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Cognitive phenotyping of wildtype and mutant mouse lines

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Zusammenfassung

Die Detektion kognitiver Phänotypen in verschiedenen Mausmodellen gewinnt immer mehr an Bedeutung, da kognitive Defizite bei der Früherkennung von neuropsychiatrischen Krankheiten eine große Rolle spielen. Um diese groß angelegte Phänotypisierung realisieren zu können, sind Lern- und Gedächtnistests nötig, die in kurzer Zeit Ergebnisse liefern und darüber hinaus in verschiedenen Labors angewandt werden können.

Der in dieser Arbeit etablierte Objekterkennungstest erfüllt diese Bedingungen, da er mit einfachen Mitteln in relativ kurzer Zeit und ohne aufwändige Lernregeln durchgeführt werden kann. Mäuse zeigen eine natürliche Tendenz, unbekannte Objekte mehr zu explorieren als bereits bekannte Objekte. Das Objektgedächtnis beruht auf der Fähigkeit, unbekannte Objekte von bekannten Objekten zu unterscheiden und mehr Zeit mit deren Exploration zu verbringen. Der Objekterkennungstest wird in vielen Labors mit den unterschiedlichsten Ausstattungen praktiziert, die Testdurchführung variiert stark bezüglich der verwendeten Geräte, Objekte und Prozeduren. Dies kann zu sich widersprechenden Ergebnissen führen. Um Ergebnisse unmittelbar miteinander vergleichen zu können, ist die Anwendung eines definierten und standardisierten Objekterkennungstests zwingend erforderlich. Mit Hilfe von Grundlagentests wie dem Objektpräferenztest, dem „Counterbalance-Test“ und dem Test zur Analyse retroaktiver Interferenz konnte ich einen Objekterkennungstest etablieren und validieren. Mäuse des Stammes C57BL/6J beiderlei Geschlechts zeigten ein intaktes Objektgedächntis nach Zeitintervallen von 3 Stunden, 24 Stunden, 48 Stunden, 72 Stunden und 7 Tage.

Der etablierte Objekterkennungstest konnte daraufhin in drei ausgesuchten Mausmodellen angewandt und Daten zur Detektion ihrer kognitiven Phänotypen erhoben werden. Zur detaillierten Analyse dieser Mausmodelle wurden zusätzlich der so genannte „Y-maze Spontaneous Alternation Test“ und der so genannte „Social Discrimination Test“ durchgeführt. Der „Y-maze Spontaneous Alternation Test“ misst das Arbeitsgedächntis bei der Exploration eines Y-förmigen Labyrinths, wohingegen der „Social Discrimination Test“ die Unterscheidung zwischen bekannten und unbekannten Artgenossen und somit soziales Gedächtnis misst. Darüber hinaus validierte ich einen futterbelohnten „Hole Board Test“ mit den Mausstämmen C57BL/6J und Balb/c, der zur Analyse von Arbeits- und Referenzgedächtnis dient.

For Ma and Pa

A prayer for the wild at heart kept in cages

(Tennessee Williams, 1911-1983)

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1. Introduction

1.1. Why do we need cognitive phenotyping?

As neurobiological research has made great progress over the last years and genetic methods allow the production of high numbers of mutant mice, time-efficient behavioural tests to analyse them are needed (Auwerx et al., 2004). For large-scale functional mouse phenotyping, learning and memory tests are necessary which allow high throughput, do not require expensive equipment, are automatizable, as time-saving and easily transferable to other laboratories as possible (Brown et al., 2005).

Tests which fulfil these claims are, beside others, the Y-maze Spontaneous Alternation Test, the Social Discrimination Test and the Food-rewarded Hole Board Test. Furthermore, I developed and validated a simple Object Recognition Test to analyze cognitive phenotypes in mice, according to the above-mentioned claims. Various aspects of cognitive performance can be evaluated via this test, e.g. long-term and short-term memory.

Cognitive phenotyping is becoming more and more important in terms of analyzing mouse models as cognitive deficits could be an early predictor for neuropsychiatric diseases (Kanazawa, 2004) such as Parkinson's disease (Whittington, 2006) and schizophrenia (Laws, 2006). Additionally, it was shown that emotional and cognitive deficits can be linked (Ohl et al., 2003). Gaining information about neuropsychiatric diseases already in early stages could be very useful for treatment and prevention.

Cognitive impairments in various neuropsychiatric diseases are described in the next chapter.

1.2. Cognitive impairments in neuropsychiatric diseases

The ability to remember is the most significant feature of our mental life.

We are who we are largely because of what we have learned and what we remember. Impairments in learning and memory can lead to disorders that range from the moderately senescent forgetfulness associated with normal aging to devastating memory losses associated with, for example, Alzheimer's disease (AD) (Mayford et al., 1999).

Learning is defined as the process of acquiring new information or skills, whereas memory refers to the persistence of learning that can be revealed at a later time. Memory is the usual consequence of learning and reflects the enduring changes in the nervous system that result from transient experiences (Johnston et al., 2003).

Dementia associated with Alzheimer's disease has a tremendous impact on a person's everyday life and many studies were undertaken to gain insight into this disease. It was shown that cognitive decline is predicted by diffuse cognitive impairment (Buccione et al., 2007). As mild cognitive impairment (MCI) represents a transitional state between cognitive changes during normal aging and very early dementia, it is recognized as a risk factor for Alzheimer's disease (Grundman et al., 2004). The question is when physiological aging is merging with cognitive deficits of AD or any other neuropsychiatric disease and at which time-point therapy should be started to delay ongoing memory decline and to preserve a patient's quality of life as long as possible.

Also in Parkinson's disease (PD), early diagnosis could be helpful to postpone the destructive symptoms of this disease. As motor deficits do usually not appear before 50 percent of dopaminergic neurons in the substantia nigra are lost (Forno, 1996; Esposito et al., 2007), diagnosis via cognitive deficits in early stages could be beneficial.

PD is primarily considered a motor disease, but neuropsychiatric complications should not be ignored. Depression is one of the most common psychiatric disorders that patients with PD suffer from (Ferreri et al., 2006). Depression is often difficult to diagnose differentially because of the clinical overlap with PD. Psychomotor slowness, concentration and sleep disturbances and social withdrawal occur in both diseases. Cognitive impairment is also a core symptom in PD. It was shown that cognitive impairment is correlated with motor symptom severity, but not with disease duration (Williams et al., 2007).

Schizophrenia is associated with neuropsychological impairments in several cognitive domains, e.g. attention and memory. As Piskulic et al. showed, especially spatial working memory is affected (Piskulic et al., 2007). Indeed, visual working memory impairments are frequently reported in schizophrenia (Tek et al., 2002).

Cognitive impairments in schizophrenia and bipolar disorders share some similarities (Green, 2006). Unipolar and bipolar depression are known to exert detrimental effects on learning and memory processes. Bipolar disorder is associated with impairment in executive functioning and spatial working memory (Glahn et al., 2007). Also deficits in verbal learning were reported (Antila et al., 2007).

In patients suffering from post-traumatic stress disorder (PTSD), impaired declarative memory performance and even smaller hippocampal volume have been observed (Golier et al., 2006). Trauma survivors with PTSD have a higher risk to develop cognitive decline later in life.

Furthermore, anxiety disorders are related to cognitive impairment. Anxious subjects were shown to be impaired in measures of short-term and delayed memory (Mantella et al., 2007). Anxiety can be a confounding factor in cognitive performance (Brooks et al., 2005).

I mentioned only some examples of neuropsychiatric disorders which are related to cognitive impairment. Nevertheless, even these few examples display the importance of an intact memory and the multiple side effects that come along with neuropsychiatric disorders. As human beings are defined through their individual memory, one can imagine the consequences of loss of this memory and the following loss of personality.

1.3. Learning and memory – classification, molecular basis and brain areas

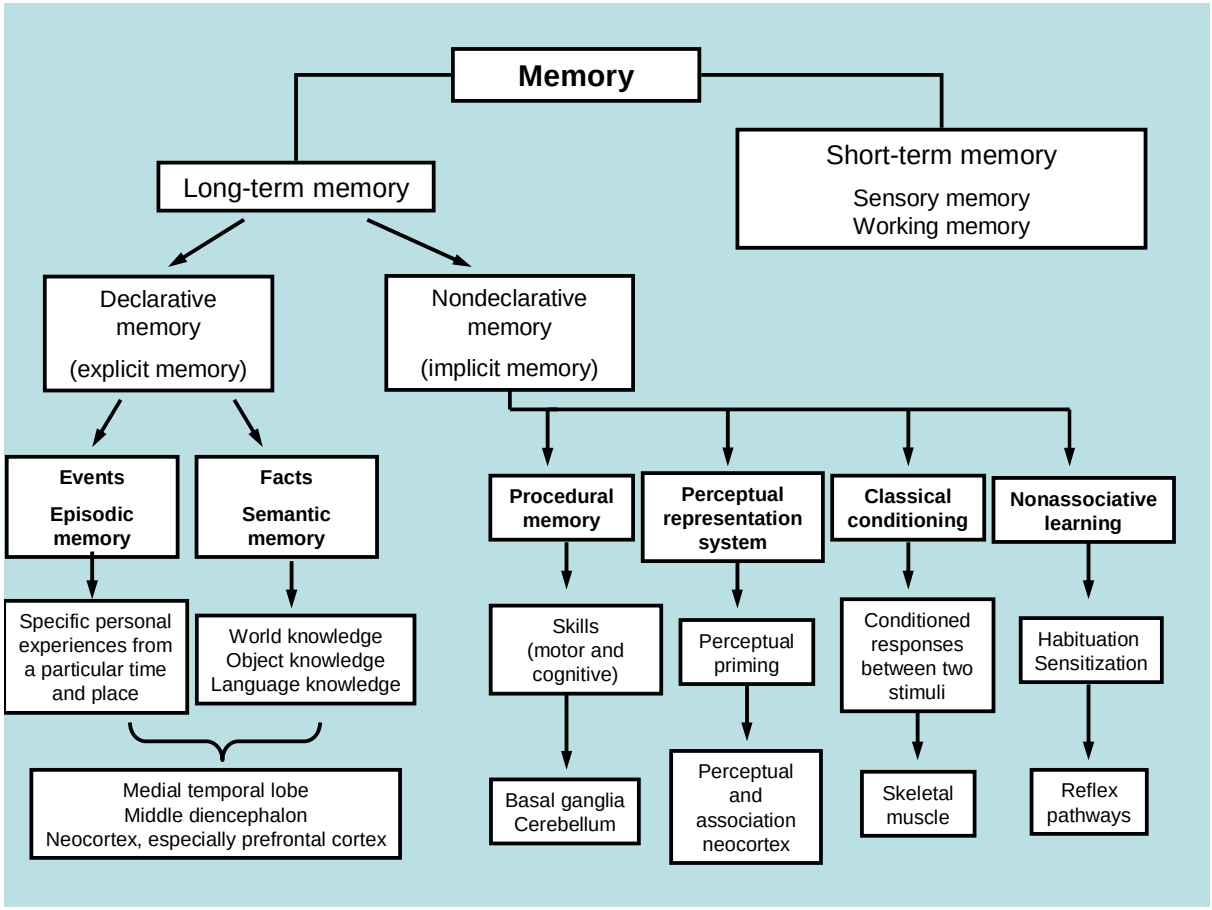

Figure 1: Classification of human memory into long-term and short-term memory and its specific divisions.

Adapted and modified from Gruber, 2006.

Memory refers to the storage, retention and recall of information including past experiences, knowledge and thoughts. Memory for specific information can vary greatly according to the individual and the individual's state of mind. Memory formation in general consists of

1. acquisition, which is the processing, encoding and combining of received information,

2. consolidation, which is the creation of a permanent record of the encoded information and

3. retrieval, which is calling back the stored information in response to some cues for their usage in a process or activity. There are many attempts to categorize different forms of memory in humans. However, trying to divide memory into its different forms

is difficult because memory systems are fluently merging into each other. One attempt is shown in Figure 1.

The first classification in human memory is between long-term and short-term memory (Figure 1). Sensory memory belongs to short-term memory (Figure 2). Sensory memory is the ability to retain impressions of sensory information after the original stimulus has ceased. It refers to items detected by the sensory receptors which are retained temporarily in the sensory registers and which have a large capacity for unprocessed information. The two types of sensory memory that have been mostly explored are iconic memory (short-term visual memory) and echoic memory (short-term auditory memory). Both are unconscious. Sensory memory allows us to take a “snapshot” about our environment and lasts only seconds.

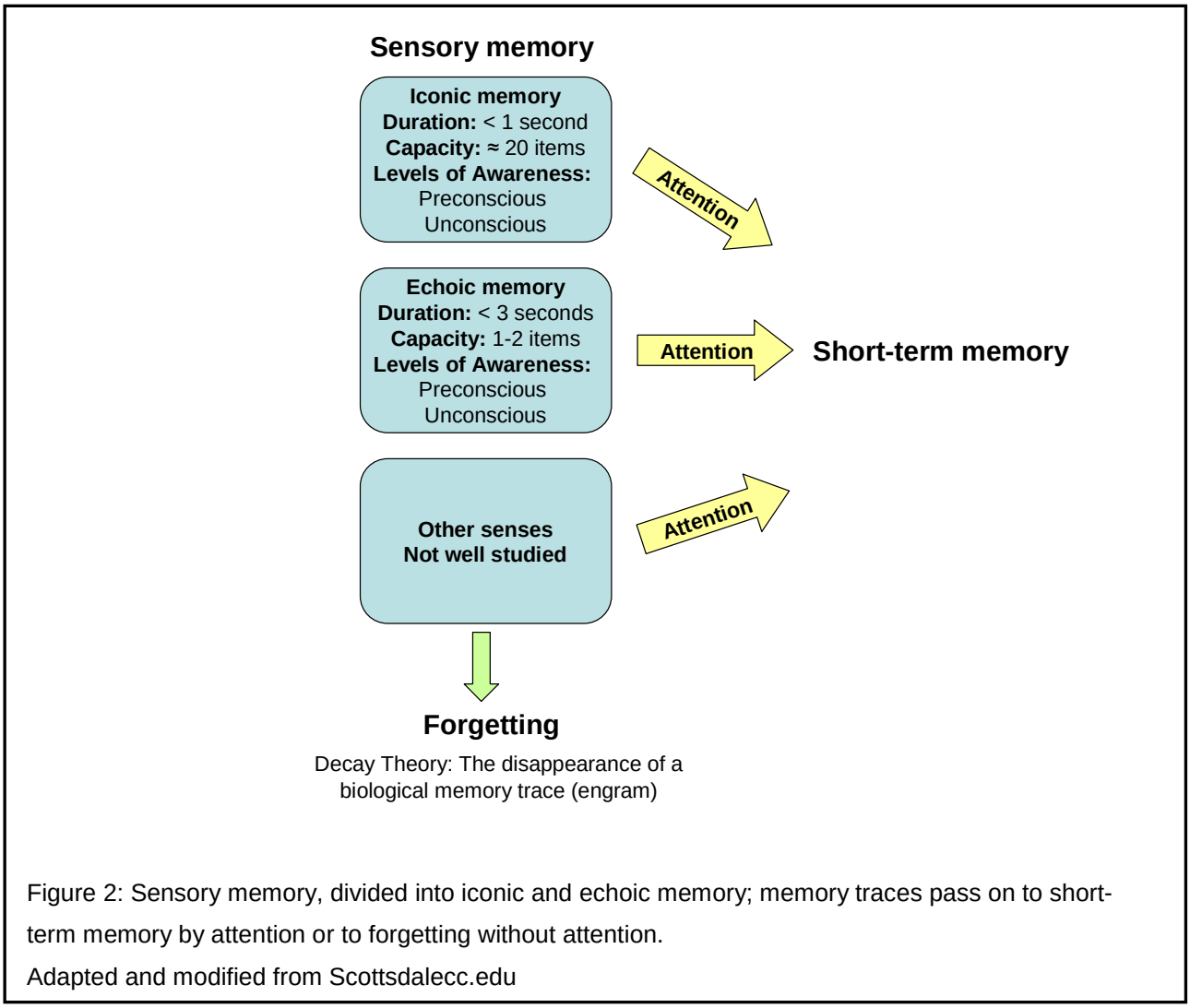

Figure 2: Sensory memory, divided into iconic and echoic memory; memory traces pass on to short-term memory by attention or to forgetting without attention.

Adapted and modified from Scottsdalecc.edu

The Decay Theory is a model of forgetting which assumes that memories fade and will gradually be lost if they are not occasionally refreshed (Conrad, 1957).

Working memory also belongs to short-term memory. It refers to the structures and processes used for temporary storage and is generally considered to have limited capacity (Ericsson et al., 1995).

Long-term memory in humans can be divided into declarative (explicit) or non-declarative (implicit) memory (Figure 1). Declarative memory is the aspect of human memory that stores facts. It is called so because it refers to memories that can be consciously discussed or declared. Declarative memory is divided into episodic memory (for events) and semantic memory (for facts). In non-declarative memory, several distinctions can be drawn. Procedural memory, which is the memory for skills or the “how to” memory, is mediated through basal ganglia and cerebellum. Also priming and conditioning belong to non-declarative memory. Priming refers to the increase in speed or accuracy of a decision that occurs as a consequence of a prior exposure to some of the information in the decision context. Classical conditioning involves repeatedly pairing of an unconditioned stimulus (for example a foot-shock) with a neutral stimulus (for example a tone or a light). An unconditioned stimulus is a stimulus that naturally evokes a certain response, which is the unconditioned response. A neutral stimulus alone would not elicit this response. However, after repetitive pairing of the neutral stimulus with the unconditioned stimulus, the neutral stimulus also elicits the same response as the unconditioned stimulus. Then the neutral stimulus has become a conditioned stimulus and the response to the conditioned stimulus is called a conditioned response.

Sensitization is the increase in the response to a harmless stimulus when that stimulus occurs after a punishing stimulus (for example an electric shock), whereas habituation is the decrease in the strength of a behavioural response that occurs when an initially novel eliciting stimulus (for example a tactile stimulus which is triggering a reflex) is repeatedly presented. Sensitization and habituation were first studied by Eric Kandel and are very well described in the seasnail *Aplysia* (Kandel et al., 1982).

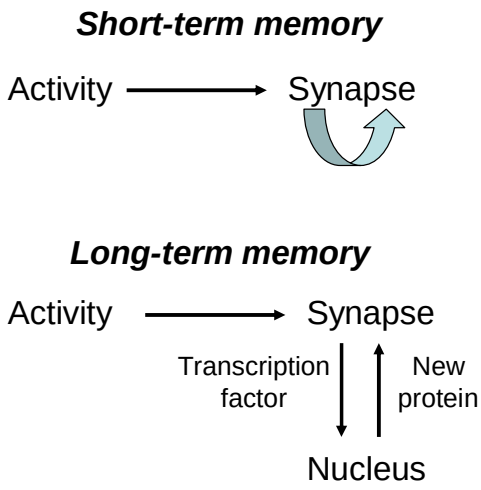

Figure 3: Short-term and long-term memory and their activation.
Adapted and modified from Johnston et al., 2003.

Number and complexity of neuronal connections distinguish the human brain from that of animals rather than fundamental chemical processes. Pathways for learning and memory are highly conserved across species and have been found in flies, snails, rodents and primates (Johnston et al., 2003).

In general, short-term memory does not require protein synthesis but results from changes in synaptic strength **within** synapses (Figure 3). The activation of CaMKII (calcium/calmodulin-dependent protein kinase II) in response to calcium/calmodulin is responsible for this type of memory and is triggered by an increased activity in alpha-**a**mino-3-hydroxy-5-**m**ethyl-4-isoxazole**p**ropionic **a**cid (AMPA) glutamate receptors. Short-term memory only requires enzyme activation and protein phoshorylation.

Long-term memory generally requires transcription and translation of new proteins which enhance the strength or number of active synapses.

A famous citation of Eric Kandel goes “Memory storage is the dialogue between genes and synapses” (Kandel, 2001).

