SA2 "unfamiliar") during the choice session of the SDT$_D$ is represented. Three different exposure intervals (EI) were used to test social memory; EI = 1 h (short-term memory), EI = 6 h (intermediate term-memory) and EI = 24 h (long-term memory).**C.** Investigation durations spent by Grb2 cKO male and Grb2 cKO females towards SA2 and SA1 during the sampling and the choice session, using an EI of 24 h. A paired *t*-test was used for the statistical analyses. In panel A and B n = 20; in panel C: male n = 13; females n = 15.

4.2.3.3.2 Latency to approach the stimulus animal during the SDT$_D$

No significant differences were observed between the latencies scored towards SA1 and SA2 during the choice session, neither in WT nor in Grb2 cKO mice in any of the EI used (Fig. 30A, WT: EI = 1 h: t = 0.6803, df = 19, p = 0.5045; EI = 6 h: t = 1.271, df = 19, p = 0.2190; EI = 24 h: t = 0.7713, df = 19, p = 0.4500; Grb2 cKO; EI = 1 h: t = 0.5620, df = 19, p = 0.5807; EI = 6 h: t = 0.7318, df = 19, p = 0.4732; EI = 24 h: t = 1.739, df = 19, p = 0.0983). Experimental subjects showed latencies from 30 to 50 s to start investigating any of the stimuli animals. A possible "EI" or "genotype" effect on the latencies to investigate the stimuli animals was also studied (Fig. 30B). A standard two-way ANOVA analysis did not detect a significant effect for any of the main factors (Fig. 30B, "genotype": $F_{(1,76)}$ = 0.2556, p = 0.6161; "exposure interval": $F_{(2,76)}$ = 0.8052, p = 0.4508), and revealed no interaction between them ($F_{(2,76)}$ = 0.4445, p = 0.6428).

Fig. 30. Latency to approach the stimuli animals for investigation showed by WT and Grb2 cKO mice during the choice session of the SDT$_D$. A. Latency recorded from WT (left) and Grb2 cKO (right) mice towards each stimulus animal (SA1 "familiar" and SA2 "unfamiliar") during the choice sessions of the SDT$_D$ using different exposures intervals: EI = 1 h (short-term memory), EI = 6 h (intermediate term-memory) and EI = 24 h (long-term memory). **B.** Total latencies (towards SA1 + towards SA2) per genotype during the choice session registered after the defined exposure intervals. A paired t-test was used to analyse the data represented in the panel A. A standard two-way ANOVA was used for the statistical analyses of the data represented in the panel B. n = 20.

4.2.3.3.3 Aggressive/sexual behaviour during the SDT$_D$

All of the experimental subjects tested, independent of the EI used, showed low levels of aggressive/sexual behaviour towards the distinct stimuli animals presented during the choice session, as in all cases they were below 3 s. However, significant differences in the duration of the aggressive/sexual behaviour towards SA2 *vs.* SA1 were observed in WT mice when tested using an EI of 24 hours (Fig. 31A, WT: EI = 1 h: t = 0.1806, df = 19, p = 0.8586; EI = 6 h: t = 0.3268, df = 19, p = 0.7474; EI = 24 h: t = 2.600, df = 19 p = 0.0176; Grb2 cKO; EI = 1 h: t = 1.972, df = 19, p = 0.0633; EI = 6 h: t = 0.6759, df = 19, p = 0.5073; EI = 24 h: t = 0.7355, df = 19, p = 0.4710). As the duration of the aggressive/sexual behaviour was found to be relatively low (less than 1.5 s in both, WT and Grb2 cKO), the number of bouts was also analysed. The analyses of this parameter revealed similar results as obtained with the analyses of the duration of the aggressive/sexual behaviour as significant differences were observed when WT mice were tested using an EI of 24 h, but also on Grb2 cKO mice tested using an EI of one hour (Fig. 31C, WT: EI = 1 h: t = 0.2107, df = 19, p = 0.8354; EI = 6 h: t = 0.0563, df = 19, p = 0.9557; EI = 24 h: t = 2.736, df = 19, p = 0.0131; Grb2 cKO; EI = 1 h: t = 2.485, df = 19, p = 0.0225; EI = 6 h: t = 0.0539, df = 19, p = 0.9576; EI = 24 h: t = 0.337, df = 19, p = 0.7398).

No significant differences were observed in the total aggressive/sexual behaviour registered during the choice session of the SDT$_D$. The standard two-way ANOVA analyses, using "genotype" and "EI" as main factors, revealed the absence of a significant effect of the factors on the aggressive/sexual behaviour duration (Fig. 31B, "genotype": $F_{(1,76)}$ = 1.242, p = 0.2720; "EI": $F_{(2,76)}$ = 1.249, p = 0.2925) and on the number of bouts (Fig. 31D, "genotype": $F_{(1,38)}$ = 0.02371, p = 0.8784; "EI": $F_{(2,38)}$ = 1.130, p = 0.3284), without interference between the main

factors (Fig. 31B and 31D, aggressive/sexual behaviour duration: $F_{(2,76)} = 0.4626$, $p = 0.6314$; number of bouts: $F_{(2,76)} = 2.252$, $p = 0.1122$).

Fig. 31. Aggressive/sexual behaviour during the SDT$_D$ showed by WT and Grb2 cKO mice. In the graph **A** are represented the duration of the aggressive/sexual behaviour the experimental subjects (left: WT littermates; right: Grb2 cKO mice) spent towards the different stimuli animals (SA1 and SA2) during the choice session tested applying different exposure intervals (EI: 1 h, 6 h and 24 h). **B.** Total aggressive/sexual behaviour duration scored during the choice session after the different exposure intervals from WT and Grb2 cKO mice, towards both stimuli animals is represented. **C.** The aggressive/sexual behaviour during choice towards each stimulus animal is represented in the number of bouts instead (left: WT littermates; right: Grb2 cKO mice). **D.** Total number of bouts of aggressive/sexual behaviour scored during the choice session after the different exposure intervals from WT and Grb2 cKO mice, towards both stimuli animals. SA1: stimulus animal 1 (familiar), SA2: stimulus animal 2 (novel juvenile, unfamiliar). A paired *t*-test was used for the statistical analyses of the data represented in the panels A and C. A standard two-way ANOVA was used to statistically analyse the data represented in panels B and D. n = 20.

4.2.3.4   Novel object recognition memory

4.2.3.4.1   Short-term memory

Grb2 cKO mice showed significantly longer travelled distances than their WT littermates (Fig. 32A, $t = 3.298$, $df = 31$, $p = 0.0025$). The standard two-way ANOVA analyses, using "trial" and "genotype" as main factors, revealed no significant effects of these factors on the exploration duration towards the objects (Fig. 32B, "trial": $F_{(1,31)} = 1.873$, $p = 0.1810$; "genotype": $F_{(1,31)} = 0.6142$, $p = 0.4392$) and no significant interaction between the main factors ($F_{(1,31)} = 0.01231$, $p = 0.9124$).

WT but not Grb2 cKO mice, spent significantly more time exploring the novel than the familiar object during the second trial or memory session (Fig. 32C, WT: $t = 2.802$, $df = 15$, $p = 0.0134$; Grb2 cKO: $t = 0.2164$, $df = 16$, $p = 0.8314$). The preference index towards the novel object was significantly higher than chance "0.5", for WT mice only (Fig. 32D, WT: $t = 2.646$, $df = 15$, $p = 0.0183$; Grb2 cKO, $t = 0.2049$, $df = 16$, $p = 0.8403$).

Fig. 32. Intact short-term object recognition memory observed in WT but not in Grb2 cKO mice. A. Total travelled distance registered during the entire NOR test from WT mice (white bar) and Grb2 cKO mice (black bar) is represented. **B.** Total exploration duration towards both presented objects spent by WT and Grb2 cKO mice, during each trial (1st and 2nd) of the NOR test. **C.** Exploration duration towards each object (familiar "white bars" and novel "black bars") during the second trial (memory session) of the NOR test. **D.** The preference Index (PI) towards each object is represented comparing it to the chance value represented at 0.5 PI. An unpaired and a paired *t*-test were used for the statistical analyses of the data represented in the panels A and C, respectively. Repeated-measures two-way ANOVA (main factors: "genotype" and "trial") and one sample *t*-test were used for statistical analyses of the data represented in panels B and D, respectively. WT, n = 15; Grb2 cKO, n = 16.

4.2.3.4.2   Long-term memory

Grb2 cKO mice travelled significantly longer distances than WT mice during the NOR test (Fig. 33A, t = 3.307, df = 17, p = 0.0042). A standard two-way ANOVA analyses with "trial" and "genotype" as main factors revealed a significant effect of the trial, but not of the "genotype" of the experimental subjects, on the exploration duration during the NOR test (Fig. 33B; "trial": $F_{(1,31)} = 13.78$, p = 0.0008, "genotype": $F_{(1,31)} = 0.09898$, p = 0.7552), without interaction between the main factors ($F_{(1,31)} = 0.1317$, p = 0.7192). During the second trial, neither WT nor Grb2 cKO mice showed significant longer exploration durations towards the novel *vs.* the familiar object (Fig. 33C, WT: t = 1.394, df = 15, p = 0.1835; Grb2 cKO: t = 1.674, df = 16, p = 0.1135). The preference index was not significantly higher than 0.5 for any of the genotypes tested (Fig. 33D; WT: t = 0.8726, df = 15, p = 0.3966, Grb2 cKO: t = 1.772, df = 16, p = 0.955).

Fig. 33. Long-term novel object recognition memory absent in WT and Grb2 cKO mice. A. Total travelled distance registered during the entire NOR test from WT mice (white bar) and Grb2 cKO mice (black bar) is represented. **B.** Total

exploration duration towards both presented objects spent by WT and Grb2 cKO mice, during each trial (1st and 2nd) of the NOR test. **C.** Exploration duration towards each object (familiar "white bars" and novel "black bars") during the second trial (memory session) of the NOR test. **D.** The preference Index (PI) towards each object is represented comparing it to the chance value represented at 0.5 PI. An unpaired and a paired *t*-test were used for the statistical analyses of the data represented in the panels A and C, respectively. Repeated-measures two-way ANOVA (main factors: "genotype" and "trial") and one sample *t*-test were used for statistical analyses of the data represented in panels B and D, respectively. WT, n = 15; Grb2 cKO, n = 16.

4.2.3.5 Fear conditioning test

4.2.3.5.1 Cued fear conditioning

<u>Fear conditioning</u>

The analyses of the overall behavioural parameters measured throughout the whole conditioning session showed no significant differences between genotypes except for the number of jumps, which was significantly higher in Grb2 cKO compared to WT mice (Fig. 34A, travelled distance: t = 1.588, df = 27, p = 0.1260; Fig. 34B, rearing episodes: t =0.1841, df = 27, p = 0.8553; Fig. 34C, number of jumps: t = 2.726 , df = 27, p = 0.0111; Fig. 34D, % time freezing: t = 0.6711, df = 27, p = 0.5079).

Fig. 34. Analyses of the behavioural parameters scored during the conditioning session of the cued fear conditioning test. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour during the conditioning session. An unpaired *t*-test was used for the statistical analyses. WT, n = 14; cKO, n = 15.

For a more detailed analysis the registered behavioural parameters during each interval of the fear conditioning session were represented (see 3.2.4.1, Fig. 35). Repeated measures two-way ANOVA with "genotype" and "conditioning interval" as main factors revealed a significant effect of the "conditioning interval" for all scored behavioural parameters (Fig. 35A, travelled distance: $F_{(3,81)} = 146.3$, p < 0.0001; Fig. 35B, rearing episodes: $F_{(3,81)} = 7.214$, p = 0.0002; Fig. 35C, number of jumps: $F_{(3,81)} = 43.41$, p < 0.0001; Fig. 35D, % time freezing: $F_{(3,81)} = 19.05$, p < 0.0001). However, no significant "genotype" effect was detected, except for the number of jumps which was significantly higher in Grb2 cKO mice compared to their WT littermates (Fig. 35A, travelled distance: $F_{(1,81)} = 2.043$, p = 0.1644; Fig. 35B, rearing episodes: $F_{(1,81)} = 0.003296$, p = 0.9546; Fig. 35C,

number of jumps: $F_{(1,81)} = 4.928$, p = 0.0350; Fig. 35D, % time freezing: $F_{(1,81)} = 0.3555$, p = 0.5560). No interaction between the main factors was revealed in any of the behavioural parameters analysed (Fig. 35A; travelled distance: $F_{(3,81)} = 0.6880$, p = 0.5620; Fig. 35B, rearing episodes: $F_{(3,81)} = 0.7657$, p = 0.5165; Fig. 35C, number of jumps: $F_{(3,81)} = 5.393$, p = 0.0020; Fig. 35C, % time freezing: $F_{(3,81)} = 0.4945$, p = 0.6871).

Fig. 35. Behavioural responses registered during the different conditioning intervals of the conditioning session from the cued fear conditioning. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour in the different conditioning intervals. The mean (of the 30 seconds measurements) + SEM are represented. Repeated-measures two-way ANOVA (main factors: "genotype" and "conditioning interval") was used for the statistical analyses. WT, n = 14; cKO, n = 15.

The behaviour of the experimental subjects during the 60 s subsequent to the presentation of each CS (post-CS) was also analysed. Repeated-measures two-way ANOVA analyses with the main factors "genotype" and "post-CS" produced the following results: (1) a significant effect of the main factor "genotype" on the number of jumps and travelled distance (Fig. 36A, travelled distance: $F_{(1,54)} = 5.120$, p = 0.0391; Fig. 36B, rearing episodes: $F_{(1,54)} = 0.3553$, p = 0.5561; Fig. 36C, number of jumps: $F_{(1,54)} = 7.784$, p = 0.0042; Fig. 36D, % time freezing: $F_{(1,54)} = 3.963$, p = 0.0567) (2) a significant effect of the main factor "post-CS" on the number of jumps, travelled distance and the percentage of time freezing (Fig. 36A, travelled distance: $F_{(2,54)} = 10.80$, p = 0.0001; Fig. 36B, rearing episodes: $F_{(2,54)} = 1.009$, p = 0.3715; Fig. 36C, number of jumps: $F_{(2,54)} = 8.850$, p = 0.0005; Fig. 36D, % time freezing: $F_{(2,54)} = 11.89$, p < 0.0001) (3) no significant interaction between the main factors was detected for any of the behavioural parameters analysed (Fig. 36A; travelled distance: $F_{(2,54)} = 0.001405$, p = 0.9986; Fig. 36B, rearing episodes: $F_{(2,54)} = 0.1596$, p = 0.8529; Fig. 36C, number of jumps: $F_{(2,54)} = 0.4644$, p = 0.6310; Fig. 36C, % time freezing: $F_{(2,54)} = 0.7623$, p = 0.4715).

Fig. 36. Behavioural changes during the 60 s after each CS, paired with the UCS, presentation. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour in response to the CS during the cued fear-conditioning training. The data was analysed using a repeated-measures two-way ANOVA (main factors: "genotype" and "post-CS"). WT, n = 14; cKO, n = 15.

Fear memory retrieval

No significant differences between Grb2 cKO mice and their WT littermates were observed when the overall behavioural parameters measured throughout the entire fear memory retrieval session were analysed (Fig. 37A; travelled distance: t = 1.898, df = 27, p = 0.0684; Fig. 37B, rearing episodes: t = 1.619, df = 27, p = 0.1172; Fig. 37C, number of jumps: t = 1.417, df = 27, p = 0.1678; Fig. 37D, % time freezing: t = 1.599, df = 27, p = 0.1215).

Fig. 37. Analyses of the behavioural parameters scored during the memory session of the cued fear conditioning test. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour during the memory session of the cued fear conditioning test. An unpaired *t*-test was used for statistical analyses. WT, n = 14; cKO, n = 15.

The different behavioural parameters measured during the fear memory retrieval session were analysed using the repeated measures two-way ANOVA with the "genotype" and the "memory retrieval interval" as main factors (see 3.2.4.1, Fig. 38). A significant effect of the "memory retrieval interval" was shown for all behavioural parameters measured (Fig. 38A; travelled distance: $F_{(3,81)} = 33.34$, $p < 0.0001$; Fig. 38B, rearing episodes: $F_{(3,81)} = 3.961$, $p = 0.0109$; Fig. 38C, number of jumps: $F_{(3,81)} = 3.082$, $p = 0.0320$; Fig. 38D, % time freezing: $F_{(3,81)} = 55.25$, $p < 0.0001$). A significant "genotype" effect was also observed in the number of rearing episodes and number of jumps, but absent in the travelled distance and the percentage of time freezing (Fig. 38A; travelled distance: $F_{(1,81)} = 2.478$, $p = 0.1271$; Fig. 38B, rearing episodes: $F_{(1,81)} = 4.286$, $p = 0.0481$; Fig. 38C, number of jumps: $F_{(1,81)} = 5.402$, $p = 0.0279$; Fig. 38D, % time freezing: $F_{(1,81)} = 2.529$, $p = 0.1234$). Interaction between the two main factors was observed on the travelled distance and number of jumps only (Fig. 38A, travelled distance: $F_{(3,81)} = 3.733$, $p = 0.0144$; Fig. 38B, rearing episodes: $F_{(3,81)} = 0.7506$, $p = 0.5252$; Fig. 38C, number of jumps: $F_{(3,81)} = 3.968$, $p = 0.0108$; Fig. 38D, % time freezing: $F_{(3,81)} = 0.2165$, $p = 0.8847$).

Fig. 38. Behavioural responses registered during the different intervals of the memory retrieval session from the cued fear conditioning. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour in the different intervals of the memory retrieval session of the cued fear condtiioning. The mean (of the 30 seconds measurements) + SEM are represented. Repeated-measures two-way ANOVA (main factors: "genotype" and "interval") was used for the statistical analyses. WT, n = 14; cKO, n = 15.

Additionally, a repeated-measures two-way ANOVA analysis was performed on the experimental subjects' freezing behaviour during the memory session with comparisons of the habituation and the CS interval,

with "interval" and "genotype" as main factors. No significant "genotype" effect was detected, while a significant "interval" effect was (Fig. 39A, "genotype": $F_{(1,27)} = 1.700$, $p = 0.2033$, "interval": Fig. 39A, $F_{(1,27)} = 111.6$, $p < 0.0001$). No significant interaction between these main factors was identified ($F_{(1,27)} = 0.1113$, $p = 0.7413$). Moreover, the progress of the freezing behaviour before and after each CS presentation was analysed in more detail (see 3.2.4.1). A repeated-measures two-way ANOVA analysis with the "CS presentation" and the "genotype" as main factors revealed only a significant effect of the main factor "CS presentation" (Fig. 39B, "CS presentation": $F_{(5,135)} = 10.33$, $p < 0.0001$; "genotype": $F_{(1,135)} = 4.094$, $p = 0.0530$), while no interaction was identified between the main factors ($F_{(5,135)} = 0.3544$, $p = 0.8787$). A Bonferroni *post hoc* test revealed significant differences in both genotypes between Pre-CS *vs.* post-CS1 and post-CS2, as well as a significant decrease in percentage of freezing behaviour between the first and the last CS presentation (for more information about the statistical analyses see 9.4.5).

Fig. 39. Progress of the percentage of time the experimental subjects showed freezing behaviour during the memory session of the cued fear conditioning test. A. Perecentage of freezing behaviour from WT mice (white bars) and Grb2 cKO mice (black bars) during the distinct intervals, habituation and CS, of the memory session from the cued fear conditioning test. **B.** The percentage of freezing behaviour during the 60 s before the CS presentation (Pre-CS) compared to the 60 s after each CS presentation (Post-CS) from WT (white squares) and Grb2 cKO mice (black squares) is represented. A repeated-measures two-way ANOVA (main factors: for the data represented in panel A "genotype" and "interval" and in panel B: "genotype" and "CS presentation") was used for statistical analyses of the data represented in panels A and B. WT, n = 14; Grb2 cKO, n = 15.

4.2.3.5.2 Contextual Fear conditioning

Fear conditioning

For the contextual fear conditioning session, the total values from the entire session were used to analyse the behaviour of the experimental subjects. No significant differences were observed in the number of rearing episodes between Grb2 cKO mice and their WT littermates (Fig. 40A, t = 1.344, df = 28, p = 0.1897). However, Grb2 cKO mice showed a significant higher number of jumps (Fig. 40B, t = 5.393, df = 28, p < 0.0001), longer travelled distances (Fig. 40C, t = 4.170, df = 28, p = 0.0003), and lower percentage of freezing behaviour compared to WT mice (Fig. 40D, t = 2.888, df = 28, p = 0.0074).

Fig. 40. Analyses of the behavioural parameters scored during the conditioning session of the contextual fear conditioning test. The following data is represented in each graph: **A**. the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour during the conditioning session of the contextual fear conditioning test. An unpaired *t*-test was used for statistical analyses. WT, n = 14; cKO, n = 15.

Repeated-measures two-way ANOVA analyses assessing the distinct behavioural parameters scored during the distinct intervals of the conditioning session of the contextual fear conditioning test with the "conditioning interval" and "genotype" as main factors was performed. A significant "conditioning interval" effect (see 3.2.4.2) was observed in all behavioural parameters (Fig. 41A; travelled distance: $F_{(2,56)} = 219.0$, $p < 0.0001$; Fig. 41B, rearing episodes: $F_{(2,56)} = 26.86$, $p < 0.0001$; Fig. 41C: number of jumps, $F_{(2,56)} = 84.73$, $p < 0.0001$; Fig. 41D, % time freezing: $F_{(2,56)} = 38.72$, $p < 0.0001$). A significant "genotype" effect was also observed in the travelled distance, the number of jumps and the percentage of freezing behaviour (Fig. 41A; travelled distance: $F_{(1,56)} = 32.36$, $p < 0.0001$; Fig. 41C, number of jumps: $F_{(1,56)} = 29.97$, $p < 0.0001$; Fig. 41D, % time freezing: $F_{(1,56)} = 8.517$, $p = 0.0069$), but not on number of rearing episodes (Fig. 41B, $F_{(1,56)} = 1.884$, $p = 0.1808$). An interaction between the two main factors was observed in all measured behavioural parameters (travelled distance: $F_{(2,56)} = 3.405$, $p = 0.0305$; number of jumps: $F_{(2,56)} = 32.61$, $p < 0.0001$; % time freezing: $F_{(2,56)} = 5.571$, $p = 0.0062$) except for the number of rearing episodes ($F_{(2,56)} = 1.168$, $p = 0.3184$).

Fig. 41. Behavioural responses registered during the different conditioning intervals of the conditioning session from the contextual fear conditioning In the graphs are represented **A.** the total travelled distance, **B.** the total number of rearing episodes, **C.** the total number of jumps and in **D.** the percentage of time during which the experimental subject showed freezing behaviour in the different conditioning intervals of the contextual fear conditioning. The mean (of the 30 seconds measurements) and SEM are represented. Repeated-measures two-way ANOVA (main factors: "genotype" and "conditioning interval") was used for the statistical analyses. WT, n = 14; cKO, n = 15.

The response of the experimental subjects during the following 60 s after each UCS presentation was analysed. Repeated measures two-way ANOVA analysis using "post-UCS" and "genotype" as main factors (see 3.2.4.2) revealed a significant "post-UCS" effect on the travelled distance, number of rearing episodes and the percentage of freezing behaviour, but not on the number of jumps (Fig. 42A, travelled distance: $F_{(2,56)} = 15.62$, $p < 0.0001$; Fig. 42B, number or rearing episodes: $F_{(2,56)} = 4.364$, $p = 0.0173$; Fig. 42C, number of jumps: $F_{(2,56)} = 0.5166$, $p = 0.5994$; Fig. 42D, % time freezing: $F_{(2,56)} = 23.91$, $p < 0.0001$). A significant "genotype" effect was revealed on the number of jumps, distance travelled and percentage of time freezing, but not on number of rearing episodes (Fig. 42A, travelled distance: $F_{(1,56)} = 20.04$, $p = 0.0001$; Fig. 42B, number or rearing episodes: $F_{(1,56)} = 0.4943$, $p = 0.4878$; Fig. 42C, number of jumps: $F_{(1,56)} = 29.90$, $p < 0.0001$; Fig. 42D, % time freezing: $F_{(2,56)} = 12.25$, $p = 0.0016$). No interaction of the main factors was observed in any of the analysed behaviours (travelled distance: $F_{(2,56)} = 0.3173$, $p = 0.7294$; number of rearing episodes: $F_{(2,56)} = 0.005493$, $p = 0.9945$; number of jumps: $F_{(2,56)} = 1.285$, $p = 0.2847$; % time freezing: $F_{(2,56)} = 3.063$, $p = 0.0547$). Moreover, the behavioural responses of the experimental subjects after each UCS presentation was further analysed using a Bonferroni *post hoc* test which revealed a significant decrease in the travelled distance and a gradual increase in freezing behaviour between post-UCS1 *vs.* post-UCS3 in both genotypes (Fig. 42; post-UCS1 *vs.* post-UCS3, Fig. 42A, travelled

distance: t = 3.866, p < 0.001; Fig. 42D, freezing behaviour: t = 3.911, p < 0.001) (to see the detailed results from the Bonferroni *post hoc* test of the percentage of freezing behaviour see 9.4.6).

Fig. 42. Behavioural changes during the 60 s after each UCS presentation during the conditioning session of the contextual fear conditioning. A. Rearing episodes, **B.** number of jumps, **C.** travelled distance and **D.** percentage of the time the mice showed freezing behaviour during the conditioning session of the contextual fear conditioning test are represented. Repeated-measures two-way ANOVA (main factors: "genotype" and "post-UCS") followed by a Bonferroni *post hoc* test were used for statistical analyses. WT, n = 15; Grb2 cKO, n = 15.

Fear memory retrieval

The analyses of the overall behavioural parameters measured during the two trials of the fear memory retrieval session (in the neutral and in the conditioned context) revealed less percentage of freezing behaviour shown by Grb2 cKO compared to their WT littermates (Fig. 43D; percentage of time freezing: t = 2.096, df = 28, p = 0.0452). No significant differences were observed in the other measured behavioural parameters (Fig. 43A: travelled distance: t = 1.738, df = 28, p = 0.093; Fig. 43B: number of rearing episodes: t = 1.255, df = 28, p = 0.2200; Fig. 43C: number of jumps: t = 0.08131, df = 28, p = 0.9358).

Fig. 43. Analyses of the behavioural parameters scored during the memory session of the contextual fear conditioning test. The following data is represented in each graph: **A.** the total travelled distance, **B.** the number of rearing episodes, **C.** the number of jumps, and **D.** the percentage of time the experimental subjects showed freezing behaviour during the both trials of the memory session of the contextual fear conditioning test. An unpaired *t*-test was used for statistical analyses. WT, n = 14; cKO, n = 15.

Repeated-measures two-way ANOVA statistical analyses with the "context" and "genotype" as main factors revealed a significant "context" effect for all the measured behaviours: rearing episodes, number of jumps, travelled distance and percentage of freezing behaviour (Fig. 44A; travelled distance: $F_{(2,56)} = 39.62$, $p < 0.0001$; Fig. 44B, number of rearing episodes: $F_{(2,56)} = 14.58$, $p < 0.0001$; Fig. 44C, number of jumps: $F_{(2,56)} = 10.11$, $p = 0.0002$; Fig. 44D, % time freezing: $F_{(2,56)} = 36.70$, $p < 0.0001$). In contrast, only a significant "genotype" effect was observed on the freezing behaviour (Fig. 44A; travelled distance: $F_{(1,56)} = 3.021$, $p = 0.0932$; Fig. 44B, number or rearing episodes: $F_{(1,56)} = 1.574$, $p = 0.2200$; Fig. 44C, number of jumps: $F_{(1,56)} = 0.0066$, $p = 0.9358$; Fig. 44D, % time freezing: $F_{(1,56)} = 4.780$, $p = 0.0373$). No interaction between the two main factors "context" and genotype" was revealed for any of the behaviours (travelled distance: $F_{(2,56)} = 0.03377$, $p = 0.9668$; number or rearing episodes: $F_{(2,56)} = 0.3120$, $p = 0.7333$; number of jumps: $F_{(2,56)} = 0.09207$, $p = 0.9358$; % time freezing: $F_{(2,56)} = 2.103$, $p = 0.1316$).

Fig. 44. Analysed behaviours during both trials of the memory session of the contextual fear conditioning test. In the graphs are represented the total **A.** travelled distance, **B.** rearing episodes, **C.** number of jumps and **D.** the percentage of time the experimetnal subjects showed freezing behaviour during each memory trial (neutral (5 min) and conditioned (10 min), the latter was divided in two parts of five minutes each). Repeated-measures two-way ANOVA (main factors: "genotype" and "context") test followed by a Bonferroni *post hoc* test were used for the statistical analyses. WT, n = 14; cKO, n = 15.

The Bonferroni *post hoc* test revealed a strong "context" effect on both genotypes when comparing the percentage of freezing behaviour scored in the neutral context to the behaviour scored during the first five minutes in the conditioned context (Fig. 45A, WT: t = 7.7378, p < 0.001; Grb2 cKO: t = 4.739, p < 0.001).

A repeated-measures two-way ANOVA analyses was performed to study, in intervals of one minute, the progress of the percentage of time the experimental subjects showed freezing behaviour during both memory trials (in the neutral and the first five minutes in the conditioned context), using as main factors "interval" and "genotype", which revealed an interval effect but not a genotype effect (Fig. 45B; "interval": $F_{(5,140)} = 18.17$, p < 0.0001; "genotype", $F_{(1,140)} = 3.552$, p = 0.0699). Interestingly, *post hoc* test revealed significant differences between the 4-5 min interval from the neutral context *vs*. the 0-1 min interval from the conditioned context between genotypes (Fig. 45B, for more detailed information from the outcome of the Bonferroni *post hoc* test see 9.4.6).

Fig. 45. Percentage of freezing behaviour during the first 5 min of both memory trials (neutral and contioned) from the contextual fear conditioning test. A. Percentage of freezing behaviour from WT (white bars) and Grb2 cKO mice (black bars) during each trial of the memory retrieval session, neutral and conditioned (Cond (1-5 min)). **B.** Percentage of time, in one min intervals, the experimental subjects showed freezing behaviour through the first five minutes of the memory trials. The data was statistically analysed with a repeated-measures two-way ANOVA (main factors: "genotype" and "interval") followed by a Bonferroni *post hoc* test. WT, n = 14; cKO, n = 15.

5   Discussion

Considering the knowledge gaps in the contemporary literature, the current research aimed to elucidate the role that different parts of the HC (dorsal *vs.* ventral) play during the acquisition of information which is essential in forming social recognition memory in mice, and is coded by the conspecific's olfactory signature. In conducting this research, the HC activity induced by the processing of the information coded by the different fractions of the olfactory signature (volatile and non-volatile) was distinguished. This was studied by analysing the protein product of *c-fos*, an IEG widely described to be involved in synaptic activity and memory formation. *In vivo* experiments were used to confirm and extend the results obtained in the first section of the study. A temporal neuronal activity inhibitor, lidocaine, was injected in the distinct parts of the HC before the acquisition session of the SDT. Changes in social memory performance of the injected experimental subjects provided new insight into the relevance of the distinct HC parts to successfully acquire and consolidate the information provided by the social cues. Furthermore, possible molecular pathways were studied and advocated as being involved in these processes. In particular, the role of Grb2, an adaptor protein from the Ras signalling cascade, was investigated using Grb2 cKO mice. In these mice, the social memory abilities were tested.

5.1   Role of the hippocampus in social memory

5.1.1   c-Fos synthesis induced by social cues

Although in the HC, a centre of declarative memory formation, rapid transcription of IEGs had been described to occur during HC-dependent learning paradigms (Wiltgen et al. 2010), we failed to find reports of HC-IEG expression distinguishing between its dorsal and ventral parts with regards to social memory. In the first section of this work, the pattern of c-Fos synthesis induced by a social encounter (sampling-like juvenile exposure) was studied in defined brain areas including the HC.

Changes in the housing conditions of the experimental subjects prior to the sampling-like session, such as overnight social isolation already in the testing cage (habituation), were introduced in order to minimize unspecific responses, also known as incidental encodings (Morris and Frey 1997, Guzowski 2002). For the same purpose, and to be able to discriminate between unspecific and specific induced c-Fos synthesis, control experimental subjects underwent the same procedural methodology as the socially stimulated, but in the absence of the stimulus animal. It is important to note that c-Fos synthesis is controlled by a negative feedback loop whereby the protein

downregulates the transcription of the gene resulting in low constitutive c-Fos levels (Minatohara et al. 2015). As such, c-Fos staining is most informative when novel stimuli are applied or when the experimental subject is stimulated after a period of sensory deprivation. Thus the isolation period used in the present experimental design was aimed to prevent the firing of areas involved in previous social interactions (for instance with cage mates), in order to enable the most accurate read out from the c-Fos synthesis pattern induced by the presentation of the novel stimulus animal during the sampling-like juvenile exposure.

The changes in neuronal activity induced by the exposure of a novel stimulus animal to an experimental subject, which either had access to the full olfactory signature (SDT_D sampling-like juvenile exposure) or only to the volatile fraction (SDT_I sampling-like juvenile exposure), were studied by analysing the synthesized c-Fos protein in different areas of the experimental subjects' brain and compared to the respective controls. However, in order to ensure that most of the quantified c-Fos expression belonged to neurons and not to glial cells, as observed in previous studies (Bennett and Schwartz 1994), double immunostaining of SDT_D stimulated experimental subjects' brain coronal sections were performed. No co-localization of c-Fos and glial cell marker GFAP was observed (Fig. 9), in contrast most c-Fos positive cells co-localized with the neuronal marker, NeuN (Fig. 10). As a result it was concluded that it was the neuronal c-Fos expression that was measured in this study.

Quantification of c-Fos positive cells in the **SCN** and **PVT** failed to provide significant differences between the exposure conditions (Fig. 11 and Fig. 12). These results indicated that the physiological condition (e.g. day-night stage; motor activity) was similar among experimental subjects, and thus differences observed in the synthesized c-Fos in the other brain areas analysed were likely due to the defined stimulation conditions. Interestingly, a higher variance was observed in the data collected from the PVT of the experimental subjects stimulated using the SDT_I sampling-like juvenile exposure, in both control and stimulated conditions, compared to the experimental subjects stimulated using the SDT_D sampling-like juvenile exposure (Fig. 12A). The PVT, besides being activated under stressful and/or physiological challenges (Colavito et al. 2015), was also identified as playing a significant role in functions related to arousal, attention, and awareness, which is often considered a nonspecific determinant of behaviour (Groenewegen and Berendse 1994, Van der Werf et al. 2002, Bentivoglio et al. 1991). Both experimental groups, control and stimulated, were tested using the SDT_I sampling-like juvenile exposure, and thus were exposed to the air flow produced by the fan units from the VFC. Thus, it seems that the air flow itself was sufficient to induce a slight generalized arousal to the experimental subjects, which contributed to the observed higher variance in the c-Fos synthesis under SDT_I sampling-like juvenile exposure conditions (see also Richter et al. (2005a)).