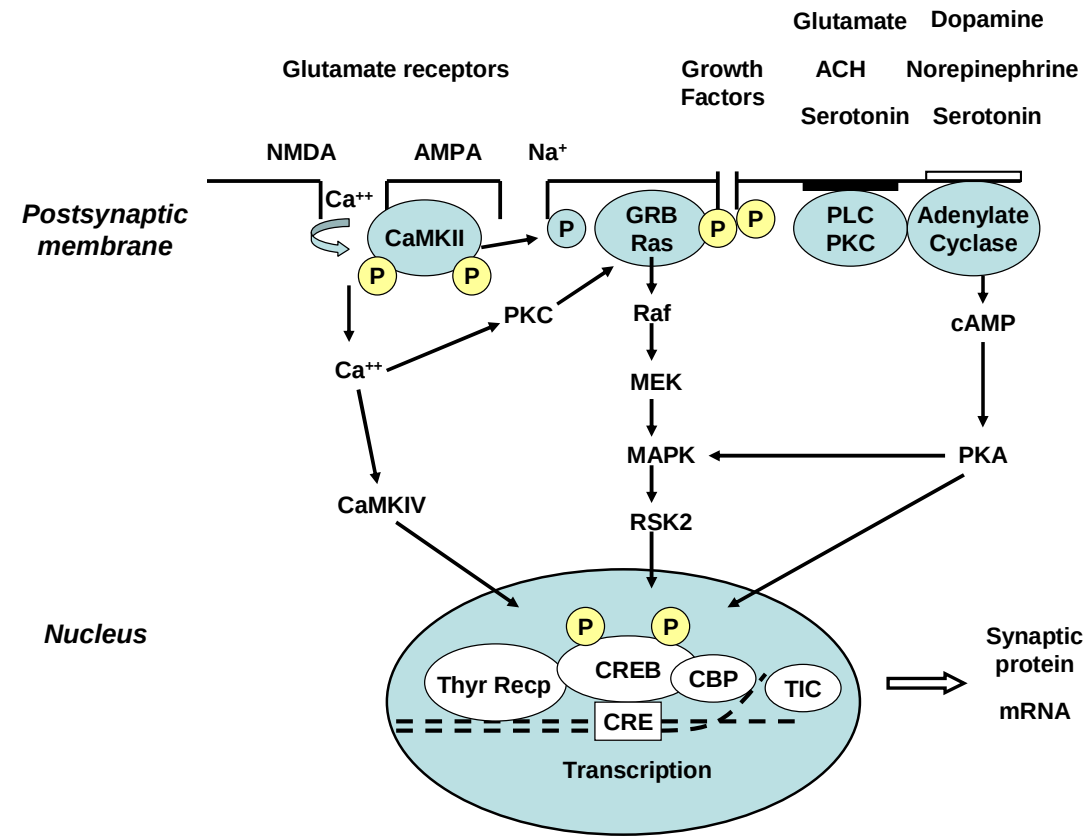

Figure 4: Signalling pathways for learning and memory.

Adapted and modified from Johnston et al., 2003.

The molecular basis of learning and memory is depicted in Figure 4.

Three types of neurotransmitter receptors are shown:

2. **ionotropic receptors** that open ion channels (**N**-**m**ethyl-**D**-**a**spartic acid (NMDA) and alpha-**a**mino-3-hydroxy-5-**m**ethyl-4-isoxazole**p**ropionic **a**cid (AMPA) receptors)
3. **metabotropic receptors** linked to synthesis of inositol phosphates and stimulation of protein kinase C (PKC)
4. **transmembrane receptors** linked to generation of cyclic adenosine monophosphate (cAMP)

Several steps of phosphorylation and dephosphorylation are initiated through different types of kinases and other enzymes, but in the end they all have the same goal:

CREB (**c**AMP **r**esponse **e**lement **b**inding protein transcription factor) is phosphorylated, it binds to CRE (**c**AMP **r**esponse **e**lement DNA sequence) and transcription of mRNA for new synaptic proteins is activated (for a review, see

Johnston et al., 2003). CBP is a **C**REB **b**inding **p**rotein, which has intrinsic histone acetyltransferase activity; the acetylation of histones neutralizes their positive charges, weakens the interaction with DNA, opens chromatin conformation and promotes therefore transcription (Lu et al., 2003).

A key word in memory formation is Long-term Potentiation. Long-term Potentiation (LTP) is an increase in the strength of a chemical synapse that lasts from some minutes to several days. It is widely considered one of the major mechanisms by which memory is formed and stored in the brain. The related region in the brain is the hippocampus which is essential for memory formation.

LTP has been observed both *in vitro* and *in vivo*. Under experimental conditions, the application of a series of short, high-frequency electric stimuli to a synapse can strengthen, or potentiate, the synapse for minutes to hours. In living cells, LTP occurs naturally and can last from hours to days, months, and years. Neurons connected by a synapse that has undergone LTP have a tendency to be active simultaneously: after a synapse has undergone LTP, subsequent stimuli applied to one cell are more likely to elicit action potentials in the cell to which it is connected.

LTP is believed to contribute to synaptic plasticity in the brain, providing a highly adaptable nervous system and it was discovered in the mammalian hippocampus by Terje Lomo in 1966 (Andersen et al., 1966).

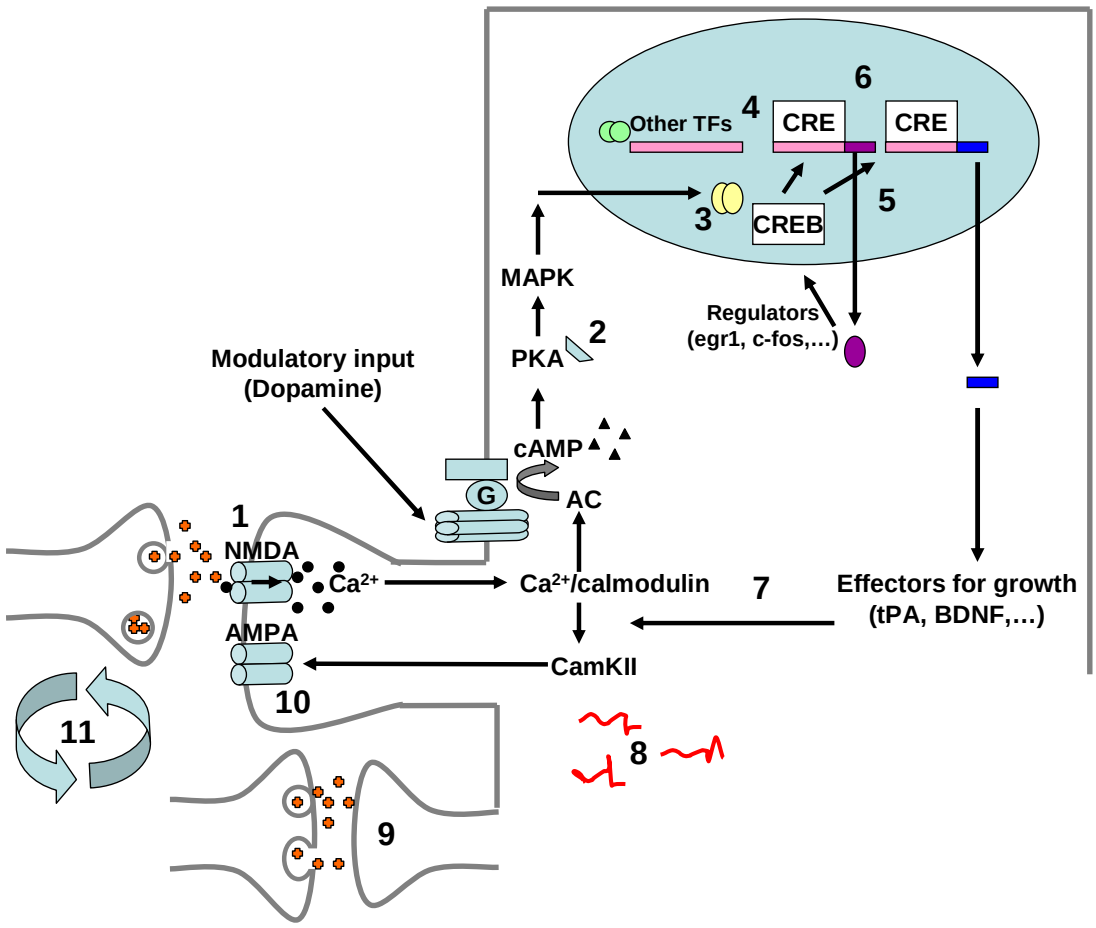

Figure 5: Memory formation in the mammalian hippocampus.
Adapted and modified from Barco et al., 2006

Figure 5 shows the pathway of memory formation in the mammalian hippocampus. Steps are numbered from 1 to 11. The main excitatory neurotransmitter in the brain is glutamate. Release of glutamate in synapses triggers calcium influx through NMDA receptors **(1)**. This leads to a local increase in cAMP through Ca^{2+}/calmodulin-mediated stimulation of adenylate cyclase (AC) and initiates the activation of cAMP-dependent protein kinase A (PKA) **(2)**. NMDA activation leads to importin translocation and retrograde transport from synapse to nucleus **(3)**. Different members of the CREB family of transcription factors can be activated **(4)** and activity-dependent induction of gene expression is initiated **(5)**. Critical chromatin changes occur during the formation of long-term memory **(6)** and newly synthesized gene products are captured at the synapse **(7)**. Local protein synthesis at active synapses leads to synaptic growth and formation of new synapses **(8, 9)**. Also silent synapses

are activated **(10)**. Self-perpetuating mechanisms are required to provide stability in memory storage **(11)** (Barco et al., 2006).

Areas involved in different learning and memory systems are difficult to characterize because memory systems are not exactly the same in humans and animals.
In general, the hippocampus is crucial for all kinds of memory, as I described above. In the next pages, I will explain the involvement of different brain areas in different kinds of memory.
Although the precise comparison to complex memory of humans is not yet established in detail, some tests and tasks were designed to classify memory in animals.

Social memory in mice can be assessed via the Social Discrimination Test (Engelmann et al., 1995; Richter et al, 2005). Social memory shares characteristics of other hippocampus-dependent memories, it was shown that ibotenic lesions of the hippocampus disrupt social recognition at 30 minutes after training (Kogan et al., 2000). This agrees with a study of Terranova et al. which also suggests the hippocampus to play a key role in social recognition (Terranova et al., 1994). Furthermore, also the amygdala differentially processes social and non-social stimuli and is therefore involved in mediating social information (Young, 2002).

To assess **working memory** in mice, several variants of mazes are used. It was shown in a Radial Maze Working Memory Task that performance of mice required the integrity of the medial prefrontal cortex (mPFC). Furthermore, it was demonstrated that working memory performance required synthesis of *de novo* proteins in the mPFC. Thus, the mPFC is involved in consolidation and storage of learning strategies used in working memory (Touzani et al., 2007). The spontaneous alternation behaviour, demonstrated by mice and rats in Y-or T-mazes, requires an intact working memory and is not only dependent on the prefrontal cortex, but also on the hippocampus, the dorsal striatum and the basal forebrain (Lalonde, 2002). Beside the maze tasks, there are also tasks which examine olfactory working memory in mice by assessing the ability of mice to remember increasing numbers of odours (Young et al., 2007).

For encoding and consolidation of **contextual memory**, the CA1 and CA3 subregions of the dorsal hippocampus play important roles (Daumas et al., 2005; Frankland et al., 2006). This is in line with previous results which displayed an impairment of hippocampus-lesioned mice in a Contextual Fear Conditioning Task (Goddyn et al., 2006). Contextual fear memory consolidation requires hippocampal ERK/CREB activation (Trifilieff et al., 2006).

Several studies have been performed to study **spatial memory** in rats and mice. Mice were tested for their ability to detect spatial displacement 24 hours after training and it could be revealed that NMDA receptor blockade within the dorsal striatum impaired spatial memory consolidation in this task (De Leonibus et al., 2003). The dopaminergic system plays an important role in information processing in the prefrontal cortex and it was shown that D1 and D2 receptor antagonist injections in this region impaired spatial learning in mice (Rinaldi et al., 2007). Furthermore, the dorsal striatum and the nucleus accumbens contribute to spatial memory (De Leonibus et al., 2005). The hippocampus is important for both spatial and object recognition memory, but it was shown that spatial memory performance requires more hippocampal tissue than does object recognition memory (Broadbent et al., 2004). Furthermore, the CA3 region of the hippocampus and its network with the dentate gyrus is essential for detecting spatial novelty, but not for detecting object novelty (Lee et al., 2005).

For **object memory**, it was shown that the perirhinal cortex plays a central role in recognizing objects in macaque monkeys. Various connections are known which give inputs or receive outputs from the perirhinal cortex (Murray et al., 2000; Murray et al., 2001). In primates and rodents, the hippocampus contributes to the memory and perception of places, whereas the perirhinal cortex contributes to object perception. Barker et al. showed that in rats the medial prefrontal cortex and the perirhinal cortex are essential for object-in-place associations and recency discriminations (temporal order memory), whereas the perirhinal cortex but not the medial prefrontal cortex is important for discriminations of familiar and unfamiliar objects (Barker et al., 2007). Lesions of the perirhinal cortex, the amygdala and the hippocampus led to impaired performance in an Object Preference Task in rats (Moses et al., 2005).

Hammond et al. suggested a delay-dependent involvement of the hippocampus in object recognition memory as lidocaine-application into the hippocampus of mice induced an impaired object recognition memory after a retention interval of 24 hours but not after a retention interval of 5 minutes. However, these data are also consistent with the view that the hippocampus is involved in object recognition memory regardless of retention intervals and that parahippocampal structures as for example the perirhinal cortex are sufficient to support object recognition memory over short (5 minutes) retention intervals (Hammond et al., 2004).

Episodic memory refers to the conscious recollection of autobiographical events and the question of whether any non-human species displays this kind of memory is controversial. However, there are several studies which suggest the existence of episodic memory in rodents. Wistar rats show episodic-like memory for unique experiences and recognized objects previously explored and remembered their order of presentation (Kart-Teke et al., 2006). Also mice displayed episodic-like memory in a “What, where and when”-object Exploration Task (Dere et al, 2005a, b). Here again the hippocampus plays a critical role for encoding events in episodic memory (Mingaud et al., 2007; Morris, 2001).

With these examples, it is obvious that the experimental design of behavioural tests is crucial. In object recognition, there are various forms of tests which can analyse object novelty, object location, object preference etc. and all these tests are related to different areas in the brains of rodents, monkeys and humans.

1.4. Aim of the work

For large-scale cognitive phenotyping of mice, learning and memory tests are required which are as time-saving and easily transferable to other laboratories as possible. The Object Recognition Task is a good option to this end, since the task can be performed in a short time span compared to e.g. operant conditioning procedures, which require training.

The Object Recognition Test has been applied in many laboratories using different arenas, objects and procedures. Contradictory results are most likely due to these discrepancies in test performance. Therefore, for comparability of results the application of a well-defined Object Recognition Procedure is recommended.

I established and validated a simple Object Recognition Task in C57BL/6 mice and applied this test in three mutant mouse models such as the GLUD2 transgenic mouse line, the MCHR1 knockout mouse line and the conditional cGKI knockout mouse line. I performed all basic tests (Object Preference Test, Counterbalance Test, Retroactive Interference Test) for the establishment of the Object Recognition Task with C57BL/6J mice, because this strain is mostly used for backcrossing genetically modified mice. With these basic tests, I could design an Object Recognition Procedure as described in ‘Material and Methods’ and assessed object recognition memory in both male and female C57BL/6J mice after retention intervals of 3 hours, 24 hours, 48 hours, 72 hours and 7 days.

For a more detailed cognitive analysis of the three mutant mouse models, I additionally performed the Y-maze Spontaneous Alternation Test for working memory assessment and the Social Discrimination Test for social memory assessment. Furthermore, I validated the Food-rewarded Hole Board Test in two inbred strains (C57BL/6J and Balb/c mice) as a cognitive test to assess working and reference memory in mice. The validation of the Food-rewarded Hole Board Test was part of the standardization program of behavioural tests within Eumorphia and the resultant Standard Operating Procedure (SOP) serves to provide comparability and reproducibility in behavioural phenotyping. Fur further information, see www.eumorphia.org.

The three mouse models available for analysis and the cognitive tests applied are described in the next pages.

1.4.1. GLUD2 mutant mice as an example of a transgenic mouse line

I analysed C57BL/6J mice transgenic for the *GLUD2* gene. This gene encodes for the glutamate dehydrogenase. The enzyme is important for recycling the chief excitatory neurotransmitter glutamate during neurotransmission and supports high neurotransmitter flux (Burki et al., 2004).

Mice have one functional, mitochondrial, housekeeping version of the glutamate dehydrogenase gene, encoded by the *GLUD1* gene on chromosome 10. About 23 million years ago, during human evolution, this gene was retrotransposed to chromosome X and evolved into brain-specific *GLUD2*. All the Apes (gibbons, orang-utans, gorillas, chimpanzees and humans) have therefore two functional glutamate dehydrogenase genes: *GLUD1*, which is mitochondria-located and a housekeeping version, and *GLUD2*, which is brain-specific and also expressed in testis (Wan et al., 1998). It was shown that *GLUD2* underwent positive selection since duplication and acquired aminoacid changes that adapted it to specific brain conditions (Burki et al., 2004). It is postulated that acquisition of this gene could be beneficial in terms of memory formation or generally enable coping with high levels of glutamate during extensive neuron firing, deduced from the biochemical characteristics and kinetics of the enzyme. Glutamate homeostasis may also have a role in neurodegenerative diseases. The gene could play a role in development of higher cognitive abilities of the hominoids. With respect to ape-specificity of *GLUD2*, this mouse line is one of the first animal models for human-specific traits.

1.4.2. MCHR1 mutant mice as an example of a knockout mouse line

MCHR1 (**m**elanin-**c**oncentrating-**h**ormone-**r**eceptor-1) is a G-protein-coupled receptor, which binds highly selectively the melanin concentrating hormone (MCH). The gene is expressed in the brain and at lower levels in various peripheral tissues such as spleen, thymus, testis and adipocytes. MCH is an orexigenic hypothalamic neuropeptide, which plays an important role in regulating energy balance, body weight and body temperature. MCHR1 antagonists have antidepressant and anxiolytic as well as anorectic effects (Borowsky et al., 2002).

MCHR1 knockout mice are known to be hyperactive during the dark phase, hyperphagic on regular chow, but the body weight is not altered. They have a lower fat mass and an increased lean mass. These mice are resistant to diet-induced obesity, i.e. on a high fat diet, they show lower body weight due to lower fat mass and lower plasma levels of leptin and insulin (Marsh et al., 2002).

I expected impaired memory (Adamantidis et al., 2005) and anxiolytic effects (Smith et al., 2006) in MCHR1 knockout mice. Adamantidis et al. (2005) showed that MCHR1 deficiency in mice lead to poor learning performance in a passive avoidance inhibitory task, whereas Smith et al. showed that MCHR1 knockout mice displayed an anxiolytic-like phenotype in behavioural models of anxiety. In rats, it was shown that MCH causes retrograde facilitation of learning and memory in a one-trial step-down inhibitory avoidance test (Monzon et al., 1999).

1.4.3. cGKI mutant mice as an example of a conditional knockout mouse line

The cGMP-dependent protein kinase type I (cGKI) is an attractive candidate mediator of cGMP signaling in the CNS (Feil et al., 2007). The analysis of mouse mutants that lack cGKI globally in all cells or specifically in the hippocampus or cerebellum showed that this protein kinase modulates various forms of neuronal plasticity, ranging from axonal pathfinding during embryogenesis to the adaptation of synaptic activity during learning and nociception. Recent immunohistochemical data indicate that the distribution and functional relevance of the cGMP-cGKI pathway in the mammalian brain is even broader than previously thought (Feil et al., 2005). Conventional cGKI null mutants are not appropriate to study the role of cGKI in the behaviour of adult animals, because they have multiple defects and a short life expectancy (Feil et al., 2007). I analysed cGKI-deficient mouse models that lack the protein in the nervous system (brain-specific cGKI mouse mutants) and in the hippocampus (hippocampus-specific cGKI mouse mutants) and can be studied throughout adulthood. The analysis of these mouse mutants indicates that cGKI is indeed involved in complex behavioural processes, such as object recognition, social discrimination and the regulation of sleep-wake activity. Hippocampal cGKI supports an age- and protein synthesis-dependent form of hippocampal long-term potentiation, whereas it is dispensable for hippocampus-dependent spatial reference and contextual memory (Kleppisch et al., 2003).

1.4.4. Y-maze Spontaneous Alternation Test

The Y-maze Spontaneous Alternation Procedure is based on the natural tendency of rodents to explore a novel environment. When placed in the Y-maze, normal mice prefer to explore the least recently visited arm, and thus tend to alternate visits between the 3 arms.

To explore the three arms successively the mouse must maintain an ongoing record of most recently visited arms and update such records. Therefore, a decrease in spontaneous alternation is used as an indication of impaired working memory (Holcomb et al., 1999; Wall et al., 2002). This task also involves some aspects of attention related to active working memory (Wall et al., 2002). Scoring other behavioural parameters such as alternate arm returns and same arm returns can assess the latter. Finally, the novelty of the maze also generates a state of anxiety, which can be assessed by scoring the time the mouse takes to exit the starting arm.

Performed in this procedure, the test attempts to investigate working memory, because the mouse has to remember **within** the task which arms it has just visited and should avoid re-entering this previously visited arm. In contrast, to test reference memory the task must be related to learning, mostly provided by rewarding a specific pattern of arms or also in an aversive way with punishment in one of the arms. The mouse has to remember what it has previously learned and then decide for the right choice.

1.4.5. Social Discrimination Test

As rats and mice are macrosmates, storage and recall of olfactory information is crucial for coping with their living and non-living environment (Richter et al., 2005). In rodents, two different neuronal pathways are known, which are different in their region of processing depending if olfactory stimuli are volatile or non-volatile.

Performance in the Social Discrimination Test is mostly based on olfactory information (Kogan et al., 2000). The two underlying neuronal systems are described in the following Figure 6.

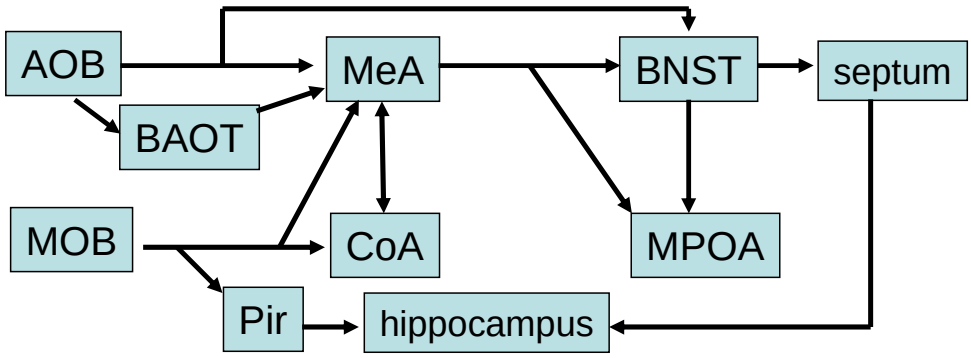

Figure 6: Main afferent areas processing olfactory information in the rodent brain. Nonvolatile stimuli are processed predominantly via the accessory olfactory bulb (AOB), volatile stimuli via the main olfactory bulb (MOB).

BAOT, bed nucleus of the lateral olfactory tract; Pir, piriform cortex; MeA, medial nucleus of amygdala; CoA, cortical nucleus of the amygdala; BNST, bed nucleus of the stria terminalis; MPOA, medial preoptic area.

Adapted and modified from Richter et al., 2005.

Mice tend to explore unfamiliar subjects for a longer duration than familiar ones. The significantly longer investigation duration of the unfamiliar stimulus animal compared to the familiar one (i.e. the conspecific previously presented during the sample phase) is taken as an evidence for an intact social recognition memory.

1.4.6. Object Recognition Test

The Object Recognition Task represents a test for natural behaviour in mice without the need of positive or negative re-inforcers such as food rewards after food restriction or electric shocks. The task belongs to common novelty-preference paradigms. This is an advantage because modifications in metabolism during food restriction can influence performance in memory tests and also electric shocks can change the natural behaviour of mice because of stress. If food restriction is needed, it should be designed in such a way that the animal's discomfort is as minimal and motivation for the task is as maximal as possible. Furthermore, it was shown that food-restricted mice show a reduction in exploration of a new environment and particular aspects of their timing memories are impaired (Tucci et al., 2006). Orsini et al. (2004) showed that food restricted C57BL/6J mice were good performers in a Spatial Novelty Test, but failed to perform a Spontaneous Object Recognition Test. As no food restriction is necessary in the established Object Recognition Procedure, I could avoid these disadvantages.

The Object Recognition Task is based on the natural tendency of rodents to spend more time exploring an unfamiliar object than a familiar one. It was first described by Ennaceur et al. in 1988 as a test for neurobiological studies in rats. In a first trial, rats were exposed to two identical objects and in a second trial they were exposed to the now familiar one and a novel object after a retention interval of 1 minute or 1 hour. Memory for objects is defined by the ability of a mouse to distinguish an unfamiliar object from familiar objects after learning, i.e. the difference in exploration time between a previously encountered object and a novel object is taken as an index of memory performance. The Object Recognition Task requires no training, does hardly imply any stress for the mice, rule learning is not necessary and it is therefore more closely related to conditions under which human recognition memory is measured (Ennaceur and Delacour, 1988). Variations of novelty-preference paradigms include tests like for example one-trial object-place recognition (Mumby et al., 2002a) or tests for episodic-like memory in rats and mice (Dere et al., 2005a). The Object Recognition Task is especially qualified to test the effects of pharmacological or genetic interventions of learning and memory in mice.

The chosen procedure with three sample phases and inter-trial intervals (ITI) of 15 minutes between the sample phases was chosen as suggested by Genoux and coworkers (2002). They showed that in general repetition in learning is essential for

the formation of long-lasting memory and that practice is most effective when distributed over time and not as efficient when closely spaced or massed. Long intervals between sessions allow better encoding than do short intervals because processing of information of one trial could be attenuated because information of the previous trial is still being processed. So a delay between sessions is essential for complete processing of information.

1.4.7. Food-rewarded Hole Board Test

The Food-rewarded Hole Board Test is a spatial learning and memory test for mice.

It is a food-rewarded searching task as well as an explorative-behavior test. Exploratory drive and neophobia can be measured.

Performance of the task is measured by recording the latency in seconds (searching time) until the mouse located all food pellets from 4 out of 16 holes, using a specific sequence for each mouse. The assessment of working memory and reference memory is possible. Reference memory errors are defined by visits to non-baited holes whereas working memory errors are defined by re-visits to baited and non-baited holes **within** one trial.

The Food-rewarded Hole Board Test is different from the Object Recognition Test, Social Discrimination Test and Y-maze Spontaneous Alternation Test because food restriction is used here to motivate the mice.

Visual cues can play an important role in learning tasks such that external visual markers can serve to orientate in space. Therefore, visual cues are used in the Food-rewarded Hole Board Test. Extra-maze cues were also shown to play an important role in the Barnes maze which requires mice to learn the position of a hole that can be used to escape the brightly lit, open surface of a maze (Harrison et al., 2006). Furthermore, it was shown in a Morris water maze task that C57BL/6J mice were able to learn both a spatial and a non-spatial version of the maze, but the non-spatial version without extra-maze cues turned out to be more difficult for the mice (Stavnezer et al., 2002).

The Food-rewarded Hole Board Test is well adapted to mice and can be used to characterize spatial memory in various genetic or pharmacological mouse models (Kuc et al., 2006).

2. Material and Methods

2.1. Mouse lines

2.1.1. Inbred strains

The animals used as wildtype controls for the establishment of the Object Recognition Test were naïve male and female C57BL/6J mice at the age of 12-14 weeks, either bred in the Charles River Laboratories or in the mouse facility of the GSF. Prior to all experiments, mice were kept for at least one week under animal holding conditions as usual in our animal facility for acclimatisation. All mice were kept under standard laboratory conditions (12:12 light-dark cycle, water and standard mouse chow (Altromin™1314, Altromin Gesellschaft für Tierernährung, Lage) *ad libitum).*

All mice were housed in groups of 3-5 in standard mouse cages and were treated identically in terms of care and behavioural testing. To minimize circadian rhythm influence, all behavioural experiments were performed between 9:00 and 15:00 am. After testing was finished, mice were sacrificed according to ethical guidelines if they were not needed for further analysis.

For the Food-rewarded Hole Board Test, 12 male C57BL/6J and 12 male Balb/c mice at the age of 12 weeks from Charles River Laboratories, France, were analysed and kept under the same conditions.

2.1.2. Mutant mouse lines

2.1.2.1. GLUD2 transgenic mice

The transgenic mice were constructed using ~180kb linearized bacterial artificial chromosome (BAC) injection into the C57BL/6J oocyte pronucleus in the Transgenic Core Facility at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden. The BAC was not modified in any way and it contains no other gene or known regulatory sequences but *GLUD2*; the promoter or other regulatory elements for *GLUD2* are not known. The transgene-positive animals were detected by PCR with BAC-specific primers in Dresden. Inbred C57BL/6 mice obtained from Harlan Lab were used for all experiments. All crosses were between hemizygous GLUD2 and wildtype mice. Non-transgenic littermates were used as controls. Mice were sent to the GSF at the age of 10-11 weeks.

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype.

In this experiment, 29 female GLUD2 mice (14 wt, 15 mutants) and 28 male GLUD2 mice (14 wt, 14 mutants) were analysed. The mice were tested for spontaneous alternation in the Y-maze Test at the age of 12 -13 weeks, for social discrimination at the age of 13-14 weeks and for object recognition memory at the age of 14-15 weeks.

2.1.2.2. MCHR1 knockout mice

The MCHR1 gene contains two exons. The first one is approximately 250 bp long and encodes for the extracellular N-terminal part of the receptor. The second exon encodes for the seven transmembrane segments and the intracellular C-terminal part of the receptor.

A *Bsp*HI-*Sac*II fragment of 690 bp of exon 2 encoding a region comprised between transmembrane segment 2 and the C-terminus has been replaced by a floxed NEO cassette. This allows the removal of five transmembrane segments and Asp^{123} which was demonstrated to be essential for MCH binding (MacDonald et al., 2000) and gives the possibility to delete later the NEO cassette, which was demonstrated to affect endogenous gene expression (Chen et al., 1999; Rucker et al., 2000). A negative selection cassette (TK) was added outside of the homologous region. R1 ES (129Sv genetic background) cells were electroporated with the targeting vector. Two positive clones, whose karyotypes were controlled, were selected and injected into blastocysts. From these two clones, a total of nine chimeras were obtained and they were crossed with C57BL/6 females. F1 animals from the same ES clone were then crossed together to give F2 and then F3 mice. The genetic background of MCHR1 knockout mice is hence a mixture of 129Sv and C57BL/6 mice. Breeding pairs were sent to the GSF from the Research Centre for Cellular and Molecular Neurobiology at the University of Liège, Belgium, and mice were bred in the mouse facility of the GSF in appropriate numbers for testing.

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype.

For the Y-maze Spontaneous Alternation Test, the Social Discrimination Test and the Object Recognition Test with retention intervals of 3 hours, 24 hours, 48 hours and 7 days, several batches were bred to use 24 female MCHR1 mice (12 wt, 12 mutants) and 24 male MCHR1 mice (12 wt, 12 mutants) for each test. Test order was Y-maze Spontaneous Alternation Test at the age of 12-14 weeks, Social Discrimination Test at the age of 13-15 weeks and Object Recognition Test at the age of 14-16 weeks.

2.1.2.3. Conditional cGKI knockout mice

In order to understand the role of a given gene product in a given cell type at a given developmental stage, genetic techniques are being developed that allow the introduction of defined mutations into the mouse genome at will, in a specific cell type and at a chosen time. Most current conditional gene targeting systems are based on the use of the site-specific recombinase Cre which catalyzes recombination between two 34 bp *loxP* recognition sites. The basic strategy for Cre/*loxP*-directed gene knockout experiments is to flank ("flox") an essential exon of the gene of interest with two *loxP* sites (by homologous recombination in ES cells), and then to "deliver" Cre to excise the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. Delivery of Cre can be achieved by crossing mice carrying the "floxed" target gene with transgenic Cre-expressing mice. Clearly, key to successful conditional gene targeting is the availability of Cre transgenic mouse strains in which Cre activity is tightly controlled in space and time.

The generation of mice carrying a conditional *loxP*-flanked ("floxed") cGKI allele (L2) or a recombined cGKI null allele (L -) and the detection of the cGKI wildtype (+) , L2 and L – alleles by PCR have been described (Wegener et al., 2002). A conditional cGKI allele (L2) was obtained by flanking exon 10 with *loxP* sites. Excision of exon 10 from the L2 allele by Cre-mediated recombination of the *loxP* sites produced an L-allele. $cGKI^{L-/L-}$ mice did not express cGKI protein in the whole nervous system.

To achieve the Cre-mediated conversion of the floxed L2 allele into the excised L– allele in the hippocampus, NEX-Cre transgenic mice were used (Schwab et al., 2000). In NEX-Cre mice, the Cre recombinase encoding sequence has been knocked into the endogenous NEX locus, which is well expressed in hippocampal neurons. Mice with modified cGKI alleles were crossed with NEX-Cre mice to generate hippocampus-specific cGKI knockout mice (genotype cGKI $^{L-/L2}$; NEX $^{+/Cre}$) and control mice (genotype cGKI $^{+/L2}$; NEX $^{+/Cre}$ or genotype cGKI $^{L-/L2}$; NEX $^{+/+}$). Mutant and control mice on a mixed 129Sv/C57BL/6 genetic background were used in all experiments. Mice were sent to the GSF from the Department of Pharmacology and Toxicology at the Technical University of Munich.

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype.

In this experiment, for brain-specific mouse mutants, 20 female NesCre x cGKI mice (7 wt, 13 mutants) and 22 male NesCre x cGKI mice (13 wt, 9 mutants) were available for analysis and in parallel 26 hippocampus-specific NEX-Cre x cGKI mice, 13 female mice (7 wt, 6 mutants) and 13 male mice (6 wt and 7 mutants) were analysed. They were tested at different ages, depending to which batch they belonged. Hippocampus-specific mutant mice were tested for object recognition memory at the age of 15-16 weeks and for social discrimination at the age of 41-42 weeks. Brain-specific mutant mice were tested for object recognition memory at the age of 16-36 weeks and for social discrimination at the age of 40-60 weeks. In both mouse lines, the Object Recognition Test was performed before the Social Discrimination Test due to demands of the mouse provider. Also the age of the mice at the time-point of testing and the age differences of the brain-specific mutants are due to the mouse provider.

2.2. Cognitive tests for analysing mice

2.2.1. Y-maze Spontaneous Alternation Test

The Y-maze is made out of grey plastic with 3 identical arms, 40 cm in length, 9 cm in width and 16 cm in height, placed at 120° from each other. Illumination in the centre of the Y-maze was 100 lux.

The mouse was put into one of the arms, facing the end wall. Starting arms were alternated within one test group of mice. After putting the mouse into the starting arm, a stopwatch was started immediately and the mouse was allowed to freely explore the maze for 5 minutes. At the end of the experimental session the mouse was carefully removed and returned to its home cage. The maze had to be cleaned thoroughly after each mouse to avoid olfactory cues.

Alternations are operationally determined as successive entries into each of the three arms as an overlapping triplet set (ABC, BCA, CAB etc.). An arm exit is defined as all 4 paws have left the length of the arm and entered the triangular space between all 3 arms. Spontaneous alternation performance percentage is defined as the ratio of actual total alternations to possible alternations ((entries-2) x100).

Number and percentage of alternate arm returns (AARs) and same arm returns (SARs) are scored for each animal in order to assess aspects of attention within spontaneous working memory.

Total entries and latency to exit the starting arm are also scored as an index of ambulatory activity and emotionality in the Y-maze.

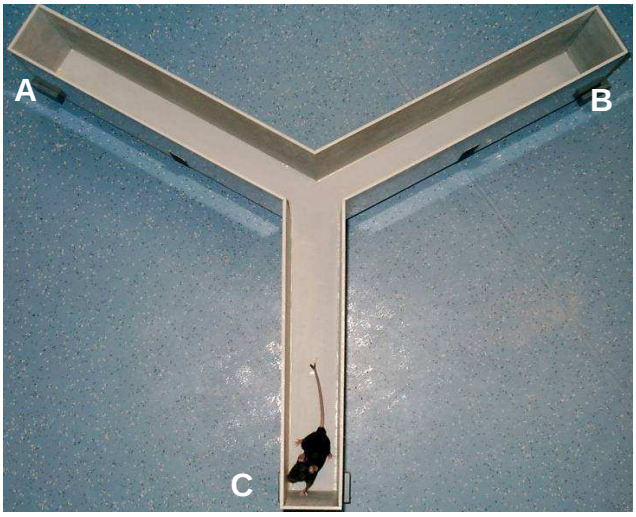

Figure 7: Y-maze Spontaneous Alternation Test

Mouse is allowed to freely explore the three arms for 5 minutes.

2.2.2. Social Discrimination Test

Social memory was assessed using the Social Discrimination Procedure previously described by Engelmann and coworkers (Engelmann, 1995; Richter et al., 2005). As stimulus animals ovariectomized 129SvJ female mice were used.

Test mice were transferred to the testing room for acclimatisation half an hour prior to testing or habituation. Afterwards, test animals were separated by transferring them to fresh cages 2 hours before starting the session. The Social Discrimination Procedure consisted of two 4 minutes exposures of stimulus animals to the test animal in the test animal's cage. During the first exposure (sample session) one stimulus animal was exposed to the test animal, and after a retention interval of 2 hours, this stimulus animal was re-exposed to the test animal together with an additional, previously not presented stimulus animal during the second (test session) exposure. During each exposure the duration of investigatory behaviour of the test animal towards the stimulus animal(s) was recorded by a trained observer blind to the genotype with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen).

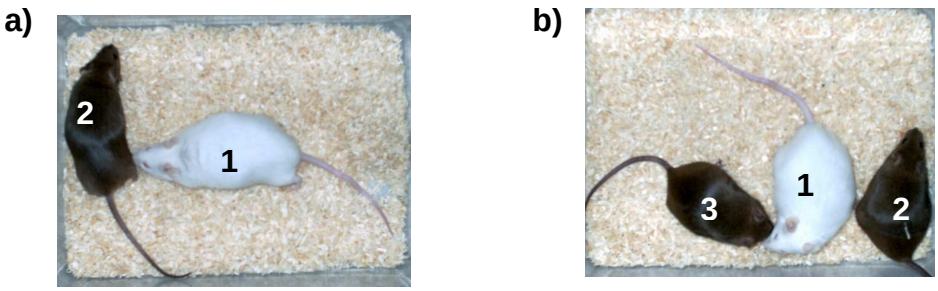

Figure 8: Social Discrimination Test

a) Sample session, first exposure of stimulus mouse (2) to test mouse (1)

b) Test session, second exposure of same stimulus mouse (2) and an unfamiliar stimulus mouse (3) to test mouse (1).

2.2.3. Object Recognition Test

Test mice were transferred to the testing room for acclimatisation half an hour prior to testing or habituation.

Mice were given two days of habituation before testing in which they were placed in the empty arena and allowed to freely explore it for 10 minutes.

On the testing day, two identical objects were placed into the arena and the test mouse was allowed to explore the two objects for 5 minutes. Exploration of an object was defined as touching the object with the nose. The mouse was then kept individually in an empty cage with litter until the next sample phase was introduced. The time spent between the sample phases (inter-trial interval) was 15 minutes and in total the two identical objects were presented three times for 5 minutes to each mouse. After retention intervals of 3 hours and 24 hours or longer intervals such as 48 hours, 72 hours and 7 days, in which mice were also kept individually in a cage, one of the previous encountered familiar objects was substituted by a new, unfamiliar one. The mouse was put back into the arena for another 5 minutes and exploration time was recorded by a trained observer blind to the genotype with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen).