Analyses in the **MeA** revealed a significant increase in the number of c-Fos positive cells under SDT_D sampling-like juvenile exposure only (Fig. 13). Despite being activated after a social encounter, as also described in previous studies (Richter et al. 2005b, Ferguson et al. 2001), the processing of social information in the MeA was shown not to be essential during the acquisition session in order to form intact long-term social memory, yet it was necessary during the recall session (Noack et al. 2015). Furthermore, MeA-lesioned mice showed an impaired social but an intact flavour recognition memory regardless of their increased social behaviours (Wang et al. 2014). These findings support the involvement of the MeA processing conspecific socio-chemical signals (Samuelsen and Meredith 2009), primarily of non-volatile olfactory cues (Baum and Bakker 2013, Cooke et al. 1998), which are only present when the experimental subjects had direct contact to the conspecifics' social cues. Thus, our results prove the hypothesis of the activation of the MeA upon social stimulation, however, only when the non-volatile fraction of the olfactory signature is present.

A downstream region of the amygdala that has also been implicated in social behaviour in mice and directly connected to the OB, is the **HC** (Sanchez-Andrade and Kendrick 2009), which is known to play an important role in social recognition memory (Kogan et al. 2000). In this area, synthesized c-Fos was analysed with distinction between the dHC and the vHC. An anatomical connectivity and functional heterogeneity along the septotemporal axis of the HC, dorsal-ventral, including differences in gene expression (Leonardo et al. 2006, Strange et al. 2014), were described in several HC-dependent cognitive tasks (Fanselow and Dong 2010). In the present study, the quantification of c-Fos positive cells in the dHC revealed significant differences between the stimulation conditions, showing significantly higher c-Fos immunoreactivity when the experimental subjects were stimulated using the SDT_D compared to when the SDT_I sampling-like juvenile exposure was used (Fig. 14). Despite this result, the quantification of c-Fos positive cells in the dHC did not reveal significant differences between the stimulated conditions *vs.* the respective controls (Fig. 14A). In the vHC instead, a significant higher number of c-Fos positive cells, compared to the respective control conditions, was detected when the experimental subjects were stimulated using the SDT_D sampling-like juvenile exposure, but not using SDT_I sampling-like juvenile exposure (Fig. 15).

Although the lack of an increased c-Fos synthesis in the dHC after social stimulation was already described in previous studies (Richter et al. 2005b, Ferguson et al. 2001), the engagement of the vHC for social memory formation has been poorly investigated, even though it shares reciprocal connections with the BLA, which is activated after social stimulation (O'Donnell and Grace 1995, Pikkarainen et al. 1999, Pitkanen et al. 2000, Kishi et al. 2006) and has a causal relationship with social behaviour (Felix-Ortiz and Tye 2014). Thus, despite the lack

of studies focusing on the vHC regarding social memory, some indication of its involvement with social memory could be found in previous publications: (1) The application of selective lesion methods suggested that the vHC, but not the dHC, was required for social interaction during a resident-intruder test (McHugh et al. 2004). (2) Moderate interconnections of the posteromedial cortical nucleus of the amygdala with the associative amygdala and the vHC were recently revealed, and suggested to be involved in emotional and social learning induced by chemical signals (Gutierrez-Castellanos et al. 2014). Therefore, our results substantiate the hypothesis that the vHC is involved in the processing of information coded by social cues which is essential for social recognition memory formation. As there was no significant increase of c-Fos positive cells observed in the dHC from stimulated *vs.* control experimental subjects, the described significant difference in this HC part may be attributed to procedural distinctions between stimulation conditions, SDT_D and STD_I sampling-like juvenile exposure. Thus, the present study provides the first direct evidence of a distinct activation pattern within the mouse HC induced only by a direct encounter with a conspecific.

A more detailed examination of the c-Fos staining from the vHC indicated, although not quantified due to technical limitations, a major increase of c-Fos synthesis in the CA1 and subiculum areas compared to the CA3 or DG of the vHC (Fig. 15). A study with hamsters described the involvement of neurons from the ventral subiculum for the encoding of relevant information necessary to distinguish and recognize odours from individual conspecifics (Petrulis et al. 2005). Furthermore, oxytocin, a neuromodulator suggested to be essential for social recognition memory in mice (Ferguson et al. 2001, Lukas et al. 2013, Choleris et al. 2007), was shown to have prominent effects on the acquisition and maintenance of this type of memory (Benelli et al. 1995, Ferguson et al. 2000) and to increase the signal-to-noise ratio of CA1 pyramidal neurons in mice in *ex vivo* preparations (Owen et al. 2013). Not only have these studies suggested an involvement of these areas in processing olfactory cues, but it is also known that VNO information, mostly from the non-volatile fraction, can reach the HC through projections from the AOB to the posteromedial cortical amygdala (Winans and Scalia 1970, Raisman 1972, Martinez-Marcos and Halpern 1999, Mohedano-Moriano et al. 2007), which in turn projects to the ventral CA1, the ventral subiculum, and the entorhinal cortex (Kemppainen et al. 2002). In addition, the ventral subiculum and ventral CA1 project to the olfactory bulb (Cenquizca and Swanson 2007, de la Rosa-Prieto et al. 2009). These connections constituted a feedback loop from the AOB to the ventral subiculum and vHC CA1 and back to the AOB. Thus, the activation of the HC subareas, CA1 and subiculum, appears to be part of the circuit which participates in recognition and memory formation of pheromones (part of the non-volatile fraction of the olfactory signature), in a similar manner to that of recognition and memory formation of odours (Eichenbaum 1998, Roman et al. 2004).

Importantly, CA1 was described to be essential for social recognition memory in rats (Garrido Zinn et al. 2016), and this area together with the DG were described to be differentially activated after the recollection of social memory in mice (Luscher Dias et al. 2016). Thus, the activation of CA1 and subiculum observed in the vHC after SDT_D sampling-like juvenile exposure was likely specific from the stimulation conditions, indicating the involvement of these areas in the processing of the non-volatile fraction of the olfactory signature. Further quantification of c-Fos in these HC areas using gene markers specific from each HC subarea, or inactivation experiments should be performed to improve the reliability of these observations.

Special attention was focused on the CA2 area of the HC, as it was recently shown to be critical for social memory formation without impacting other forms of HC-dependent memory (Stevenson and Caldwell 2014, Hitti and Siegelbaum 2014). This area was suggested to provide the morphological substrate underlying, at least partially, the social cognition deficits seen in some psychiatric and neurodevelopmental disorders (Piskorowski et al. 2016). More detailed studies on HC CA2 excitatory neurons and their circuits, revealed that CA2 cells appear to be at the centre of the septotemporal circuit covering the whole longitudinal axis of the HC (Kohara et al. 2014). Thus, CA2 cells may provide, not only a direct link between the dHC and the vHC, but also a fast route to directly influence downstream regions connected to ventral CA1, such as the prefrontal cortex and the BLA (Cenquizca and Swanson 2007, Andersen et al. 2007). Due to the recent progress on the functionality of this area, and its high apparent involvement with social memory, c-Fos positive cells in the CA2 area of the dHC and the vHC from the brain slices of experimental subjects stimulated with the SDT_D sampling-like juvenile exposure were also studied. While dorsal CA2 is located between the dorsal one-third of the CA1 and CA3, the ventral CA2 overlaps partially with the intermediate CA1, located between the dorsal and ventral CA1, being difficult to discriminate without any specific CA2 pyramidal neurons staining, such as RGS14 or PCP4 (Lee et al. 2010, Fanselow and Dong 2010, Lein et al. 2005). Thus, only an approximate quantification of c-Fos positive cells in the CA2 area from the dHC and the vHC were performed. No significant differences between stimulated and control conditions were revealed on the dHC, however a significantly higher number of c-Fos positive cells were quantified in the vHC from stimulated experimental subjects compared to their respective controls (for results and analyses see 9.2). Regarding the results obtained from the dHC, a similar outcome was described in a study with rats, in which no increase in neuronal firing rates or c-Fos expression, upon social stimulation was observed in the CA2 area from the dHC (von Heimendahl et al. 2012). Moreover, a recent study which also analysed the c-Fos synthesis in mice brains after a novel and a familiar conspecific presentation, did not observe an increase of c-Fos positive cells in CA2 for any of the described situations (Luscher Dias et al. 2016). However, in this latter study the entire CA2, including from the

dorsal to the "ventral" part of the HC (- 0.58 / - 3.08 from Bregma) was analysed. Thus, our results emphasize the relevance to discriminate between the dorsal and the ventral part of the HC due to its distinct functionality and involvement on processing the information coded by social cues.

The detection of a significant increase of c-Fos positive cells in the CA2 area of the vHC supports the overall activation of this HC part, however it does not show a selective and/or distinct involvement of the CA2 area compared to other HC areas, such as CA1 or subiculum. Remarkably, the studies mentioned above, which reported a selective involvement of CA2 in long-term social memory were achieved by means of non-reversible inactivation of CA2 before acquisition. However, it is possible that this selective involvement in social memory formation takes part in the later stages, as CA2 has been described as being crucial for the consolidation of socially relevant information into long-term memory (Dudek et al. 2016). Thus, CA2 could be playing a modulatory and/or synchronizing role in the process. A more detailed analysis using CA2 neuronal markers to study the participation of this area in social memory formation should be performed by investigating the synthesis of c-Fos or other IEGs at distinct stages of memory formation.

Unexpectedly, despite the described involvement of the HC in the social recognition memory formation in mice, no neuronal activity (interpreted from the increased synthesized c-Fos) was observed in either the dHC or the vHC, after the presentation of the volatile fraction of the olfactory signature (Fig. 14 and Fig. 15). It was described that the HC plays a crucial role on both short-term and long-term social memory formation, however it is based on different mechanisms (Kogan et al. 2000). While permanent lesions to the entire HC impaired short-term social recognition memory, the inhibition of protein synthesis blocked only the long-term social recognition memory (Kogan et al. 2000). The presentation of either the volatile fraction or the non-volatile fraction, separately, was described to be sufficient to form short-term recognition memory in mice. In addition, only access to the volatile fraction of the olfactory signature, but not the non-volatile fraction, allowed recognition of a conspecific in a long-term manner (Noack et al. 2010). The composition of the stimulus, not only can determine the type of memory which can be formed, but the subset of IEGs that are induced and how/when their protein products are modified translationally (Curran and Morgan 1986). Moreover, volatile odours, although being able to activate both, the MOB and the AOB (Noack et al. 2010), are mainly processed through the MOB. While the AOB is activated mostly by non-volatile substances and has direct connections with the vHC (de la Rosa-Prieto et al. 2009), the MOB sends the information to the primary olfactory cortices (Noack 2012), to further communicate with the HC. Thus, given these anatomical connections (see 1.1.2.1) the lack of an increase of c-Fos immunodetection in the whole HC from the exposure of the volatile fraction of the olfactory signature might be due to a delayed and/or

prolonged c-Fos induction in this area. This could explain the undetected HC response at the time used in this study, however may be detectable at later time-points as its processing also seems to be more relevant for long-term rather than short-term social memory formation (Noack et al. 2010). Nonetheless, it is also possible that the exposure to the volatile fraction of the olfactory signature induces the expression of other IEGs such as *arc* or *egr-1*, instead of *c-fos* (Wacker et al. 2010). Both, Arc and Egr-1 expressions were described as being involved in neural plasticity and memory processes (Bozon et al. 2002, Soule et al. 2008, Okada et al. 2014, Guzowski 2002, Minatohara et al. 2015), however there are no studies investigating their expression in the HC after a social encounter or presentation of social odours. Hence, further experiments focused on the aforementioned aspects are indeed needed to determine how the information coded by social volatile odours is processed in the HC to form long-term social memories.

In summary, our c-Fos analyses provided evidence of the vHC involvement in social learning, consistent with the idea that the HC is functionally differentiated along its septotemporal axis. It also supported the role of the vHC in rapid encoding, necessary for acquisition of complex memory representations such as the identity of a stimulus animal. As already stated, no increase of synthesized c-Fos was observed in the dHC after social stimulation. Thus, using IEG imaging we identified candidate structures associated with social behaviour. Nevertheless, local interventions such as region-specific temporal inactivation are needed to confirm the essential role for those structures. Therefore in order to get further insight into the functionality of the dHC and the vHC during social memory formation local temporal inactivation experiments were performed.

4.1.1 Hippocampal inactivation

The bilateral HC injections of lidocaine were used to block the generation and propagation of action potentials in either the dHC or the vHC during the learning session of the SDT, in order to study its impact in short-term and long-term social recognition memory. The injection procedure, including the insertion of the injection cannulae, the injected fluid volume and the subsequent increased pressure within the tissue, did not cause major damage in the HC as shown by the *post mortem* histological reconstruction of the injections sites (Fig. 16). No impact was observed on the experimental subjects' behaviour neither after the cannula implantation, nor after the injection procedures as subjects showed intact long-term social recognition memory in both cases (Fig. 17 and Fig. 18).

The approach of using lidocaine to inactivate the HC is advantageous as its effects are temporary in the subject, with inactivation lasting approximately 20 min at a concentration of 20 µg/µl (0.5 µl were injected /

hemisphere) which was used in the current experiments (Sandkuhler et al. 1987). This approach thus crucially allowed for HC inactivation in discrete memory phases (e.g. encoding *vs.* retrieval). In this study, intra-HC lidocaine was administered prior to the memory encoding event (sampling session, acquisition of the information contained in the conspecific's olfactory signature), and the effects of HC inactivation were tested with short (1 h) and long (24 h) EIs. During the choice session, significantly longer investigation duration towards the novel stimulus animal was interpreted as intact social memory of the experimental subject to the familiar stimulus animal. Regarding the effects of the injected substances, there were no differences between treatment groups in the aggressive/sexual behaviour towards the stimuli animals (Fig. 19 and Fig. 21), latency to investigate the stimuli animals, or the time invested investigating them. Thus, intra-hippocampal aCSF and lidocaine administration *per se* did not have disturbing sensory, motor or motivational influences.

5.1.1.1 Behavioural consequences of the temporal dHC inactivation during acquisition

Experimental subjects showed almost no aggressive/sexual behaviour during the sampling session after both aCSF and lidocaine injections (Fig. 19). This behaviour was significantly more frequent and thus longer registered during the choice sessions independently of the EIs used (1 h and 24 h) (Fig. 19A and Fig. 19B). As the low levels of aggressive/sexual behaviour during the sampling session were observed after the injection of both substances, a specific effect of the dHC inactivation on aggressive/sexual behaviour was unlikely. As described previously (see 3.3.3), sampling took place shortly after the injections for which the experimental subjects underwent mild anaesthesia (~ 3 % isofluorane during ~ 3 min). It has been described that long lasting exposures to high doses of inhalation anaesthetics, including isoflurane, can interfere with memory through the modulation of N-methyl-D-aspartate-type glutamate receptor signalling (Liu et al. 2014), however the exact mechanism is still unclear. Despite the mild and brief anaesthesia used in our experiments, we cannot entirely exclude the presence of lasting associated effects which may have reduced the aggressive/sexual behaviour of the experimental subjects during the sampling session only. Interestingly, this effect was specific for the aggressive/sexual behaviour as it did not affect the length of time the experimental subjects' spent investigating the presented stimulus animal during the sampling session (See 9.3).

Any lasting effects of the anaesthesia would most likely have impacted on the behavioural performance of the experimental subjects during the choice session while testing short-term rather than long-term memory due to the longer EI used in the latter case. In our experiments, aCSF itself was not expected to produce major changes in the HC functionality, and yet short-term memory was impaired when tested with the SDT_D (Fig. 20A). Our hypothesis is that these results are a consequence of the residual effects from the anaesthesia, which might have

impaired the ability of the dHC to integrate the complex information during recollection (Misic et al. 2014), as acquisition processes do not seem to be disturbed. This could explain the absence of the "anaesthesia effects" when only the volatile fraction of the olfactory signature was presented due to its unimodal nature and thus no need for complex HC integration (Fig. 20C). Moreover, inactivation of the dHC via lidocaine injections impaired short-term memory when tested under SDT_I stimulation conditions, thus indicating that this area is essential to acquire and build the neuronal engram from unimodal stimulus (volatile fraction only), as this ability was not impaired by the lasting effects of the anaesthesia. Surprisingly, although after receiving dHC aCSF injections short-term memory formation was impaired while long-term memory remained intact, when tested with the SDT_D (Fig. 20B). These results suggest that the formation of long-term memory is possible even when the recall or access to the short-term memory is impaired. The interpretation of these observations and their impact will be discussed in more detail in the following section. Finally, inactivation of the dHC during sampling impaired long-term social memory only when the whole olfactory signature was presented (SDT_D), but had no effects when tested on the SDT_I (Fig. 20D).

When all is considered, the data suggests that the dHC is essential not only to process the information of a conspecific's social cues, but also to integrate the afferent information from the different sensory modalities activated after direct contact with the stimulus animal in order to successfully form both short- and long-term memory. In contrast, the processing of the information coded by the volatile fraction of the olfactory signature seems to be additionally independent from the dHC, mainly in the formation of long-term recognition memory.