Day 1	**habituation 10'**	**singly housed during inter-trial and retention intervals**					
Day 2	**habituation 10'**						
Day 3	**sample 1 5'**	**ITI 15'**	**sample 2 5'**	**ITI 15'**	**sample 3 5'**	**retention 3h**	**test 5'**
Day of testing	**retention 24h, 48h, 72h, 7 days**					**test 5'**	

Table 1: Time flow of the Object Recognition Procedure

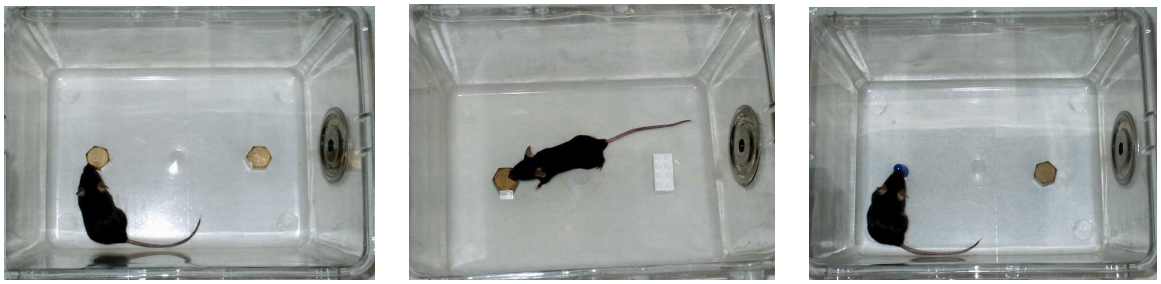

Figure 9: Test mouse in three different exposures

a) Two identical objects (brass hexagons) in sample phases

b) Substitution of one of the familiar objects for an unfamiliar one (white rectangle) after the first retention interval (3 hours)

c) Substitution of one of the familiar objects for an unfamiliar one (blue toy) after the second retention interval (24 hours, 48 hours, 72 hours, 7 days)

Figure 10: The three objects (brass hexagon, white rectangle, blue toy) in comparison

Retention intervals could be expanded to other durations, depending on the experimental question (e.g. 7 days for long-term memory), but all animals performed three sample sessions and the test after a retention interval of 3 hours. With this procedure, all data can be compared, because all mice underwent the same time flow and only the second retention interval varied in its duration.

Before starting to establish the Object Recognition Procedure, three basic tests were performed, called in this study

1. **Object Preference Test**
2. **Counterbalance Test**
3. **Retroactive Interference Test**.

The Object Recognition Task and all three basic tests were performed in a standard type II mouse cage (IVC, VentiRacks^{TM}, BioZone, Margate, UK, 20cm x 14cm x 26cm) out of transparent plastic. The objects to be discriminated were made of either metal or plastic and consisted of a brass hexagon (2cm x 2cm), a white rectangle (4cm x 2cm x 1cm) and a blue toy (2,5cm x 0,5cm). The objects were fixed to the bottom of the apparatus by double-sided adhesive tape to ensure that they could not be moved by the mice. Objects that can be manipulated would attract more attention. As far as could be ascertained, the objects had no natural significance for the mice and they had never been associated with a re-inforcer. For each experiment, the role (familiar versus unfamiliar object) and the position of the two objects were counterbalanced and randomly assigned to each mouse as far as it was necessary for the test.

The Object Recognition Test was performed in a quiet room, illuminated with 110 lux.

Object Preference Test

Each pair of objects was tested for spontaneous preference for one of the chosen objects, i.e. do mice naturally prefer one of the objects and therefore spend more time exploring it. Male and female C57BL/6J mice were habituated on two days prior to testing in the empty arena and allowed to freely explore it for ten minutes. On the testing day, each pair of objects (rectangle vs. toy, toy vs. hexagon, hexagon vs. rectangle) was presented once for ten minutes and exploration time was measured by a trained observer with a hand-held computer. In total, 36 male and 36 female C57BL/6J were tested, because for each of the three object configurations 12 naïve male and female mice were analysed. With this test I wanted to assess natural object preference.

Counterbalance Test

The Counterbalance Test was performed to exclude a confounding influence of any natural preference for the chosen objects in their specific configurations and to

ensure that performance in the object recognition test is due to novelty preference and not based on natural object preference. For this purpose, any object configuration was presented in a way that every object was unfamiliar in at least one case, e.g. familiar object was the rectangle; unfamiliar objects were first brass hexagon and second the blue toy. With this test, I wanted to find out if the test will work no matter which objects were assigned familiar or unfamiliar.

Retroactive Interference Test

Retroactive interference often occurs in learning and memory tests when new learning is disrupting previously learned information. As holding space is limited in mouse facilities, it is important to know whether group-housing of mice during inter-trial intervals and retention intervals is impairing object recognition memory in C57BL/6J. For practical reasons, group-housing the animals during the whole test procedure would be more efficient, first of all because of space and second, because males can not be put together after longer periods of separation (territorial fights). To study this, mice were put back into the home cage after sample sessions and test sessions, i.e. in inter-trial and retention intervals. Mice were immediately socially investigated by their littermates and often littermates started to attack the animal which was put back after testing. The goal of this test was to check if these social investigations were severe enough to affect previously learned object familiarity and to impair therefore object recognition memory.

In all basic tests and in the established Object Recognition Test, the following parameters were measured: total number of object exploration, latency to first object exploration, total duration of object exploration and also percentage of total duration of object exploration.

Results were analysed by Student's t-test, paired two samples for mean ($p < 0.05$).

2.2.4. Food-rewarded Hole Board Test

The Hole Board Arena is an enclosed square arena (custom-built by EMBL, Heidelberg, Germany) of dimensions of 70 cm length, 70 cm width and 45 cm height. The walls of the arena are made of black PVC with a removable grey PVC floor-plate. The floor-plate contains four rows of four equidistant holes (2.5 cm in diameter and 2 cm in depth). A transparent plastic starting chamber with sliding doors to put the mouse in and to open the gate to the arena is situated outside the box.

Visual cues in form of coloured posters were placed on the wall. Also the experimenter served as a visual cue and therefore it was necessary that the experimenter maintained a constant position during the test.

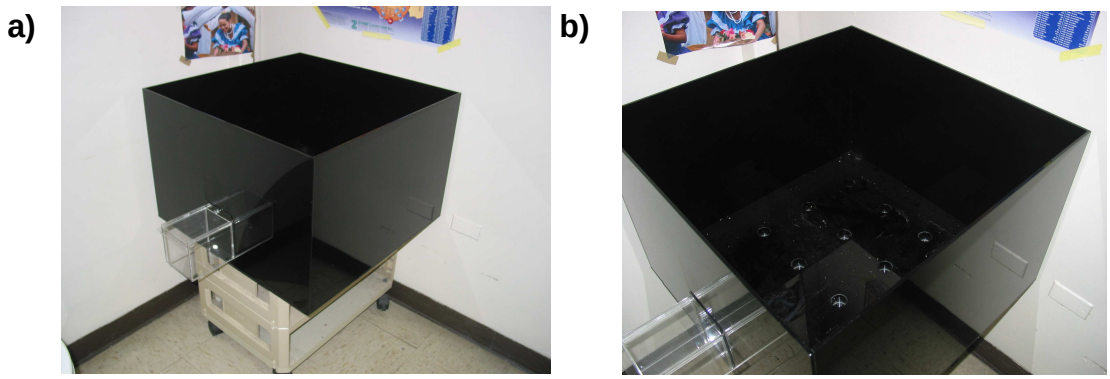

Figure 11: Food-rewarded Hole Board Test
a) Arena with transparent starting chamber outside.
b) View inside the arena with 16 holes inside.

During the test mice were kept three per cage to reduce time between trials and competition between mice.

Each mouse was assigned a specific test sequence and this sequence remained unchanged throughout acquisition. A trained observer scored the latency in seconds to locate sweet food pellets (Fruit Crunchies™, Bio-Serv, Frenchtown, NJ) from 4 out of 16 holes. In this test, mice had to be food-restricted for motivation to perform the task. Food restriction started two days prior to habituation. Each mouse performed two sessions, consisting of one trial, during each of the two habituation days. So on day 3 and 4, habituation started in which mice were allowed to explore the fully baited holes for 5 minutes, for the first time in groups per each cage and then individually. So in session 1 of habituation 1 on day 3, all mice of one cage were placed into the centre of the arena and allowed to explore the fully baited maze. In session 2 of habituation 1 on day 3, mice were placed individually into the centre of

the arena and allowed to explore the fully baited maze. The procedure of this second session was repeated on day 4 in session 1 and in session 2, but exploration time was now limited to 3 minutes.

On day 5, 6 and 7, acquisition sessions started. 2 sessions per day were performed, with each session consisting of 5 trials. In each trial, one mouse was placed in the centre of the arena and once all 4 paws have entered the arena and the entrance was closed, the mouse was allowed to locate the 4 food pellets in their specific pattern in the maze. The mouse was removed if all of the 4 pellets have been located or if an exploration time of 3 minutes elapsed. In these 3 days of acquisition mice learned “their” specific distribution of 4 sweet pellets in the arena.

On day 8 and 9, food restriction was maintained and on day 10, a retention test was performed with 5 trials (3 minutes each) in summary, in which the specific pattern in the baited maze was presented to the mouse after a retention interval of 48 hours.

Body weight of each mouse had to be monitored carefully throughout the whole experiment to ensure that the body weight did not fall below 85% of the preliminary weight measured.

Habituation, acquisition and retention sessions were performed during the light phase of the day between 9:00am and 16:00 pm.

After each trial of a mouse, the holes were emptied if necessary and the arena and holes were cleaned with a disinfectant (Pursept™, Merz Pharma GmbH, Frankfurt).

Errors in reference memory were defined as visits to non-baited holes. Reference Memory Ratio (RMR) was calculated as total visits to baited (TVB) holes divided by total visits of baited and non-baited holes (TV). With this design of the test, 12 mice per week could be tested.

Data were statistically analysed using repeated measures, accepted level of significance of $p < 0.05$ (SPSS software, SPSS Inc, Chicago, USA).

a) **b)**

Figure 12: Test mouse in the arena of the Food-rewarded Hole Board

a) Mouse is searching for food rewards

b) Mouse is nose-poking in one of the holes

Day	Food	Experiment
Day 1	Food restriction	none
Day 2	Food restriction	none
Day 3	Food restriction	Habituation 1
Day 4	Food restriction	Habituation 2
Day 5	Food restriction	Acquisition 1
Day 6	Food restriction	Acquisition 2
Day 7	Food restriction	Acquisition 3
Day 8	Food restriction	none
Day 9	Food restriction	none
Day 10	Food restriction	Retention

Table 2: Scheme of procedure performed in the Food-rewarded Hole Board Test.

Mice were food-restricted for 10 days, starting 2 days prior to habituation.

Body weight had to be measured during the whole test procedure.

3. Results

3.1. Object Recognition Task in C57BL/6J mice

3.1.1. Object Preference Test

In the Object Preference Test, male C57BL/6J mice equally distributed exploration times on all object configurations and did not prefer one object over any other (Figure 13). Female C57BL/6J mice showed a natural preference for one of the chosen objects, i.e. exploration time on one of the objects (rectangle) was significantly enhanced compared to the others in one specific configuration (rectangle versus toy) (Figure 14).

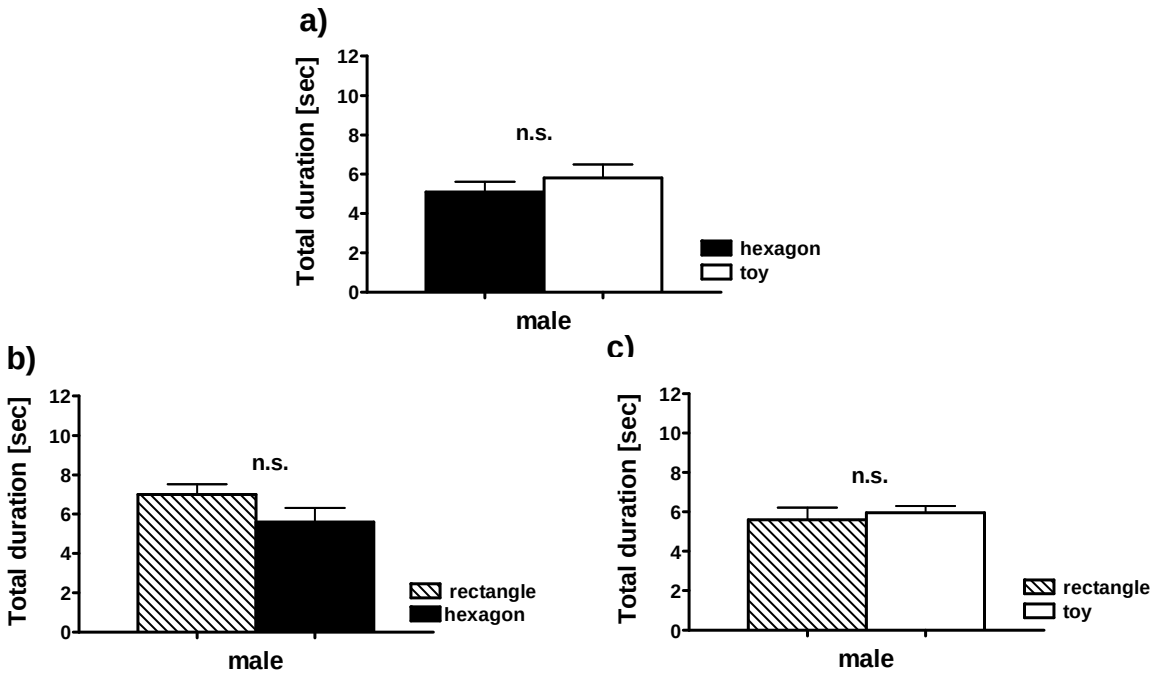

Figure 13: Object Preference Test in male C57BL/6J mice

Total duration of investigation time in seconds in the 3 object configurations:

a) hexagon vs. toy

b) rectangle vs. hexagon

c) rectangle vs. toy; of 12 male C57BL/6J mice each (n = 36 in total)

n.s. non-significant

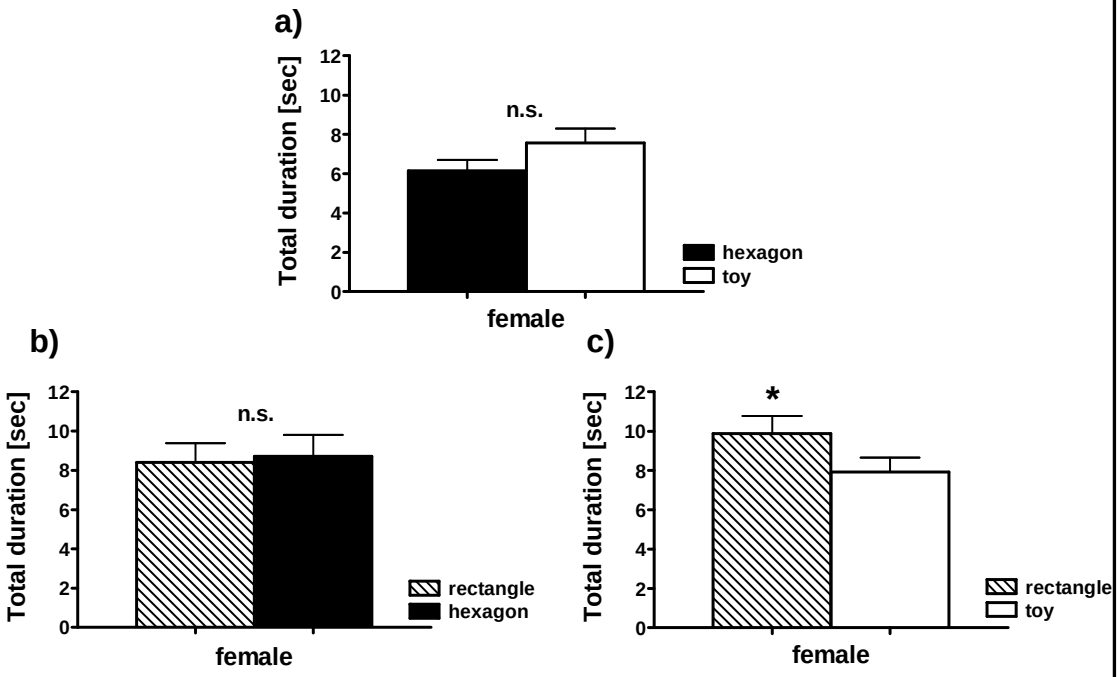

Figure 14: Object Preference Test in female C57BL/6J mice

Total duration of investigation time in seconds in the 3 object configurations:

a) hexagon vs. toy

b) rectangle vs. hexagon

c) rectangle vs. toy; of 12 female C57BL/6J mice each (n = 36 in total)

n.s. non-significant, * $p < 0.05$

3.1.2. Counterbalance Test

To assess if such a natural preference could confound the test, it was crucial to know if for female C57BL/6J mice the natural preference would be more important than unfamiliarity. For this purpose, the preferred object (rectangle) was presented as the familiar object and another non-preferred object was presented as unfamiliar during the Object Recognition Procedure.

Figure 15 shows the results of this test design in which both male and female C57BL/6J mice could discriminate between familiar object (rectangle) and unfamiliar objects (hexagon and toy) after retention intervals of 3 hours and 24 hours. Within the sample sessions, the test did not reveal any extraordinary results. In male C57BL/6J mice, exploration time became less from sample session 1 to sample session 3. Female C57BL/6J mice also displayed habituation and exploration time became less from sample session 1 to sample session 3 and also from sample session 2 to sample session 3.

As female C57BL/6J mice explored significantly longer the unfamiliar object over the preferred one, I concluded that natural preferences can be neglected in this test design.

Also male C57BL/6J mice could discriminate between familiar and unfamiliar object and spent more time exploring the unfamiliar one after retention intervals of 3 hours and 24 hours when the rectangle was chosen as familiar object.

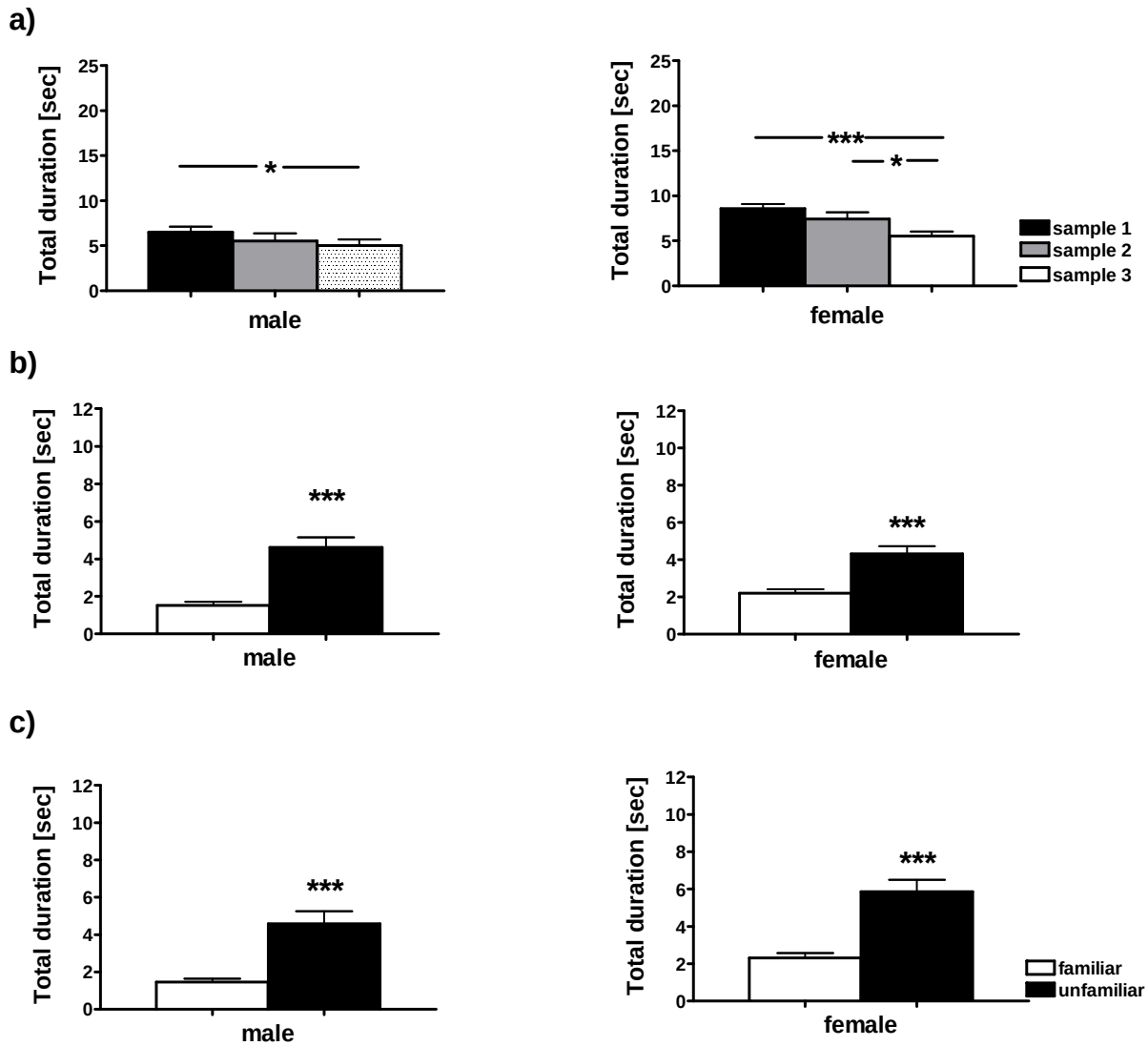

Figure 15: Counterbalance Test in male and female C57BL/6J mice

Total duration of investigation time in seconds:

Rectangle was the familiar object; unfamiliar objects were hexagon after 3 hours and toy after 24 hours.

a) Sample sessions, in which two identical objects were presented

b) Object Recognition Test after a retention interval of 3 hours

c) Object Recognition Test after a retention interval of 24 hours

* p< 0.05, *** p< 0.001

3.1.3. Retroactive Interference Test

Retroactive Interference is a well-known phenomenon in learning and memory tests in humans and also in rodents.

As Figure 16 shows, both male and female C57BL/6J mice were not able to perform the Object Recognition Task any more when they were group-housed during inter-trial and retention intervals. After a retention interval of 3 hours and of 24 hours, both sexes did not discriminate between familiar and unfamiliar objects. Both sexes habituated to the objects. In both sexes, exploration times became less from sample session 1 to sample session 3 and from sample session 1 to sample session 2. Male C57BL/6J mice spent more time in object exploration than female C57BL/6J mice ($p < 0.001$).

I could show that singly housing in inter-trial and retention intervals is crucial for memory formation in this test procedure. The social interaction during these intervals was indeed disturbing test mice in such a way that they could not perform the test anymore.

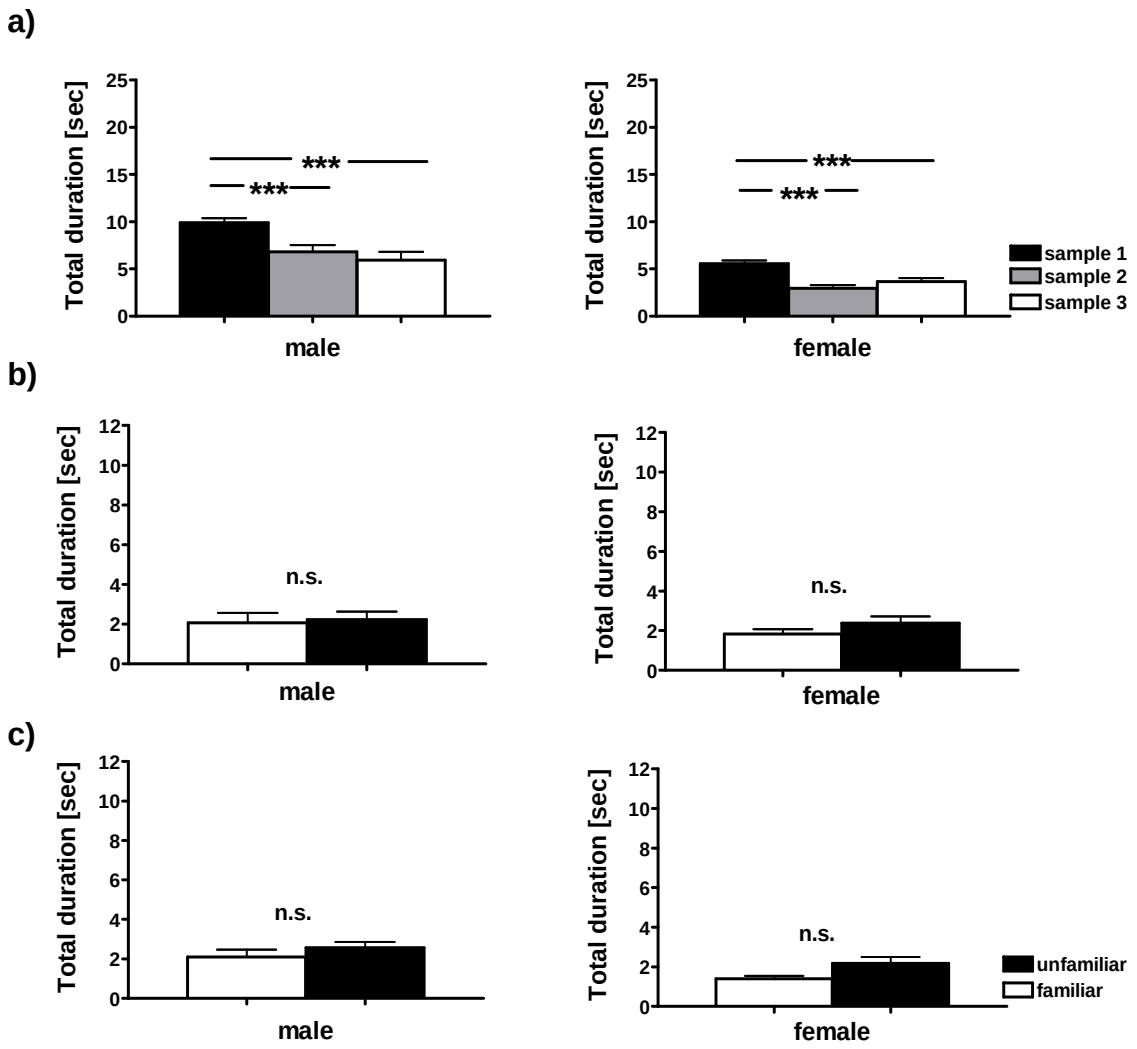

Figure 16: Retroactive Interference Test in male and female C57BL/6J mice

Total duration of investigation time in seconds:

Mice were group-housed during inter-trial and retention intervals. The hexagon was presented as familiar object, rectangle and toy as unfamiliar objects after 3 hours and 24 hours.

a) Sample sessions, in which two identical objects were presented

b) Object Recognition Test after a retention interval of 3 hours

c) Object Recognition Test after a retention interval of 24 hours

n.s. non-significant, *** p< 0.001

3.1.4. Object Recognition Test

Figure 17 shows exploration times of male and female C57BL/6J mice in three sample sessions in which two identical objects were presented. These sample sessions were chosen exemplarily for all the sample sessions performed. Exploration time became less from sample session to sample session and in general, female C57BL/6J mice explored more than males ($p < 0.05$). The tendency towards a decrease in exploration time from sample session to sample session in both sexes and the increased exploration time in female C57BL/6J mice compared to males could be observed repeatedly in all tests performed.

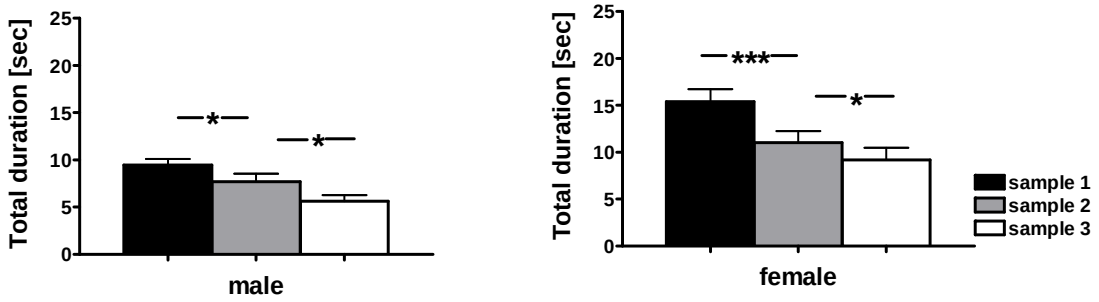

Figure 17: Object Recognition Test in male and female C57BL/6J mice

Total duration of investigation time in seconds in the sample sessions in which two identical objects were presented.

* $p < 0.05$, *** $p < 0.001$

To investigate the time spans of object recognition memory, I performed the same test procedure with retention intervals of 3 hours, 24 hours, 48 hours, 72 hours and 7 days.

Figure 18 shows the exploration times of male and female C57BL/6J mice after a retention interval of 3 hours and 24 hours (a), 3 hours and 48 hours (b), 3 hours and 72 hours (c) and 3 hours and 7 days (d). At each time-point, both sexes discriminated between familiar and unfamiliar object, by spending significantly more time exploring the unfamiliar object than the familiar one.

Figure 18 compares the results regarding sex, performance after the same retention intervals of male and female C57BL/6J mice are depicted in one graph each.

In female C57BL/6J mice, the exploration time after a retention interval of 48 hours was increased regarding the exploration times in all the other tests.

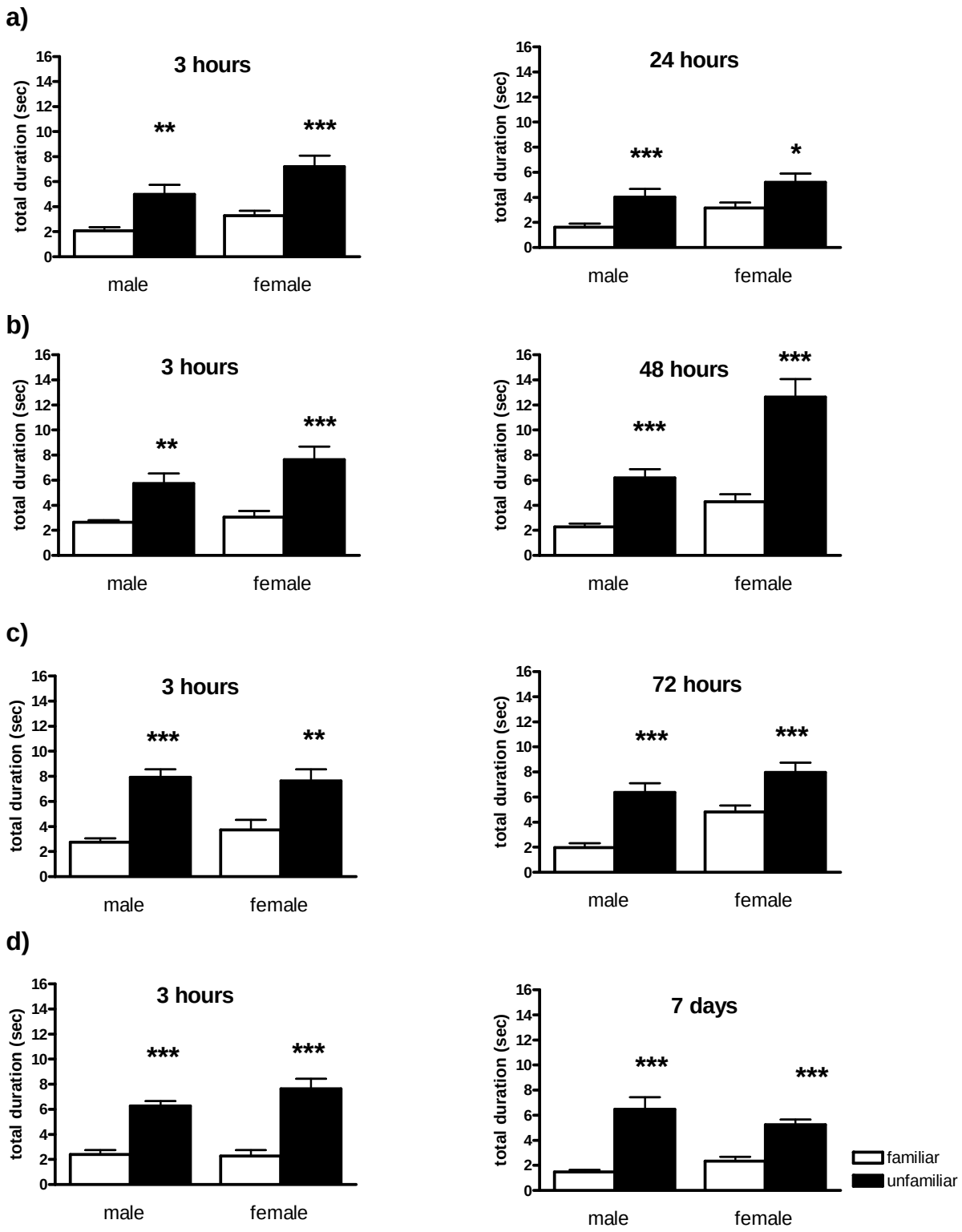

Figure 18: Object Recognition Test in male and female C57BL/6J mice

Total duration of exploration time on familiar and unfamiliar objects, after retention intervals of a) 3 hours and 24 hours b) 3 hours and 48 hours c) 3 hours and 72 hours d) 3 hours and 7 days. Male and female C57BL/6J mice significantly discriminated between familiar and unfamiliar object and spent more time exploring the unfamiliar object after each of the selected retention intervals.

* p< 0.05, ** p< 0.01, *** p< 0.001

Figure 19 shows the same results as in Figure 18 with the purpose to give another overview of all performed retention intervals. It shows the performance of each group of mice after the retention interval of 3 hours and accordingly of 24 hours, 48 hours, 72 hours and 7 days. After every retention interval, both male and female C57BL/6J mice spent more time exploring the unfamiliar object compared to the familiar one.

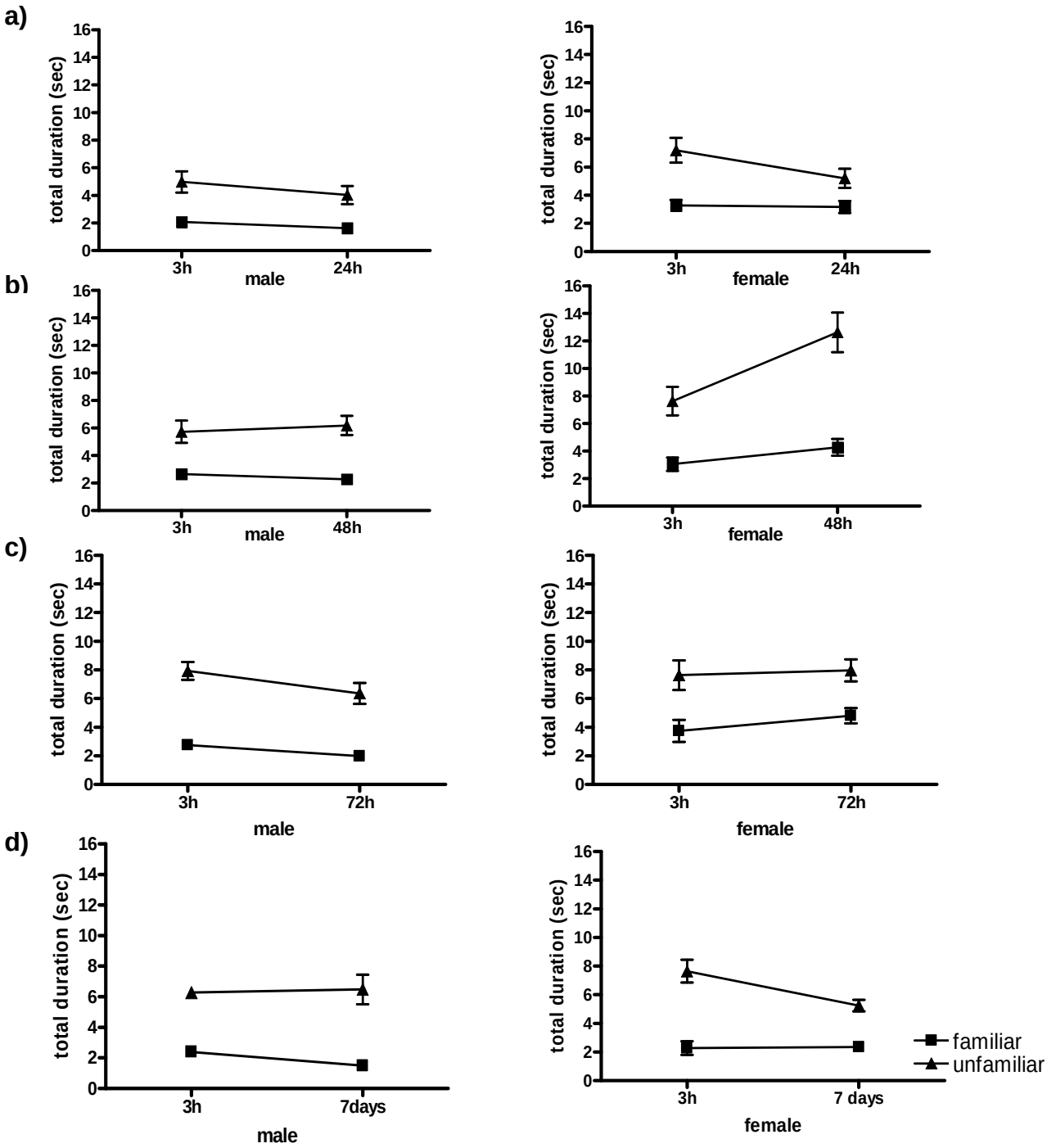

Figure 19: Object Recognition Test in male and female C57BL/6J mice
Total duration of exploration time in seconds, after retention intervals of
a) 3 hours and 24 hours b) 3 hours and 48 hours c) 3 hours and 72 hours d) 3 hours and 7 days

In general, results of all tests showed the same tendency. Exploration time of female C57BL/6J mice on the unfamiliar object in the test after a retention interval of 48 hours was relatively high, as already described in Figure 18. To further investigate this result, I calculated the Object Recognition Index for all tests. The Object Recognition Index is a common way to describe results in Object Recognition Tests and is defined as the ratio of the duration in which the novel object was explored over the duration in which both objects were explored (Time B/(Time A + Time B)). 0.5 is defined as chance level. The Object Recognition Index provides a more precise interpretation of results because it calculates exploration time on the unfamiliar object in respect to the total exploration time.

Figure 20 shows the Object Recognition Indices of all performed retention intervals in male and female C57BL/6J mice. All indices showed tendency towards the same levels and there were no significant differences between male and female mice. Also in the test after a retention interval of 48 hours in female C57BL/6J mice, which showed a very high duration in object exploration, one can see that in total the Object Recognition Index is nearly the same as for male mice. This means that here in this test phase, in general the exploration time was higher for unknown reasons, but this did not change the index and can therefore be neglected.