5.1.1.2 Behavioural consequences of temporal vHC inactivation during acquisition

Similar aggressive/sexual behavioural patterns were observed during the sampling sessions which followed both the dHC and the vHC injections (Fig. 21), thereby supporting the likelihood that this behaviour was affected by the residual effects of the anaesthesia inhalation. As discussed in the previous section, the lasting effects of the anaesthesia on the experimental subjects ability to integrate the information of different sensory modalities during the choice session could explain the short-term memory impairment revealed after aCSF injections (Fig. 22A), although this effect was eliminated by the inactivation of the vHC (Fig. 22A). This implies that the vHC inactivation reduced the information transmitted to the dHC and thus limited it to the non-volatile fraction of the olfactory signature of the presented stimulus animal. This would make the information which reaches the dHC "simpler" and thus independent of a complex integration for its recall. Hence in this context, it is likely that information from the non-volatile fraction could reach the dHC independently of the vHC, most likely through the septum (Khakpai et al. 2013) (Fig. 1), and thereby still allowing the formation and/or recollection of a "simple" short-term memory. According to the hypothesis previously introduced, when only the volatile fraction of the

olfactory signature was presented, no effects of the anaesthesia were observed (i.e. control experimental subjects showed intact short-term memory, Fig. 22C). Yet inactivation of the vHC impaired this ability and thus implied its involvement in the initial processing of the information acquired from the volatile fraction for an intact short-term social memory (Fig. 22C).

The vHC appears to also be involved in long-term memory formation when the whole olfactory signature is presented (Fig. 22B). This outcome could be related to the inability of the information coding for the volatile fraction of the olfactory signature to reach the dHC during the sampling session, but not during the choice session, due to the inhibition of the vHC neuronal activity transmission only during the sampling session. Consequently, as described by Noack et al. (2010), mice failed to recognize a conspecific stimulus (short-term and long-term) when different fractions of its olfactory signature were presented in the sampling and choice sessions. Interesting results were obtained using the SDT_I when long-term memory was tested, and although the statistical analysis failed to reveal significant differences, a tendency towards a longer investigation duration of the unfamiliar stimulus animal could be measured after both, aCSF and lidocaine injections (Fig. 22D). These results may indicate a mild impairment of the recognition of the stimulus animal's volatile fraction, however independent of the effect of the injected substances, but affected by the alteration of the physiological conditions by the injections *per se*, as a similar effect was observed under aCSF and lidocaine injections.

The results obtained from the vHC inactivation experiments indicate that this area is essential for short-term social memory, in order to transmit the information coding from the volatile fraction of the olfactory signature to the dHC. In contrast, when testing long-term memory, vHC inactivation might impair consolidation under both SDT testing conditions, when only the volatile fraction (SDT_I) and when the whole olfactory signature is presented (SDT_D), although inducing a more severe impairment in the latter situation.

5.1.2 Synthesis I

The dose and amount of the injected substances were as per the descriptions presented in previously published studies (Wanisch et al. 2005, Noack et al. 2015). The volume of aCSF and lidocaine injections was small (0.5 µl / hemisphere) and only animals with cannula placement verified either in the dHC or the vHC were included in the analyses, thus any potential effects of lidocaine spreading into the surrounding cortical tissue were reduced as much as technically possible. Interestingly, analyses of the registered aggressive/sexual behaviour indicated lasting effects of the anaesthesia inhalation. Although these effects had no impact on the investigation duration of the experimental subjects towards the stimulus animal during the sampling session, short-term social

memory tested by presenting the whole olfactory signature in the SDT$_D$ was impaired after receiving aCSF injections in both the dHC and the vHC (Fig. 20 and Fig. 22). The dHC CA1 area was described as being essential for the integration of the poly-sensory inputs from distributed association areas throughout the neocortex acting as a critical convergence zone (Misic et al. 2014). Thus, our hypothesis to explain the impairment observed in short-term social recognition memory after aCSF injections is that the lasting effects of anaesthesia affected the recall of short-term "multi-modal" social memories due to the short EI used (1 h). The lasting effects of the anaesthesia might have temporally impaired the ability of the dHC (CA1) to integrate the complex neuronal representation (poly-sensory) of the social cues (including the olfactory signature) of the previously encountered conspecific and thus blocked its successful retrieval during the choice session tested 1 hour after the injections. Accordingly, when only unimodal social cues (e.g. volatile fraction, SDT$_I$) were presented after receiving aCSF injections, experimental subjects showed an intact short-term social recognition memory. In contrast, under the same testing conditions, inactivation of either the dHC or the vHC by lidocaine during the sampling session impaired short-term social memory. On the one hand, the impaired short-term memory due to the dHC inactivation during the sampling session may indicate the need for the intact information processing in this area in order to form, on the basis of the volatile fraction only, short-term memory (an ability which was not impaired by the lasting effects of the anaesthesia). While on the other hand, the results indicate that the impairment of short-term memory observed by the inactivation of the vHC might be due to the inability to appropriately process the information from the parahippocampal cortices to the dHC. It has been described that the connectivity between the HC proper, the adjacent cortex and the vHC is particularly important for information flow, as the parahippocampal and perirhinal cortices are thought to be the primary route by which information is exchanged between the whole HC formation and the neocortex (Misic et al. 2014). Overall, the dHC is thought to play an important role on the processing of the olfactory signature in order to form the "neuronal engram" which is essential for recollection. While the vHC appears to be less important for the processing of the information contained in the olfactory signature, but definitely involved in modulating the information flow (volatile fraction) to the dHC (Fig. 46).

When analysing long-term social memory, intact dHC and vHC functioning during sampling was confirmed as being essential to acquire and build the proper neuronal representation for a successful long-lasting memory of a conspecifics' multi-modal social cues including the olfactory signature (Perna et al. 2015). In contrast, acquisition of the neuronal representation formed only by the volatile fraction, showed a tendency of being HC independent, with this pattern mostly observed when testing long-term social memory. This might be explained by the supportive role of the entorhinal, perirhinal and parahippocampal cortices play during the acquisition of the

information coding for the volatile fraction (unimodal). Nonetheless, the HC might become more relevant during consolidation and recollection of those memories, as previously suggested (Ross and Eichenbaum 2006, Noack et al. 2010). Moreover, it is important to note that short-term social memory, in contrast to long-term social memory, does not rely on protein synthesis, although it needs the functionality of the HC for its successful formation in mice (Kogan et al. 2000). This indicates that the short-term social memory formation is more dependent on the fast induced changes triggered during the sampling session which might partially explain the social recognition memory impairment, likely induced by the anaesthesia prior to the learning session and observed only when testing short-term memory.

Interestingly, the results obtained from the HC inactivation experiments support the outcome of the c-Fos immunohistochemistry, as neuronal activation in the vHC was observed only when both fractions of the olfactory signature were presented. In agreement with those results, the inactivation of the vHC during the acquisition of the conspecific's social cues revealed this area to be involved essentially in the formation of long-term social memory under direct exposure (SDT$_D$), but not when only the volatile fraction of the olfactory signature was presented under indirect exposure (SDT$_I$). Thus, these results support the involvement of the vHC in processing the information acquired from the non-volatile fraction of the olfactory signature, specifically to form long-term social memory. This is in agreement with recent findings which suggest that long-term social memory primarily depends on initial social olfactory processing in the HC (Luscher Dias et al. 2016). The relevance of the vHC for consolidation of social memories was also supported by a recently published study which showed that a phosphodiesterase, PDE11A, enriched in vHC neurons was required for the proper consolidation of recognition and associate social memories, but not for short-term or non-social odours recognition (Hegde et al. 2016).

It is important to note that the synthesis of the results from the c-Fos immunocytochemistry and the data from the inactivation experiments supports the fact that the absence of c-Fos imaging does not necessarily preclude the involvement of a cell group in a functional circuit. c-Fos synthesis is a generic marker of neuronal activation, and not exclusive of neuronal activity. Thus, only in response of external transient acute stimuli c-Fos is likely to be expressed. This could be one of the reasons why we failed to detect differences induced by the volatile fraction of the olfactory signature. Rodents are constantly "exposed" to volatile substances due to their easy propagation. This might hinder the detection of the c-Fos synthesis induction in response to an "acute" stimulation with only a conspecific's volatile fraction. A similar situation could be experienced in the dHC, which is known to be involved in the integration of different stimuli, not only olfactory but spatial or visual, and thus this area might previously be already stimulated or induced by the mere presence or movement of the experimenter in the testing room. Hence, it

might be particularly useful to investigate the expression of different IEG under those conditions and in the mentioned areas.

Fig. 46. Hippocampal signalling transduction during acquisition of a conspecifics' olfactory signature. It appears that the different fractions of the olfactory signature are processed differently not only in the olfactory system but also in the HC. While part of the information coded by the non-volatile fraction seems to be directly sent to the dHC, the information coded by the volatile fraction needs to be processed first by the vHC to later access the dHC. The dHC appears to be more relevant for short-term memory formation, quite "independently" from the vHC, although the latter becomes more relevant in long-term memory formation. The ability to integrate the information coming from multi modal sensory modalities of the dHC was impaired by the exposure to the light anaesthesia during the injection procedure, when testing short-term memory only. Other sensory modalities are not considered in the design of this scheme. Abbreviations: dHC, dorsal hippocampus; vHC, ventral hippocampus; STM, short-term memory; LTM, long-term memory.

Based on the data analysis the following conclusions were drawn: vHC activation is involved in early social memory processes (such as encoding or early time points of consolidation), which are necessary mainly for long-term social memory formation. Moreover, this HC part seems to be essential to ensure the information flow from the parahippocampal cortices to the dHC during the acquisition of social cues. The dHC, as previously described, is engaged in building the neuronal representation belonging to the information acquired from the different sensory modalities. It is likely this function which makes the dHC essential after the acquisition of the multi-modal social cues, including the olfactory signature, in order to ensure its later successful recall. Thus, depending upon the perceived social cues or fractions of the olfactory signature (non-volatile or volatile), distinct HC parts are required to form social memory. The interpretation of the results though is limited as very little is known about the molecular basis of social memory acquisition and consolidation that is tested in the social discrimination paradigm (van der Kooij and Sandi 2012, Camats Perna and Engelmann 2015). Hence in future studies, administering intra-HC lidocaine at various time points after the sampling session (e.g. consolidation, retrieval) would permit testing the involvement of the distinct parts of the HC (i.e. dHC and vHC) in the suggested sequence of their involvement leading to social memory. However, in the present study we focused on selected signalling pathways that might be involved in the formation of social recognition memory in the HC.

5.2 Grb2 conditional knock-out mice

To investigate in more detail the involvement of Grb2 in social recognition memory formation, Grb2 cKO mice were used. These mice were created using the Cre/*LoxP* system, which allows spatial and temporal control of the deletion of genes. This approach avoids potential developmental defects and allows deletion to be restricted to brain areas and cell types known to be important for learning and memory. To inactivate Grb2 in specific brain regions we used Grb2$^{flx/flx}$ mice carrying a CaMKIIα-Cre transgene (see 1.3.1). The CaMKIIα promoter is only activated postnatally (at or after P20) in excitatory neurons of the forebrain (Burgin et al. 1990, Mayford et al. 1996b), thus restricting the deletion of the Grb2$^{flx/flx}$ gene to adulthood and only to excitatory neurons of the forebrain. Experimental subjects with a post-natal forebrain neuron-specific ablation of Grb2, Grb2$^{flx/flx}$ CaMKIIαCre $^{+/-}$ mice (referred as Grb2 cKO mice), were on a mixed BALB/c - C57BL/6 background. Previous studies from our laboratory showed behavioural changes on the SDT depending on the mice mixed genetic background (Hadicke and Engelmann 2013). Hence, to control and discard this possibility, animals Grb2$^{flx/flx}$ CaMKIIαCre$^{-/-}$ mice (referred as control or wild-type mice) were used as controls for all the biochemical analyses and behavioural tests performed in the current research.

5.2.1 Screening of Grb2 cKO mice

In order to ensure that there were no defects on the brain development following the ablation of Grb2 in our mice line, the brain anatomy of WT and Grb2 cKO experimental subjects were studied. Grb2 cKO mice did not show gross morphological abnormalities or reduced brain size compared to control littermates (Fig. 23), and the expected Mendelian ratio of genotypes was observed suggesting no developmental defects in these mice. Importantly, it has previously been shown that mice expressing Cre-recombinase in the forebrain do not exhibit learning and memory deficits (Chen et al. 2006).

The downregulation of Grb2 expression in the studied brain areas from Grb2 cKO mice was characterized at the ages corresponding to the ages of the experimental subjects in the course of the behavioural tests (10 - 24 weeks old) *vs.* their control littermates. Using western blot analysis, a significant reduction of the HC Grb2 expression was observed in Grb2 cKO mice (Fig. 24). Under basal conditions no significant differences on phosphorylation levels of Erk1 and Erk2 (pERK1 "p44" and pERK2 "p42") were found (Fig. 24). Once confirmed that the reduction of Grb2 expression was in the HC, further brain areas were also studied. Significantly reduced levels of Grb2 protein were observed in the olfactory bulb, frontal cortex and striatum, but not in cerebellum of Grb2 cKO mice compared to their control littermates, as has been previously described (Minichiello et al. 1999)

(Fig. 25). The broad detection of Grb2 in control mice reflected its wild type expression, which demonstrated that the introduced *loxP* sites do not interfere with the Grb2 protein expression.

These results supported the correct functionality of the Cre/*loxP*-recombination system in the Grb2 cKO mice. Following the study of the possible behavioural impact of the reduced forebrain Grb2 protein levels in Grb2 cKO mice, their learning and memory abilities were assessed.

5.2.1.1 Influence of Grb2 downregulation in distinct behaviours

5.2.1.1.1 Open field

First, the presence of locomotor defects or anxiety-like phenotypes was investigated in Grb2 cKO mice using the open field test, as such phenotypes could potentially confound the interpretation of the animals' performance during memory tasks. Grb2 cKO mice and their control littermates spent similar amounts of time inspecting the centre area (~ 16 %) and the periphery (~ 84 %) of the open field arena (Fig. 26A and Fig. 26B). Although both genotypes spent comparable time in each area, Grb2 cKO mice crossed the dividing lines of the field more often, with this metric being significantly higher during the time spent in the periphery only (Fig. 26E). While no differences were observed in the grooming episodes between WT and Grb2 cKO mice, significantly higher rearing episodes were registered in the Grb2 cKO mice (Fig. 26C and Fig. 26F). The number of puddles or streaks of urine registered during the open field test were not significantly different between the genotypes (Fig. 27). The greater number of crossed lines in the open field arena exhibited by the Grb2 cKO *vs.* their control littermates indicated elevated activity levels, thus Grb2 cKO mice explored the new environment more persistently. Similarly to the inspection of the open field rearing is a behaviour of exploratory nature, which indicates the subjects to be less anxious and thus more interested in investigating the immediate environment (Fig. 26F).

Despite Grb2 cKO mice showing a mild hyperactive phenotype, no significant differences were observed in the anxiety-like phenotype of these mice as they spent similar times in the centre and periphery of the arena compared to their control littermates (Fig. 26B).

5.2.1.1.2 Odour habituation and dishabituation

In the following experiments, subjects underwent the odour habituation and dishabituation test in order to assess the functionality of the olfactory system of the created cKO mice, and thus ensure that the downregulation of Grb2 expression observed in the olfactory bulb did not affect olfactory abilities (Fig. 28). Both genotypes showed a significant decrease in the investigation time towards the same odour after consecutive exposures, thus

showing intact odour habituation and a subsequent significant increase when the mix of the previously exposed odour together with a novel one was presented, further illustrating intact dishabituation (Fig. 28A and 28B). However, the analyses based on the number of bouts indicated intact habituation and dishabituation for the WT mice but only significant habituation to a familiar odour for the Grb2 cKO mice, without significant dishabituation (Fig. 28C and Fig. 28D). As reflected by the investigation duration towards the presented odours, Grb2 cKO mice spent more time than WT mice investigating them, however in less number of bouts, probably due to their less anxious-like, and thus, more pronounced exploratory behaviour phenotype. Hence, considering the described phenotype from Grb2 cKO mice, the interpretation of the number of bouts tended to suggest a misleading outcome (Fig. 28). Overall, Grb2 cKO and WT mice had intact olfactory functionality being able to habituate and discriminate to and between odour/s.

5.2.1.1.3   Social recognition memory

Social recognition memory was tested on the experimental subjects, WT and Grb2 cKO mice, using three different EI's between sampling and choice; 1 h, 3 h and 24 h, thus assessing short-term, intermediate-term, and long-term social recocognition memory, respectively.

During choice, WT mice showed a significantly longer investigation duration towards the unfamiliar stimulus animal, SA2, in all tested conditions, whereas Grb2 cKO mice demonstrated a significantly longer investigation duration towards SA2 only when tested using EI's of 1 h and 3 h, but not 24 h (Fig. 29A and Fig. 29B). The deficit of Grb2 cKO mice to form long-term social recognition memory was not dependent on the sex of the subjects, as neither females nor males were able to discriminate between stimuli animals during the choice session (Fig. 29C). In addition, although some significant differences were observed between the aggressive/sexual behaviour towards SA2 *vs*. SA1 under defined EI's in both WT and Grb2cKO mice (Fig. 31A and Fig. 31B) (likely due to the higher interest towards the novel juvenile, SA2), no significant differences between genotypes were observed either on the total length of the latency to approach the stimuli animals (Fig. 30B), or on the total aggressive/sexual behaviour (Fig. 31C) during the SDT$_D$. The lack of differences with regards to the general behaviours between WT and Grb2 cKO mice evaluated during the SDT$_D$ supported the absence of unspecific effects due to the deficit of Grb2. Thus, these results indicate that the impairment to form long-term social recognition memory observed in Grb2 cKO mice may be linked to the downregulation of Grb2 protein expression in the forebrain of those mice.