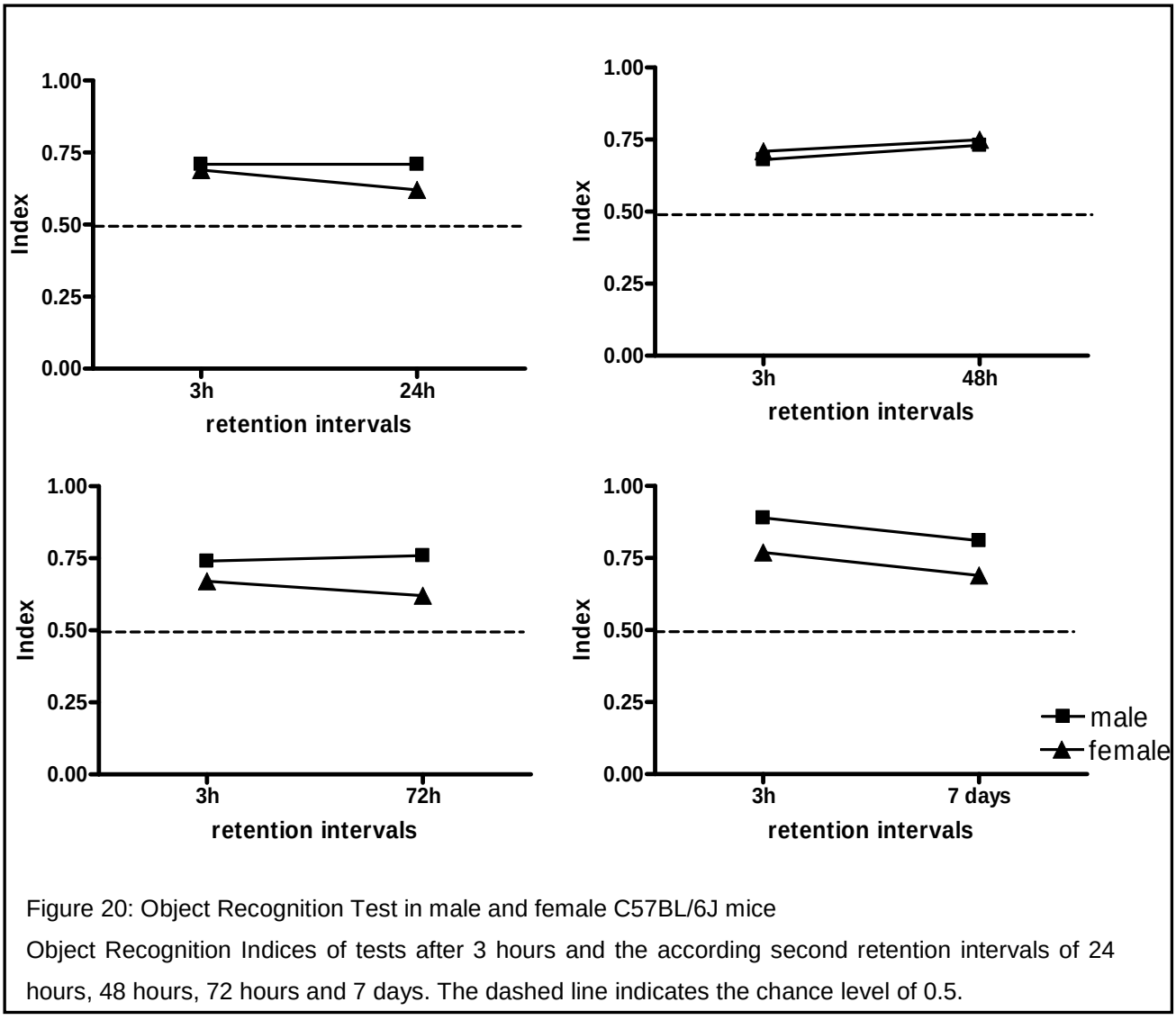

Figure 20: Object Recognition Test in male and female C57BL/6J mice

Object Recognition Indices of tests after 3 hours and the according second retention intervals of 24 hours, 48 hours, 72 hours and 7 days. The dashed line indicates the chance level of 0.5.

3.2. GLUD2 transgenic mice

3.2.1. Y-maze Spontaneous Alternation Test

GLUD2 wildtype and transgenic mice were analysed in the Y-maze for spontaneous alternation behaviour.

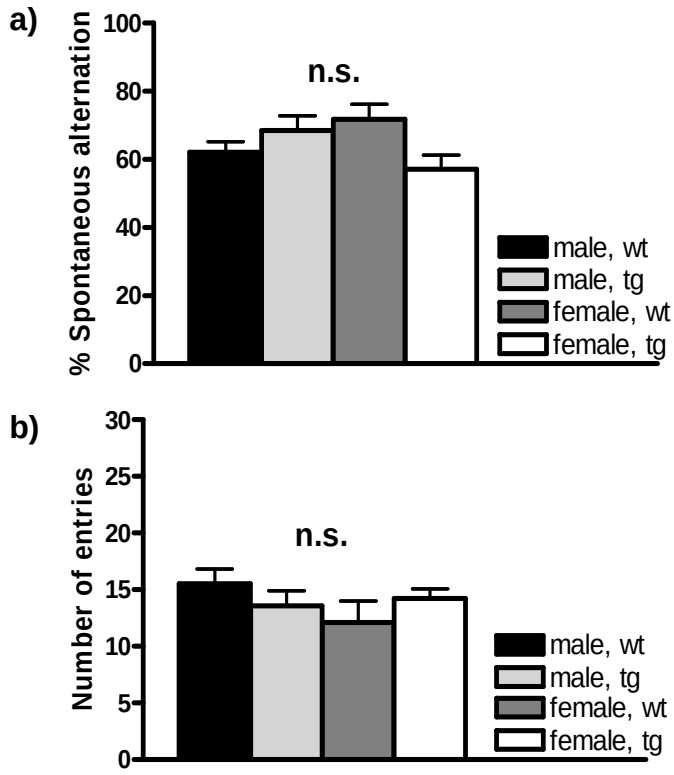

Figure 21: Y-Maze Spontaneous Alternation Test in wildtype and transgenic GLUD2 mice, both sexes.
a) Spontaneous alternation in percent.
b) Number of entries
n.s. non-significant

The Y-maze test did not reveal any genotype-related differences between wildtype and transgenic male and female mice. There was no significant difference concerning spontaneous alternation behaviour in both genotypes (Figure 21a). Neither wildtype or transgenic mice showed any difference in activity as measured by the number of arm entries (Figure 21b).

3.2.2. Social Discrimination Test

GLUD2 wildtype and transgenic mice were also analysed in the Social Discrimination Test.

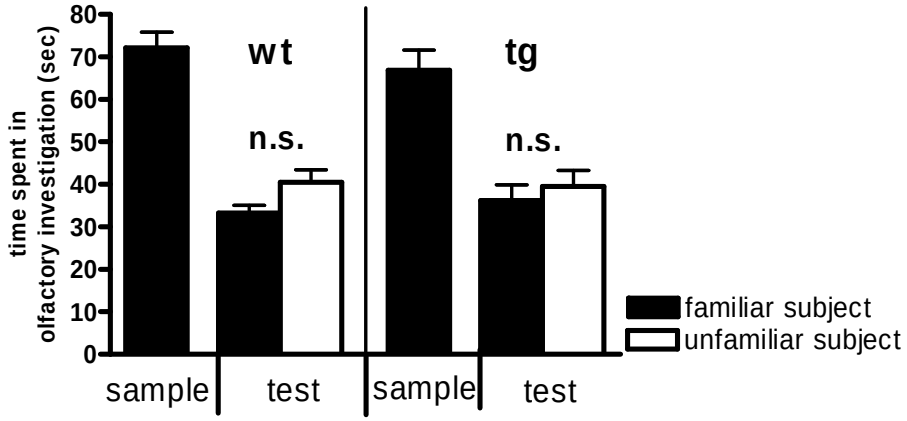

Figure 22: Social Discrimination Test in wildtype and transgenic GLUD2 mice

Pooled data of male and female mice; time spent in olfactory investigation in sample and test session on familiar and unfamiliar subject.

n.s. non-significant

Both wildtype and transgenic GLUD2 mice did not spend more time in olfactory investigation towards the unfamiliar subject in the test session (Figure 22). There were no significant differences in investigation times between familiar and unfamiliar subject in both genotypes.

3.2.3. Object Recognition Test

GLUD2 wildtype and transgenic mice were analysed in the Object Recognition Test.

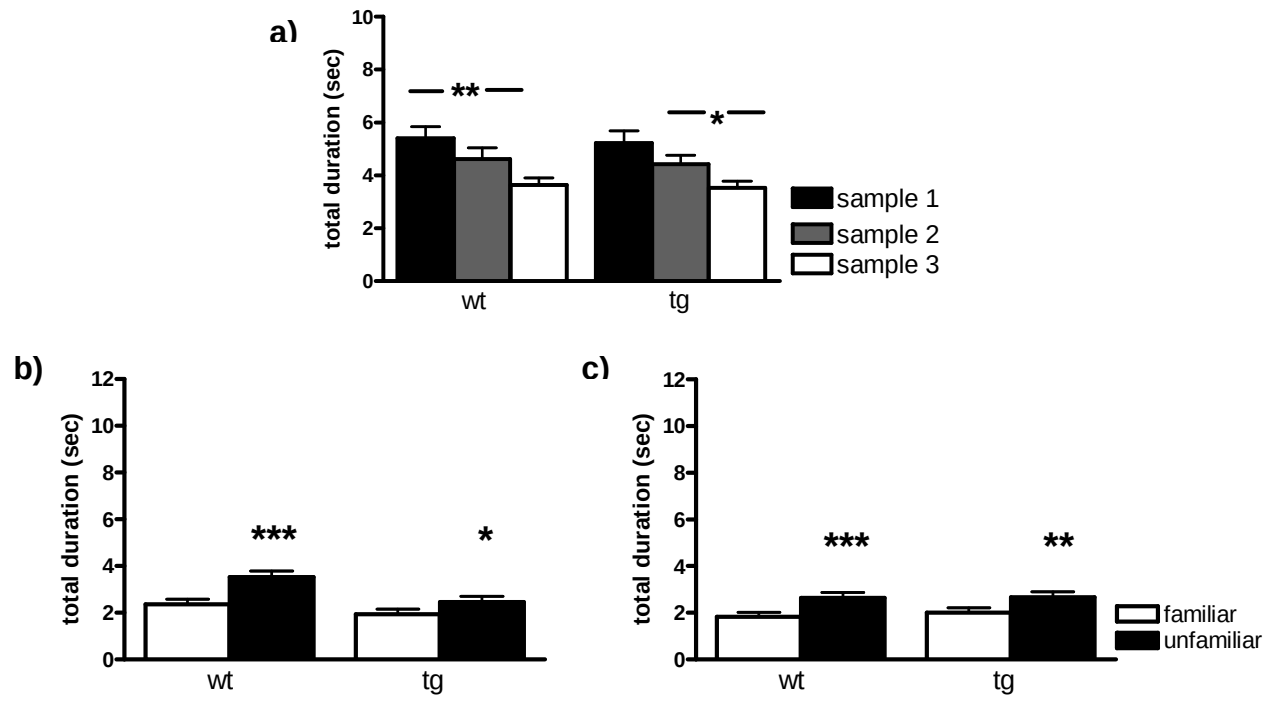

Figure 23: Object Recognition Test in wildtype and transgenic GLUD2 mice
Pooled data of male and female mice; total duration of exploration times in
a) Sample sessions
b) Test after a retention interval of 3 hours
c) Test after a retention interval of 24 hours
* p< 0.05, ** p< 0.01, *** p< 0.001

In the sample sessions, GLUD2 wildtype and transgenic mice showed habituation to the objects and exploration times became less with sample experience (Figure 23a). GLUD2 mice of both genotypes spent less time in object exploration than C57BL/6J mice.

Both wildtype and transgenic mice could discriminate the unfamiliar object from the familiar one after a retention interval of 3 hours and also after a retention interval of 24 hours (Figure 23b, c). The test worked in both genotypes and no genotype-related differences were detected.

3.3. MCHR1 knockout mice

3.3.1. Y-maze Spontaneous Alternation Test

MCHR1 wildtype and knockout mice were analysed in the Y-maze for spontaneous alternation behaviour.

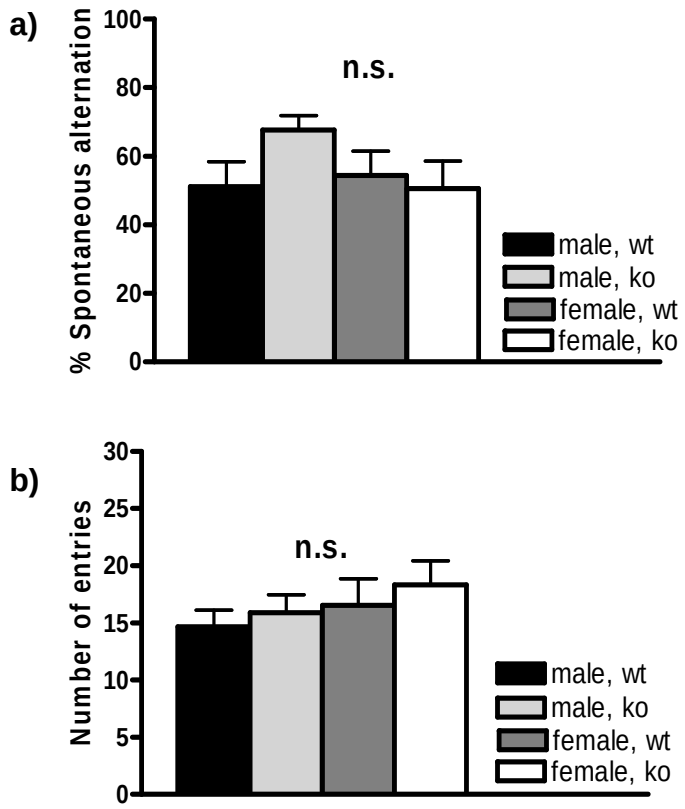

Figure 24: Y-Maze Spontaneous Alternation Test in wildtype and knockout MCHR1 mice, both sexes.
a) Spontaneous alternation in percent.
b) Number of entries
n.s. non-significant

The Y-maze test did not reveal any genotype-related differences between wildtype and knockout male and female mice. There was no significant difference concerning spontaneous alternation behaviour in both genotypes (Figure 24a). Neither wildtype or knockout mice showed any difference in activity as measured by the number of arm entries (Figure 24b).

3.3.2. Social Discrimination Test

MCHR1 wildtype and knockout mice were analysed in the Social Discrimination Test.

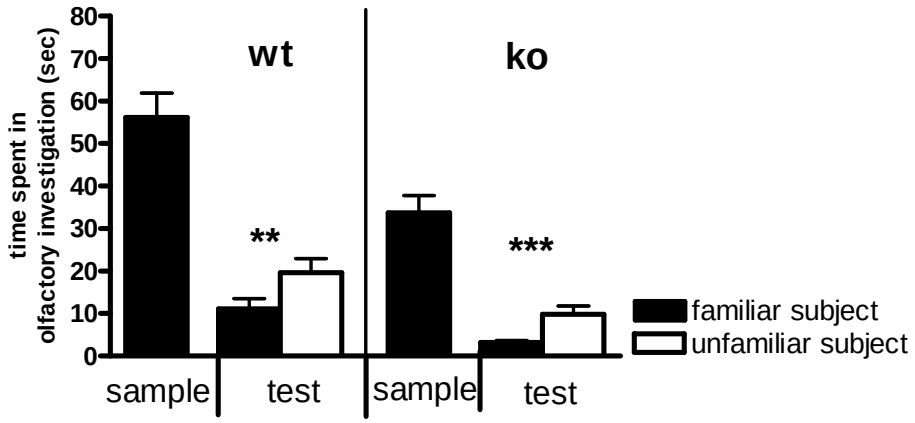

Figure 25: Social Discrimination Test in wildtype and knockout MCHR1 mice

Pooled data of male and female mice; time spent in olfactory investigation in sample and test session on familiar and unfamiliar subject.

** p< 0.01, *** p< 0.001

MCHR1 wildtype and knockout mice could perform the task and spent both more time in olfactory investigation towards the unfamiliar subject (Figure 25). Both genotypes displayed social recognition memory in this test.

3.3.3. Object Recognition Test

MCHR1 wildtype and knockout mice were analysed in the Object Recognition Test.

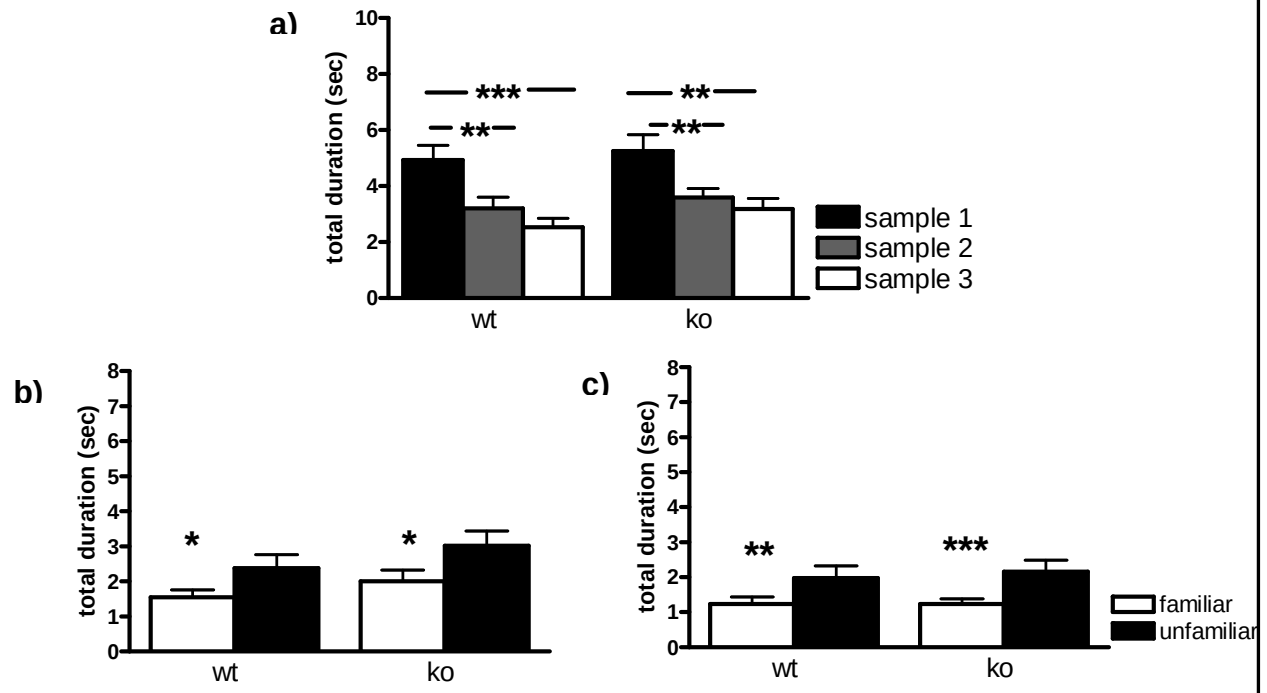

Figure 26: Object Recognition Test in wildtype and knockout MCHR1 mice
Pooled data of male and female mice; total duration of exploration times in
a) Sample sessions
b) Test after a retention interval of 3 hours
c) Test after a retention interval of 24 hours
* p< 0.05, ** p< 0.01, *** p< 0.001

Sample sessions revealed a decrease in exploration time with increasing sample experience; mice habituated to the objects and explored them less from sample session to sample session (Figure 26a). MCHR1 mice of both genotypes spent less time in object exploration than C57BL/6J mice.

After retention intervals of 3 hours and 24 hours, both wildtype and knockout mice were able to significantly discriminate the unfamiliar object from the familiar object and could perform the task (Figure 26b, c).

For retention intervals of 48 hours and 7 days, another batch of MCHR1 mice was bred so that 24 female MCHR1 mice (12 wt, 12 knockout) and 24 male MCHR1 mice (12 wt, 12 knockout) could be analysed.