Previous studies reported the involvement of Grb2 in cognitive and memory functions. For instance, downregulation of Grb2 in the HC was already previously linked with cognitive deficits (Shin et al. 2007). Moreover, after training, in response to the insulin receptor activation, Grb2 was described as having accumulated

in the HC (Zhao et al. 1999, Rozakis-Adcock et al. 1992, Sasaoka et al. 1996). Nonetheless, despite the significant role that Grb2 seems to play in memory, this is the first study which investigates its function in social recognition memory. The reported results demonstrated that mice with a significant deficit of the adaptor protein Grb2 in the forebrain showed intact short-term and intermediate-term memories, but were unable to consolidate and thus form long-term social memory. Interestingly, previous studies described that the formation of long-term social recognition memory required two stages of protein synthesis (Richter et al. 2005b). Thus, the phenotype observed in Grb2 cKO mice, could be due to the dysregulation of these transcriptional events which might be modulated by Grb2, and are essential in forming long-term social memory.

5.2.1.1.4   Novel object recognition

As observed already in the open field, Grb2 cKO mice showed significantly higher locomotor activity compared to their control littermates. These mice travelled longer distances and thus explored the field where the NOR task took place more extensively (Fig. 32A and Fig. 33A). Despite this difference, the exploration duration towards the objects was similar between genotypes (Fig. 32B and Fig. 33B). Nonetheless, during the long-term NOR test, significantly lower exploration duration towards the objects was registered during the second trial compared to the first trial for both genotypes (Fig. 33B). As long-term memory was tested one week before short-term memory, the first trial from the long-term NOR test was the first time that the subjects were placed in that context as no habituation session was performed before training. Hence, the significantly longer exploration duration during that trial reflected the higher interest of the experimental subjects to explore, likely both, the context and the objects due to novelty (Arias et al. 2015).

With regards to the ability for recognising a familiar object, memory tested 1 h after training revealed differences between the genotypes (Fig. 32C). Control mice showed a significant preference towards the novel object, while Grb2 cKO mice did not, indicating that the mutant mice had impaired short-term object recognition memory (Fig. 32C and Fig. 32D). In contrast, when tested 24 h after the training session, none of the experimental subjects showed significant preference for the novel object (Fig. 33C and Fig. 33D). The lack of long-term NOR memory in WT littermates could be due to technical or procedural reasons, as despite the high number of studies performing NOR, there are no consistencies in the training criterion (Leger et al. 2013). The novelty factor present during the first trial of the long-term NOR test might have influenced the attention of the experimental subjects focused not only on the objects but also on the new context. Hence, possible disturbing effects, such as context novelty could be easily avoided for instance by introducing an additional habituation session as demonstrated in other studies (Leger et al. 2013).

In our mice model the deletion of Grb2 was controlled by the promoter of CamKIIα, whose highest expression is in the HC (DG and pyramidal layers CA1 and CA3), although it has also been described to be expressed in the perirhinal cortex (Wang et al. 2013). As previously introduced (see 1.1.3.2.1), when using delays longer than 10 min between the training and the memory session, the HC was described as being essential for the retention of object recognition memory. Due to the use of longer delays, the memory impairment observed in Grb2 cKO mice was likely a consequence of the lower levels of the Grb2 protein in the HC, which might have caused the dysregulation of protein synthesis pathways essential to form object recognition memory in the dHC (Wang et al. 2016, Orr et al. 2012, Pereira et al. 2014). Previous studies with rats, showed the impairment of both short-term (10 min) and long-term (24 h) object recognition memory after inhibition of Trk receptors (Callaghan and Kelly 2013). However, no single member of the neurotrophin family of proteins was essential for the acquisition of this task as its inhibition, using activity-blocking neutralising antibodies did not impair short-term memory (10 min) but were required for the consolidation of object recognition memory (Callaghan and Kelly 2013). Thus, these results together with those obtained in our study suggest a possible effect of Grb2 downregulation on the signalling transmission downstream Trk receptors. However, the role neurotrophins play in short-term object recognition memory remains unclear as Grb2 cKO mice showed impaired short-term memory, although tested using an EI of 1 h *vs*. the 10 min used in the mentioned study. Thus, further research is required to confirm the mechanisms by which Grb2 might affect Trk and neurotrophins molecular signalling during object recognition memory.

5.2.1.1.5 Associative fear conditioning memory

In the final experiments, the performance of the subjects' associative memory was assessed using the Pavlovian fear conditioning paradigm. Experimental subjects underwent the contextual or the cued fear conditioning tests. Each session of those tests (conditioning and memory retrieval) was divided into different intervals depending on their respective positions to the UCS presentation (see 3.2.4). A significant effect of the different intervals was observed in all measured behavioural parameters in both sessions of the tests, and thus discarded the possibility of sensory impairment (cued fear conditioning: Fig. 35 and Fig. 38; contextual fear conditioning: Fig. 41 and Fig. 44).

During the conditioning session of the **cued fear conditioning** test, Grb2 cKO mice showed a significantly higher number of jumps compared to their control littermates, while all other parameters measured (travelled distance, rearing episodes and percentage of time freezing) were not significantly different between the genotypes when analysing the overall session (Fig. 34). A significant UCS (foot shock) paired with the CS (tone) effect was

observed as an increase in the percentage of time that the experimental subjects showed freezing behaviour when analysing the 60 s interval after its presentation, which was linked to a significant reduction of travelled distance and number of jumps with each subsequent UCS-CS presentation (Fig. 36). During the memory retrieval session, 24 h later, no significant differences were observed between genotypes when analysing the overall measured behaviours (Fig. 37). However, when analysed in response to the CS presentation, all experimental subjects showed a significant increase in the percentage of time the experimental subjects showed freezing behaviour proving the presence of an intact association with the UCS, and thus intact long-term memory for both genotypes (Fig. 39).

In the case of the **contextual fear conditioning** test during the conditioning session, Grb2 cKO mice showed significantly (1) longer travelled distances, (2) a higher number of jumps, and (3) a lower percentage of freezing behaviour compared to their control littermates (Fig. 40). When analysing the behavioural response during the 60 s following the presentation of the UCS, both genotypes significantly decreased the distance travelled and increased the percentage of time freezing after each UCS presentation (Fig. 42). The response pattern after each UCS presentation compared between genotypes was alike for all measured behavioural parameters, except for the freezing behaviour. WT mice showed a significant increase in the time spent freezing after the second UCS presentation (compared to the previous one, post-UCS1 *vs.* post-UCS2), while Grb2 cKO mice showed a significant increase after the third UCS presentation (post-UCS2 *vs.* post-UCS3) (Fig. 42D) (for statistical analyses see 9.4.6). During the fear memory retrieval session, with an interval exposure of 24 h, Grb2 cKO mice spent a significantly lower percentage of time showing freezing behaviour than their control littermates (Fig. 43). In addition, a similar delayed pattern showing an increase in the freezing response, as observed during the conditioning session, was also detected when analysing the timing of the freezing behaviour through the memory trials (Fig. 45B) (for statistical analyses see 9.4.6). However, when analysing the overall behavioural responses during the first five minutes of the memory retrieval sessions discriminating between contexts (neutral *vs.* conditioned) both WT and Grb2 cKO mice exhibited significant differences on all measured behaviours including the percentage of time freezing (Fig. 44 and Fig. 45), thus showing intact long-term memory.

Fearful experiences are rapidly acquired and easily consolidated into long-term memory. This is plausible as it is of vital importance to recall information regarding danger in the environment that is critical to survival. Long-term fear memory formation is thought to be induced by late long-term potentiation, a long-lasting RNA- and protein-synthesis-dependent phase of plasticity (Huang et al. 2000) that requires the cAMP-dependent protein kinase A (PKA) and the ERK/MAPK (Schafe et al. 1999, Sindreu et al. 2007). Infusions of inhibitors of the

activation of these two signalling pathways in the amygdala impaired consolidation of fear memory (Schafe et al. 2000, Schafe and LeDoux 2000, Bailey et al. 1999). Different lines of evidence suggested an involvement of the HC in fear memory: virally-delivered miRNAs targeting R-Ras in the CA1 region of the dHC produced an impairment in context discrimination (Darcy et al. 2014); PKA inhibition in the dHC was also able to impair contextual, but not cued fear conditioning memory (Ahi et al. 2004). Moreover, the expression of the early growth response protein 1 was increased in dHC CA1 neurons after the retrieval of contextual, but not cued, fear memories, while it was increased after both tests in the amygdala (lateral, basal and central nuclei) (Hall et al. 2001). Contextual and cued fear memory formation have been associated with a rapid and transient hyper-phosphorylation of Erk1 and Erk2 in different subfields of the HC (Sananbenesi et al. 2002, Besnard et al. 2014, Trifilieff et al. 2007, Schafe et al. 2000) and amygdala (Schafe et al. 2000, Radwanska et al. 2002, Trifilieff et al. 2007, Athos et al. 2002), respectively. Interestingly, an additional cross-talk between ERK and ROCK (Rho kinase) able to modulate cued fear memory formation was described in neurons (Hensel et al. 2014, Sahai et al. 2001). Furthermore, inhibition of ROCK in the lateral amygdala during training impaired the long-term, but not the short-term memory formation of the tone-shock association (Lamprecht et al. 2002, Zhao et al. 1999). Although Grb2 has been described as being able to interact and/or modulate the activation of all the introduced pathways, very little is known about its involvement in fear memory formation, as the most downstream molecular events involved or regarding the dynamics of their activation in consolidation has yet to be investigated.

Grb2 cKO mice showed a higher number of jumps and a longer travelled distance, but a lower percentage of freezing behaviour compared to WT mice. As previously reported, HC-neural manipulations are associated with an increase in locomotor activity, which reduced the performance of freezing behaviour without affecting associative learning or the fear responses *per se* (Maren et al. 1998, Anagnostaras et al. 1999). The assessment of locomotor activity in Grb2 cKO mice provided essential information to aid in the interpretation of the behavioural responses during the fear conditioning tests. Mice with reduced levels of Grb2 adaptor protein did not show a significant long-term memory impairment in any of the fear conditioning procedures tested (Fig. 39 and Fig. 45). On one hand, most of the differences observed in the behavioural parameters measured during the <u>cued fear conditioning</u> are likely to be a consequence of the higher locomotor activity of the Grb2 cKO *vs.* their control littermates. Despite these differences between the genotypes, all experimental subjects showed an increase in the percentage of freezing behaviour after each UCS presentation during the conditioning session, which could be interpreted as intact cued immediate-term or short-term memory (Fig. 36D). During the cued fear memory retrieval session, 24 h after the conditioning, both Grb2 cKO and WT animals showed a significant increase in the

percentage of freezing behaviour when the tone was presented, implying an intact long-term cued fear memory (Fig. 39). While, on the other hand, significant differences were detected between genotypes, on the measured behavioural parameters during the contextual fear conditioning (Fig. 40). For instance, a difference which seems to be independent of the observed higher activity of Grb2 cKO mice, is the pattern regarding the increased percentage of time spent freezing during the conditioning session after each UCS presentation (Fig. 42D). Grb2 cKO mice showed a delayed increased of freezing behaviour after the UCS presentation compared to their control littermates. As described by Blanchard and Blanchard (1969) post-shock freezing is not directly elicited by shock, but linked to a contextual stimuli (or cue) that have been paired with the shock (Blanchard et al. 2013, Fanselow 1980). Thus, the delayed increase of freezing behaviour after the UCS presentations observed in Grb2 cKO mice may indicate a slight deficit in acquiring contextual associative memory. Despite this observation, 24 h after the conditioning session, both genotypes displayed a significant increase in the percentage of freezing behaviour when placed in the conditioned context compared to a neutral one, thus showing intact contextual fear associative long-term memory (Fig. 45). However, when looking closer at the timing of the freezing behaviour through the memory trials, Grb2 cKO mice showed a delayed freezing behavioural response when placed in the conditioned context, which may indicate that Grb2 cKO mice need a longer time to recall the association between the UCS and the context (CS) (Fig. 45B) (for statistical analyses see 9.4.6). The slight, but specific deficits on the acquisition of associative contextual fear and on the response during its recollection are likely to be attributable to the downregulation of Grb2 in the HC. However, once this association was learned, reduced Grb2 protein levels did not affect its consolidation to form long-term contextual fear memory. Interestingly, comparing these results to the obtained in the cued fear conditioning test, in the latter no effects from Grb2 downregulation were observed. Thus, confirming the independent HC-character of this test and the likely wider impact of the lack of Grb2 in the HC than in the amygdala.

Regarding the cause of the mild deficits observed in Grb2 cKO mice, it is hard to attribute them to the dysregulation of a specific biochemical pathway on the basis of our behavioural results alone; however some possible explanations in accordance with the contemporary literature may be offered. It has been described that inhibition of ROCK through intracerebroventricular injections affects anxiety- and/or fear-related behaviours (Saitoh et al. 2006), however the behavioural response we measured was opposite to the one observed in that study: where ROCK inhibitor-treated mice froze significantly more often and longer than their controls during an auditory fear conditioning task (Saitoh et al. 2006). A possible explanation of these findings is that ROCK inhibition might enhance the acquisition of contextual fear conditioning, although this is unlikely as the inhibition

of ROCK in the lateral amygdala impaired long-term memory formation (Lamprecht et al. 2002). Moreover, the enhancement of Rho activation in the HC is understood to increase the long-term spatial memory (Dash et al. 2004, Hill et al. 1995). Thus, the alternative and more likely interpretation is provided by the fact that mice may become more fearful after the administration of the ROCK inhibitor. Since this phenotype was not observed in the Grb2 cKO mice, it was assumed that this cascade was not severely affected by the Grb2 downregulation. The PKA and MAPK cascades are known to modulate common processes in the cell and share multiple levels of cross-talk (Gerits et al. 2008). While, inhibition of PKA in the HC impaired the contextual fear memory, it was recently described that the stimulation of MAPK during training for contextual memory was in part depending on Ca^{2+}-stimulated adenylyl cyclase activity (thus also PKA) and lead to the activation of the CREB, mitogen and stress activated kinase 1 (Bailey et al. 1999, Darcy et al. 2014, Ahi et al. 2004). However, due to the close crosstalk between the defined pathways it is difficult to state which of them was more affected, if any, by the downregulation of the expression of Grb2 adaptor protein. However, our experimental approach was not aimed at unravelling these details *a priori*.

Overall, despite the broad involvement of Grb2 modulating different signalling cascades, its specific downregulation in the forebrain of mice did not impair the ability to form associative long-term fear memory. Our explanation suggests that the crosstalk of the Ras-signalling cascade with other biochemical pathways may compensate the lack of Grb2 in the HC.

5.2.2 Synthesis II

Grb2 cKO mice showed neither an anatomically nor a functionally altered development, despite the postnatal low levels of Grb2 protein observed in their olfactory bulb, frontal cortex, striatum and HC. In fact, the only phenotypical apparent derangement in the Grb2 cKO mice was an increase in the locomotor activity, first measured in the open field, but generally present in most of the behavioural tests performed. This increase in locomotor activity was likely due to the downregulation of Grb2 protein in the HC, since this behaviour has been previously associated with HC-neuronal manipulations (Maren et al. 1998, Anagnostaras et al. 1999). However, it is important to note that we did our best to exclude the enhanced locomotor activity observed in the Grb2 cKO mice as a confounding factor in our behavioural studies, as it was always taken into account during the interpretation of the behavioural parameters measured during each behavioural test. Hence, the behavioural and physiological characterization of these animals should provide a reliable assessment of the role Grb2 plays in learning and memory processes in mice.

Using Grb2 cKO mice, the relevance of Grb2 in HC-dependent tasks was assessed. The HC is one of the major players in distinct memory- and learning-related tasks including exploration of new environments (Daenen et al. 2001), social memory (Pena et al. 2014, Kogan et al. 2000), novel object recognition (Broadbent et al. 2004, Barker and Warburton 2011) and fear-associated contextual learning (Kim and Jung 2006, Maren 2001). Exploration of new environments was assessed with the open field test, which allowed at the same time, the study of anxiety related behaviours of the experimental subjects. Grb2 cKO mice did not show altered exploration of the new environment nor anxiety behaviours in the open field. Both, Grb2 cKO mice and their control littermates did show intact olfactory habituation and dishabituation to a volatile odour, an essential ability to acquire the social cues and thus, to build social memory. Intact short-term and intermediate-term, but impaired long-term social recognition memory were observed in Grb2 cKO mice. Regarding object recognition, a deficit was already detected when testing short-term memory in Grb2 cKO mice. Long-term fear conditioning memories were intact despite the mild deficits detected during the contextual fear conditioning. Thus, the forebrain downregulation of Grb2 protein expression appeared to affect the behavioural performance in a different manner depending on the nature of the memory being studied (Table 6).

Table 6. Summary of the memory abilities showed by Grb2 cKO mice.

Type of memory tested / Exposure interval (EI)	1 h	6 h	24 h
Object recognition	✗	n.r.	✗
Social recognition	✓	✓	✗
Associated fear conditioning (contextual and cued)	n.r.	n.r.	✓

✓ : Presence of memory   ✗ : absence of memory   **nr**: no results. It has not been tested.