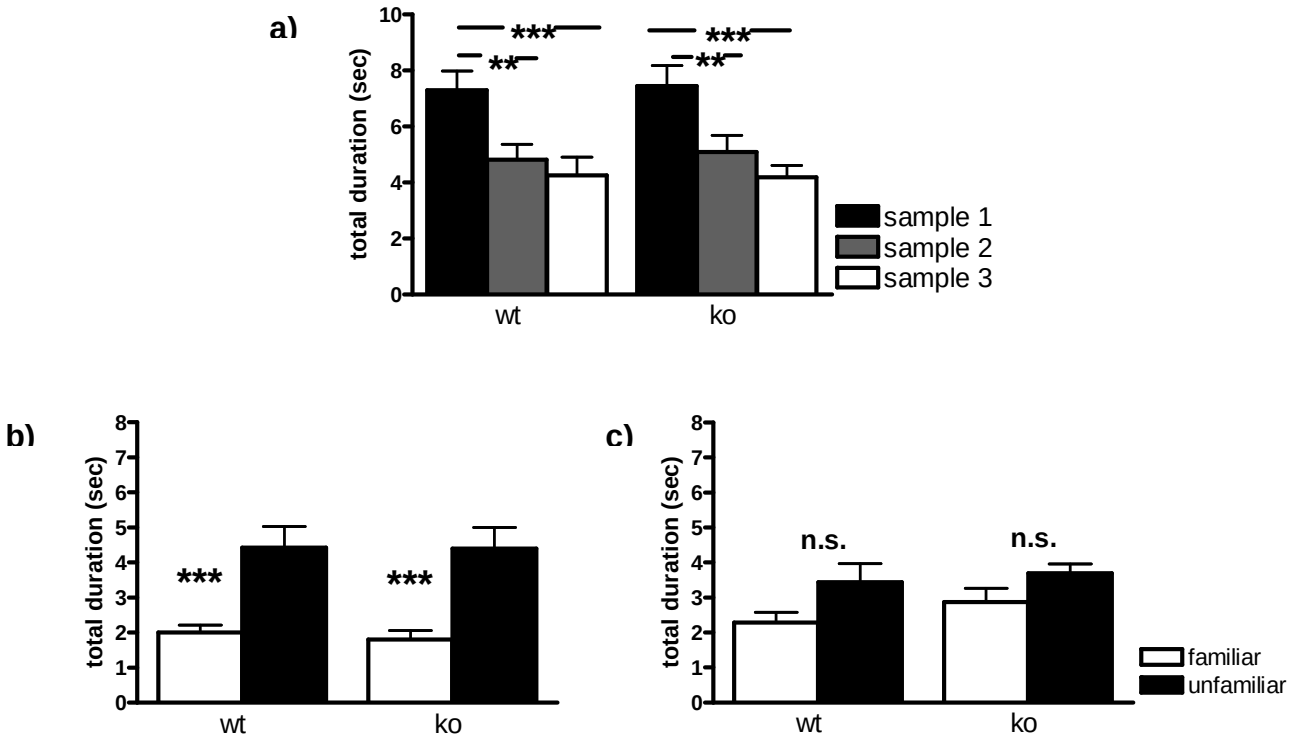

Figure 27: Object Recognition Test in wildtype and knockout MCHR1 mice
Pooled data of male and female mice; total duration of exploration times in
a) Sample sessions
b) Test after a retention interval of 48 hours and
c) Test after a retention interval of 7 days
n.s. non-significant, * p< 0.05, ** p< 0.01, *** p< 0.001

Wildtype and knockout mice showed habituation to the objects in sample sessions and exploration time became less with sample experience (Figure 27a).
After a retention interval of 48 hours, wildtype and knockout mice could significantly distinguish the unfamiliar object from the familiar one. After a retention interval of 7 days, neither wildtype or knockout mice could perform the task, indicating that the memory span for this mouse line lies between 48 hours and 7 days (Figure 27b, c).

3.4. Conditional cGKI knockout mice

3.4.1. Social Discrimination Test

Brain-specific cGKI wildtype and mutant mice were analysed in the Social Discrimination Test.

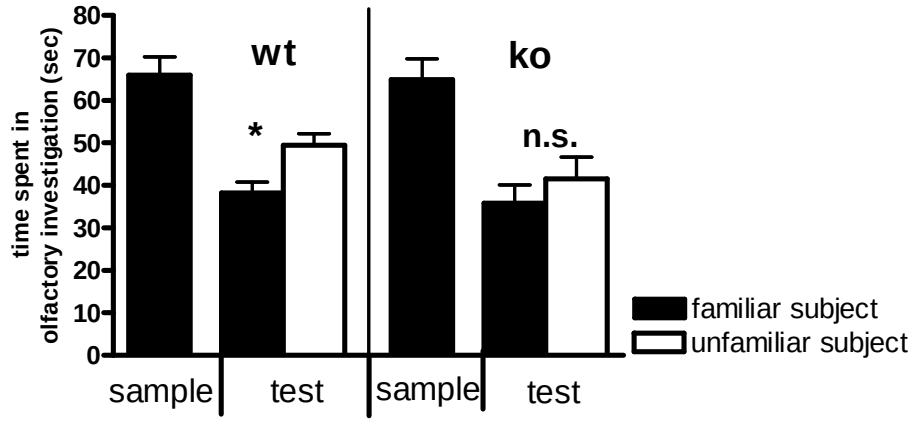

Figure 28: Social Discrimination Test in brain-specific cGKI wildtype and mutant mice
Pooled data of male and female mice; time spent in olfactory investigation in sample and test session on familiar and unfamiliar subject.
n.s. non-significant, * p< 0.05

Wildtype mice significantly distinguished between familiar and unfamiliar subject and spent more time in olfactory investigation towards the unfamiliar subject. **Brain-specific** cGKI knockout mice displayed a deficit in performing the task (Figure 28). **Brain-specific** knockout mice did not spend more time in olfactory investigation towards the unfamiliar subject in comparison to the familiar subject.

Hippocampus-specific cGKI wildtype and mutant mice were analysed in the Social Discrimination Test.

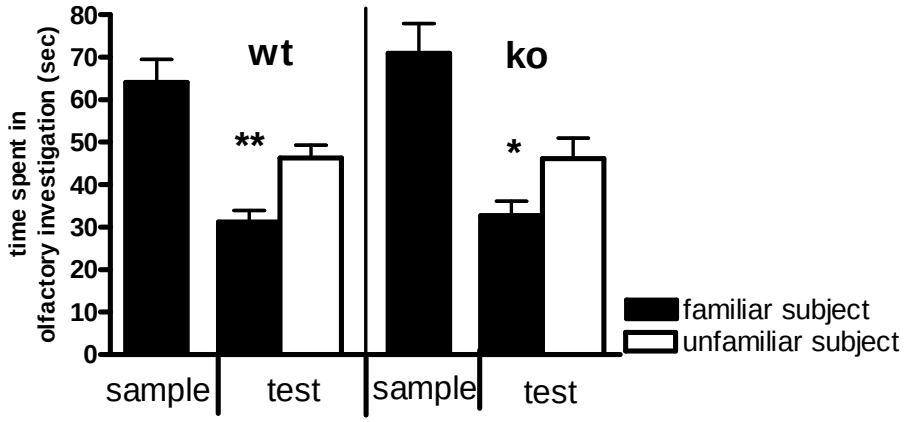

Figure 29: Social Discrimination Test in hippocampus-specific cGKI wildtype and mutant mice
Pooled data of male and female mice; time spent in olfactory investigation in sample and test session on familiar and unfamiliar subject.
* p< 0.05, ** p< 0.01

The Social Discrimination Test in **hippocampus-specific** knockout and wildtype cGKI mice did not reveal any genotype-related differences (Figure 29). Both genotypes could perform the test and spent more time in olfactory investigation towards the unfamiliar subject.

3.4.2. Object Recognition Test

Brain-specific cGKI wildtype and mutant mice were analysed in the Object Recognition Test.

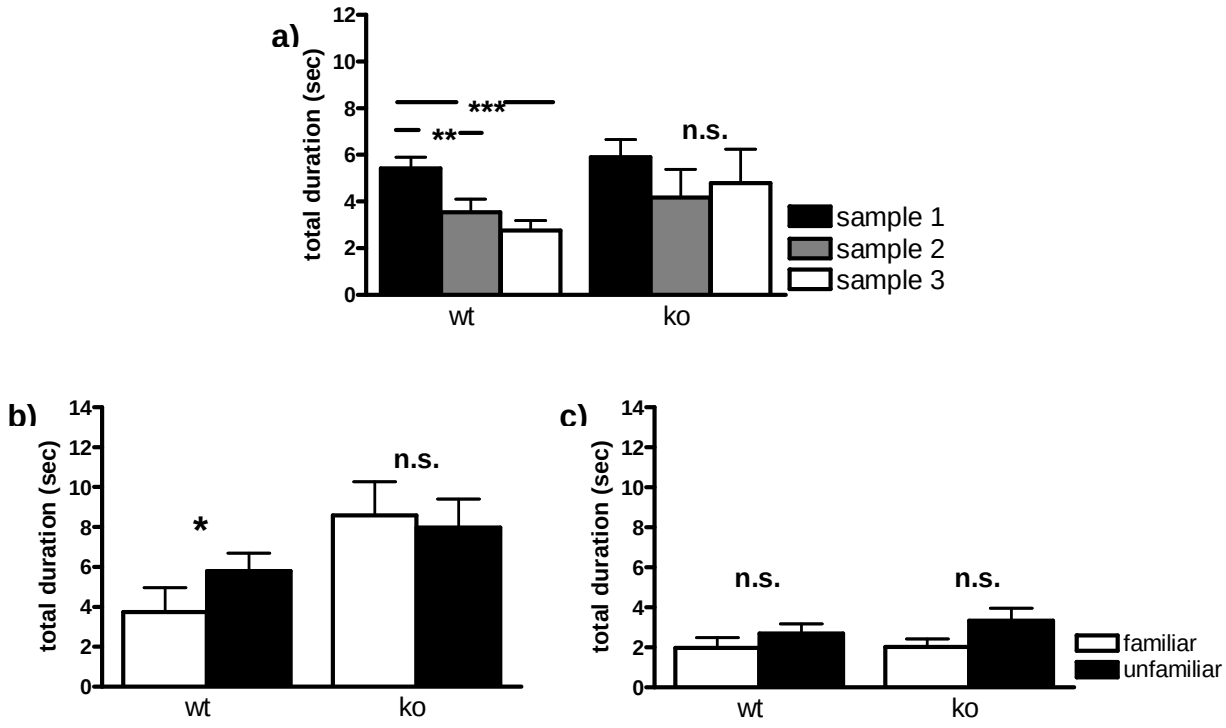

Figure 30: Object Recognition Test in brain-specific cGKI wildtype and mutant mice
Pooled data of male and female mice; total duration of exploration times in
a) Sample sessions
b) Test after a retention interval of 3 hours and
c) Test after a retention interval of 24 hours
n.s. non-significant, * p< 0.05, ** p< 0.01, *** p< 0.001

Wildtype cGKI mice showed habituation to the two identical objects and exploration time decreased significantly from sample session to sample session, whereas **brain-specific** mutant cGKI mice did not display such habituation and for them, no significant decrease in exploration times could be revealed (Figure 30a).

After a retention interval of 3 hours, **brain-specific** mutant mice displayed a deficit in object recognition memory compared to their wildtype littermates and could not discriminate the unfamiliar object from the familiar one (Figure 30b).

After a retention interval of 24 hours, neither wildtype nor mutant mice were able to perform the task (Figure 30c).

Hippocampus-specific cGKI wildtype and mutant mice were analysed in the Object Recognition Test.

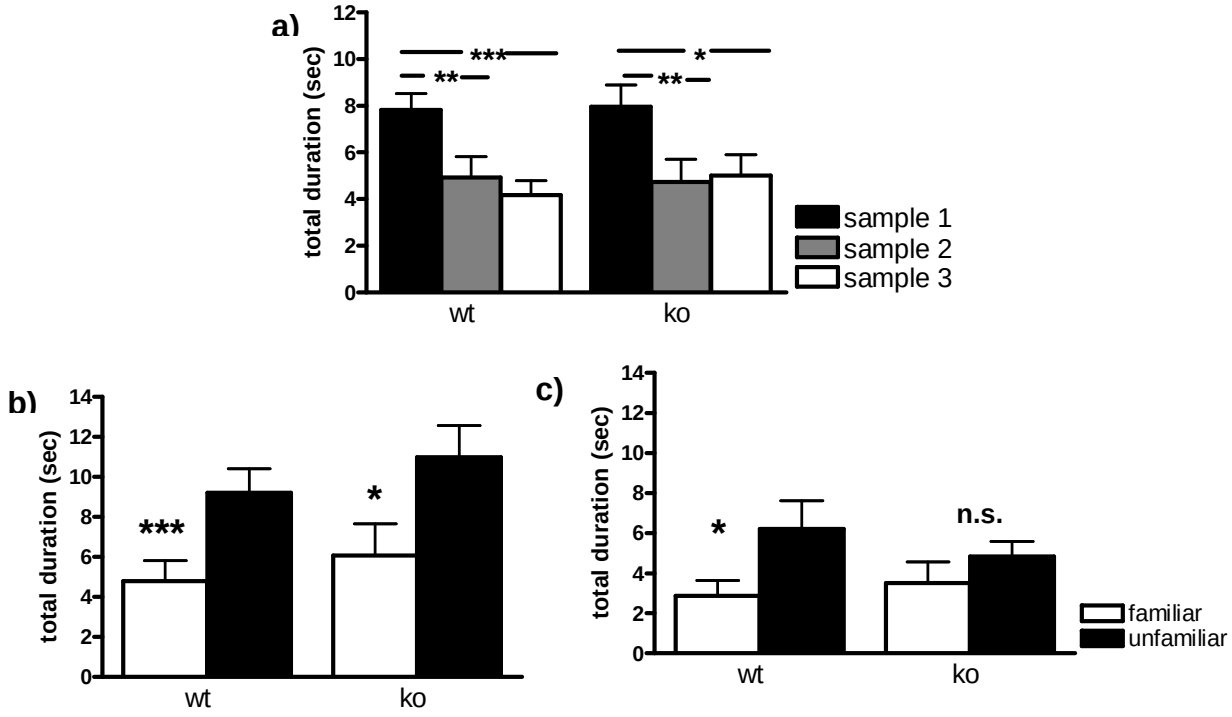

Figure 31: Object Recognition Test in hippocampus-specific cGKI wildtype and mutant mice
Pooled data of male and female mice; total duration of exploration times in
a) Sample sessions
b) Test after a retention interval of 3 hours and
c) Test after a retention interval of 24 hours
n.s. non-significant, * p< 0.05, ** p< 0.01, *** p< 0.001

In both **hippocampus-specific** cGKI wildtype and mutant mice, exploration times became less from sample session to sample session and both genotypes showed habituation to the two identical objects (Figure 31a).

After a retention interval of 3 hours, both genotypes could significantly distinguish the unfamiliar object from the familiar one (Figure 31b).

After a retention interval of 24 hours, wildtype mice could perform the task, whereas **hippocampus-specific** mutant cGKI mice showed a deficit in object recognition memory (Figure 31c).

3.5. Food-rewarded Hole Board Test in C57BL/6J and Balb/c mice

12 male C57BL/6J and 12 male Balb/c mice were tested in the Food-rewarded Hole Board Test.

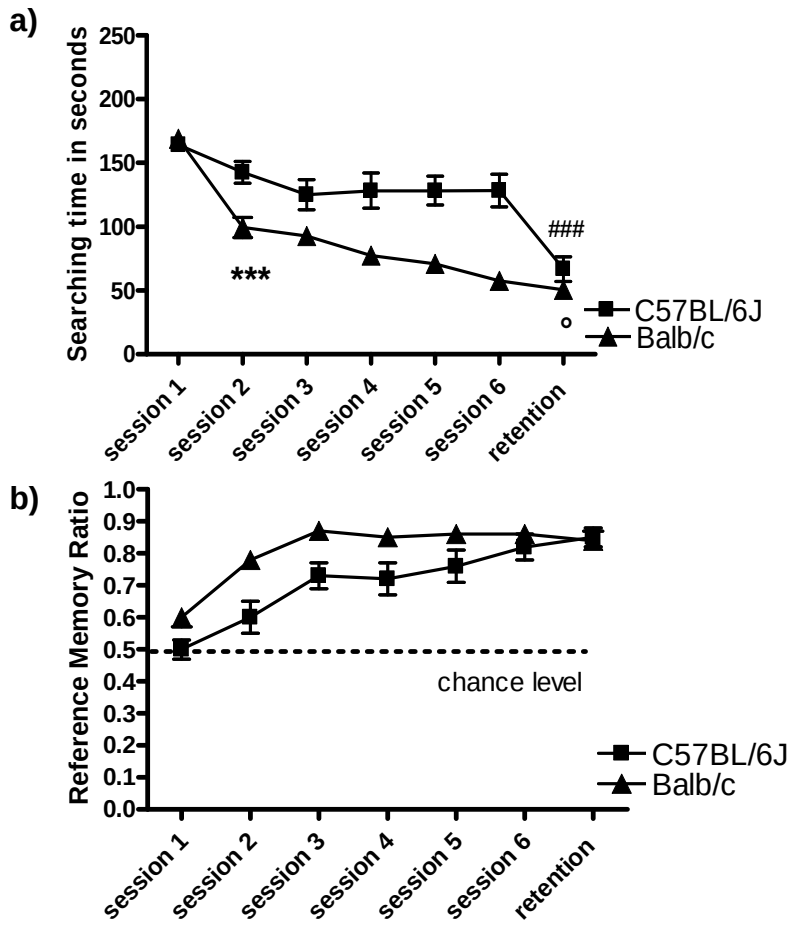

Figure 32: Food-rewarded Hole Board Test in C57BL/6J and Balb/c mice.

a) Searching time in seconds

b) Reference Memory Ration

* session 1 vs. session 2 Balb/c, # retention vs. session 6 C57BL/6J, °retention vs. session 6 Balb/c

*** p< 0.001, ## p< 0.01, °p< 0.05

During acquisition, i.e. from session 1 to session 6, searching time was reduced from session to session and this reduction was stronger in Balb/c mice than in C57BL/6J mice. Searching time was further reduced in the retention test, with C57BL/6J mice showing a bigger difference to session 6 than Balb/c mice (Figure 32a).

Working memory can be assessed with this test, but it was not demanded for the validation of the Food-rewarded Hole Board Test according to the Standard Operating Procedure (SOP) provided by Eumorphia.

Reference Memory Ratio was calculated for both strains. Balb/c mice learned more quickly than C57BL/6J during acquisition from session 1 to session 6 ($p < 0.01$). Both strains could remember the task in the retention test as the Reference Memory Ratio was above chance level (0.5) (Figure 32b).

4. Discussion

4.1. Object Recognition Test

The Object Recognition Test has been applied in many laboratories using different arenas, objects and procedures. It is not only used for acquiring basic knowledge about strain differences but also for demonstrating alterations in novelty processing due to drug application or brain lesions. The test is also sensitive enough to analyse mutant mouse lines with specific deficits in genes involved in the pathways for memory formation.

C57BL/6J mice have been analysed in several variants of the Object Recognition Test in many studies. Contradictory results in all these tests might be due to discrepancies in performing the test. A study of Frick et al. tested spatial and object novelty in C57BL/6 mice. Male C57BL/6 mice preferred displaced and substituted objects over unchanged objects, but females showed preference in only object novelty (Frick et al., 2003). The Object Recognition Task in Frick's study consisted of three phases in total such as habituation, sample and choice phase which were each completed on separate test days. Female C57BL/6 mice failed to perform the task after retention intervals of 24 hours and 7 days. Male C57BL/6 mice significantly spent more time exploring the unfamiliar object after a retention interval of 24 hours, but failed to do so after a retention interval of 7 days. The difference to the Object Recognition Test I established lies not only in the size of the arena and the objects (arena 60cm x 60cm x 45cm; objects 10cm x 6 cm x 6cm). Differences in the performance of the task might likely be due to the different way of acquisition and consolidation as I used three sample sessions, in which I presented the two identical objects, instead of only one sample session as performed in the study of Frick.