As mentioned above, different types of memories require the activation of distinct brain areas for its successful formation and consolidation, including the activation of different intracellular signalling cascades. This might be the reason why the imbalance caused by the reduction of the Grb2 adaptor protein, mainly in the HC, affected the distinct types of memories tested differently, by impairing the formation of an object memory and the consolidation of a social memory. However, it failed to significantly affect fear conditioning memory.

Interestingly, previous studies which inhibit the MAPK cascade at different levels, from forebrain-specific TrkB-receptor knockout mice, to mice lacking the Erk1 isoform of MAPK, showed a common hyperactive

phenotype and intact associative fear memory formation (Zorner et al. 2003, Selcher et al. 2001). Moreover, the dysregulation of Erk1 and Erk2 by producing a protein tyrosine phosphatase receptor type R deficient mice, exhibited increased exploration of new environments and impaired object recognition, similar to the phenotype observed also in the current Grb2 cKO mice (Erkens et al. 2014). Thus, these results point towards a likely disturbed MAPK signalling cascade activation in the HC as the cause of the impaired object and long-term social recognition memory function observed in Grb2 cKO mice. However, preliminary results from our laboratory failed to reveal significant differences between genotypes (unpublished results). In this context, western blot analyses were conducted to detect the phosphorylation levels of Erk1 and Erk2 within the distinct parts of the HC, dHC and vHC, at 15 min and 30 min after a social encounter.

Although, typically too little is known about how Grb2 functions within neurons to discuss its likely actions during the process of learning and memory with any certainty, the present results supported by the literature suggests Grb2 and its downstream molecular cascades do play an essential role in HC-memory formation and/or consolidation.

6 Conclusions

The present work provided new data supporting the different contributions of the distinct HC parts, dHC and vHC, during the processing of the information coded by the conspecific's social cues, including each fraction of the conspecific's olfactory signature, and its relevance to form social recognition memory. In addition, the current findings suggest that the Grb2 adaptor protein is involved in the formation of long-term social recognition memory, which is likely modulated by the Ras-MAPK signalling cascade.

Our results imply a functional separation of the HC into a dorsal and a ventral part. These distinct parts were studied separately and were shown to be engaging differently during the processing of the information coded by the social stimuli used to recognize a conspecific. Additionally, the activation of the two distinct HC parts was found to be linked to the fraction of the olfactory signature presented (volatile *vs.* non-volatile fraction). According to the results, the involvement of each HC part in the acquisition of the information coded by the conspecific's distinct social cues, which are essential to form social recognition memory, follows a complex scheme. The dHC appears to be critical in successfully acquiring the information coded by a conscpecific's social cues, including the olfactory signature, and responsible to later integrate it during the recall session, for both, short-term and long-term social memory formation. However, this HC part was less important for the acquisition of the mono-modal information, such as the volatile fraction, and was dispensable for long-term social memory. Regarding the vHC, it appears to be engaged with the transmission of the information coded by the volatile fraction to the dHC during its acquisition, in order to be later able to integrate it together with the processed information from the other sensory modalities during recollection. Accordingly, the vHC was essential in forming long-term memory when the whole olfactory signature was presented. Similarly to the results obtained from the dHC, the vHC did not appear to be highly relevant during acquisition in order to form long-term social memory when only the volatile fraction of the olfactory signature was presented. Thus, these findings suggest that the entire HC is essential for the acquisition of complex (multi-modal) social cues in order to create the engram which later will enable the experimental subject to remember, whereas mono-modally coded information seemed to be more HC-independent. This pattern was mostly observed when assessing long-term memory. Thus, although each HC part might be selectively involved in specific tasks or processes, both the dHC and the vHC are tightly connected and influencing each other in order to successfully form social recognition memory.

An interesting observation concerning the fact that impaired short-term memory did not predict an impaired long-term social recognition memory further supports the existence of two different mechanisms, one for each type

of memory. While similar results supporting these conclusions have been previously reported and obtained in other tasks (one-trial avoidance) and tested in rats(Vianna et al. 1999), further studies are encouraged to investigate this –very interesting- phenomenon in more detail.

The study was extended by investigating which intracellular HC-pathways are activated in order to form and consolidate social recognition memory. The c-Fos expression and synthesis, used to study the neuronal activity after a social encounter, is regulated among others, by the Ras-MAPK pathway. This pathway can be modulated by the binding protein Grb2 (Miyamoto 2006, Monje et al. 2005). Interestingly, using Grb2 cKO mice, we also confirmed the involvement of this adaptor molecule in the generation of long-term social memory. The forebrain-specific disruption of Grb2 expression produced a mild hyperactive phenotype in Grb2 cKO mice, nonetheless, they did not show altered anxiety-like behaviours, as no other differences in the general behaviour were identified. However, when testing their memory abilities, some deficits were detected. For instance, short-term object recognition memory and long-term social recognition memory were impaired, whereas short-term and intermediate-term social recognition memories were intact, as well as long-term fear conditioning memory. Hence, our findings suggest that the adaptor molecule Grb2 probably through the modulation of the Ras-MAPK cascade is essential to acquire and/or consolidate specific types of memories. This might happen in an HC-dependent manner since no significant effects were observed neither in cued, nor in contextual, long-term fear conditioning memory.

The simultaneous increase of c-Fos immunoreactivity in the MeA and the vHC indicated a link between these two brain parts during the direct presentation of a social stimulus. The temporal HC inactivation experiments revealed that the vHC seems to be essential to form an intact long-term social recognition memory, and interestingly the dysregulation of Grb2, predominantly in the HC, may have caused the disruption of this type of memory. Although more studies are needed to confirm this hypothesis, the present results suggest that the c-Fos synthesis observed in the vHC was partially gated via the MeA, in context with the acquisition of the non-volatile fraction of the olfactory signature. This would support the consolidation of the social recognition memory involving the induction of HC c-Fos synthesis partially through Grb2 dependent mechanisms, which are likely to be impaired in Grb2 cKO mice. Thus, the forebrain-specific disruption of Grb2 expression might affect the information processing in the HC and, thus, impair long-term social recognition memory, while maintaining short-term and intermediate-term social recognition memory. In support of the idea that IEG expression, such as *c-fos*, in HC neurons can be modulated by distinct brain areas, Huff et al. (2006) demonstrated that BLA inactivation with muscimol attenuated the increase in *c-fos* mRNA in the HC after contextual fear conditioning, but not after context exploration. Moreover, when comparing the effects of the forebrain downregulation of the Grb2 expression in the

different types of memory tested, memories known to be partially or mainly modulated by other brain areas distinct from the HC, such as the MeA or the lateral amygdala, seem to be able to compensate the downregulation of the Grb2 expression, likely in a Grb2-independent manner. Thus, the connectivity of the HC with other brain areas might facilitate long-term social recognition memory by adjusting the HC response. This then may involve the activation of the Ras-MAPK cascade and, thereby modulate the "durability" of the memory.

Social recognition memory is based on the acquisition of the information coded by the unique combination of social cues including the conspecifics' olfactory signature. Its information is processed, among other brain areas, by the HC in order to shape the neuronal engram required for its later recall. This information carried by the different fractions of the olfactory signature, volatile and non-volatile, is processed differently in the HC, with the dHC and the vHC involved in different aspects of the process. Thus, social recognition requires a combination of fined tuned connections within the HC, in order to process the information coded by each fraction of the olfactory signature to be able to form and consolidate a social memory. The HC activity driven by the acquisition of this information induces different molecular pathways, which are involved in memory formation via the adaptor molecule Grb2 which contributes to the modulation of some of these pathways.

7 Outlook

The current results obtained in the course of this research have raised additional questions that should be addressed and further investigated in future research projects. Some of the aroused questions have already been mentioned previously, while others are outlined below:

Despite investigations involving the c-Fos synthesis allowed to reveal the distinct involvement of the different parts of the HC (dHC *vs*. vHC) on the processing of the conspecific's olfactory signature, they failed to detect an increase neuronal activity induced by the presentation of only the volatile fraction of a conspecific's olfactory signature in any of the HC parts studied. Nonetheless, the HC was described as being essential during the acquisition of the volatile fraction of a conspecific's olfactory signature to form social memory. Moreover, with the current experimental approach the effects of the inactivation of the dHC and the vHC neuronal transmission during the acquisition of the information coded by the distinct fractions of the olfactory signature in forming social recognition memory were studied. However, how these areas are involved at later points in time including consolidation or recollection one hour or 24 hours after learning is not known. To address these questions further experiments are required. The study of the synthesis of other IEGs proteins, which have also been described as essential for memory formation, such as Egr-1 or Arc, after the presentation of the different fractions of the olfactory signature could provide an insight into the neuronal activity induced by the volatile fraction not detected by the analysis of the synthesis of c-Fos. Furthermore, investigating their expression, including c-Fos, at later time points such as during consolidation and/or recollection might provide some hints regarding the IEG expression pattern in the different phases of memory formation and thus help in its interpretation. Alternatively, an option to identify a marker at this stage of the process and under the defined stimulation conditions would be to perform a protein microarray. This would provide information regarding which proteins increase their expression in response to the different fractions of the olfactory signature in the defined brain areas, and thus identify possible markers and even pathways for further studies. Whether Egr-1, Arc or another protein candidate identified by the microarray, its HC expression pattern could be evaluated by immunohistochemistry or immunofluorescence. Finally, in a similar manner as performed in the present study these results could be complemented with *in vivo* experiments, e.g. blocking their expression, for instance inhibiting the transmission of neuronal activity in the areas where they were mostly expressed, and thus study its effects in social recognition memory formation. This should be performed at the same time points used for the study of IEG expression/microarray to reveal its functional relevance.

Regarding the procedure to inactivate neuronal transmission with lidocaine injections, further research investigating the exact timing of the effects of this substance at the concentration used as well as its diffusion and efficiency in the HC would help to more accurately predict the mechanisms which are altered and thus involved in the behavioural responses affected. Moreover, a possible milder dosage of anaesthesia or a different anaesthetic which does not produce lasting effects that can affect social recognition memory should be investigated for its application during the injection procedure. This should be considered specifically when studying short-term memory.

The second part of this study provided first evidence that the Grb2 adaptor molecule may be involved in the formation of HC-dependent memories, such as object recognition and social recognition memory. However its relevance appears to be less important in memories linked to a pronounced "emotional factor", such as fear conditioning. Due to the described defined effects in the different types of memories produced by the downregulation of Grb2 in the mouse forebrain, new challenging questions arose.

On one hand, it would be of high interest to test whether linking the object to be remembered in the NOR test, to a social (i.e. adding a social odour to the object) or fear (i.e. adding an odour related to an innate threat cue, such as 2,5-dihydro-2,4,5-trimethylthiazoline (Rosen et al. 2015)) would enhance object recognition memory in Grb2 cKO mice. This would aim to rescue the deficit observed in Grb2 cKO mice through the activation of additional brain areas, which might support the induction of memory formation processes in the HC (Roozendaal et al. 2008, Dornelles et al. 2007). Additionally, Grb2 cKO mice showed only a mild impairment in the acquisition and recollection of the association context-footshock, however a deficit in social recognition memory consolidation was indicated, whereas under NOR test conditions Grb2 cKO mice showed impaired short-term object recognition memory. Hence, more detailed research focusing on the phase of the memory formation affected by the downregulation of Grb2 expression in the forebrain might provide helpful information in order to direct towards the molecular cascade/s downstream Grb2 affected in each type of memory, if not the same. In this line, the analyses of c-Fos synthesis in the vHC from Grb2 cKO mice induced by a social encounter compared to their WT littermates would provide helpful information regarding a possible defective HC activation pattern.

On the other hand, is it of crucial importance that future investigations study the mechanisms used by Grb2 adaptor protein to activate downstream signalling cascades upon social stimulation in order to understand its effects. For this purpose, techniques such as two-dimensional blue native polyacrylamide gel electrophoresis followed by mass spectrometry from HC homogenates obtained from stimulated experimental subjects, could

provide detailed information regarding the interaction between the adaptor protein Grb2 and other candidates' proteins upon social stimulation. Thus, these techniques could provide with new insight indicating which signalling cascades are essential for long-term social memory formation. As an alternative, and in order to characterize the HC-molecular mechanisms affected and responsible for the observed memory deficits in the Grb2 cKO mice, primary cultures of HC neurons from the Grb2 cKO mice could be studied, since deficits in neuronal morphology or signalling could be more easily detected *in vitro*. Although it is likely that the deficits observed in Grb2 cKO mice are due to the dysregulation of the MAPK cascade, we failed to provide evidence supporting this hypothesis. Finally, in an advanced stage of the investigation, the involvement of the candidate molecular pathways, likely downstream of Grb2, could be proven to be involved in social memory formation by the injection of inhibitors in the distinct HC parts (dHC and vHC) and further analyse the consequences in social learning and memory.

On the whole, the present work further confirms the use of the social discrimination test model as a convenient and promising tool to study the brain areas as well as the underlying processes and/or molecular pathways involved in the different phases of social memory formation. However, despite the present results contribution to the improved understanding of the role the HC plays in social memory formation and the suggested underlying signalling cascades that are likely involved in this process, further research is recomended to be able to better comprehend the underlying molecular mechanisms modulating perhaps the most important attribute of the "social brain", social recognition, which is essential to build proper social behavioural responses able to determine the fate of an individual in a social community.

8 Literature

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9   ANNEX

9.1   Grb2 cKO mice – genotyping

In this section more detailed information is provided regarding the PCR mixture used to amplify the DNA fragments of interest, together with the respective cycler program and its outcome. This process allowed to knowledge the genotypes of the experimental subjects. On a 1.5 % agarose gel, the presence of CaMKIIα-Cre transgene was determined (Table 7). A band of 320 bp revealed its presence, while a 300 bp fragment belonged to the wt CaMKIIα (Fig. 47B). At the same time DNA samples from the same experimental subjects were processed in order to determine the presence of the wt or the floxed Grb2 gene (Table 8). On a 2 % agarose gel, DNA fragments of either 241 bp length occurred, characterizing the wt Grb2 allele, or of 209 bp length for the mutant floxed Grb2 allele (Fig. 47A). A third band of ~280 bp present only for heterozygous mice was due to the formation of an heteroduplex of the two DNA strands amplified in the PCR, wt Grb2 and floxed Grb2, however not linear annealed thus slower running on the gel, without providing accurate information regarding their size.

Table 7. Master Mix and cycler program used to check the presence of CaMKIIα-Cre transgene

Master mix **CaMKIIα-Cre** PCR:

Ingredients	Final concentration	Volume (µL)		Cycler program **CaMKIIα-Cre** PCR:		
ADN		2				
Primer 1	0,25 mM	1,5		**Cycles**	**Temp (°C)**	**Duration (s)**
Primer 2	0,25 mM	1,5		1	95	300
Primer 3	0,25 mM	1		30	95	30
Primer 4	0,25 mM	1			60	30
10x PCR buffer	1x	2			72	30
dNTP mix	200 mM	2		1	72	420
Taq-Polymerase		0,2		1	4	∞
MgCl$_2$	50 mM	1				
Betaine	5M	4				
H$_2$O		3,8				
Volume$_{TOTAL}$		20		**1,5 % Agarose gel elecrotphoresis**		

Table 8. Master Mix and cycler program used to check the presence of floxed Grb2

Master mix **floxed Grb2** PCR:

Ingredients	Final concentration	Volume (μL)		Cycler **floxed Grb2** program**:**		
ADN		2				
Primer 1	0,25 mM	1,5		**Cycles**	**Temp (°C)**	**Duration (s)**
Primer 2	0,25 mM	1,5		1	94	240
10x PCR buffer	1x	2,5		35	94	20
dNTP mix	200 mM	2,5			63	20
Taq-Polymerase		0,2			72	30
MgCl$_2$	50 mM	0,8		1	72	10
Betaine	5M	3		1	4	∞
H$_2$O		11				
Volume$_{TOTAL}$		**25**		**2% Agarose gel electrophoresis**		

Fig. 47. Representative PCR products after genotyping mice samples from the Grb2 cKO mouse line. A. The fragment containing CaMKIIα-Cre was 320 bp while the endogenous CaMKIIα and thus wt allele was 300 bp big. **B.** DNA samples from mice with double floxed Grb2 showed a single band 209 bp of length. Heterozygous mice, showed a band at 209 bp, belonging to the floxed Grb2 and another band at 241 bp, identified as the wt allele. Thus, for WT mice only a band at 241 bp was detected. The third band detected at ~280 bp belongs to the heteroduplex complex. In the left side of the panels A and B the length in base pairs (bp) of the markers used are indicated.

9.2 Quantification of c-Fos positive cells in the CA2 area of the dHC and the vHC

The c-Fos positive cells were quantified in the HC area CA2 distinguishing between the dorsal and the ventral part. Only brain slices from experimental subjects stimulated with the SDT$_D$ sampling-like juvenile exposure and their respective controls were included in this study, as no differences in c-Fos synthesis were observed either in the dHC or the vHC of those experimental subjects stimulated using the SDT$_I$ sampling-like juvenile exposure.

Due to the lack of specific CA2 neuronal markers in the brain slices used in the present work, a defined small area of the CA2 from the dHC and the vHC were quantified. The c-Fos positive cells that were present within a

200 μm length area of the CA2 pyramidal layer were measured in the dHC (from - 2.06 to - 2.30 mm from the Bregma), while the ones present within a 500 μm length area of the stratium oriens were measured in the vHC (from - 2.92 to - 3.16 mm from the Bregma) (Fig. 48). Significant differences were observed between control and stimulated conditions only in the vHC part, but not in the dHC part of the HC (dHC, p = 0.0771, Mann-Whitney U = 6.500; vHC, p = 0.0152, Mann-Whitney U = 3.0).