It was shown by Genoux and coworkers that one single sample trial of 25 minutes, in which the to-be-familiarized objects were presented, or distributed training with brief intervals between the sample trials (five 5 minutes trials with inter-trial intervals of 5 minutes) were not as effective for memory formation as five 5 minutes trials with inter-trial intervals of 15 minutes (Genoux et al., 2002). So repetition in learning together with longer inter-trial intervals is essential for the formation of accurate and long-lasting memory. Most memories fade with time without further recall. The molecular basis for this time-dependent weakening of memory traces is not yet clear. Genoux et al. showed that protein phosphatase 1 (PP1) is limiting acquisition and

favouring memory decline, it is a repressor of learning and memory in mice and promotes forgetting (Genoux et al., 2002).

The hypothesis that multiple training sessions separated by defined time intervals is beneficial for the formation of long-lasting memory was also supported by Waddell (Waddell, 2003). Precise steps of consolidation are crucial for long-enduring memory, and consolidation proceeds slowly to support the adaptable modulation of memory strength (McGaugh, 2000).

Repetitive learning is different from one-time learning (Cammarota et al., 2005). It was shown that one-time learning requires activation of glutamate receptors in the CA1 region of the hippocampus for consolidation, whereas additional learning steps require molecular processes in the striatum.

Another study attempted to investigate the performance of different mouse strains in an Object Recognition Task (Sik et al., 2003). Performance in many cognitive tasks is strain-dependent (Voikar et al., 2001). Sik et al. (2003) showed that individually housed C57BL, Swiss, BALB/c and 129/Sv mice discriminated between the unfamiliar and the familiar object after a delay of 1 hour, but did not discriminate after a delay of 24 hours. After a delay of 4 hours, the discrimination performance was at an intermediate level, which might suggest a delay-dependent forgetting in this test design. This result could be explained by the use of only one sample session for acquisition and consolidation. The question now arises why I found different results in my study. C57BL/6J mice in my study did not display delay-dependent forgetting, which might be due to single-housing the animals during inter-trial intervals and retention intervals. Singly housed mice are not submitted to retroactive interference. No disturbing or new occurring input interacted with the previously learned object information and so this information was maintained in memory for a retention interval of even 7 days. But also in the study of Sik et al. mice were individually housed. The interaction between sampling three times and single-housing the animals during inter-trial and retention intervals might be an explanation for the good performance of C57BL/6J mice in my Object Recognition Procedure.

It is crucial at which time-point and how long mice are individually housed. In my study, I started single-housing with beginning of the test and the good performance of C57BL/6J mice in the procedure supported my way of performing the Object Recognition Test. Nevertheless, it was shown that single-housing impaired novel object recognition in C57BL/6J mice, but in this study, mice were separated and

individually housed at the age of 4 weeks (Voikar et al., 2001). Long-term individual housing has strong strain-and test-specific effects on behaviour and can impair memory in certain tasks, so the time-point in which single-housing is started must be chosen critically.

Another critical point in performing the Object Recognition Procedure is the age of the animals. It was shown that old (18-20 months) male and female C57BL/6J mice performed worse than young (3-4 months) or middle-aged (10-12 months) in Novel Location, but not Novel Object Recognition Tasks (Benice et al., 2006). But here again the Object Recognition Procedure was different to my procedure in acquisition and consolidation and also in the number of objects which were presented. It is debatable to which extent all the different Object Recognition Procedures are comparable and therefore accurate choice is even more important.

With this Object Recognition Test I could show that C57BL/6J mice are an appropriate strain to establish a learning and memory test for high throughput cognitive phenotyping of mutant mouse models. There might be some mutant mouse strains which show a deficit in performing the Object Recognition Test after certain retention intervals under the same experimental conditions in which a wildtype mouse strain (in my case C57BL/6J) does not show this deficit. If such a deficit could be found, I suggest the further investigation of activity levels, locomotion, perceptual processes, neophobia or stress reactivity. A lack of discrimination between familiar and unfamiliar objects can not only be due to memory deficits, but also due to non-cognitive factors.

In summary, the Object Recognition Test is successfully established and validated for C57BL/6J mice. However, regarding all the above described discrepancies in performing the test, the appropriate procedure must be chosen critically and further tests would be necessary for a complete and precise behavioural analysis of mutant mouse models.

Object recognition is influenced by many external factors:

- Environmental conditions: single-housed or group-housed mice, time-point and duration of single-housing
- Test-specific factors: size of the arena, number and size of the objects, number and duration of sample sessions, duration of inter-trial and retention intervals

- Experimental design: mouse strain or genetic background, age of the test mice, treatment/handling and therefore stress level of the test mice, previously performed behavioural tests.

4.1.1. Object Preference and Counterbalance Test

The preference for novel stimuli like objects or places is called neotic preference (Hughes, 2007). It refers to an active choice of stimuli depending on their novelty value. Choice of the more novel stimulus is called neophilia (Cowan, 1977) whereas choice of the more familiar stimulus is called neophobia (Barnett, 1958). Novelty is described as the expression that refers to an interaction between stimulus and perceiver. Beside the colour, texture and shape of an object itself, also the context in which the object was presented is essential for object recognition. Rats and mice are attracted by novel objects and approach and explore them with their vibrissae, nose and forepaws. A single explorative approach can already leave a complex memory trace with information about features of the object explored, but also about where and even when the object was encountered (Dere et al., 2007). Object Recognition Tests work because of this phenomenon.

It is relatively easy to assign novel objects for laboratory animals, but more difficult to find behavioural Novelty Preference Tests for humans when the past experiences of the individual are not known. Novelty of a stimulus depends on the context in which it was presented, on the delay between exposures of that stimulus and the number and duration of each exposure to the stimulus (Hughes, 2007).

There is a conflict for every individual between approach (neophilia) and avoidance (neophobia) (Montgomery, 1955). Intense novelty can provoke avoidance because of fear, whereas mild novelty can provoke approach because of curiosity. This conflict is described in Figure 33.

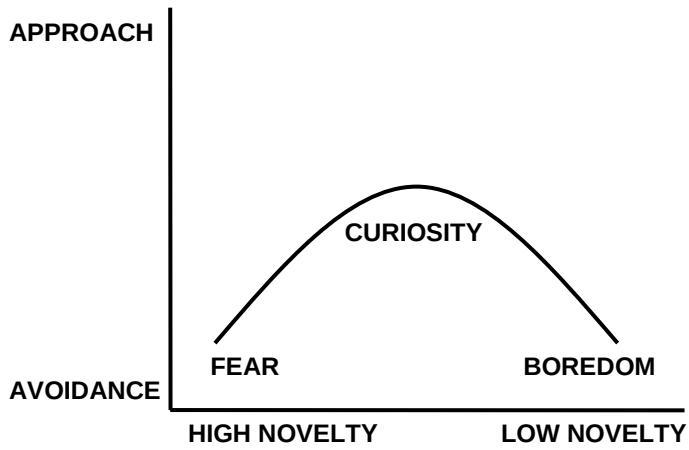

Figure 33: Possible relationship between decreasing novelty and avoidance or approach of novel stimuli as determined by fear, curiosity or “boredom”.
Adapted and modified from Hughes, 2006.

Mouse models of novelty seeking are widely used and measure parameters such as activity in a novel environment, novel environment preference but also preference for food, odours, drinking solutions or, as performed in this study, for objects (Kliethermes et al., 2006).

Novelty reward is also used as a measure of anhedonia (Bevins and Besheer, 2005). Anhedonia is a major symptom of depression in humans and refers to the decrease in sensitivity to pleasurable stimuli (Kliethermes et al., 2006). Bevins and Bardo showed that rats displayed an increase in preference for an environment that had been previously paired with novel objects (Bevins and Bardo, 1999).

The degree of object exploration is determined by the complexity of the object itself. An object that has several different features such as holes or varying textures will induce greater exploration than a less complex object. In addition, objects that can be manipulated are more attractive for mice. An object that can be carried or dragged will attract more attention (Bevins and Besheer, 2006). To avoid this non-cognitive criterion I fixed the objects with double-sided adhesive tape.

C57BL/6J mice equally distributed exploration times on all objects used in this test, except females which preferred one of the objects (rectangle) in one specific configuration (rectangle versus toy).

However, by means of the Counterbalance Test, I could approve novelty preference, because all C57BL/6J mice clearly preferred the novel object compared to the familiar one after retention intervals of 3 hours and 24 hours. In my procedure, it did not play a role which object was assigned familiar or unfamiliar. Even if mice preferred one of two objects when they were presented for the first time, they did not focus on this preferred object anymore as soon as an unfamiliar object was presented simultaneously. I concluded that natural preferences can be neglected in my Object Recognition Procedure and considered the chosen objects as validated.

4.1.2. Retroactive Interference Test

In the Retroactive Interference Test, C57BL/6J mice could not perform the Object Recognition Test anymore when they were group-housed during inter-trial and retention intervals.

I could show that group-housed mice focused on newly learned information or input (e.g. coping with attacking littermates) and didn't remember the objects.

Retroactive interference is well studied in humans. It was shown that in patients suffering from amnesia, severe forgetting of word lists and stories occurred in activity-filled delays and that forgetting was less distinctive when these patients spent the delays between sessions in dark, quiet rooms (Cowan et al., 2004). Furthermore, also spatial memory in humans is subject to interference effects, which is also true for verbal memory and motor memory (Elmes, 1988).

It was shown in rats performing an Object Recognition Task that the presentation of an object between sample and test sessions disrupted subsequent recognition of the sample object (Winters et al., 2007).

A long time has passed since Müller and Pilzecker hypothesized in 1900 about the perseveration-consolidation steps in memory formation (McGaugh, 1999). They were the first to state that memory of newly learned information was disrupted by the learning of other information just recently presented after the original learning, and showed for the first time that new information can interfere with to-be-remembered items. It was confirmed that forgetting can be induced by any subsequent mentally effortful task, the interfering information does not have to be similar to the to-be-remembered stimuli (Dewar et al., 2007). This finding would support my theory. In my Object Recognition Procedure no objects were presented to group-housed mice in the delays, but social interaction occurred during inter-trial and retention intervals which impaired performance in the Object Recognition Task.

The role of retroactive interference in the spatial memory of normal rats and rats with hippocampal lesions was studied in 1982 and it was shown that retroactive interference even disturbed control animals (Jarrard et al., 1982). Beside this, rats were significantly impaired in performing a visual discrimination task when a high-interference treatment was given before discrimination training (Winocur, 1984).

Retroactive Interference can occur in several tasks and in different species and should therefore be minimized during behavioural testing.

4.2. Cognitive phenotyping of mutant mouse lines

4.2.1. GLUD2 transgenic mice

In attempt to characterise the behavioural phenotype of GLUD2 mice in more detail, several tests were performed.

In the Y-maze Spontaneous Alternation Test, no differences between wildtype and transgenic mice in spontaneous alternation could be found, so I can say that GLUD2 mice showed no working memory deficit concerning this test. There was also no significant difference in the number of entries, so there is evidence that the amount of activity in this test was the same for transgenic and wildtype mice.

In the Social Discrimination Test, both GLUD2 transgenic and wildtype mice showed a deficit in social recognition. No genotype-related differences could be revealed.

The Object Recognition Test was performed to assess object memory. Exploration times in all three sample sessions were similar in GLUD2 mice, i.e. there were no significant differences in exploration times between genotypes. After a retention interval of 3 hours, wildtype and transgenic mice could discriminate the unfamiliar object from the familiar one. After a retention interval of 24 hours, again wildtype and transgenic mice distinguished between familiar and unfamiliar object. The hypothesis that *GLUD2* is beneficial in terms of memory formation and that it plays a role in higher cognitive abilities could not be corroborated in this mutant mouse line. GLUD2 transgenic mice did not show an improvement in object recognition memory compared to wildtype mice.

Glutamate dehydrogenase (GDH) is important for recycling the main excitatory neurotransmitter in the brain. *GLUD2* is hominoid-specific which makes it very interesting for investigation as it probably contributed to enhanced brain function in humans and apes. Nevertheless, GLUD2 transgenic mice did not display any enhanced memory performance in the applied cognitive tests. Testing GLUD2 mice in some other cognitive tests would be advisable. Perhaps cognitive tests with different experimental designs would reveal some genotype-related differences, e.g. cognitive tests based on external motivation, reward or punishment. It could be possible that the Y-maze Spontaneous Alternation Test, the Social Discrimination Test and the Object Recognition Test are too weak to reveal a detailed cognitive phenotype in these mice.

4.2.2. MCHR1 knockout mice

In the Y-maze Spontaneous Alternation Test no genotype-related differences in spontaneous alternation and number of entries in the three arms could be found, i.e. MCHR1 mice did not show a deficit in working memory or an alteration in locomotor activity levels in this test situation.

In the Social Discrimination Test, both wildtype and mutant MCHR1 mice showed social recognition memory, i.e. both genotypes spent more time in social investigation with the unfamiliar subject.

In the Object Recognition Test, exploration times in sample sessions were similar and there was no significant difference in exploration times between wildtype and mutant MCHR1 mice, so exploration activity levels were comparable in this test. After retention intervals of 3 hours, 24 hours and 48 hours both wildtype and mutant mice showed significant discrimination between familiar and unfamiliar object, indicating intact object recognition memory. After a retention interval of 1 week wildtype as well as mutant MCHR1 mice did not discriminate anymore between familiar and unfamiliar object, which means that the memory span in this test lies between 48 hours and 1 week for both genotypes.

Many studies were done for investigation of the MCH receptor 1 and taken together, the results suggest an involvement of melanin-concentrating hormone (MCH) in mood disorders.

In aversively motivated associative learning tests, it was shown that disrupting the melanin-concentrating hormone receptor 1 in mice leads to cognitive deficits (Adamantidis et al., 2005; Monzon et al., 2002).

As MCHR1 mutant mice did not show any deficits in working memory, object memory and social memory, which are not aversively motivated, the results imply that MCHR1 plays a specific role in aversively motivated learning and therefore stressful tests, but not in positively motivated forms of learning. This is in line with previous results which suggest an involvement of MCH in the regulation of emotion and stress by increasing the hypothalamus-pituitary-adrenal (HPA) axis activity (Shimazaki et al., 2006).

4.2.3. Conditional cGKI knockout mice

In attempt to characterise the behavioural phenotype of conditional cGKI mutant mice in more detail, several tests were performed.

In the Social Discrimination Test, NesCre x cGKI mutants showed a deficit in social recognition, mainly due to performance in females. This deficit was not evident in hippocampus-specific NEX-Cre x cGKI mutants.

The Object Recognition Test was performed to assess object memory. Exploration times in all three sample phases were similar in NesCre x cGKI mice and also in hippocampus-specific NEX-Cre x cGKI mice, i.e. there were no significant differences in exploration times. After a retention interval of 3 hours, mutant NesCre x cGKI mice did not discriminate the unfamiliar object from the familiar one, whereas hippocampus-specific NEX-Cre x cGKI mutants did. After a retention interval of 24 hours, neither NesCre x cGKI mutant mice nor hippocampus-specific NEX-Cre x cGKI mutant mice distinguished between familiar and unfamiliar object.

Taken together, both NesCre x cGKI and hippocampus-specific NEX-Cre x cGKI mutants showed a deficit in the Object Recognition Test in comparison to their controls. This was evident after a 3 hours retention interval in the former and after a 24 hours retention interval in the latter.

Taking the results of social memory and object recognition memory together, NesCre x cGKI mutants exhibit both social and object memory deficits, whereas hippocampus-specific NEX-Cre x cGKI mutants exhibit only an object recognition deficit. In hippocampus-specific NEX-Cre x cGKI mutants, the cognitive phenotype was less distinctive and more limited than in NesCre x cGKI mutants, in which the whole brain is affected. The extent of the cognitive phenotype correlated with the extent of the affected brain region. It would be interesting to know if there are also alterations in electrophysiology (LTP) and protein biosynthesis in these mutants. The hippocampus is involved in object recognition memory as lidocaine-treatment in the hippocampus of male C57BL/6J mice resulted in impaired novel object preference after a retention interval of 24 hours (Hammond et al., 2004). cGMP-dependent protein kinase I (cGKI) is expressed in the hippocampus and supports an age- and protein synthesis-dependent form of long-term potentiation (Kleppisch et al., 2003).

4.3. Food-rewarded Hole Board Test

In this test, searching time was measured during acquisition and retention sessions. Balb/c mice showed reduced searching time compared to C57BL/6J mice, in session 1 both strains started at the same level and also in the retention session both strains reached the same level of searching time. The performance during the acquisition sessions from session 2 to session 6 differed. Reference Memory Ratio was calculated for both strains. Balb/c mice learned more quickly than C57BL/6J during acquisition from session 1 to session 6. Both strains could remember the task in the retention session.

In conclusion, both strains reached the same level of performance, but used different strategies of learning.

In general, Balb/c and C57BL/6J mice show differences in the performance of several learning and memory tasks.

In a rewarded discrimination learning task using the olfactory tubing maze, Balb/c mice consistently remembered the odour-reward association whereas C57BL/6J mice took more sessions to learn the task and to reach the same level as Balb/c mice (Restivo et al., 2006).

In a radial maze study, it was shown that Balb/c mice learned the task by using olfactory cues, but C57BL/6J mice seemed to use spatial cues. This study is similar to the performed Food-rewarded Hole Board Test as in the radial maze study also food rewards were used to motivate the mice (Roullet et al., 1993). In the Food-rewarded Hole Board Test mice could navigate through the arena either by using olfactory cues within one trial or by using spatial cues or by using both, but I did not further investigate in the strategies used. However, it is likely that both strains acted with similar behavioural plans as described in the study of Roullet et al.

Male Balb/c mice show different risk assessment and exploration behaviour compared to male C57BL/6J mice. Balb/c mice avoided risk areas and showed high risk assessment whereas C57BL/6J mice were more explorative and risk taking and showed little risk assessment (Augustsson et al., 2004).

It is known that Balb/c mice, which are albinos, bear the hippocampus lamination defect (Nokakowski, 1984; Lassalle et al., 1994) and suffer from variable corpus callosum agenesis (Wahlsten, 1989; Filgueiras et al., 2004). Although Balb/c mice showed some deficits in other behavioural tests, they were not impaired in the Food-

rewarded Hole Board Test and displayed even better results during acquisition. The genetic defects did not handicap Balb/c mice in this performed test.

There are a variety of behavioural differences in mouse strains, including Balb/c and C57BL/6J mice, shown in a study of Kalueff et al. Behavioural differences range from sensory behaviour (olfaction, hearing), anxiety (neophobia, freezing), aggression (inter-male aggression) to grooming behaviour (Kalueff et al., 2007).

Taken together, Balb/c and C57BL/6J mice proved to be appropriate strains for the establishment and validation of the Food-rewarded Hole Board Test and the test can now be used in terms of analysing mutant mouse models.

4.4. Summary and outlook

With this work, I could successfully establish and validate a simple Object Recognition Procedure in male and female C57BL/6J mice. The resultant standard operating procedure (SOP) allows the appliance of the test in different experiments and in other laboratories. Reproducibility can be ascertained. I applied this newly developed test in three mutant mouse models and further investigated in cognitive phenotyping of these mutants by means of the Y-maze Spontaneous Alternation Test and the Social Discrimination Test. Results in working memory, social memory and object recognition memory could be obtained.

Furthermore, I validated a more time-consuming but also more profound learning and memory test in C57BL/6J and Balb/c mice, called the Food-rewarded Hole Board Test. With this test, it is not only possible to investigate working and reference memory, but also to assess learning curves and memory decline of mice.

For a spatial version of the Object Recognition Test, I would suggest an experimental setting as described by Roullet et al. in 1996. In this test procedure five objects are presented and allow the investigation of spatial and non-spatial memory in one experiment (Roullet et al., 1996). With five objects presented, both displacement (spatial change) and substitution (non-spatial change) can be applied.

Cognitive phenotyping plays an important role in the characterization of wildtype and mutant mouse lines. For precise behavioural phenotyping, I would suggest a primary screening of the mouse line with tests such as the Modified Hole Board Test or the Open Field Test. These tests provide an overview of locomotion and exploration. In a secondary screening, I would suggest tests which investigate specific behaviours such as the Y-maze Spontaneous Alternation Test, the Social Discrimination Test and the Object Recognition Test. The simple procedure underlying the Object Recognition Test allows the analysis of mutant mouse lines in object recognition memory in a relatively short time-span compared to other learning and memory tests.

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