Fig. 48. Quantification of c-Fos positive cells in the dorsal and ventral parts of the CA2 HC area. A. Quantification of the c-Fos positive cells in the experimental subjects' dHC CA2 area under control conditions (white squares) or stimulated conditions (black squares). **B**. Quantification of the c-Fos positive cells in the experimental subjects' vHC CA2 area under control conditions (white squares) or stimulated conditions (black squares). Mann-Whitney U test was used for the statistical analyses. n = 6.

9.3 Social investigation duration after receiving dHC- or vHC - bilateral injections

All the experimental subjects showed a robust social investigation duration towards the stimulus animal (SA1) during the sampling session after receiving bilateral injections in either the dHC or the vHC. No significant differences were observed between the injected substances, aCSF and lidocaine, in any of the injected areas neither when tested using the SDT_D (Fig. 49: dHC, t = 1.329, df = 78, p = 0.1877; vHC, t = 0.3642, df = 78, p = 0.7167) nor when using the SDT_I (Fig. 50: dHC, t = 0.1288, df = 70, p = 0.8979; vHC, t = 0.3519, df = 73, p = 0.7259).

Fig. 49. Experimental subjects' investigation duration towards the stimulus animal during the sampling session of the SDT$_D$. A. Time the experimental subjects spent investigating the presented stimulus animal after receiving bilateral injection in the dHC of either aCSF or lidocaine. **B.** Time the experimental subjects spent investigating the presented stimulus animal after receiving bilateral injections in the vHC of either aCSF or lidocaine. An unpaired *t*-test was used for the statistical analyses. n = 40.

Fig. 50. Experimental subjects' investigation duration towards the stimulus animal during the sampling session of the SDT$_I$. A. Time the experimental subjects spent investigating the presented stimulus animal after receiving bilateral injections in the dHC of either aCSF or Lidocaine. **B.** Time the experimental subjects spent investigating the presented stimulus animal after receiving bilateral injections in the vHC of either aCSF or Lidocaine. An unpaired *t*-test was used for the statistical analyses. n = 40.

9.4 Supplementary statistical data

9.4.1 Analyses of the quantification of the c-Fos expression induced by social stimulation

A standard <u>one-way ANOVA followed by Tukey HSD multiple comparison</u> *post hoc* test was used to analyse the c-Fos positive cells from the MeA, the dHC and the vHC as all data groups followed a Gaussian distribution.

9.4.1.1 Statistical analyses from the quantification in the MeA

One-way ANOVA: Factor "treatment" $F_{(3,21)}= 15.81$ $p < 0,0001$ ***

Table 9. *Post hoc* statistical analyses of the quantified c-Fos positive cells under distinct conditions in the MeA

Tukey's Multiple Comparison Test	Control Indirect	Stimulated Indirect	Control direct	Stimulated direct
Control Indirect	0	1.058	1.676	8.557 ***
Stimulated Indirect	1,058	0	0,5717	7.452***
Control Direct	1,676	0,5717	0	7,216***
Stimulated Direct	8,557***	7,452***	7.216***	0

9.4.1.2 Statistical analyses from the quantification in the dHC

One-way ANOVA**:**   Factor "treatment" $F_{(3,21)}= 3.698$ p = 0.0311 *

Table 10. *Post hoc* statistical analyses of the quantified c-Fos positive cells under distinct conditions in the dHC

Tukey's Multiple Comparison Test	Control Indirect	Stimulated Indirect	Control direct	Stimulated direct
Control Indirect	0	0,7734	0,6915	3,537
Stimulated Indirect	0,7734	0	1,499	4,345*
Control Direct	0,6915	1,499	0	2,984
Stimulated Direct	3,537	4,345*	2,984	0

9.4.1.3 Statistical analyses from the quantification in the vHC

One-way ANOVA**:**   Factor "treatment" $F_{(3,21)}= 6.806$ p = 0.0029 **

Table 11. *Post hoc* statistical analyses of the quantified c-Fos positive cells under distinct conditions in the vHC

Tukey's Multiple Comparison Test	Control Indirect	Stimulated Indirect	Control direct	Stimulated direct
Control Indirect	0	0,2351	1,141	5,464 **
Stimulated Indirect	0,2351	0	0,8954	5,219 **
Control Direct	1,141	0,8954	0	4,534 *
Stimulated Direct	5,464 **	5,219 **	4,534 *	0

9.4.2 Analyses of the investigation duration before surgery, after surgery and after injections

The results from the statistical analyses of the investigation duration measured during each session of the SDT$_D$ at different time points: before surgery, after surgery and after injections, for both the dHC and the vHC are below presented (Table 12 and Table 13). A repeated-measures one-way ANOVA test was used for the statistical analyses of the investigation duration measured during each session, sampling and choice, with discrimination of the latter between the investigation duration towards SA1 or SA2.

Table 12. Results from the repeated-measures one-way ANOVA analysing the investigation duration of the experimental subjects towards the stimulus animal/s (SA1 and SA2) during the Sampling and Choice sessions between different time points of the experimental subjects' manipulations in the dHC.

SDT_D test	df	F-value	p-value	Significance
Sampling (towards SA1)	2	2,933	0,0600	n.s.
Choice (towards SA1)	2	1,751	0,1814	n.s.
Choice (towards SA2)	2	3,096	0,0517	n.s.

df: degrees of freedom

Table 13. Results from the repeated-measures one-way ANOVA analysing the investigation duration of the experimental subjects towards the stimulus animal/s (SA1 and SA2) during the Sampling and Choice sessions between different time points of the experimental subjects' manipulations in the vHC.

SDT_D test	df	F-value	p-value	Significance
Sampling (towards SA1)	2	1,535	0,2273	n.s.
Choice (towards SA1)	2	0,8381	0,4396	n.s.
Choice (towards SA2)	2	2,167	0,1272	n.s.

df: degrees of freedom

9.4.3 *Post hoc* analyses of the sexual/aggressive behaviour after hippocampal injections

9.4.3.1 After bilateral injections in the dorsal hippocampus

Table 14. Results from a standard two-way ANOVA test analysing the sexual/aggressive behaviour duration using as main factors: "session" and "injected substance", from the experimental subjects which received dHC injections.

FACTOR	df	F-value	P value	Significance
Session	2	33,92	P<0,0001	***
Injected substance	1	0,2456	0,6209	n.s.
Interaction	2	1,689	0,1880	n.s.

df: degrees of freedom

Table 15. Results from the Bonferroni *post hoc* test of the sexual/aggressive behaviour:

COMPARISON BETWEEN TRIALS	Substance	P value	Significance
Sampling *vs*. Choice 1 h	aCSF	P<0,001	***
	lidocaine	P<0,001	***
Sampling *vs*. Choice 24 h	aCSF	P<0,01	**
	lidocaine	P<0,05	*
Choice 1 h *vs*. Choice 24 h	aCSF	P>0,05	n.s.

lidocaine	P<0,01		**

9.4.3.2 After bilateral injections in the ventral hippocampus

Table 16. Results from a standard two-way ANOVA test analysing the sexual/aggressive behaviour duration using as main factors: "session" and "injected substance", from the experimental subjects which received vHC injections.

FACTOR	Degrees of freedom	F-value	P value	Significance
Session	2	14,19	P<0,0001	***
Injected substance	1	0,3953	0,5305	n.s.
Interaction	2	2,208	0,1134	n.s.

Table 17. Results from the Bonferroni *post hoc* test of the sexual/aggressive behaviour:

COMPARISON BETWEEN TRIALS	Substance	P value	Significance
Sampling *vs*. Choice 1 h	**aCSF**	P<0,05	*
	lidocaine	P<0,001	***
Samping *vs*. Choice 24 h	**aCSF**	P<0,001	***
	lidocaine	P<0,05	*
Choice 1 h *vs*. Choice 24 h	**aCSF**	P>0,05	n.s.
	lidocaine	P>0,05	n.s.

Since similar results from the analyses of the aggressive/sexual behaviour duration and the number of bouts were obtained, only the *post hoc* analyses of the aggressive/sexual behaviour duration is shown.

9.4.4 Habituation and dishabituation olfactory test

A repeated-measures <u>one-way ANOVA test followed by a Tukey HSD multiple comparison</u> *post hoc* was used to analyse the differences between the distinct trials of the habituation and dishabituation olfactory test.

> ➢ WT littermates:

Investigation time:

<u>Repeated-measures one-way ANOVA</u> : Factor "trials" $F_{(4,99)} = 17.66$ $p < 0.0001$ ***

Table 18. Results from the Tukey HSD multiple comparison *post hoc* test analysing the investigation time towards the presented odour in each trial of the olfactory habituation and dishabituation task in **WT** mice.

	H1	H2	H3	H4	Dishabituation
H1	0	2.598	8.575 ***	9.805 ***	2.941
H2	2.598	0	5.977 ***	7.207 ***	0.3427
H3	8.575 ***	5.977 ***	0	1.230	5.634 **
H4	9.805 ***	7.207 ***	1.230	0	6.864 ***

Dishabituation	2.941	0.3427	5.634 **	6.864 ***	0

Number of bouts:

Repeated-measures one-way ANOVA: Factor "trials" $F_{(4,99)} = 12.25$ $p < 0.0001$ ***

Table 19. Results from the Tukey HSD multiple comparison *post hoc* test analysing the number of bouts towards the presented odour in each trial of the olfactory habituation and dishabituation task in **WT** mice.

	H1	H2	H3	H4	Dishabituation
H1	0	0.1374	5.907 ***	7.419 ***	1.511
H2	0.1374	0	6.045 ***	7.559 ***	1.649
H3	5.907 ***	6.045 ***	0	1.511	6.396 *
H4	7.419 ***	7.559 ***	1.511	0	5.907 ***
Dishabituation	1.511	1.649	6.396 *	5.907 ***	0

> ➤ Grb2 cKO mice:

Investigation time:

Repeated-measures one-way ANOVA: Factor "treatment" $F_{(4,99)} = 9.803$ $p < 0.0001$ ***

Table 20. Results from the Tukey HSD multiple comparison *post hoc* test analysing the investigation time towards the presented odour in each trial of the olfactory habituation and dishabituation task in **Grb2 cKO** mice.

	H1	H2	H3	H4	Dishabituation
H1	0	2.245	4.752 *	7.223 ***	0.02458
H2	2.245	0	2.507	4.978 **	2.220
H3	4.752 *	2.507	0	2.471	4.727 *
H4	7.223 ***	4.978 **	2.471	0	7.198 ***
Dishabituation	0.02458	7.223 ***	4.752 *	2.245	0

Number of bouts:

Repeated-measures one-way ANOVA: Factor "treatment" $F_{(4,99)} = 3.895$ $p < 0.0001$ ***

Table 21. Results from the Tukey HSD multiple comparison *post hoc* test analysing the number of bouts towards the presented odour in each trial of the olfactory habituation and dishabituation task in **Grb2 cKO** mice.

	H1	H2	H3	H4	Dishabituation
H1	0	0.7645	2.421	5.224 **	2.803
H2	0.7645	0	1.656	4.460 *	2.039
H3	2.421	1.656	0	2.803	0.3823
H4	5.224 **	4.460 *	2.803	0	2.421

Dishabituation	2.803	2.039	0.3823	2.421	0

9.4.5 Cued fear conditioning test

Results from the <u>repeated-measures two-way ANOVA</u> test analysing the percentage of time the experimental subjects showed freezing behaviour during the memory session of the cued fear conditioning test, using as main factors "CS presentation" and "genotype":

<u>Repeated-measures two-way ANOVA:</u>

Factor "CS presentation"	$F_{(5,135)} = 10.33$	$p < 0.0001$ ***
Factor "genotype"	$F_{(1,135)} = 4.094$	$p = 0:0530$
Interaction	$F_{(5,135)} = 0.3544$	$p = 0.8787$

Table 22. Results from the Bonferroni *post hoc* test of the percentage of time the **WT** experimental subjects showed freezing behaviour after each CS-presentation.

WT	Pre-CS	Post-CS1	Post-CS2	Post-CS3	Post-CS4	Post-CS5
Pre-CS	0	5.129 ***	3.697 ***	2.994 **	2.115	2.742 *
Post-CS1	5.129 ***	0	1.432	2.136	3.014	2.388 *
Post-CS2	3.697 ***	1.432	0	0.7034	1.582	0.9555
Post-CS3	2.994 **	2.136	0.7034	0	0.8787	0.2521
Post-CS4	2.115	3.014	1.582	0.8787	0	0.6266
Post-CS5	2.742 *	2.388 *	0.9555	0.2521	0.6266	0

Table 23. Results from the Bonferroni *post hoc test* of the percentage of time the **Grb2 cKO** experimental subjects showed freezing behaviour after each CS-presentation.

Grb2 cKO	Pre-CS	Post-CS1	Post-CS2	Post-CS3	Post-CS4	Post-CS5
Pre-CS	0	4.026 ***	4.161 ***	2.090	1.743	2.502 *
Post-CS1	4.026 ***	0	0.1341	1.936	2.284	1.525
Post-CS2	4.161 ***	0.1341	0	2.070	2.418 *	1.659
Post-CS3	2.090	1.936	2.070	0	0.3477	0.4112
Post-CS4	1.743	2.284	2.418 *	0.3477	0	0.7589
Post-CS5	2.502 *	1.525	1.659	0.4112	0.7589	0

9.4.6 Contextual fear conditioning test

Results from the <u>repeated-measures two-way ANOVA</u> analysing the percentage of time the experimental subjects showed freezing behaviour using as main factors "Post-UCS" and "genotype", during **the conditioning session of the contextual fear conditioning test**:

Two-way ANOVA: Factor "post-UCS" $F_{(2,56)} = 23.91$ $p < 0.0001$ ***

Factor "genotype" $F_{(1,56)} = 12.25$ $p < 0.0016$ **

Interaction $F_{(2,56)} = 3.063$ $p = 0.0547$

Table 24. Results from the Bonferroni *post hoc* test of the percentage of time the **WT** experimental subjects showed freezing behaviour after each CS-presentation.

WT	Post-UCS1	Post-UCS2	Post-UCS3
Post-UCS1	0	4.366 ***	6.178 ***
Post-UCS2	4.366 ***	0	1.812
Post-UCS3	6.178 ***	1.812	0

Table 25. Results from the Bonferroni *post hoc* test of the percentage of time the **Grb2 cKO** experimental subjects showed freezing behaviour after each CS-presentation.

Grb2 cKO	Post-UCS1	Post-UCS2	Post-UCS3
Post-UCS1	0	1.034	3.583 **
Post-UCS2	1.034	0	2.549 *
Post-UCS3	3.583 **	2.549*	0

Results from the <u>repeated-measures two-way ANOVA</u> analysing the percentage of time the experimental subjects showed freezing behaviour during the **memory session of the contextual fear conditioning test** using as main factors "interval" and the "genotype",:

Two-way ANOVA: Factor "interval" $F_{(5,140)} = 18.17$ $p < 0.0001$ ***

Factor "genotype" $F_{(1,140)} = 3.552$ $p = 0.0699$

Interaction $F_{(5,140)} = 1.1179$ $p = 0.3227$

Table 26. Results from the Bonferroni *post hoc* test of the percentage of time the **WT** experimental subjects showed freezing behaviour in each minute interval of the neutral and the conditioned context (first five minutes).

WT	0 -1 N Cx	2 - 3 N Cx	4 – 5 N Cx	0 -1 C Cx	2 - 3 C Cx	4 – 5 C Cx
0 -1 N Cx	0	2..130	1.433	5.484 ***	6.513 ***	4.488 ***
2 - 3 N Cx	2..130	0	0.6971	3.353 **	4.382 ***	2.358 *

4 – 5 N Cx	1.433	0.6971	0	4.050 ***	5.079 ***	3.055 **
0 -1 C Cx	5.484 ***	3.353 **	4.050 ***	0	1.029	0.9956
2 - 3 C Cx	6.513 ***	4.382 ***	5.079 ***	1.029	0	2.025
4 – 5 C Cx	4.488 ***	2.358 *	3.055 **	0.9956	2.025	0

N Cx: Neutral context; C Cx: Conditioned context

Table 27. Results from the Bonferroni *post hoc* test of the percentage of time the **Grb2 cKO** experimental subjects showed freezing behaviour in each minute interval of the neutral and the conditioned context (first five minutes).

Grb2 cKO	0 -1 N Cx	2 - 3 N Cx	4 – 5 N Cx	0 -1 C Cx	2 - 3 C Cx	4 – 5 C Cx
0 -1 N Cx	0	1.345	1.555	2.630 *	5.191 ***	3.258 **
2 - 3 N Cx	1.345	0	0.2094	1.285	3.845 ***	1.913
4 – 5 N Cx	1.555	0.2094	0	1.076	3.636 ***	1.704
0 -1 C Cx	2.630 *	1.285	1.076	0	2.560 *	0.6281
2 - 3 C Cx	5.191 ***	3.845 ***	3.636 ***	2.560 *	0	1.932
4 – 5 C Cx	3.258 **	1.913	1.704	0.6281	1.932	0

N Cx: Neutral context; C Cx: Conditioned context

9.5   Materials

9.5.1   Chemicals

Chemicals	Manufacturer / Source
aCSF	Harvards apparatus, Massachusetts, USA
Avidin-Biotin-Peroxidase complex (kit)	Vector Laboratories, Inc., Bulingame, USA
Biotinylated secondary antibody (goat anti rabbit IgG)	Vector Laboratories, Inc., Bulingame, USA
3,3'-Diminobenzidin-Tetrahydrochlorid	Sigma-Aldrich, Steinheim, Germany
DePex	Serva Elecrophoresis GmbH, Hidelberg, Germany
Disodium phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Domitor®	Pfizer Pharmacia, Berlin, Germany
Embedding medium	Leica Microsystems Nussloch GmbH, Germany
Ethanol, 96 %	Otto Fischer GmbH j& Co. KG, Saarbrücken, Germany

Ethanol, 100 %	Zentralapothecke, Uniklinik Magdeburg
Gelatine	Serva Electrophoresis GmbH, Heidelerg, Germany
Glycin	Merck, Darmstadt, Germany
Goat normal serum	PAN-Biotech GmbH, Aidenbach, Germany
H_2O_2, 30 %	Merck KGaA, Darmstadt, Germany

Chemicals	**Manufacturer / Source**
Heparin-Natrium (25000IE/5 ml)	Raiopharm GmbH, Ulm, Germany
Histoacryl	Aesculap AG, Tuttlingen, Germany
Immumount	Thermo Electron GmbH, Dreieich, Germany
Isofluorane	Baxter Deutschland GmbH, Unterschleißheim, Germany
Isoamyl acetate ($\geq$ 97 %)	Sigma-Aldrich, Steinheim, Germany
Isopropanol (2-propanol)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Isopentane	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ketavet® (100 mg/ml)	Pfizer Pharmacia, Berlin, Germany
Kresylbiolett	Merck KGaA, Darmstadt
Lidocaine (Xylocitin®-loc 2 %)	Mibe Vertriebsgesellschaft mbH, Jena, Germany
Metacam® (Meloxicam, 5 mg/ml)	Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany
Methanol	VWR International GmbH, Darmstadt, Germany
NaCl solution (0,9 %)	B.Braun Melsungen AG, Melsungen, Germany
Ponceau S solution	Sigma-Aldrich, Steinheim, Germany
R-(-)- Carvone (for synthesis)	Merck KGaA, Darmstadt, Germany
Sodium azide	Serva Electrophoresis GmbH, Heidelberg, Germany
Sodium borohydride	Sigma-Aldrich, Steinheim, Germany
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe

Monosodium phosphate (NaH$_2$PO$_4$)	Merck KGaA, Darmstadt, Germany
Sodium hydroxide (NaOH$_{(aq)}$)	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Serva, Heilderberg, Germany
Paraformaldehyde (PFA)	Merck KGaA, Darmstadt, Germny
Bovine serum albumine	PAA Laboratories GmbH, Pasching, Austria
Rompun®	Bayer Vital GmbH, Leverkusen, Germany

Chemicals	Manufacturer / Source
Hydrochloric acid (HCl $_{(aq)}$)	Merck KGaA, Darmstadt, Germany
Sucrose	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
TRIS (Tris (hydroxymethyl) aminomethan)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Triton-X-100	Ferak Berlin GmbH, Berlin, Germany
Xylol	VWR International GmbH, Darmstadt, Germany

9.5.2 Solutions

Solutions, buffers	Composition	
Phosphate buffered saline (PBS)	140 mM	NaCl
	3 mM	KCl
	10 mM	NaH$_2$PO$_4$
	5 mM	NaOH
Avidin-biotin-Peroxidase complex- solution (with PBS)	0.1 % Kit component A	
	0.1% Kit component B	
	0.2 % Bovine serum albumin	
	mixed gently during at least 30 min	
Formaldehyde solution	4 % PFA in 0.1 M NaPi-buffer, pH 7.4	
Isofluorane-anaesthesia inhalation	2 % isofluorane	
	100 % oxygen	
Gelatine object slide	0.5 g Gelatine	
	100 ml distilled water	
	warm up and gently mixed	
	object slide shortly submerge on the solution	
	led the object slide dry	
Kresylviolett	5 gr Kresylviolett	
	100 ml destilled water	
	filtered	
	dilute to the wished colour intensity	

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Solution for a deep Anaesthesia	2 mL Ketavet 0.5 ml Rompun 7.5 ml NaCl-solution
Metacam- solution (0.5 mg / Kg body weight)	0.05 mL Metacam (0.25 mg Meloxicam) 4 ml Nacl-solution
Methanol-H_2O_2-solution	1:1 Methanol:PBS 1 % H_2O_2 (30 %)

Solutions, buffers	**Composition**
NaCl solution (0,9 % in distilled water)	9 g NaCl / l
NaCl-heparin-solution	1mL heparin-Natrium 1 L NaCl solution
BSA-solution (in PBS)	0.2 % BSA 0.1 % Triton-X-100 0.1 % Sodium azide
Sucrose solution	1 M in 0.1 M NaPi-buffer
Tris buffer (0,5 M, pH 7,6)	30.25 g TRIS 500 ml distilled water
GNS solution	10 % goat normal serum 0.3 % Triton-X-100 0.1 % Sodium azide

NaPi buffer	Solution I:
	    0.1 M Na_2PO_4
	    adjust pH 7.4 with:
	Solution II:
	    0.1 M NaH_2PO_4

RIPA-DOC lysis buffer	150 mM NaCl 2.5 mM EDTA 1 % NP40 0.5 % Sodium-deoxycholate 0.1 % SDS 1 mM Na_3VO_4 50 mM Tris pH 7.4 adjust with HCl + 1 tablet of proteases inhibitors / 10 ml (Roche) + 1 tablet of phosphatases inhibitors/10 ml (Roche)
1% Cresyl violte solution	1 g Cresyl violet acetate 50 mL 96 % ethanol Bring to 100 mL with distilled water stir for 7 h at RT (protected from the light)

	filter	
	store protected from the light	

Electrophoresis Buffer:	25 mM	Tris
	250 mM	Glycine
	0.1 %	SDS

Transfer Buffer:	48 mM	Tris
	39 mM	Glycine
	0.0375 %	SDS
20 %		Methanol

Solutions, buffers	**Composition**

Acrylamid 5-20 % gradient gel:

	Separating gel		Stacking gel
	20%	5%	5%
Buffer [a]	2,75 ml	2,75 ml	2,7 ml
Acrylamide	5,5 ml	1,4 ml	1,3 ml
Glycerol (100%)	2,0 ml	0,6 ml	2,3 ml
APS (100%)	26,0 µl	40,0 µl	57,1 µl
TEMED	6,5 µl	6,5 µl	7 µl
Bromophenol blue	50 µl		
Phenol red			5 µl
Nanopure H_2O	0,62 ml	6,2 ml	3,6 ml

a. Buffers:

Separating gel buffer:	1.5 M	Tris
	0.4 %	SDS
	1.03 mM	EDTA
	pH 8.8	adjust with HCl

Stacking gel buffer:	0.5 M	Tris
	0.4 %	SDS
	1.03 mM	EDTA
	pH 6.8	adjust with HCl

TRIS-Buffer	0.5 M	Tris

TRIS Buffered Saline (TBS)	50 mM	Tris-HCl, pH7.5
	150mM	NaCl

TBS-T	1x	TBS
	0.1 %	Tween

6x sample buffer	0.25 %	Bromophenolblue
	0.25 %	Xylenecyanol FF
	15 %	Ficoll 400

9.5.3 Equipment

Equipment	Manufacturer / Provider
Anaesthesia Delivery Unit S/5	Datex Ohmeda Division, Instrumentarium AB SE-161 O2 Bromma, Sweden
Axio Imager. M1 (fluorescence and bright field microscope)	Zeiss, Jena
Cryostat (Leica CM3050 S)	Leica Microsystems GmbH, Wetzlar, Germany
Microinjections pump	CMA Microdialysis AB, Stockholm, Sweden

Equipment	Manufacturer / Provider
Perisaltic Pump P-1	Pharmacia LKB Biotecnhonology AB, Uppsala, Sweden
Power Supply, model 1000/500	Bio-Rad Laboratories, Inc. California, USA
SE250 Mighty Small II vertical electrophoresis unit	Hoefer, Inc. Holliston, MS 01746, USA
TE22 Mighty Small Transfer Tank	Hoefer, Inc. Holliston, MS 01746, USA
TSE Fear Conditioning System	TSE, Bad Homburg, Germany

9.5.4 Computer programs

Computer Programs	Manufacturer / Source
Anymaze Video tracking system	Stoelting Co., Wood Dale, IL, USA
Axio Vision (Release 4.8)	Carl Zeiss MicroImaging GmbH, Göttingen
Eventlog (Eventlog Recorder, Version 1.0, 1986)	Design: Robert Hendersen
GraphPad Prism (version 5.04)	GraphPad Software Inc., San Diego, USA
Microsoft Office	Microsoft, Redmond, WA, USA
Image J (Fiji)	ImageJ 1,50b, Wayne, Rasband, National Institute of Health, USA
TSE Fear Conditioning System Software	TSE, Bad Homburg, Germany
WinRat (Version 2.30, 1995)	*Copyright*© by Dr.-Ing. H. Barthelmes, Max Planck Institute for Psychiatry, Munich

9.5.5 Consumables, supplies, accessories

Consumables, supplies, accessories	Manufacturer / Provider
24-Well-Plate	Greiner Bio-One GmbH, Frickenhausen, Germany
Anaesthesia machine	(MLW Medimorph System) VEB MLW, Leipzig, Germany
Bolts	(DIN 84, 1,0 x 2,0 mm) Paul Korth GmbH & Co. KG, Lüdenscheid, Germany
Cannulae (23 G)	Becton Dickinson GmbH, Heidelberg, Germany

Consumables, supplies, accessories	Manufacturer / Provider
Cotton-tipped Applicators (Raucotupf)	Lohmann & Rauscher International, GmbH & Co. KG, Rengsdorf, Germany
Cold light source	A. Krüss Optronic GmbH, Hamburg, Germany
Coverglass	Gerhard Menzel GmbH, Braunschweig, Germany
Dental drill	VEB MLW Dentalfabrik, Treffurt, Germany
Dental cement (Dual$^®$ Cement, *Base & Catalyst*)	Ivoclar Vivadent AG, Schaan, Liechtenstein
Edding (30, neutral odour)	Edding International GmbH, Ahrensburg, Germany
Eye gel (Vidisic)	Dr. Mann Pharma und Bausch & Lomb, GmbH, Berlin, Germany
Food	Ssniff Spezialdiäten GmbH, Soest, Germany
Hyperfilm ECL autoradiographic	Amersham BioSciences, Little Chalfont, UK
Litter	J. Rettenmayer & Söhne GmbH & Co. KG, Rosenberg, Germany
Guide cannula (0.55 x 0.08 x 9.00 mm)	Injecta GmbH, Klingenthal, Germany
Injection cannula (0.36 x 0.08 x 20.00 mm)	Injecta GmbH, Klingenthal
Insect pins (Stärke 0)	Ento Sphinx s.r.o., Pardubitz, Tschechische Republik
Ocular sticks (Pro-Ophta$^®$)	Lohmann & Rauscher International, GmbH & Co. KG, Rengsdorf, Germany

Pads of cork	VWR International GmbH, Darmstadt, Germany
Phenol Red Concentrated 2000x	Sigma-Aldrich, Steinheim, Germany
Plastic hose	(Tygon$^{®}$ R3607, VWR International GmbH, Darmstadt, Germany, Inner diameter: 0.19 mm, Wall thickness: 0.91 mm)
Slides	Gerhard Menzel GmbH, Braunschweig, Germany
Shaker	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Scalpel Blade (*Feather No. 11*)	Pfm Medical AG, Cologne, Germany
Syringe	B. Braun Melsungen AG, Melsungen, Germany
Consumables, supplies, accessories	**Manufacturer / Provider**
Syringe (Hamilton)	Hamilton Bonaduz AG, Bonaduz, Switzerland
Stereotaxic frame	TSE Systems GmbH, Bad Homburg, Germany
SuperFrost$^{®}$ Plus-Slides	Gerhard Menzel GmbH, Braunschweig, Germany
Type II cage (Makrolon$^{®}$, 22 x 16 x 14 cm)	E. Becker & Co. GmbH, Castrop-Rauxel, Germany
Type III cage (Makrolon$^{®}$, 37 x 21 x 15 cm)	E. Becker & Co. GmbH, Castrop-Rauxel, Germany
VFC	Built by Prof. Dr. M. Engelmann, Magdeburg, Germany
Vortex	Scientific Industries, Inc., Bohemia, MY, USA
Water bath	Windaus Labortechnik, Clausthal-Zellerfeld, Germany
Rotilabo®-round filters, type IIIA	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Acknowledgements

In this section I would like to thank everybody who contributed to make this project possible and to all of them who shared a little or big part of this path with me, I will not be able to name them all but they are in my thoughts.

First of all, I would like to express my most sincere gratitude to my "doctor father", Prof. Dr. Mario Engelmann, not only for giving me the opportunity to join his group, for his supervision, his productive discussions and excellence guidance throughout the PhD, but also for his patience, kindness and friendship we shared during this time. He taught me to be critic, to grow as a scientist, to look at all results with optimism and never see any as "negative" but as challenging, in order to improve or to update our hypothesis. Thank you for your guidance and your support.

Special thank goes also to Rita Murau, for her excellent technical and personal support, but mostly for her endless patience with my German. I thank Prof. Dr. Klaus-Dieter Fischer for his co-supervision and as the director of the Institute of Biochemistry and Cell Biology, for allowing me to perform most of the experiments included in this thesis. I would also like to sincerely thank Prof. Dr. Oliver Stork for his contributions to our collaborations and for always being there in case further discussions were needed. Also to our collaborator, Dr. Thomas Endres, for letting me perform some of the behavioural experiments in their animal facility and for providing valuable discussions when they were required. I thank Dr. Dora Zelena for her very positive contributions in our collaborations, and her support and short visits with energy bars that helped me to survive the late evenings in the office.

I also would like to thank the Leibniz Institute and the LGS graduate school for financing the first three and a half years of my PhD and offering the chance to join interesting international workshops, conferences and retreats. For four months I got a "Promotionsstipendium" from the Otto von Guericke University, for which I am really thankful. Durign the last year, I have been financed by a collaboration between the Otto von Guericke University and the laboratory of Prof. Dr. Gert Lubec from the Vienna University. I sincerely thank the people involved for giving me the chance to finish writing my thesis in addition to the new experimental work.

Very importantly, I want to thank all the members of the Institute of Biochemistry and Cell Biology for providing me with a very welcoming work environment from the first day I arrived in Magdeburg. I specially would like to thank Dr. Karin Richter, Dr. Kristina Langnäse and Dr. Thomas Schlüter for their endless patience in answering all my questions and helping me to commence my research. Special thanks go to the wonderful

secretary of our Institute, Mrs Julia Czerney, for her unwavering positive attitude and for being always willing to help me when I needed it. Also to those who made the lunch break a real escape from work, Dr. Anna Gottfried, Dr. Anne-Christin Lehmann, Dr. Laura Tech and Julia Czerney.

To Dr. Julia Noack, my guardian angel ever since I arrived in Magdeburg, I cannot thank you enough for all that you helped with and offered me from the first day I arrived in the laboratory until my submission, however I will thank you again for the most precious offering I got from you, thanks for your friendship. Also to my favourite German girls, Dr. Anne Gieseler, Susi Bonifatious, Dr. Josi Hildebrandt and Dr. Julia Noack, thank you all for your wonderful friendship and constant support.

I also would like to express my deepest thanks to Andrew Kaus, who has been a great support during the final years of this PhD and the most welcomed company after a long day at work. Thanks for always supporting me and for your enthusiasm for the language correction of my thesis. I am glad I have you by my side.

Finally, to those people who not only supported me during the PhD but throughout my life, my friends, Teresa Torra, Katy Farrus, Neus Jordana and Laura Garcia, but most importantly my family, my mum Mª Dolors Perna Parra, my dad Ramon Camats Macià and my sister Eli Camats Perna. Because being far from you also made me realize how important you are and how much I need you, thanks for your unconditional support and for always believing in me.

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List of publications

1- Parra-Damas A, Chen M, Enriquez-Barreto L, Ortega L, Acosta S, Perna JC, Fullana MN, Aguilera J, Rodriguez-Alvarez J and Saura CA (2017). CRTC1 function during memory encoding is disrupted in neurodegeneration. Biological Psychiatry. Available online 11 July 2016. [In Press, Corrected Proof] DOI:10.1016/j.biopsych.2016.06.025

2- Camats Perna J and Engelmann M. Recognizing Others: Rodent's Social Memories. Current Topics in Behavioral Neuroscience pp 1 – 21. DOI:10.1007/7854_2015_413. [Epub ahead of print]

3- Perna JC, Wotjak CT, Stork O and Engelmann M (2015). Timing of presentation and nature of stimuli determine retroactive interference with social recognition memory in mice. Physiology & Behaviour. 14, 10-14. DOI:10.1016/j.physbeh.2015.02.029

4- Bhardwaj D, Náger M, Camats J, David M, Benguria A, Dopazo A, Cantí C and Herreros J (2013). Chemokines induce axon outgrowth downstream of Hepatocyte Growth Factor and TCF/β-catenin signalling. Frontiers in cellular neuroscience. 30 April 2013. DOI:10.3389/fncel.2013.00052

Declaration of Authorship

Hiermit erkläre ich, Judith Camats Perna, dass ich die von mir eingereichte Dissertation zum Thema

"Distinction between dorsal and ventral hippocampus in processing social cues and the underlying mechanisms essential for social recognition memory in mice"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und alle benutzten Quellen und Hilfsmittel vollständig angegeben habe.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades *doctor rerum naturalium* (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Datum: 22.11.2016

Unterschrift: