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Learning induced neuronal activation pattern measured by c-fos expression in murine hippocampus and nucleus accumbens

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I GENERAL INTRODUCTION

The question, whether mental processes can be localized within the brain was first addressed in the early 19th century by Franz Josef Gall (1758-1828), the founder of the phrenology. He postulated a variety of discrete cognitive and behavioral functions that directly correspond to discrete areas of the brain. Although his idea that every human trait was to be localized by the form of the skull surface turned out to be wrong, he established the concept for an important feature of the brain, the principle of functional localization.

The extreme view of the phrenology aroused many doubts and initiated different attempts of a scientific rebuttal of the principle of functional localization. Pierre Flourens (1794-1867) removed functional centers of the brain according to Gall's classifications in experimental animals to isolate the different contributions. From his results he concluded that there are no discrete brain areas for specific behaviors but rather a concerted participation of the cerebral cortex in all kinds of mental functions.

These findings stood in strong contrast to the first neuropsychological studies based on lesions and brain damages in humans. Scientists like Pierre Broca (1824-1880) or Kinnier Wilson (1878-1937) correlated brain pathology of post mortem studies of patients with their behavioral or cognitive deficits. Broca localized an area responsible for the motor control of speech. Combining these results with his own identification of a sensory speech area, Carl Wernicke (1848-1905) established a theory of brain function that is known as cellular connectionism. Considering Ramòn y Cajals (1852-1934) insights in neuron anatomy he supposed that individual neurons are the signaling units of the brain. They connect in a defined way to form functional groups. Wernicke pointed out that cognition is a complex function of various components carried out by the functional groups, which can lie in different brain areas that are organized in a network interconnected via neural pathways.

Trying to investigate functional localization from the morphological side Brodmann (1868-1918) divided the cerebral cortex into 52 distinct areas based on cytoarchitectonical differences. These so called Brodmann areas still fit partially to later defined divisions of the cortex (O'Keefe and Nadel, 1978).

The debate between localized and unitary function went on for more than a century. Karl Lashley (1890-1958) still negated functional localization in the cortex. Via a maze-learning paradigm in rats he showed by applying systematic lesions that there was no direct connection of learning impairment and area. Stating the so-called 'law

of mass action' he assumed that the debilitating effects of brain damage depended more on the extent than on the locus of the damage.

The rapid increase in technical and methodical possibilities enabled an explanation for these contradictory findings. The idea that certain brain areas process and store certain memory contents was given up in favor of complex interacting spacious neuronal networks that itself represent memory. A main contribution towards this change of concept was made by Donald Hebb (1949). He postulated that memories are formed when repeated firing of connected neurons strengthens their connection by an increase of the efficiency of signal transmission on a synaptic level. At a higher level of neuronal organization, he postulated that independent neurons, which repeatedly fire in close temporal proximity, could form associations with dependency of their firing pattern (Buonomano and Merzenich, 1998). New memory contents can be stored as reorganizations of the network by strengthening or weakening existing connections or building new ones. However, the fact that neurons and neuronal ensembles can take part in several distinct networks aggravates the identification of their individual role and contribution (Fuster, 1998).

Neuroanatomical studies using retro- and anterograde tracers and various histochemical stainings where able to identify afferent and efferent connections in the brain systems (Groenewegen et al., 1987; Groenewegen and Van Dijk, 1984; Kelley and Domesick, 1982). Systematic lesion and pharmacological studies were used to distinguish the contribution of different brain areas to different behavioral functions (Aggleton et al., 2000; Buonomano and Merzenich, 1998; Cubero et al., 1999; Everitt et al., 1999; Everitt et al., 2003; Hollup et al., 2001; Moser et al., 1993; Packard and Knowlton, 2002).

In addition, biochemical and molecular biological methods were used to investigate the cellular processes involved in cognition. One important finding is that a cascade of phosphorylation and enzyme activation leading to new gene expression is required for the stabilization of information into long-term memory (LTM) (for review see Bozon et al., 2003; Sheng and Greenberg, 1990; Silva et al., 1998b; Tischmeyer and Grimm, 1999). Inducing this signaling cascade therefore links external stimulations and a cellular response that can lead to synaptic plasticity and thereby LTM. Several constituents of the signaling cascade (such as MAPK, CREB, various immediate-early genes (IEG) including c-fos) were shown to be crucially involved in memory consolidation and therefore used to correlate cognitive functions with cellular

processes (Bourtchouladze et al., 1998; Bozon et al., 2003; Cammarota et al., 2000; Guzowski et al., 2001; Izquierdo and Medina, 1997; Silva et al., 1998b). A common marker for neuronal activation is the c-fos gene. Mapping neuronal activation by c-fos expression pattern has several profound advantages. Due to its rapid and transient expression pattern, c-fos provides a precise temporal and spatial resolution frame for monitoring experience induced gene activation, which can be investigated in several brain areas in parallel. In contrast, neuroimaging techniques of Positron Emission Tomography (PET) and functional Nuclear Magnetic Resonance Imaging (fMRI) visualize neuronal substrates *in vivo* that are activated during the execution of mental functions but cannot reach resolutions down to cellular levels (Kim and Ugurbil, 1997; Raichle, 1998). Electrophysiological recordings in behaving animals allow to closely relate firing pattern with differentiated proportions of stimuli respectively behaviors, but are restricted to certain cells in a distinct area (Frank et al., 2004; Holscher et al., 2003; Lavoie and Mizumori, 1994; McNaughton et al., 1989; Taube, 1995). In addition, activity mapping by electrophysiological recordings and neuroimaging helps to show how individual cell- or cell ensemble-activity can change acutely as a direct consequence of behavioral challenges or external stimuli and how different neuronal elements operate together as an ensemble (Moser and Paulsen, 2001). However, these methods show a very acute event and until now there are just indirect conclusions on the following up processes, which lead to the transition into LTM. Several discrepancies are found, since changed firing pattern or activity related to cell metabolism not necessarily lead to a transition into LTM (Abel et al., 1997; Blair et al., 2001; Maguire, 1997; Trullier et al., 1999). In contrast, increased gene expression serves as a marker for a neuronal activation with a direct relevance for LTM and in this goes one step further than methods of neuroimaging and in vivo recordings.

Lesion studies or transgenic mouse models are other frequent tools to investigate the mechanisms underlying cognitive functions. However, both methods have consequences for the whole system of various kinds that can influence, change or mask the direct effects of the manipulation and thereby make interpretations very difficult (Freitag et al., 2003; Gerlai, 1996; Gerlai, 2000; Gerlai and Clayton, 1999; Gingrich and Hen, 2000; Lipp and Wolfer, 1998). For instance, non-hippocampal systems could take over the function of a lesioned hippocampus (Silva et al., 1998a). Such studies always just indicate the remaining abilities together with the alternative

strategies, which a disturbed system can use (Lipp and Wolfer, 1998). In contrast, the non-invasive investigation of c-fos expression reveals an undisturbed process with a normal contribution of all memory systems.

As evidence for multiple memory systems increases, the interaction between these systems and their brain areas come more and more into focus. Multiple memory systems may act independently, cooperatively, competitively or in temporal sequence (Colombo et al., 2003; Lavoie and Mizumori, 1994; Packard and McGaugh, 1996; Poldrack and Packard, 2003). The interconnection and serial circuitry can partly be addressed by disconnection lesions (Floresco et al., 1997) but deals with difficulties if areas interconnect different systems or are part of several circuitries. Localized functional measurement of transcription factors as c-fos and other signaling molecules during memory formation are useful for describing relationships among multiple memory systems during normal cognitive processes in the intact brain (Aggleton et al., 2000; Biegler and Morris, 1996; Colombo et al., 2003).

Mapping neuronal activation as c-fos expression induced by a learning paradigm enables to connect an ethologically analyzable behavior with cellular events initializing LTM storage. The behavioral analysis offers the possibility to distinguish different temporal stages to have a closer look to pattern shifts in a continuous learning process. At the same time it is possible to compare different sub-regions for different activation due to possible functional segmentation. Another possibility is to dissect different factors in learning on the behavioral level to differentiate their contributions. Factors like attention or novelty are not to separate from learning since they serve as preconditions for any kind of learning. Although no learning can take place without the animal paying attention to a novel stimulus, novelty detection by itself does not necessarily lead to a learning process (Fyhn et al., 2002; Vinogradova, 2001). Therefore it is of interest to compare novelty induced and learning induced c-fos expression.

In addition to learning, c-fos is also activated in response to sensorimotor input, arousal, stress and other stimuli (Kaczmarek and Robertson, 2002; Tischmeyer and Grimm, 1999). Thus mapping neuronal activation by c-fos is a helpful tool but has to be handled with care regarding unspecific side stimuli. Therefore an important requirement for the current PhD project was to design a learning paradigm in consideration of the best control for unavoidable side stimuli. At the same time due to the rapid expression pattern of c-fos the learning should take place within a short and

defined time-window. A spatial learning paradigm was employed to specifically address hippocampus-function (Mizumori et al., 1999; O'Keefe and Nadel, 1978; Redish and Touretzky, 1998). The hippocampus is known to play a fundamental role in a wide range of memory functions. The integrative potential of the hippocampus with its various cortical connections assign the hippocampus a key role for several functions in the memory consolidation (Alvarez and Squire, 1994; Squire and Zola-Morgan, 1991). Spatial learning especially requires the integration of many different inputs and therefore is often seen as an animal model for declarative memory (Holscher, 2003).

Due to the rather differing aspects in the aims the current PhD project was divided in two studies. The first study describes the establishing of the spatial learning paradigm and the development of a protocol appropriate for the necessities of an investigation of c-fos expression. The second study investigates the c-fos expression induced in the hippocampus and the nucleus accumbens (NAc) by relearning and novelty. The investigation focuses on these regions, since both are involved in spatial learning and suggest to have different roles in novelty-induced responses.

I.1 Declarative Memory

Knowledge, which is encoded as LTM, is generally distinguished into declarative or explicit memory and several non-declarative or implicit forms of memory.

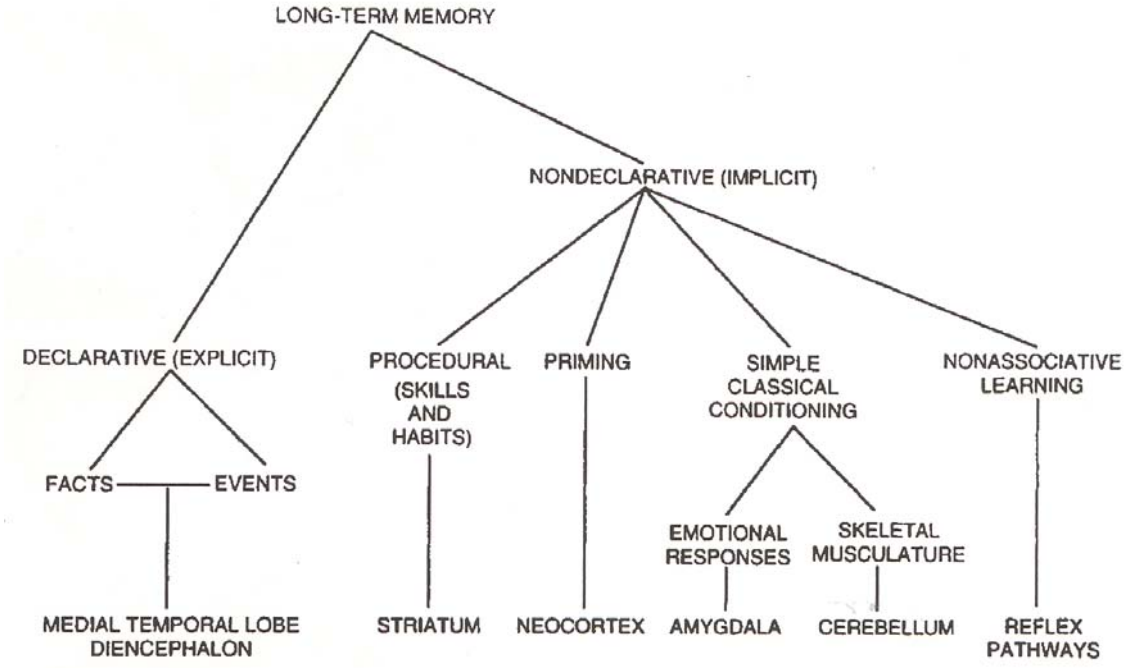


Fig. I.1: A taxonomy of mammalian memory systems
This taxonomy lists the brain structures and connections thought to be especially important for each kind of declarative and non-declarative memory (from Milner et al. 1998)

Herein the declarative memory can be recollected consciously and can be subdivided into semantic (concerning facts) and episodic memory (concerning events). Therefore the term of memory commonly used refers to declarative memory. In contrast, the non-declarative memory provides no conscious access and is characterized by changes in skilled behavior and the ability to respond appropriately to stimuli through practice. Studies on amnesic patients revealed that explicit and implicit forms of memory are governed by independent memory systems (Fig. I.1) and that the loss of either form of memory does not concern of the other. These studies also indicated that severe declarative memory loss is connected with damages in the Medial Temporal Lobe (for a comprehensive overview see Milner et al., 1998). Within this area the hippocampus was long time seen as the main region being responsible for a transition of information into LTM.

Therefore, the temporal lobe and the hippocampus attracted to investigate processes concerning memory consolidation in various different approaches, such as electrophysiological, pharmacological or morphological studies. One reason for this strong focus to the mnemonic function of the temporal lobe is certainly the case of patient H.M. suffering severe anterograde and retrograde amnesia following bilateral surgical removal of the medial temporal lobe (Scoville and Milner 1957).

That the hippocampus plays an important role in memory processes is unquestioned. Although in recent times an increasing amount of research reveled that hippocampal lesions can achieve impairments but not the total abolishment of cognitive functions. In addition, lesions in several regions surrounding the hippocampus, such as the rhinal cortices, induced similar or even more severe impairment in declarative memory functions. These findings suggested that the declarative memory system comprises a broad network of several areas, which possess allocated as well as supplementary functions for each other, so that no complete loss of function is achieved, especially in restricted lesions (for comprehensive overview see Holscher, 2003).

Since the concept of conscious recollection is central for declarative memory, difficulties appear, how this form of memory can be addressed in animal models. To refer to the episodic characteristic of this cognitive function, an important feature is a fast creation of flexible representations of temporal and spatial configurations (Eichenbaum, 1999; Milner et al., 1998; O'Reilly and Rudy, 2000; Tulving and Markowitsch, 1998).

The inference from experiences in novel situations and the relational learning of either spatial configurations or sequences of events are addressed by various different learning tasks. For instance, recognition tasks as 'delayed nonmatch to sample' tasks, radial maze tasks or contextual conditioning tasks were shown to require the hippocampus-dependent, i.e. declarative memory system (Eichenbaum, 2000; Fanselow, 2000; Hodges, 1996). Although pavlovian conditioning itself is a non-declarative memory-function, conditioning paradigms achieve hippocampus-dependency, when the animal has to remember the context, in which the conditioning took place (Gerlai, 1998; Holland and Bouton, 1999). An important role underlies the spatial learning, which comprises the integration of multimodal stimuli and landmark configurations to achieve a representation that allows localization and navigation in space. Herein the Morris Water Maze (MWM) is the most common paradigm to investigate declarative memory functions and memory consolidation (D'Hooge and De Deyn, 2001). Hippocampal dependency is also assigned to phenomena as pattern completion, which requires to substitute incomplete configurations, and latent inhibition, in which former experience is influencing associative learning (Mizumori et al., 1989; Nakazawa et al., 2002; Radulovic et al., 1998; Sotty et al., 1996).

Although some forms of learning e.g. response or habituation learning are seen as separated from the hippocampus dependent declarative memory system and assigned to an independent memory-system (Jog et al., 1999; Milner et al., 1998), many studies show a parallel and often integrated function of memory systems in learning (O'Reilly and Rudy, 2000; Packard and McGaugh, 1996; Pratt and Mizumori, 1998; Warburton et al., 2001). Many of the investigations in recent times comprise either the interplay of different memory systems, the conditions for their contributions or the time course of involvement (Gall et al., 1998; Packard and McGaugh, 1996). Others concentrate on the functional differentiation within the sub-areas of the memory systems (Floresco et al., 1997; Gall et al., 1998; Redish and Touretzky, 1998; Vinogradova, 2001; Zahm, 1999)

Two structures, namely the hippocampus itself and the nucleus accumbens (NAc) are in the focus of this study. They both are involved in the performance of behaviors that require declarative memory processing, although they are assigned for different functions in different memory-systems. In the following, both structures are described for their morphological and suggested functional features.

I.2 Hippocampus

The hippocampus is an archicortical brain structure that due to the relative simplicity of neural architecture and stratified axonal projections represents an attractive goal for investigations in several fields of neuroscience, e.g. for neurophysiological and neurochemical experiments (Bliss, 2003).

The hippocampus can be subdivided into the *cornus ammoni* regions CA1-3, often called hippocampus proper, and the dentate gyrus (*fascia dentata*, DG). The often found term 'hippocampus formation' includes additionally the subiculum, the entorhinal cortex (EC) and in some nomenclatures even the pre- and parasubiculum (the later three as multilaminate structures are also termed parahippocampal cortex in differentiation to the allocortical areas with less than six layers, see Amaral and Witter, 1995).

The hippocampus itself is a three-layered structure. The middle layer contains the principal cells and, corresponding to their cell types, is called pyramidal cell layer or *stratum pyramidale* in the CA regions and granule cell layer or *stratum granulosum* in the DG.

The principal cell layers are surrounded by layers, which contain relative few cells and mainly the dendritic and axonal fibers (*stratum oriens* and *stratum lacunosum-moleculare*, *stratum radiatum*, *stratum lucidum* in the CA, *stratum moleculare* and polymorph layer in the DG). A majority of these non-pyramidal and non-granule cells are GABAergic and take part in a local recurrent feedback-loop (for more details see Amaral and Witter, 1995; O'Keefe and Nadel, 1978)

The main pathway through the hippocampal formation is seen in the so-called trisynaptic circuit. In this circuit axons from the EC enter the hippocampus through the perforant path (*tractus perforantis*) ending in excitatory glutamatergic synapses on the granule cells of the DG. The axons of the granule cells form the connection termed mossy fibers with the pyramidal cells of the CA3 region. The axons of the CA3 pyramidal cells split into efferents, which exit the hippocampus via the fornix to the contralateral side, and the so-called *Schaffer Collaterals* that prolong the circuit towards the CA1 pyramidal cells. The CA1 pyramidal cells themselves send efferents to the subiculum and the EC. Like this the hippocampus shows a kind of feedback loop since the projections come from and go to the EC and the EC itself receives and sends projections from various areas of the association cortices.

The serial processing via the trisynaptic circuitry is not the only important connectivity within the hippocampus. Since the sub-regions of the hippocampus receive in addition direct inputs from the EC, the hippocampus allows parallel and serial information transfer to most of its fields. In case of the CA1 region this system provides for monosynaptic, disynaptic and trisynaptic influences of CA1 neurons by the same entorhinal activation. Therefore this region can compare and integrate information coming from the EC that is differentially processed and modulated depending on its way through the hippocampal formation (Amaral and Witter, 1995).

Apart from the trisynaptic circuit described above, there are other important projections in the hippocampal formation. The septal projections arising from the medial septum/diagonal band run to the DG and to the CA1. They enter the hippocampus mainly via the fornix and fimbria.

Additional projections provide dopaminergic input from the ventral tegmental area (VTA) and the substantia nigra as well as input from amygdala, hypothalamus and anterior thalamic nucleus. The hippocampus itself is directly projecting to the NAc and back to the amygdala and anterior thalamic nucleus (Gasbarri et al., 1994; Goldsmith and Joyce, 1994; Roozendaal et al., 2001; Taube and Muller, 1998).

Additionally to the extrinsic afferents that reach the hippocampus, the cells are connected to several types of intrinsic afferents, which can come from cells of the same sector, from other sectors and commissural afferents from the contralateral hippocampus (O'Keefe and Nadel, 1978). The intrinsic afferents comprise inhibitory as well as excitatory circuits. Especially in the CA3 field are seen many direct monosynaptic excitatory connections between the pyramidal cells. This strongly build field of recurrents suggests a function for the CA3 region as an association field (Amaral and Witter, 1995; O'Keefe and Nadel, 1978).

Another important feature of the hippocampus is the fact that the connectivity in the dorsal and ventral part is rather different (Moser and Moser, 1998). Lesion studies as well as activation pattern find several differences in impairments and responses of these parts of the hippocampus (Bertaina-Anglade et al., 2000; Korzus, 2003; Moser et al., 1993). Several researchers suggest that the dorsal part is more strongly involved in spatial learning while the ventral part is important for motivational and emotional aspects, in line with its strong connectivity to the basolateral amygdala (BLA) or NAc (Moser and Moser, 1998; Zahm, 1999) as well as the distinct

distribution of receptor types related to reward, emotional or stress responses (opiate, dopamine or corticosteroid receptors, see Holscher, 2003 for overview)

The various connections with sensory sources as well as association cortex regions clearly assign the hippocampus a role in integration of different types of information. Though a specific function of the hippocampus remains less clear. Hippocampal dysfunction shows its involvement into different processes. The selective attention becomes unstable and highly sensitive to interference from irrelevant stimuli (Vinogradova, 2001). Even more in focus is the disturbed transfer of information into LTM storage. Several different functions are suggested for the different forms of learning as well as for its contributions as part of a memory system.

The finding of principal cell of the CA proper that fire with respect to the position of the animal in space (O'Keefe and Dostrovsky, 1971) stressed the meaning of the hippocampus for spatial learning. However, since other cells are found to react not simply to position in space but to several factors as heading direction or even in relation to reward expectation, the function certainly extents the suggestion that the hippocampus serves a simple mapping of the spatial surrounding. Investigations e.g. in darkness, revealed that the 'place cells' in addition show firing correlates in respect to information about moving speed, directionality, smell or timing. It was shown that neurons in the hippocampus associate spatial and task relevant information and change their firing pattern, when different forms of learning take place in the same familiar spatial surrounding. Other cells reveal firing correlation to matches or mismatches of expected reward locations (for comprehensive overview see Holscher, 2003).

In addition, lesion studies revealed that situational or environmental changes influence the activity of place cells differently in the sub-areas of the hippocampal formation. However, lesion studies restricted to hippocampal sub-areas revealed differential effects on places cell, as well as distinct impairments in learning (Lee and Kesner, 2003; Mizumori et al., 1989).

Cells that reveal place or directional codes of firing are reported from various brain regions, e.g. the striatum, anterior thalamic nucleus (ATN) or amygdala. (Lavoie and Mizumori, 1994; Pratt and Mizumori, 1998; Taube, 1995).

However, a striking difference compared to hippocampal place cells is that these cells almost immediately change their firing pattern after environmental changes, while around half of the hippocampal place cells maintain their place fields or reveal

delayed changes (Mizumori et al., 1999). The maintenance and delayed changes suggest a function for the hippocampus, to integrate memory-driven expected with current spatial context information. This comparison could, in turn, account for the ability to generalize across spatial situations.

Comparative functions of the hippocampus also suggest the anatomical features with its largely unidirectional transverse loop of excitatory pathways coming from and going to the EC, together with the large recurrent fiber field of the DG and CA3 region (Amaral and Witter, 1995).

Many authors support the idea of hippocampus as comparator of current and memorized states (Fyhn et al., 2002; Lee et al., 2004; Mizumori et al., 1999; Moser and Paulsen, 2001; Vinogradova, 2001; Wallenstein et al., 1998). As this the hippocampus receives a functional meaning for detection of novelty. However, Vinogradova (2001) suggests that this novelty detection is not a simplistic notice of new, but concerns the filtering of relevant information. Even small but relevant changes compared to a stable background can elicit stronger responses of the hippocampus than a novelty that is not or not yet associated to former experience. This concept is supported much by investigations that compare the response to different forms of novelty, as Wan et al. (1999) reporting of hippocampal activation by experience of familiar items in novel arrangements but not by a novel item itself.

Many investigations suggest that the sub-regions of the hippocampus play a differential role. Especially the differences in connectivity in the sub-areas suggest a functional distinction. The recurrent excitatory loops predestine the CA3 for an associative function that may allow the comparison of expected and current spatial context within a dynamical process for changing information during movement (Mizumori et al., 1999). This goes in a similar direction as suggestions of Redish and Touretzki (1998), who see a function of the CA3 region in self-localization and routing, therefore the dynamic comparison of spatial position in movement. Others suggest a meaning for filtering relevant sensory inputs by matching old and new inputs and thereby finding relevant changes in the surrounding (Vinogradova, 2001). The CA3 region reveals more specific place fields of the place cells compared to the CA1 and at the same time more sensitivity to environmental changes (Mizumori et al., 1999). Lee et al. (2004) suggest a key role for the CA3 in rapid formation of spatial representations of new spatio-temporal sequences. In addition the collateral fiber system allows the embodiment of computational processes in pattern

completion and sequential learning (Lee et al., 2004; Nakazawa et al., 2002). Investigations of different researchers indeed show that the CA3 has a crucial role in early stages of new learned tasks (Bertaina-Anglade et al., 2000; Gall et al., 1998), therefore at time points, when the impact of new surroundings and situations is large.
Also the CA1 region is often assigned a function in finding matches and mismatches. Especially since it receives information of the same regional source (EC) directly and after processing and thereby modulation by DG and CA3 (Amaral and Witter, 1995). This suggests that the CA1 region may have the capacity to compare incoming sensory information from the EC with information stored within the hippocampus and respond to incongruities between these sources of information (Fyhn et al., 2002; Lee et al., 2004).
However, the CA1 region reveals a lesser focus on spatial representation. CA1 place cells fire with less spatial selectivity (Mizumori et al., 1999) and many of them reveal more complex firing patterns with multimodal discharges. Therefore the CA1 comprises many cells that discharge corresponding to task-relevant features, reward-expectation, expectation mismatch and other, non-spatial correlates (Holscher et al., 2003; Moser and Paulsen, 2001; Wood et al., 1999). Mizumori et al. (1999) suggest a role for CA1 in the temporal organization of hippocampal efferent messages for a patterned output of the relevant discrepancies found e.g. in the CA3 comparisons. The special meaning as output structure of the hippocampus is as well stressed by findings of Lee and Kesner (2003) showing that CA1 lesions exclusively elicit impairments in a non-matching-to-position task with long delays of 5 min. In contrast, lesions in the recurrent areas of the CA3 and DG as well reveal impairments in short delay (10 s) versions. They suggest that the CA1 coordinates between the recurrent areas of CA3 and DG, which keep information for short delays, and extra-hippocampal regions as requirement for longer delays. The involvement of the recurrent regions in more acute or dynamical processing fits as well with the concept of a dynamical self-localization for the CA3, as introduced before, and a pattern separation or filtering function for the DG.
Anatomical investigations indicate that the afferents reaching the DG are very widespread in comparison to the efferents the DG sends towards the CA3 region, which are spatially and numerically limited (Amaral et al., 1990). Inputs running from the EC towards the CA3 region receive a secondary simplification resulting in a less variable and selective patterning for the outgoing signals (Vinogradova, 2001).

Though the concept of the trisynaptic loop for successively processing and refinement of information from the EC over the DG towards the CA1 region is revised in its strict sense. An important contribution herein was that the clear and reliable spatial selectivity that is found especially in CA3 and as well in CA1 pyramidal cells is missing in DG granule cells. The fact that the EC projects also directly to CA3 and CA1 raises the question if the DG at all takes part in the direct assembly line for spatial representation (for overview see Barnes et al., 1990). In accordance with this, the place fields of CA3 and CA1 were unchanged by lesion of the DG (McNaughton et al., 1989). However, at the same time the spatial learning performance in the MWM was impaired due to this lesion, which proves that the DG is required for spatial learning in another function than creating the spatial representation via place cells.

The segregation from the CA proper is as well supported by reports for different mechanisms for long-term potentiation (LTP) in the CA regions and the DG (Tecott et al., 1998) and an opposite effects of transgenic enhanced potential for LTP for performance in the MWM (Okada et al., 2003). Okada et al. (2003) report that enhancing LTP via transfection with AMPA receptors leads to enhanced performance, if applied to CA1, but to impaired performance for the DG. This finding may stress the meaning of inhibitory functions within the DG. Recent evidence points to an important role for interneurons in mediating synaptic changes and plasticity (Paulsen and Moser, 1998). Moser et al. (1996) show that in an exploration task different subsets of interneurons within the DG reveal differential inhibition pattern in time course and strength. They suggest an inhibitory control of impulse flow through the DG when unfamiliar information enters the hippocampus. The integration of exciting and inhibiting signals could serve the attenuation of background noise with parallel focus and amplification of newly incoming inputs. The hippocampal function, stressed by Vinogradova (2001), for selective attention with inhibitory control protecting the processing of information from interference, may therefore be attributed to the DG region.

An inhibitory form of control may also explain the exclusively strong activation of the DG in the latent inhibition phenomenon, a form of attenuation of conditioning learning due to pre-exposure of the conditioning stimulus (Sotty et al., 1996).

The ventral part of the hippocampus is often seen as less critical for spatial learning, due to findings of lesions comparing the dorsal and ventral hippocampus, as well as

the differences in anatomical connectivity or in the nature of their place cells (Jung et al., 1994; Moser et al., 1993; Moser and Moser, 1998). Jenkins et al. (2004) found activation to novel spatial arrangements in the rostral part but not the caudal part of the hippocampus. Since the current study investigates specifically the involvement in spatial learning, the ventral part of the hippocampus was not taken in consideration.

I.3 Striatum and nucleus accumbens

The striatum serves as the primary entrance structure of the basal ganglions. This group of anatomically delimited subcortical nuclei form a functional unit, which coordinate the selection, adaptation and initiation of voluntary motility in a context-dependent manner. This planning of movements includes the integration process of sensimotor information with motivational and emotional aspects.

The basal ganglia are part of a functional loop system that includes the thalamus and the cerebral cortex. Cortical signals arrive in the striatum and are send via two differently processing ways to the output structures (*substantia nigra pars reticulata* and *entopeduncular nucleus*). They project to the thalamus that itself influences the cortical activity (Alexander and Crutcher, 1990).

An important feature of the signal processing in the basal ganglia represents the neuromodulation by dopamine that is released by the neurons projecting from the *substantia nigra pars compacta* and the ventral tegmental area (VTA). A special meaning has the dopamine modulation in the striatum where it can have inhibitory and excitatory influence depending on the type of neuron it reaches. In this function the striatum plays a central role as a main integrator for various information due to the afferents from different transmitter systems that coverge at the same neurons (Smith and Bolam, 1990).

The telencephalic *striatum* itself can be subdivided functionally and anatomically into a dorsal and a ventral part. In primates the dorsal part is separated by the fiber tract of the *capsula interna* in the two well-defined structures *nucleus caudatus* and *putamen*. In all other mammals the missing fiber tract leaves a uniform dorsal striatum called *caudate putamen*.

For a long time the meaning of the striatum for the coordination of locomotion was in the center of interest due to the connection to pathological phenomena occurring in the striatal area that had most obvious symptoms concerning motoric disturbances. Though many of the locomotor controlling effects result of an integration of

information coming from the different functional loops meeting in the striatum, therefore integrating motivational, emotional and motoric processing. More recent findings indicate a dorso-striatal contribution as well in learning, e.g. in the hippocampus independent response learning (Packard and McGaugh, 1996).

The ventral part of the striatum consists of the nucleus accumbens (NAc) and the *tuberculum olfactorium*. The NAc itself is subdivided into the more dorsolaterally located core and the more medioventrally located shell due to its neurochemical and connective properties. Sometimes the rostral pole is distinguished as third subdivision, where the shell and core are not yet separated (Zahm and Heimer, 1993).

The core reveals many parallels in its connectivity to the dorsal striatum and with the typical projections to the basal ganglia. In contrast, the shell exhibits greater neurochemical and neuroanatomical diversity and input from 'limbic' cortical areas, such as hippocampal formation, BLA, prefrontal cortex (PFC) and rhinal cortices in addition to afferents from several thalamic nuclei and the VTA. As well the efferents of the shell region are less typical for striatal connectivity and more typical for the extended amygdala (for overview see Setlow, 1997; Zahm, 1999). Via this connectivity the shell appears to have the receptive capacity for current and remembered stimulus-reward associations (Zahm, 1999). In addition, a dopamine increase was found with initial entry into a novel environment only in the shell, which supported the involvement of this sub-region in the novelty-induced behaviors as increased locomotion and exploration (Rebec et al., 1997).

A special meaning of the NAc regarding spatial learning is seen by the integration of spatial information coming from the hippocampal memory system and the information about reward expectations and cues for signifying reward coming from VTA and BLA (Lavoie and Mizumori, 1994; Pratt and Mizumori, 2001; Redish and Touretzky, 1998). Recent investigation repeatedly showed that manipulation within the nucleus accumbens could influence the performance in spatial learning paradigms (Annett et al., 1989; Gal et al., 1997; Ploeger et al., 1994; Setlow, 1997; Smith-Roe et al., 1999). Whether this influence is governed by a contribution of an independent memory system that concerns more the procedural contributions of the learning process or by modulations within the hippocampal memory system, e.g. mediated by glucocorticoids as suggested by Roozendaal et al. (2001), is not clear (Sargolini et al., 2003).

II BEHAVIORAL INVESTIGATION OF THE CIRCULAR MAZE (CM)

II.1 Introduction

Learning can be defined as any change in behavior in response to the environmental situation and therefore as adaptation to changes in the environment. The behavioral response is an externally observable phenomenon expressed by a system that integrates many processes. These processes depend on the external and internal conditions of the animal and are influenced by experience and expectations.

To investigate learning processes, paradigms are performed that create a situation that should provide controlled learning conditions with similar preconditions for the animals. However, a learning paradigm can address different forms of learning depending on the conditions it presents and the demands it requires. Since the current study focuses on hippocampus dependent learning processes, the paradigm was chosen to address spatial learning as a model for rodent episodic memory.

As mentioned before, declarative memory and thereby hippocampus-dependent learning was always a central field of investigations concerning memory processes.

However, there are several different debates on the hippocampus-dependent memory system. One concerns the differentiation of declarative memory into episodic and semantic memory, seen as more or less independent to each other. While a declarative theory strongly connects the semantic and episodic memory and therefore argues that hippocampal impairment concern both forms of memory, the episodic theory strongly supported by Tulving suggests episodic memory as specific extension of semantic memory therefore including the possibility of impairments in episodic coding sparing semantic coding but not vice versa (for review see Tulving and Markowitsch, 1998). Tulving and Markowitsch (1998) as well state that true episodic memory is exclusive for humans and is strongly connected with lingual abilities.

The more general definition as encoding of spatio-temporal relations allows the extension of episodic memory functions to animal models and their investigation by cognitive functions such as spatial or contextual learning.

However, since behavior is a final output of all ongoing cognitive processes in a certain situation, different memory systems naturally operate together to support the great majority of behavioral performances. Thus the animal can often respond to learning situations with different strategies that involve different memory systems to

different degrees. E.g. in a MWM the animals can search the submerged platform by a hippocampus-dependent spatial strategy, approaching it directly, or by a more procedural strategy, such as circling in a certain distance to the walls, which is a non-declarative form of learning (Hodges, 1996). Thus it is crucial to create task conditions that require the declarative memory - in this case spatial learning - abilities and to control that the animals indeed use this form of memory with the corresponding strategy to solve the task (Eichenbaum et al., 1992; Hodges, 1996).
Therefore, in this study a Circular Maze (CM) paradigm was established, which provided task conditions for spatial learning and in addition the possibility to analyze the performance and chosen strategy via several behavioral parameter.

II.1.1 The circular maze as hippocampal learning paradigm

Hippocampal lesion studies together with the finding that principal cells in the hippocampus fire in correlation to the position in space (so called place cells) supported the function of the hippocampus in spatial learning. In many cases spatial learning is performed in different forms of maze learning paradigms (Hodges, 1996). Other important paradigms that address the spatial aspect of hippocampus-dependent learning are conditioning paradigms that test contextual memory abilities. As in the case of spatial maze learning contextual memory is seen as higher cognitive function with multi-factorial integration of different stimuli concerning spatio-temporal configurations of the environmental features, such as configuration of landmarks. Classical paradigms testing contextual memory are fear conditioning and passive or active avoidance paradigms. These paradigms deliver an electrical foot-shock (unconditioned stimulus), which is to associate with a neutral stimulus e.g. a tone (conditioned stimulus) or a neutral behavior e.g. exploratory behavior (conditioned response). In addition to the high stress component connected to the foot-shock, mice are confronted with an extremely artificial situation, which reveals few considerations of the natural adaptation or response repertoire of the animals.
However, the necessity of using ethologically relevant behavioral tasks instead of 'nature blind' ones is stressed by many researchers (Gerlai and Clayton, 1999; Lipp and Wolfer, 1998). This especially is important since behavioral relevance influenced the learning abilities of the animals. A related danger lies in the misinterpretation of displayed behavior. Animals may pay attention to stimuli, which seem irrelevant to the experimenter, or ignore scheduled ones. Other behavioral factors may influence

the behavioral output to a larger degree if learning paradigms ignore the behavioral ecology of the species, which may in consequence be less sensible for differences in the addressed abilities (for review see Gerlai and Clayton, 1999). This is especially true for paradigms such as the fear conditioning, where many experiments revealed inconsistent or contradictory results due to difficulties in behavioral interpretation or the strong influences of non-mnemonic factors as emotionality or activity-status of animals (Bach et al., 1995; D'Hooge and De Deyn, 2001; Gerlai, 2001; Holland and Bouton, 1999; McNish et al., 1997)

Altogether, to achieve a certain ethological relevance that takes in consideration the natural behavioral repertoire and species-specific behavioral characteristics is an important feature for experimental designs in an anyway artificial situation of laboratory conditions. For instance, spatial maze learning paradigms achieve some ethological relevance demanding the animals to orientate in search for food or safe places in a spatial surrounding, which is typical in rodents live (Floresco et al., 1997)

The MWM is a spatial learning paradigm that is used in many investigations as standard procedure. It offers the advantage that in the water-filled arena olfactory cues or intra-maze cues, which would interfere the spatial orientation with extra-maze cues, are ruled out (Morris, 1984). The confrontation with the water lays a strong force for reaction and moving (Gerlai and Clayton, 1999) and ensures a motivation without food deprivation (Morris, 1984). Although the overall approach is ethologically based, this paradigm was designed for rats, a species, which is confronted with water in their normal habitat, whereas mice are typical inhabitants of dry grassland areas. Many researchers describe the weak performance of mice in MWM compared to rats (Gerlai and Clayton, 1999; Morris, 1984; Whishaw and Tomie, 1996). The search for a platform is herein not the most natural behavior and learning performance can be interfered since the animals first have to be trained not to search a direct escape from the pool near the walls but via reaching a platform from which they get rescued (Deacon and Rawlins, 2002). In addition, the swimming puts very high physical demands on mice and increases the stress-releasing component of this maze (Crawley, 2000; Deacon and Rawlins, 2002).

A dry variant for a spatial learning paradigm, which offers the same flexibility to test different components as cue and spatial learning (Morris, 1984), is the Barnes Maze. This paradigm was repeatedly shown to be specific for hippocampus dependent learning (Bach et al., 1995; Barnes, 1979; Mansuy et al., 1998). In parallel to the

MWM the mice can use extra-maze landmarks or in an alternative design a direct visual cue to find the goal and thereby escape a circular arena. The goal in the Barnes Maze consists of a dark escape box underneath the platform, reachable through a hole. The low physical demands even allow the investigation of senescent animals (Barnes, 1979; Pompl et al., 1999). In addition the exposure to an open field causes much smaller increases in corticosterone and corticotropin than swim stress (Sternberg et al., 1992).

The Barnes Maze offers a choice of parameters to investigate the spatial performance of mice. In contrast, the MWM observes the acquisition of learning normally via the escape latency, though this parameter is influenced strongly by factors independent of orientation, as physical abilities or motivation (Contet et al., 2001; Hodges, 1996). Upchurch and Wehner (Upchurch and Wehner, 1988a; Upchurch and Wehner, 1988b) suggest that measurements of latency are not a good measure of cognitive performance for the MWM in mice, since mice tend not to take the direct path to the hidden platform as rats. E.g. Inman-Wood et al. (2000) suggest attentional alterations as reason for prolonged latencies in the first two days of acquisition, in this case in a Barnes Maze with prenatal-cocaine-treated mice. In the Barnes Maze the learning performance during acquisition can alternatively and more directly be observed by errors the mice do or the searching strategy the mice use (Bach et al., 1995; Fox et al., 1998; Inman-Wood et al., 2000; Mansuy et al., 1998).

The circular maze in this study is an adaptation of the Barnes Maze. The main alteration is given by a tunnel leading into the home-cage of the animal instead of an escape box beneath the platform. By this change the task takes advantage of the high motivation of the animals to escape an unpleasant situation into the safety of the home environment. Therefore the set-up can abstain additional aversive stimuli as fans or buzzers, which are used to increase the motivation to search the escape box (Bach et al., 1995; Pompl et al., 1999).

Low aversive pressure leaves the option for different performances and therefore a variance in behavioral response that is naturally expressed in complex behaviors. Behavioral complexity is especially important since simplifying procedures (as in conditioning paradigms) do not allow the observation and score for multiple behavioral variables that may separately be linked to specific behavioral traits and the resulting simplified behavioral responses do not adequately represent the

complexity of the influencing factors that caused it (Kaczmarek and Robertson, 2002).

II.1.2 Aims of the study

The main purpose for establishing a spatial learning task in this study is the application for an investigation of c-fos expression pattern. Since c-fos expression is a very general marker of activation that can be elicited by several different stimuli in addition to learning the requirements for the learning paradigm are very specific. The task must reliably induce a pronounced learning process under conditions with minimal unwanted side stimuli and maximal control of those factors that influence gene expression. It is important to provide high similarities in the preconditions of all animals and to form control groups with high comparability to the experimental groups.

The CM by itself serves for an appropriate ethological relevance in task demands and form of reward as prerequisite to achieve a reliable spatial learning process. In addition the possibility to avoid aversive and physically demanding conditions minimizes the influence of factors that can interfere the learning-induced c-fos expression. The various behavioral parameters enable a detailed behavioral analysis of the progressing learning process under different conditions. This is important to ensure that spatial strategies are used and to identify conditions for a protocol that obtains high similarity and comparability between mice and groups, which are necessary for the c-fos investigation.

II.2 Animals & Methods

II.2.1 Animals and husbandry

The animals were raised in a breeding facility under pathogen free conditions. After transferring the animals to a different animal facility they were maintained in an inverted 12 h **:** 12 h light **:** dark cycle (light on at 19:00) under standard housing conditions with 21 ± 1°C, 40-50 % humidity, food and water *ad libitum*. They were single-housed in 22 x 16 x 14 cm cages for at least two weeks before behavioral testing to allow them to adapt to the changed conditions. Behavioral tests were performed within the middle 8 h of the dark period of the animals. The experimental

room, which was situated next to the animal facility, was illuminated with red light except for the experimental setup itself (see below). Three separate experiments were conducted with three different batches of animals.

Experiment 1 - Establishing CM: At the age of 3 - 4 months 14 male C57Bl/6J mice were transferred to the animal facility for the training in a circular maze.

Experiment 2 - Relearning CM: A group of 19 FLZ (fos-lacZ transgenic) mice consisting of 10 females 6 - 10 months old and 9 males of 4 - 6 months age was transferred to the animal facility for training in a circular maze.

Experiment 3 - Reinforced Relearning CM: 47 male C57Bl/6J mice with an age of 2 ½ - 3 months were transferred to the animal facility in three batches. 42 animals were trained in a circular maze, the additional 5 mice served as cage controls for the morphological study and therefore remained naïve.

The FLZ mice originated from a breeding line of transgenic mice carrying an additional fos-lacZ reporter gene expressing β-Galactosidase governed by the regulator region of the c-fos gene (Smeyne et al., 1992). The animals were at least 4 generations backcrossed into the C57Bl/6J breeding line.

II.2.2 Description of the circular maze set-up

The circular maze consisted of a circular platform 100 cm in diameter made of white PVC elevated 1.20 m from the ground on a rotating stand. Either 36 holes (Fig. II.1A) or 12 holes (Fig. II.1B) were regularly spaced in a row close to the rim with a distance to each other of either 4.2 or 19.6 cm, respectively. The holes were 3.5 cm in diameter located in a distance of 4 cm to the rim. To achieve the 12-hole platform the 36-hole platform was covered with an equally sized 1 mm thin PVC sheet matching every third hole of the platform (for the Novelty Exploration Task (NET) a sheet without holes was used, Fig. II.1C see chapter III.2.3).
All holes, except for one, led into a dead end wire grid tunnel. These were accessible for 3 cm and closed with a wire grid wall. One hole led to the 'escape tunne', a 50 cm long freely accessible wire grid tunnel. It led into the home-cage that was placed on a board in the center beneath the maze platform. The hole leading into the escape tunnel is from now on referred to as the 'Target'. The escape tunnel could be fixed to

every hole. Since the platform could be rotated it allowed the use of different holes as Target in a fixed position (Fig. II.1D).

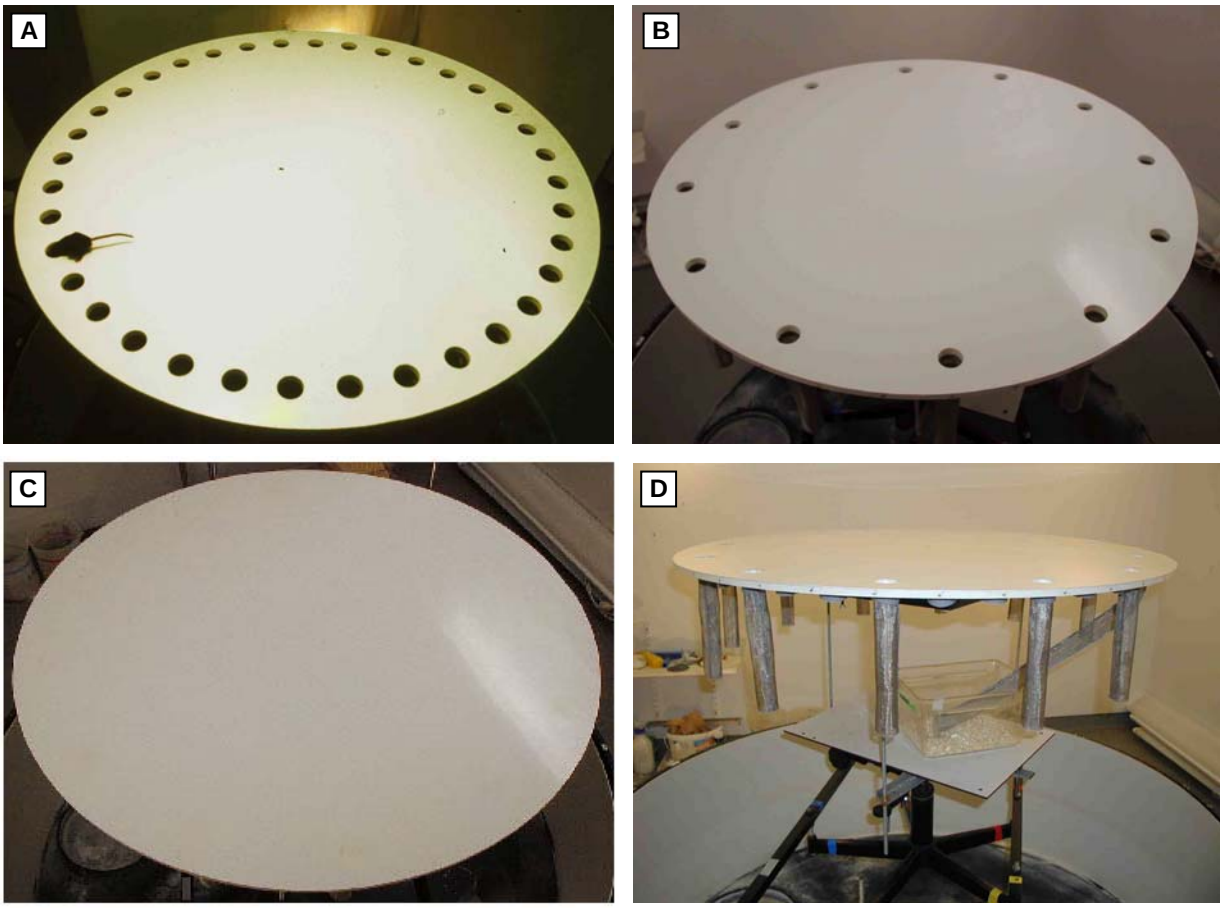


Fig. II.1: The Circular Maze apparatus
The three different platform versions with 36 holes **(A)**, 12 holes **(B)** for the CM experiments and without holes **(C)** for the NET are all mounted on a rotatable stand equipped with a board to place the home-cage **(D)**. For the CM experiments dead end grid tunnels and an escape tunnel leading into the home-cage are fixed beneath the holes.

The maze was placed in the center of a room (3.5 x 3.5 m, one corner cut-off by a curtain) with white walls provided with 5 visual cues serving as extra-maze landmarks. These prominent landmarks represented approximately 50 x 60 cm sized distinctive shapes colored in black or with a high contrast black and white pattern placed at a height of about 150 cm.

In some of the maze tests the edges of the room were covered with 20 cm broad black cloth stripes in order to stress the asymmetric shape of the room (Novelty Exploration Task, Reinforced Relearning CM). To mask the landmarks for a visible cue test or for working with a new set of landmarks it was possible to surround the platform with a dark brown curtain hanging in a square with a distance of 40 cm, 30 cm, 70 cm and 80 cm to the maze. The maze was brightly illuminated (500 lux) with two neon lamps hanging above it. To introduce the mice onto the maze an

opaque Plexiglas cylinder of 10 cm in diameter and 15 cm in height was used. The platform was cleaned with 70 % ethanol before every session. In-between trials within a session the platform was rotated and a new hole used as Target. Thus animals were prevented from using olfactory tracks or intra-maze cues to find the Target. Wire grid tunnels at every hole were used to avoid that the smell of the home-cage funneled through the escape tunnel and guided the mice.

A video camera, hanging above the center of the maze, was connected to a video recorder and a computer located in the adjacent room. In addition to the live observation via monitor all trials were video recorded and analyzed post training with the video tracking system EthoVision.

II.2.3 Wire grid Habituation

To habituate the animals to the wire grid tunnels, a wire grid tube of 12 cm length and 6 cm diameter was placed into their home-cage for one week before the experiment started. The animals often used the inside of the tube as resting place. Habituation to the wire grid was just applied to mice trained in a CM.

II.2.4 Visible Cliff Task

The visible cliff task was used to test the mice for visual deficits or poor attention to visual stimuli (Brandewiede et al., 2004). The apparatus consisted of a wooden box (50 x 50 x 80 cm). A platform (50 x 25 cm) was installed adjacent to a wall in 50 cm height above the floor, which covered half the box area, leaving a physical cliff along the centerline. Platform and inner surface of the box were covered with black and white checkerboard pattern to emphasize the ledge drop-off. A piece of clear Plexiglas in the size of the box (50 x 50 cm) covered the platform and spanned over the ledge, so that there was no physical drop-off but only a virtual cliff. The apparatus was illuminated by 10 lux and recorded by a video camera installed sideways above facing the platform side.

Two protocols were used on two consecutive days. On day 1 the mouse was placed onto the platform close to the wall opposite the cliff with the head facing the wall. The mouse was then observed exploring the box. Mice that immediately crossed the cliff showing neither hesitation nor risk assessment were considered to have bad sight or pay not enough attention to visual stimuli. On day 2 a 10 cm square gray platform

was placed on the Plexiglas part, so that three sides of the platform were bordering the cliff. This was leaving access to "safe ground" just at one side. The mouse was placed into a cylinder on the center of the platform and after lifting the cylinder the side to which the animal stepped-down was observed. Animals choosing the "safe" side towards the checkerboard platform to step down were considered to have good sight and pay attention to visual stimuli. The Visible Cliff Task was performed with all animals except those used for Establishing CM. None of the animals showed deficits in sight or attention. Therefore all were considered to be able to use a visual stimulus for orientation and were used for the different maze trainings.

II.2.5 Pre-training

In all experiments (with the exception of the Establishing CM) the animals underwent a Pre-training to get used to enter the wire grid tunnel through a platform hole to escape into the home-cage. For Pre-training a square platform (30 x 30 cm) was used with just two holes located at diagonally opposite corners 15 cm apart. One of the holes led to a dead end tunnel, the other to an escape tunnel running into the home-cage. The home-cage was placed next to the platform, so that it was visible for the animal but just reachable through the escape tunnel.

The Pre-training consisted of three successive trials on two consecutive days. The mice were placed into an opaque Plexiglas cylinder (10 cm in diameter, 15 cm in height) situated in the middle of the platform. After lifting the cylinder the mouse could explore the platform and enter the hole within a maximal trial duration of 3 min. If the animal did not enter the hole by then, it was placed into the hole by hand. Usually this was just necessary, if at all, in the first trial of the Pre-training, when animals had not yet experienced that the tunnel led into the home-cage.

II.2.6 Establishing Circular Maze (ECM)

The ECM experiment consisted of different training phases serving different purposes (for overview see Fig. II.2):

1. Visually cued Target CM task: Training with a visually cued Target to accustom the mice to the escape tunnel and check their visual abilities (5 days of training)
2. Spatial CM task: Training animals to orientate with extra-maze landmarks
    a. Learning: First training to a fixed Target position (28 days of training)

b. Relearning 1: Training to a new Target position with unchanged environmental surroundings (9 days of training)
c. Relearning 2: Training to another new platform position with changed environmental and landmark surrounding (8 days of training)

Step Down Avoidance Task: Training in the hippocampus dependent one trial learning task to test if there is a correlation between the performances in the CM and the Step Down Avoidance Task.

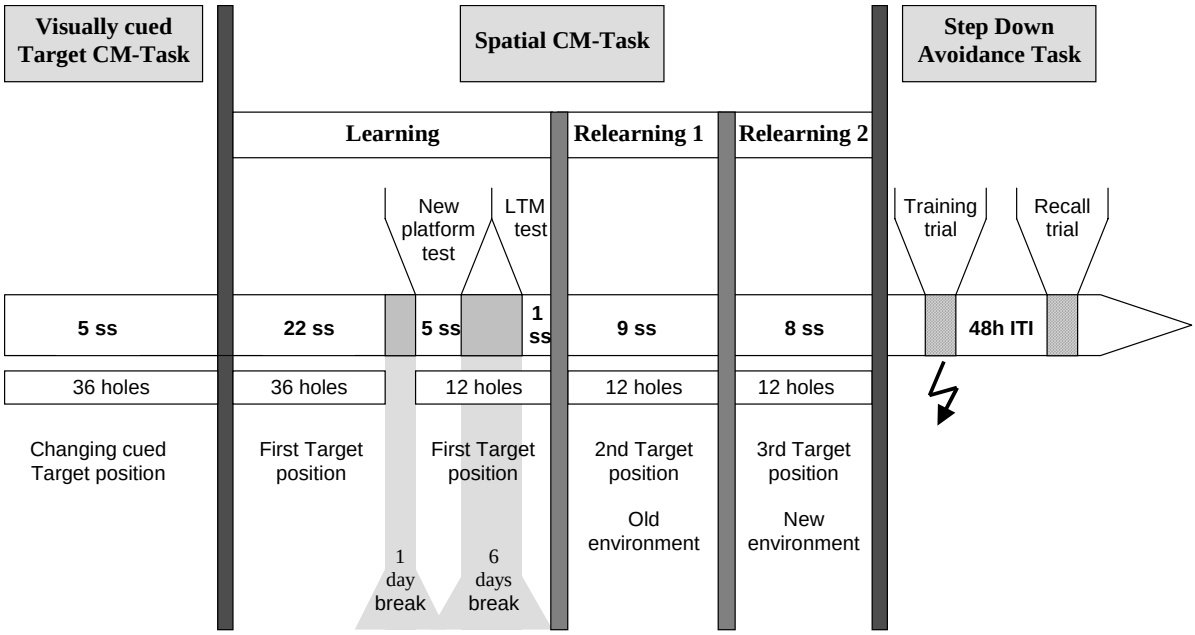


Fig. II.2: Overview for the different training phases of the ECM
The ECM experiment is depicted with all training phases. A time scale arrow indicates the duration of the different training phases. Beneath the time scale arrow the corresponding platform version and training conditions are indicated. LTM=long term memory, ss = sessions, ITI = inter-trial-interval, ⇨ =Time scale arrow, ▭ =CM training break, ▨ =SDA trial, ↯ electrical shock.

II.2.6.1 Visually cued Target CM task

The animals of this first CM were not subjected to the Visible Cliff Task or the Pre-training. Instead they started training with a visible cue at the Target, which served both to habituate them to the rewarding effect of the escape tunnel and to show their ability to use a visual stimulus for orientation. The animals were trained on a platform presenting 36 holes.

On the first day the mice were trained on the platform with an escape tunnel fixed on top of the hole, thereby leading visibly into the home-cage. Animals trying to climb on top of the tunnel were forced back onto the platform. After a maximal trial duration of

15 min mice that did not enter the tunnel were placed into it by hand. The session consisted of four consecutive trials.

On the following day, the escape tunnel was fixed beneath the Target hole but the Target was marked by a salient cue (a black and white striped cylinder of 16 cm height and 4 cm diameter standing behind the Target). Animals performed four trials with a maximal trial duration of 15 min. From day 3 on the maximal trial duration was reduced to 5 min. During the whole training a dark-brown curtain surrounded the maze in order to mask the landmarks at the walls of the room.

II.2.6.2 Spatial CM task – Learning

The spatial version of the CM using the 36-hole platform was started directly following the visual version. The animals were trained to find the Target using the extra-maze landmarks for spatial orientation. For this the Target was determined at a fixed position that was not in a direct line with a landmark seen from the center. The animals were trained in a daily session consisting of up to four consecutive trials with a maximal trial duration of 5 min. To increase the rewarding effects of a direct approach of the Target, the session was terminated after the trial, in which the animal approached the Target or the adjacent hole at each side first.

The mice were placed into an opaque Plexiglas cylinder (10 cm in diameter, 15 cm in height) in the middle of the platform for about 30 s. Lifting the cylinder started the measurement for latency to reach the Target and latency to enter the escape tunnel completely. The first hole approached was noted and all approaches to other holes than the Target were counted as mistakes. Also multiple approaches to Target were noted. An approach was counted if an animal dipped its head towards the hole. The approach ended when an animal moved away from the hole with at least both front paws. An entrance into escape tunnel was counted when a mouse disappeared with all paws from the platform. Fecal boli and urine were counted as index of anxiety and stress. Additional parameters were analyzed with EthoVision, namely distance moved, mean velocity, time spent in different zones and mean distance to Target. The trials ended with entering the escape tunnel. Very rarely animals reached the maximal trial duration of 5 min and were placed into the Target hole by hand. After reaching the home-cage they were left undisturbed for approximately 1 min before starting the next trial.

Between trials and sessions the platform was turned, so that a new hole came into the fixed Target position chosen in a pseudo-random order. Before every session the platform was cleaned with 70 % ethanol. This form of training was performed for 22 days.

After the performance reached an asymptotic level showing no more increase, the 36-hole platform was exchanged with a 12-hole version. After a break of one day the animals performed 5 additional sessions training with the 12-hole platform. Long-term memory was tested in a final session with the 12-hole platform performed after a break of 6 days.

II.2.6.3 Spatial CM task – Relearning 1

This second spatial training phase served to investigate whether the mice were able to perform a fast relearning when trained with a new Target position. Apart from the new position of the Target the training was carried out in the same manner to the learning phase with the 12-hole platform. The training was performed for 9 days, until the animals showed an increase of performance to a level comparable to that of the learning phase.

II.2.6.4 Spatial CM task – Relearning 2

This third spatial training was an alternative form of relearning. Therefore the environmental conditions and landmark composition were changed profoundly in addition to another new Target position. To achieve this the maze was surrounded by a dark-brown curtain equipped with 5 new landmarks in white or light gray color. The training was performed with the 12-hole platform for 8 days until it was obvious that the performance did not ameliorate comparable to relearning 1.

II.2.6.5 Step Down Avoidance Task (SDA)

A Step Down Avoidance task was performed to test whether the learning performance in the CM correlated with another hippocampus dependent form of learning not requiring spatial orientation. In this one-trial learning paradigm, the mice were conditioned with an electrical shock immediately after they stepped down from a little platform. The latency to step down in the conditioning trial was taken as baseline. In a recall trial the animals were put again into the same context and the change in latency was measured. Mice showing an enhanced latency to step down were considered to have learned the task.

The step down apparatus consisted of a 30 cm square metal grid surrounded by 50 cm high Plexiglas walls and wired to a transformer that could electrify the grid with a choice of current ranging between 90 µA and 250 µA and a shock duration between 0.5 s to 5 s. For this test a shock of 125 µA for 0.5 s was chosen. The apparatus was illuminated at 25 lux and recorded by a video camera. The platform (10 x 10 x 2 cm) was placed in a corner on the opposite side. For the introduction an opaque Plexiglas cylinder of 10 cm diameter and 15 cm height was used. The apparatus was cleaned before and after each trial with water and 70 % ethanol.
In the conditioning trial the animal was placed into an opaque Plexiglas cylinder (10 cm in diameter, 15 cm in height) located on the platform and left to accustom for 1 min. Latency to step down was measured from lifting the cylinder until the mouse touched the grid with all four paws. As mice stepped down they received a foot shock and were immediately taken back into their home-cage.
After an inter-trial interval (ITI) of 48 h mice were tested for recall. The recall trial was performed in exactly the same way as the training except that no shock was delivered after stepping down. The time to step down was recorded as recall latency. In both trials the amount of fecal boli and urine was noted. Additional records were made for tail rattling, jumping and stepping down backwards.

II.2.7 Relearning Circular Maze (RCM)

The RCM experiment was performed to analyze c-fos expression after a relearning event in the CM. In addition, the behavioral data were used for a detailed analysis of the previously optimized protocol, which was adapted to the necessities of a mapping study. Therefore the mice were trained for several days for a successful learning performance, followed by the relearning event, which consisted of one day of training with a new platform position (for overview see Fig. II.3).
Prior to the RCM the animals performed the Visible Cliff Task and the Pre-training as described previously. The training on the 12-hole platform was carried out as described for the ECM in chapter II.2.6.2 except that a session always consisted of four trials so that all animals underwent an equal number of trials per session.
At day 7 two probe trials were performed, exchanging the escape tunnel with a dead end tunnel. The mouse could explore the platform for 1 min. Afterwards it was placed back into the home-cage and left undisturbed for one minute with the home-cage situated on the board beneath the platform. The following final trial occurred as

normal training trial with the escape tunnel fixed beneath the usual Target hole to restore reward in the expected position.

All animals were trained until they reached the learning criterion, namely approaching as first choice the Target or one adjacent hole at each side in three consecutive trials. When the criterion has been achieved the animal finished the training. The day after reaching criterion, mice were subdivided into two groups in a pseudo-random fashion so that both groups consisted of comparable numbers in gender and animals that reached criterion fast or slowly.

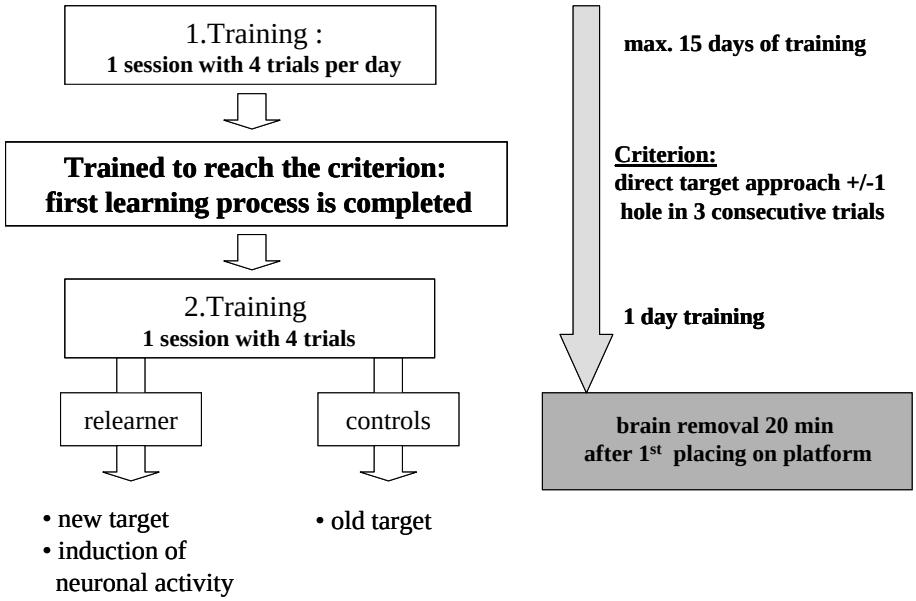


Fig. II.3: Schematic schedule for the RCM

The RCM starts with a first training consisting of a daily session with 4 trials. The first training ended when the mice reached the criterion of approaching the Target +/-1 hole in 3 consecutive trials. The second training was performed on the following day, consisting of a single session in which the mice were divided into two experimental groups with different treatment. For the relearner group an induction of neuronal activity was achieved by a new Target position. The controls performed an unaltered training with the old Target position. Brain removal occurred 20 min after session start.

One group of animals served for induction of relearning (relearner group), the other as controls. Animals of the relearner group were presented a new Target position with no other change in environment or protocol. Animals of the control group were again presented the old Target position, therefore receiving no change at all as compared to the previous training protocol. After four trials the animals were left undisturbed for 20 min starting from the beginning of the first trial. Thereafter the brain was removed within 3 min. Depending on the performance of the individual animals the training ranged between 8 and 15 days.

II.2.8 Reinforced Relearning Circular Maze (RRCM)

The RRCM was performed to investigate c-fos expression in different stages of the relearning. The experimental conditions were as in the RCM experiment with the exception that black vertical stripes (20 cm wide and extending from the floor to the ceiling) were located at the five corners of the room to stress the asymmetric shape of the room. In addition animals not reaching the criterion within 10 days of training were excluded.

Prior to the RRCM the animals performed the Visible Cliff Task and the Pre-training as described previously.

The training protocol was identical to that of the RCM (see chapter II.2.7) except that the probe trial was omitted. The animals received a daily training session on the 12-hole platform with four trials separated by ITI of 1 min. When a mouse reached the criterion the training ended and the induction phase started on the following day. For the induction phase the mice were divided pseudo-randomly into three groups, namely relearner, reinforced relearner and control group.

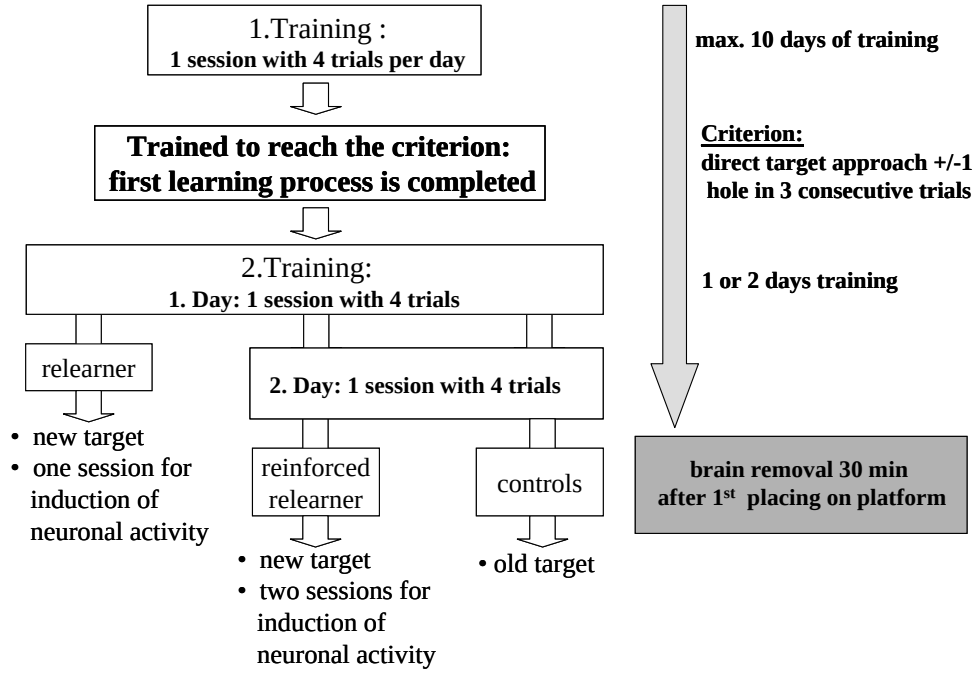


Fig. II.4: Schematic schedule for the RRCM
The RRCM started with a first training consisting of a daily session with 4 trials. The first training ended when the mice reached the criterion of approaching the Target +/-1 hole in 3 consecutive trials. The second training started on the following day, consisting of a single session for the relearner and two daily sessions for the reinforced relearner and control group. For the relearner and reinforced relearner group an induction of neuronal activity was achieved by a new Target position. The controls performed an unaltered training with the old Target position. Brains were removed 30 min after session start.

The relearner group performed one relearning session in which a new Target position was the only alteration. The group of reinforced relearner mice performed two

relearning sessions on two consecutive days with the new Target position as only alteration. The control group performed two sessions with the same Target position under identical conditions as in the previous training. Mice from the relearner group were sacrificed and their brains removed 30 min after starting the session on the first day of the induction phase, whereas mice from the control and reinforced relearner group underwent the same procedure after the session at the second day (Fig. II.4).

II.2.9 Data analysis and statistics

Data are presented in box plots showing median, quartiles (25^{th} and 75^{th} percentile) and maximum and minimum value unless indicated differently.

The comparison of two independent groups was tested with a Man Whitney-U test for significance. More than two independent groups were compared with a Kruskal-Wallis test, followed by a post hoc analysis using the Dunn's test for Multiple Comparisons if appropriate. Differences in two dependent groups of values were tested by a Wilcoxon Matched Pairs test. For more than two dependent groups a Friedman test was performed. The Friedman test was followed by a post hoc comparison with a Dunn's test for Multiple Comparisons if appropriate.

Since there is no non-parametric procedure for multi-factorial analysis, a parametric analysis of variance (ANOVA) for repeated measurements was performed. The ANOVA was followed by a post hoc analysis using a Duncan's Multiple Range Test & Critical Ranges if appropriate. All ANOVA tests were done with repeated measures, unless otherwise stated.

Differences to a hypothetical value as chance level were tested by the Wilcoxon Signed Rank test.

Distributions as the frequencies of first approaches for all holes of a platform were tested with the Observed Versus Expected Frequencies test against the according chance level frequency (=number of trial/number of holes). In addition the fitting for a rectangular distribution was tested with Kolgomorov-Smirnov Distribution Fitting test.

II.3 Results

For the establishing of a learning paradigm and design of an experimental protocol several behavioral parameters were analyzed to reveal the motivation and ability of mice to learn the spatial orientation and to find a measurement for the performance

level that can be considered as successful. In consideration of the c-fos investigation a special emphasis laid on a minimized influence of side stimuli on the learning process and a high comparability between individuals and experimental groups.

II.3.1 Establishing Circular Maze (ECM)

The training in the ECM started with a visual cued Target version in which the animals were accustomed to use the escape tunnel in order to reach their home-cage. At the same time the vision abilities of these animals were thereby tested for their capability to orientate within the maze. All 14 mice showed the ability to find and use the escape tunnel within several trials and were therefore trained in the spatial version of the ECM.

II.3.1.1 Escape Latency

To prove that the mice were able and motivated to find the Target and enter the escape tunnel, the escape latencies were measured. For detailed analysis two different latencies were distinguished, namely searching latency and entering latency. Searching latency defined the time the mouse needs to reach the Target and dip the head inside the Target hole. Entering latency measures the time span between reaching the Target and entering the escape tunnel with all four paws.

While searching latency can be influenced by the motivation as well as by the efficiency to find the Target, entering latency is independent of searching abilities. Therefore the latter serves as better measure for motivation, since the mice have already been trained to use the tunnel during the Pre-training in the visual cued Target version of the CM. The latencies of the first trials of each day are particularly indicative for the behavioral response of the mice to introduction into the CM arena. Therefore the focus was on the first trials per daily session (ss). Latencies of the following trials are given as examples.

During the first two days almost half of the mice showed very high searching latencies over 100 s in the first trial of each daily session (Fig II.5). The searching latency decreased strongly within the first four training sessions to a rather stable low level with median values of around 25 s. Accordingly the Friedman test showed a strong effect of training sessions ($p<0.001$).

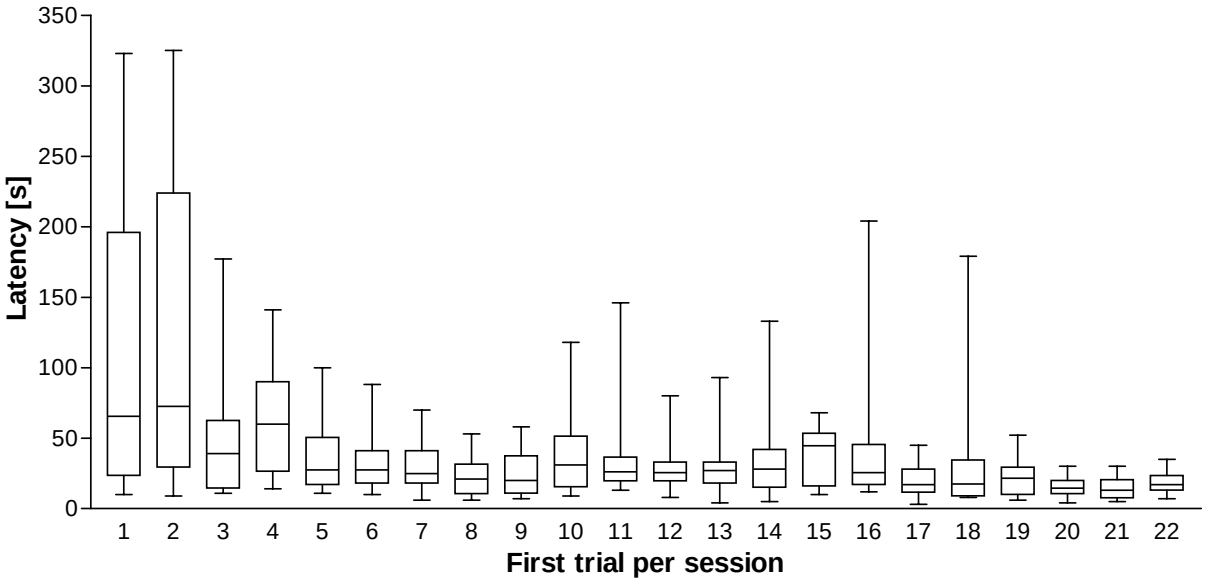


Fig. II.5: Searching latency of the first trials per session
Searching latencies of the first trials of each session are presented as box plots. Over consecutive sessions the mice decreased latencies, indicating that they familiarize with the CM arena and the protocol (Friedman test p<0.001).

The comparison of the latency of the four trials within one session revealed a changing pattern. In the first days of training animals needed more time to reach the Target in trial 1 than in trial 2-4 (Fig. II.6). Whereas at the end of training, mice showed minimal searching latencies already in the first trial, revealing no further decrease in the following trials. However, this observation was not tested for significance since the number of animals trained in the second to fourth trial varied.

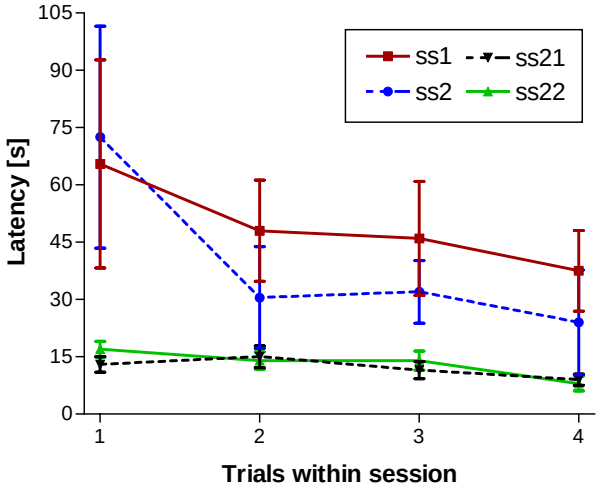


Fig. II.6: Searching latency within 4 trials
Searching latency in the 4 trials of a session is presented as median +/- SEM for the first and last two sessions of training. While in the first two sessions a decrease is seen within sessions, the latencies within the last two sessions show a stable level.

Similar results are given by the analysis of entering latency. Just in the first trial of ss 1 the mice needed about 10 s to enter the tunnel, showing large individual differences (Friedman test $p < 0.001$, see Fig. II.7).

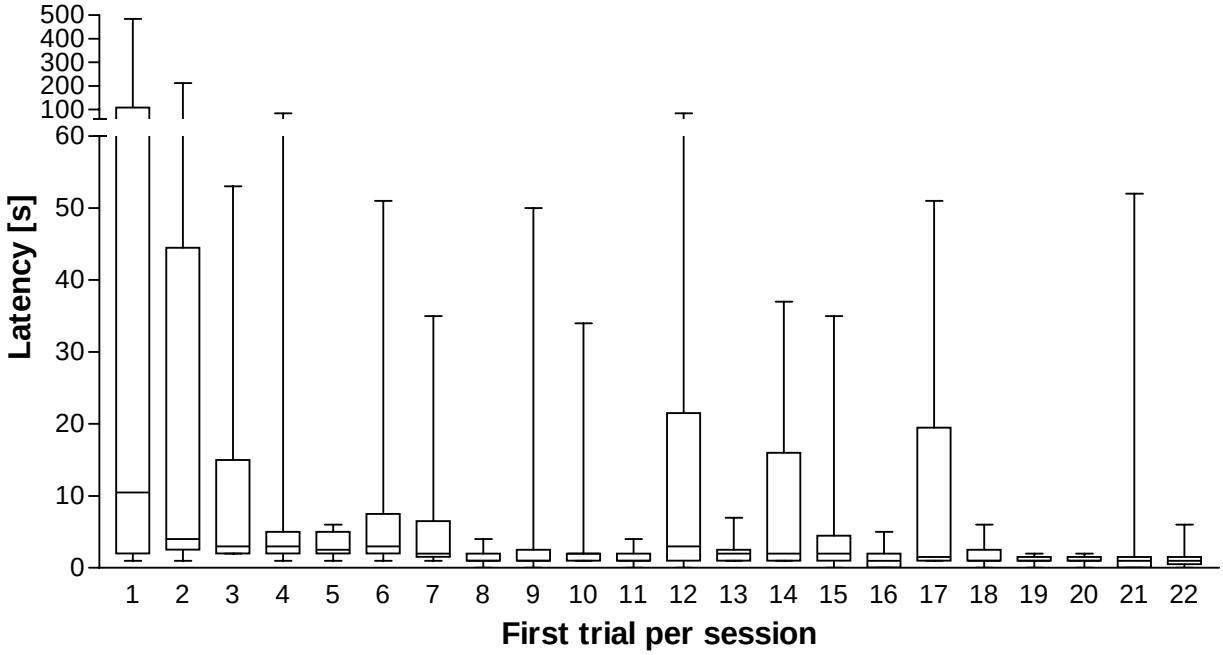


Fig. II.7: Entering latency of the first trials per session
The entering latencies of the first trials of every session are presented as box plots. After the first session the mice enter the escape tunnel within few seconds indicating a high motivation to leave the platform (Friedman test $p<0.001$).

The comparison of the first trial of each session (Fig. II.7) as well as the four trials within sessions (Fig. II.8) showed that most animals entered the tunnel immediately after reaching the Target within less than 5 s. Mainly during the first trials of ss 1 and ss 2 some of them hesitated for longer time. These cases became more rare in the second to fourth trial as well as with successive sessions. Due to the different number of animals trained in the second to fourth session no statistical analysis was performed.

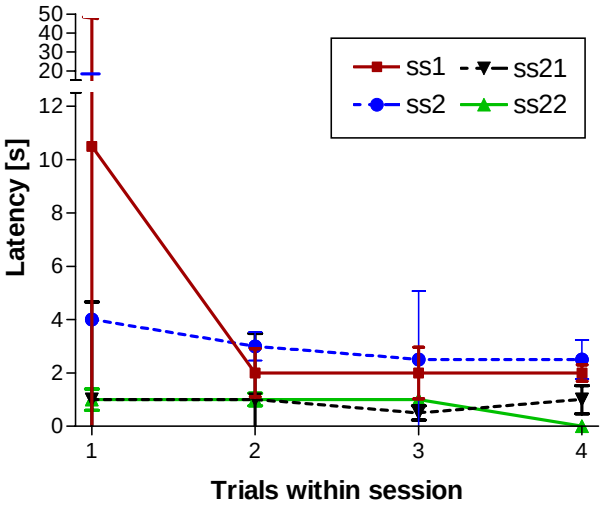


Fig. II.8: Entering latency within 4 trials
The entering latency in the 4 trials of a session is presented as median +/- SEM for the first and last two sessions of training. Except for the first trial of session 1 the mice quickly enter after reaching the Target indicating a high motivation to use the tunnel.

II.3.1.2 Search strategy

The CM was chosen as a spatial learning paradigm. Therefore it was important to demonstrate that the mice use spatial orientation to find the Target. There are three different possibilities for the animals to search for the Target as shown in Fig. II.9A. They can visit different holes in a randomly chosen sequence. Thereby they would repeatedly change the direction of movement and traverse the platform. This can be called a random search strategy. A second possibility is to run immediately to the row of holes and visit the consecutive holes in a serial manner. The mice would run along the holes until they reach the Target without changing the direction of movement. This can be called a serial search strategy. For the third possibility, that can be called spatial search strategy, the mice directly approach the Target. A typical example for each search strategy is shown as track run by a mouse in Fig. II.9B.

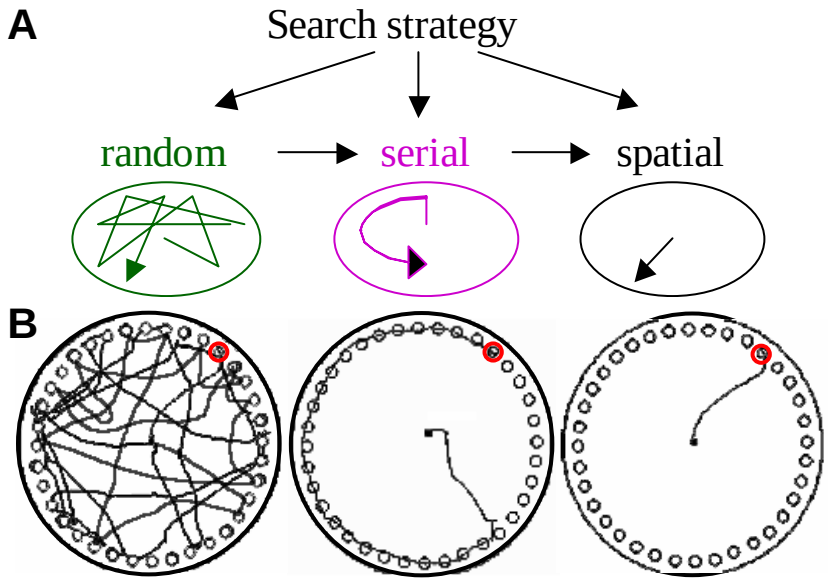


Fig. II.9: Demonstration of the different search strategies
(A) The three possible search strategies illustrated as pictograms are normally used in a typical sequence going from random over serial to spatial. **(B)** Typical examples for all search strategies displayed as path run by a searching mouse recorded by EthoVision tracking system within the arena of the CM. The red ring marks the Target. The black dot indicates the starting position. Left to right: random, serial and spatial.

Since mice normally progress through these strategies from random to spatial indicating increasing spatial abilities (Barnes, 1979; Mansuy et al., 1998), the course of the different strategies throughout training was investigated. For this the paths of all animals classified into the three possible types of search strategies, were defined as follows:

1. Random strategy: The mouse traverses the platform and/or changes moving direction to reach the Target.

2. Serial strategy: After reaching the first hole the mouse visits the consecutive holes without changing moving direction.
3. Spatial strategy: The mouse directly approaches the Target. Approaching the adjacent holes was still counted as spatial strategy if the mouse afterwards went immediately to the Target.

Fig. II.10 presents the distribution of searching strategies for the 4 trials of each session during the course of training.

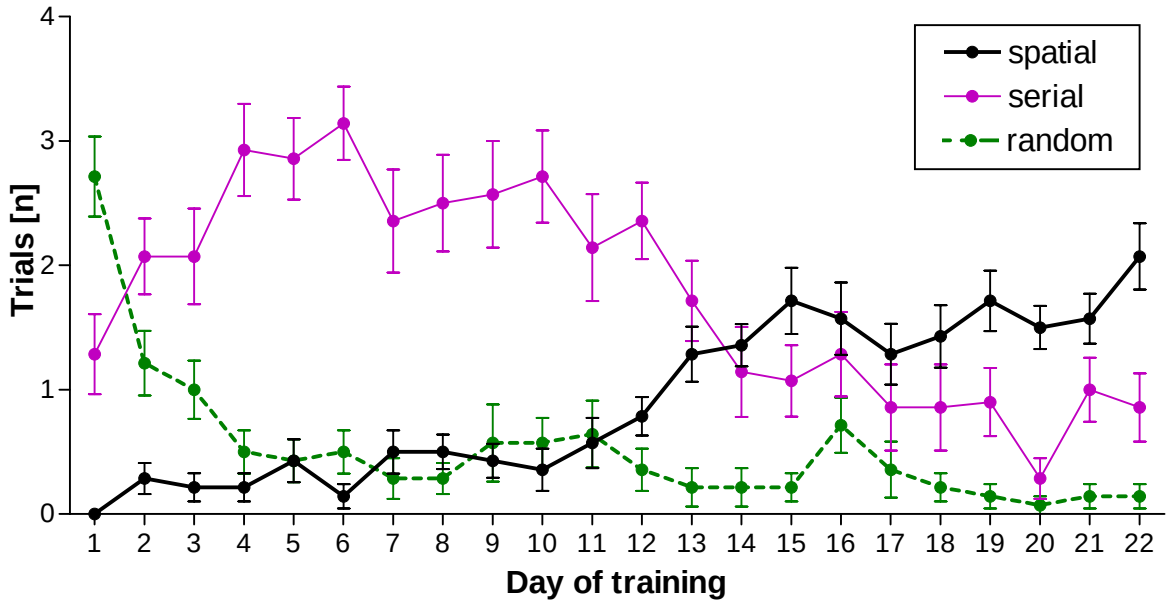


Fig. II.10: Distribution of search strategies in progressing training
For each strategy the number of trials per day it was used in is presented as mean +/- SEM. The mainly used strategy changed from random on the first day to serial from day 2 until day 13 and to spatial from day 14 on. Note: Friedman Test for spatial ($p<0.001$), serial ($p<0.001$) and random strategy ($p<0.001$).

The strategies used changed in a typical manner during the course of training. On day 1 the mice mainly searched the Target by random search. The spatial search strategy was not used at all. Already at day 2 the serial search strategy was predominant. A closer look to the strategies within the first sessions revealed that this shift already started immediately after the first trial. In trial 1 all except one mouse performed the random strategy. In trial 2 already 3 mice searched in a serial manner and in trial 3 and 4 half of the mice used the serial strategy. The mice continued searching mainly in a serial manner until day 12. From day 12 to day 13 the mice strongly shifted towards the spatial strategy instead of the serial one. From day 15 on the spatial strategy was predominant. The random strategy appeared continuously in a few cases. The Friedman test corroborated the effect over sessions to be significant in all three strategies ($p < 0.001$ for all).

II.3.1.3 Learning performance

The increase of learning performance during the acquisition of a learning paradigm can be measured in several different ways. In the CM an appropriate possibility is to count the mistakes as the number of holes visited before reaching the Target. Fig. II.11 presents the number of mistakes for blocks of 2 training days.

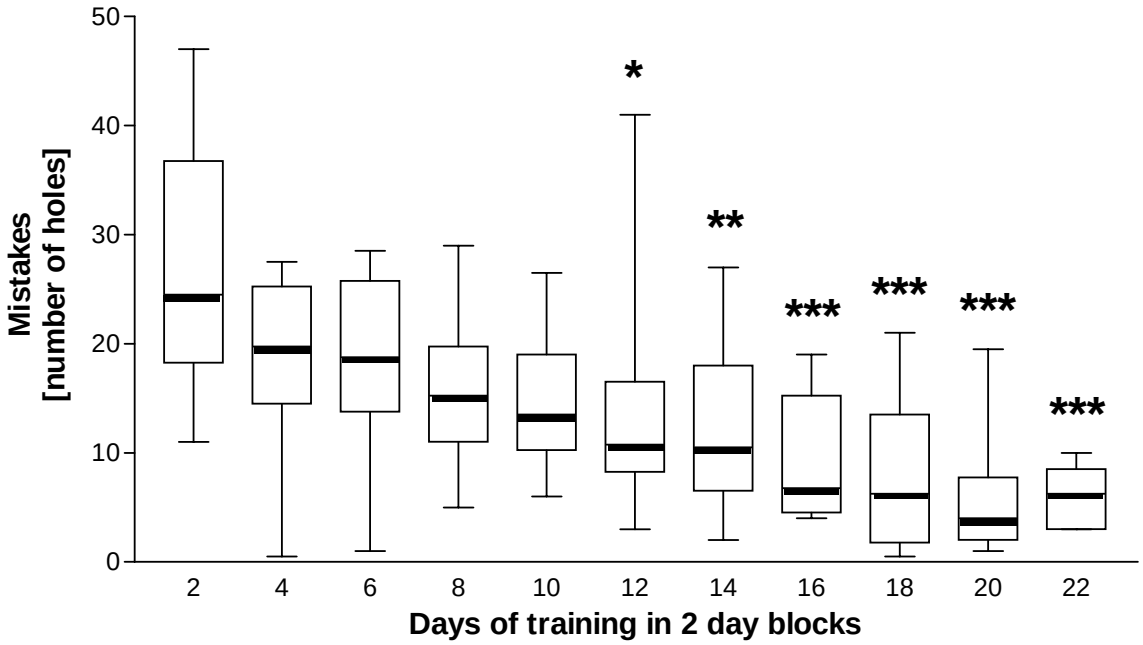


Fig. II.11: Performance of learning measured as mistakes
The number of mistakes in blocks of 2 days of training is presented as box plots. The mice decrease the mistakes with ongoing training revealing an increasing performance in searching the Target. *-p<0.5, **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

The mice continuously decreased the number of mistakes until day 16. From then on the errors remained stable until the end of training, decreasing rather in variability of values than in median. The effect of decreasing mistakes within the training is highly significant ($p < 0.001$, Friedman test). The Dunn's test revealed that mice made fewer mistakes compared to the first two training days starting from day 11-12 on.

Although the number of mistakes gave a good representation of the improved learning performance, this parameter did not consider the spatial aspect. In order to investigate more specifically the spatial orientation abilities of the mice, an additional parameter for performance was analyzed.

This parameter consisted of the number of holes lying between the first approached hole and the Target, which is defined as 'distance to Target' (DTT). Fig. II.12 represents an example for the determination of this parameter.

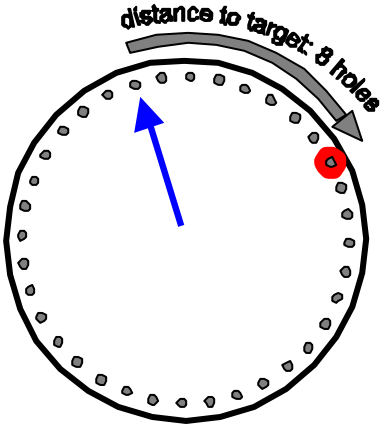


Fig. II.12: Example for determination of distance to Target
The blue arrow indicates the hole the animal approached at first. The red ring marks the Target. The 8 holes counted include the first hole.

In Fig. II.13 DTT is shown in training blocks of two days. It resembled the decrease found for mistakes. After a plateau at the beginning, the DTT decreased continuously with the largest gain in performance between day 12 and 14. A stable level of performance was seen from day 18 on. The Friedman test showed a highly significant decrease within the training blocks ($p<0.001$). The Dunn's post hoc analysis comparing the first training block with the others revealed that the DTT is significantly lower from day 14 on.

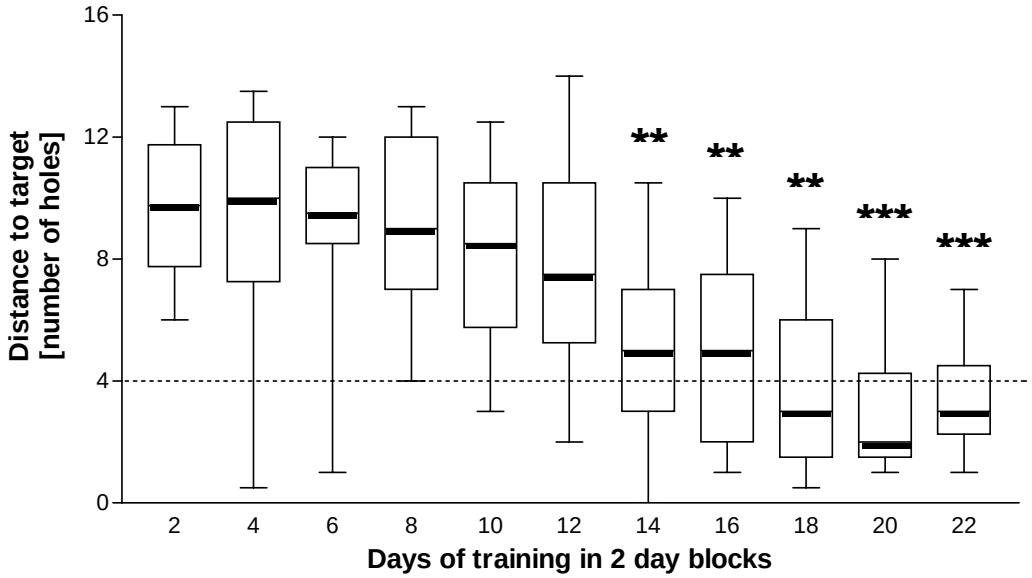


Fig. II.13: Performance of learning measured as distance to Target
The distance to Target, given as number of holes is presented for blocks of 2 days of training as box plots. The decrease in distance to Target indicates an increasing spatial orientation in searching performance. The broken line indicates the distance that matches criterion (see below). **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

II.3.1.4 First approaches

The main indicator for spatial orientation is the first choice of direction for moving. It reveals if mice have a preference for certain holes and are able to guide directly

towards them. However, since DTT is averaging values for number of holes, it cannot reveal any certain preference. This can be achieved by analyzing the first approaches for all holes of the platform. If all holes are of the same attraction value or mice visit them equally due to a missing knowledge of direction, they should approach each hole with the same probability around chance level, which lies at 2.8 % for 36 holes. A distribution differing from this rectangular form indicates that mice preferred or rejected certain holes. To do so the animals need to be able to discriminate, at least roughly, between holes.

If the distribution shows a preference for an area surrounding a certain hole, this indicates that mice are able to discriminate the direction but not exactly one hole. Thereby this distribution indicates how precisely the mice can localize the Target. The precision abilities determine the level of maximal performance a mouse can reach, since the mouse visits all of those holes, which it cannot discriminate.

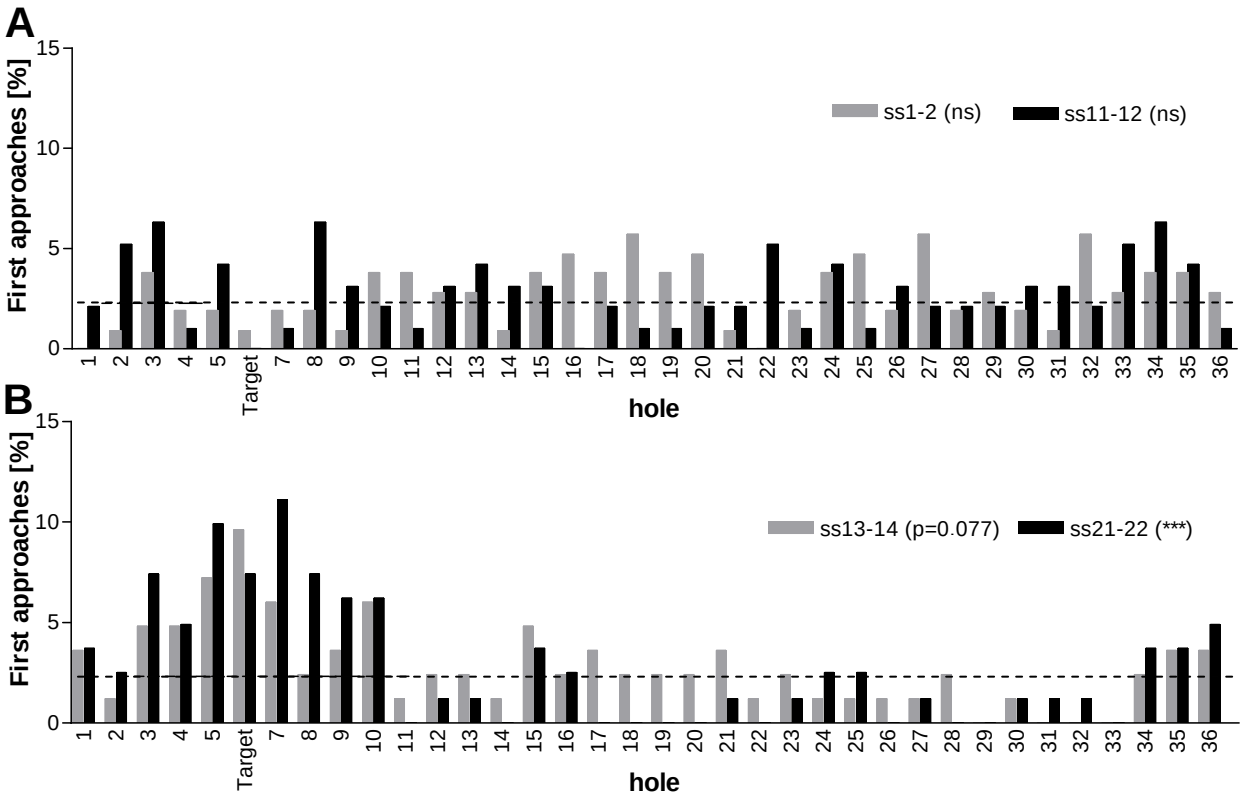


Fig. II.14: Distribution of first approaches for all holes of the platform.
The frequency of first approaches given in percentage is presented for all holes. **(A)** The first approaches of ss1-2 and ss11-12 show a rather random distribution. **(B)** The distribution of ss13-14 and ss21-22 shows that the mice prefer to approach the Target and 3 to 4 neighboring holes at each side. Chance level (=2,8%) is indicated as broken line. Significance levels are given for the Observed Versus Expected Frequencies test (***-p<0.001). Note: Kolgomorov-Smirnov Distribution Fitting test for rectangular distribution (ss1-2/ss11-12 ns; ss13-14/ss21-22 p<0.01).

Fig. II.14 shows the distribution of first approaches for all holes given as percentage for 2 training sessions. Together with the first (ss 1-2) and last (ss 21-22) training

sessions, the ss 11-12 and ss 13-14 are presented. As seen for DTT the strongest change of behavior could be observed at the latter time point.

The distribution was tested with an Observed Versus Expected Frequencies test with measured frequencies versus the expected frequency of chance level. This test showed that neither in ss 1-2 nor in ss 11-12 the approaches significantly differed from the expected chance level. In the following ss 13-14 a tendency ($p = 0.077$) for a distribution differing from chance level was found. The mice focused their approaches onto the Target and the 3-4 neighboring holes at both sides. The holes of the opposite side (hole 24 and the neighboring holes) are approached below chance level. In the last block of ss 21-22 this distribution pattern is strengthened and correspondingly differs highly significant from the expected chance level ($p < 0.001$). The Kolgomorov-Smirnov Distribution Fitting test for a rectangular distribution showed that both ss 13-14 and ss 21-22 differed from a rectangular distribution that would correspond to a distribution around a chance level ($p < 0.01$ for both).

II.3.1.5 Learning criterion

One of the aims of the ECM was to investigate in detail the processes occurring during learning in CM and to show that mice can learn the spatial location of the Target. Another important aim is to develop an optimal design for mapping the c-fos expression induced by a relearning event. Therefore it is necessary to ensure that the first learning process is completed and a stable situation is achieved before inducing relearning as a new learning process. To judge when the mouse reaches an asymptotic level of performance and thereby a stable situation a criterion had to be defined. This was achieved considering the results of mistake and DTT evaluations. In addition the analysis of first approaches was assumed to reflect the precision of search in a most reliable way. Based on these assumptions was defined that a mouse reached the criterion, when the first hole it approached in three consecutive trials was the Target or one of the 4 adjoining holes. This corresponds to an allowed DTT of 4 holes as seen for the stable level of performance in Fig. II.13 and the distribution pattern seen in Fig. II.14. In this way the area that matches the criterion covers 25 % of the platform, defining a Target quadrant similar to many MWM designs.

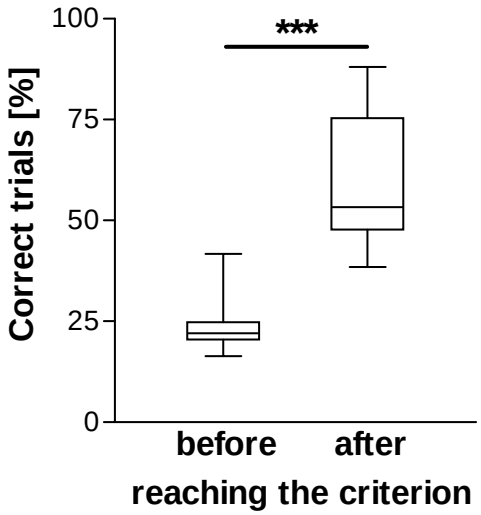


Fig. II.15: Performance comparison before and after reaching the criterion.
The correct trials as percentage of trials before and after reaching the criterion are shown as box plots. The mice doubled the correct trials after reaching the criterion. *** =p<0.001 (Wilcoxon signed rank test).

In order to test the validity, the criterion was applied to the ECM. For every mouse the day, in which the criterion was reached, was determined. Thereafter the trials with correct performance (DTT ≤ 4 holes) before and after criterion day were calculated. The number of correct trials was compared to see whether the criterion separated bad and good performance.

Fig. II.15 presents the correct trials (Target +/- 4 holes) as percentage of the total number of trials before and after the criterion was reached. It indicates that the performance before and after reaching criterion differed highly significant (Wilcoxon signed rank test, p = 0.001), revealing that the defined criterion was indeed indicative of acquired and stable spatial learning in the mice.

II.3.1.6 The 12-hole platform

The acquisition curve for the parameter DTT and the distribution of first approaches showed that even a stable level of performance did not reach a precision higher than Target +/-4 holes. However, the experimental design was not chosen to test precision abilities but to judge the spatial learning of all animals. Therefore to reduce the mistakes due to precision and simplify the decision for a correct hole the platform was reduced to 12 holes. In this way the criterion is reduced to Target +/-1 hole although the matching area remains unchanged (Fig. II.16).

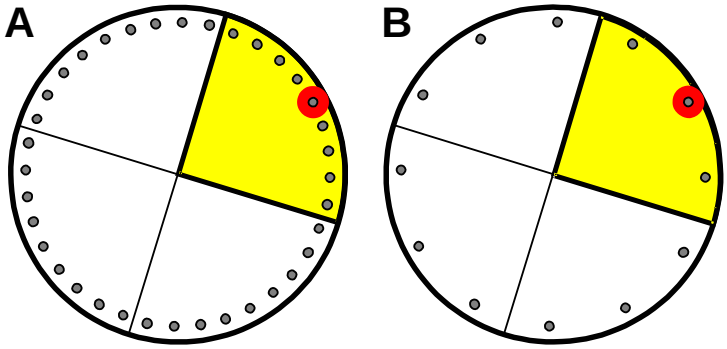


Fig. II.16: 36-hole and 12-hole platform version
(A) Pictogram of the 36-hole platform. **(B)** Pictogram of the 12-hole platform. The red ring marks the Target. The area matching the criterion (Target+/-4holes, Target+/-1hole, respectively) is marked in yellow. This area corresponds to the Target quadrant.

On this 12-hole platform the mice were trained another 6 days to accustom to the new platform before the relearning started. By this procedure it was possible to test whether the reliability of all parameters was enhanced. In Fig. II.17 to II.18 a comparison of the results for training on the 36- and 12-hole platform is shown for various parameters.

Although the last training day was spaced from the others through a break of 6 days, none of the parameters differed from the other training days. For comparison reason all training days were presented in 2-day blocks as for the training with the other platform version. Fig. II.17 shows that the change in platform affected neither the searching nor the entering latency.

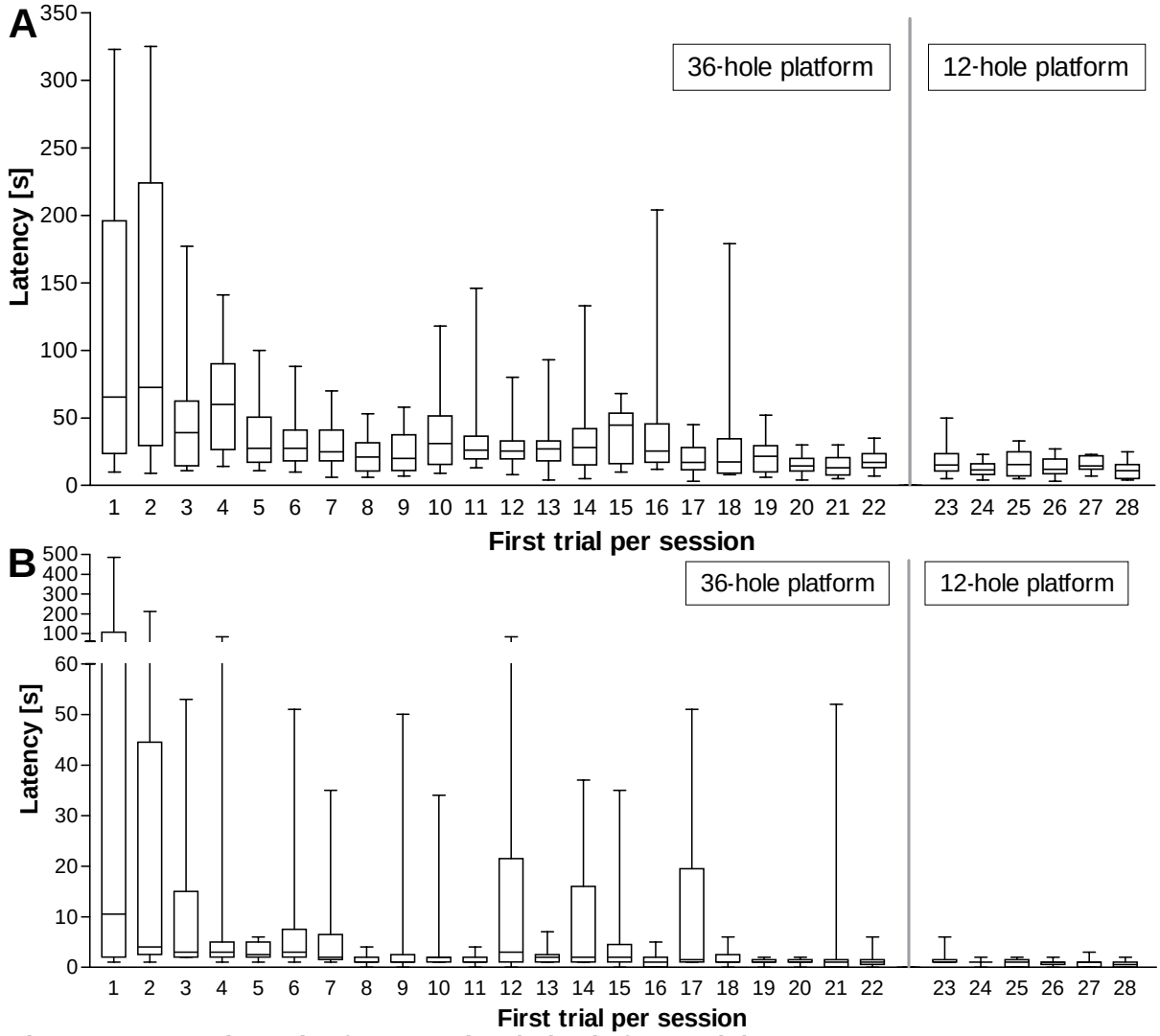


Fig. II.17: Escape latencies for 36- and 12-hole platform training
(A) Searching latency for the training with the 36- and the 12-hole platform for the first trial of each session. Comparison of training with the new platform (12-hole) for ss 1 and ss 22 of the old platform (36-hole): Friedman test $p < 0.001$. Dunn's test shows differences to ss 1 (ss 23-28: all $p<0.001$), no differences to ss 22. **(B)** Corresponding entering latency. Friedman test $p < 0.001$. Dunn's test shows differences to ss 1 (ss 23: $p < 0.01$, ss24-28: all $p < 0.001$), no differences to ss 22.

Interestingly not even at the first day of training with the 12-hole platform a difference could be observed. Outliers, as seen occasionally in the training with the former 36-hole platform, were not found any more for both parameters. The comparison of the new

training sessions (ss 23-28) with ss 1 and ss 22 of the old version revealed a significant difference just for ss 1 but not for ss 22 (Friedman test $p < 0.001$; Dunn's test for ss 1: ss 23 $p < 0.01$; ss24-28 $p < 0.001$).

Also DTT was unaffected by the change of 36- to 12-hole platform (Fig. II.18A). The comparison of training with the 12-hole platform (day 24-28) to first and last training block of the 36-hole version confirmed a significant difference just for first (day 2) but not for last (day 22) block (Friedman test $p < 0.001$; Dunn's test for day 24-28 $p < 0.001$).

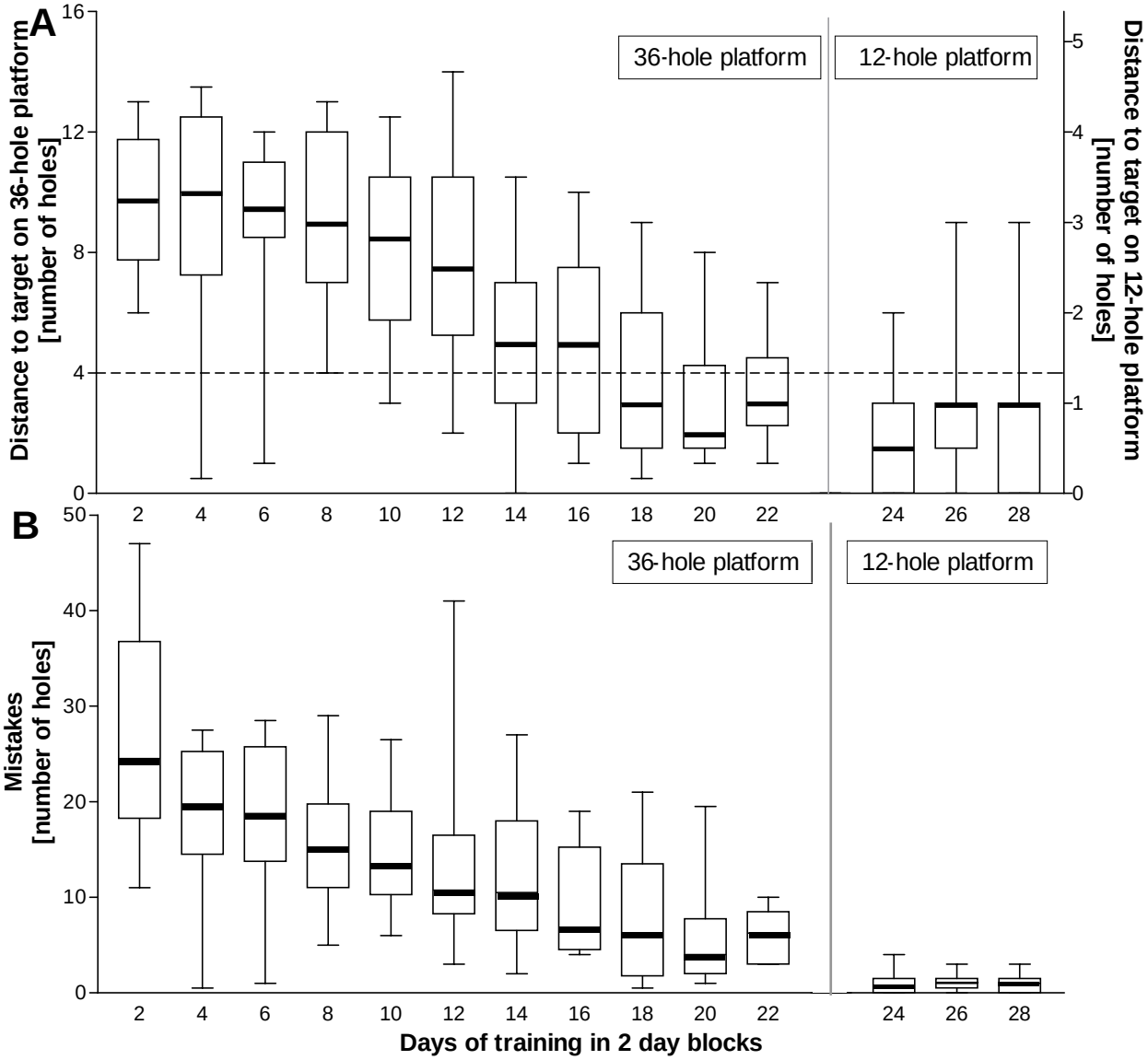


Fig. II.18: Learning performance for 36- and 12-hole platform training
The number of mistakes **(A)** and distance to Target **(B)** for the 36- and the 12-hole platform is presented in blocks of 2 days of training. **(A)** The y-axes indicate the corresponding holes for the 36-hole (left) and the 12-hole (right) platform version. This shows that the mice visit the corresponding holes on the 12-hole platform showing no change in behavior due to platform exchange. Note: Comparison of training with the new platform with day 2 and day 22 of the old platform: day 23-28 all $p<0.001$ to day 2, no differences to day 22. **(B)** The number of mistakes is reduced on the 12-hole platform according to the reduced number of holes. Note: Comparison of training with the new platform with day 2 and day 22 of the old platform: day 23-28 all $p<0.001$ to day 2, day 23-28 all $p<0.01$ to day 22 (Dunn's test after Friedman test).

As seen in Fig. II.18B the mistakes were reduced in the training with the 12-hole platform as compared to the previous training on the 36-hole platform, reaching a

median level of about 1 mistake per trial. Indeed, the mistakes during the training with the new platform (day 24-28) were fewer than on the first (day 2) and last (day 22) training block with the old version (Friedman test $p < 0.001$; Dunn's test for day 2: day 24-28 $p < 0.001$; Dunn's test for day 22: day 24-28 $p < 0.01$).

II.3.1.7 Relearning 1

The relearning 1 was performed to investigate the acquisition of new information in a familiar situation. The difference to the training in the spatial learning consisted exclusively of a new position for the Target.

On day 1 of relearning, the mice showed a median value of 7.5 mistakes, which they then reduced strongly on day 2, followed by a slower decrease from day 3 on, as shown by the Friedman test ($p < 0.001$). At day 9 the mice showed a median number of mistakes about 1 that is comparable to the level seen during the final training in the learning phase.

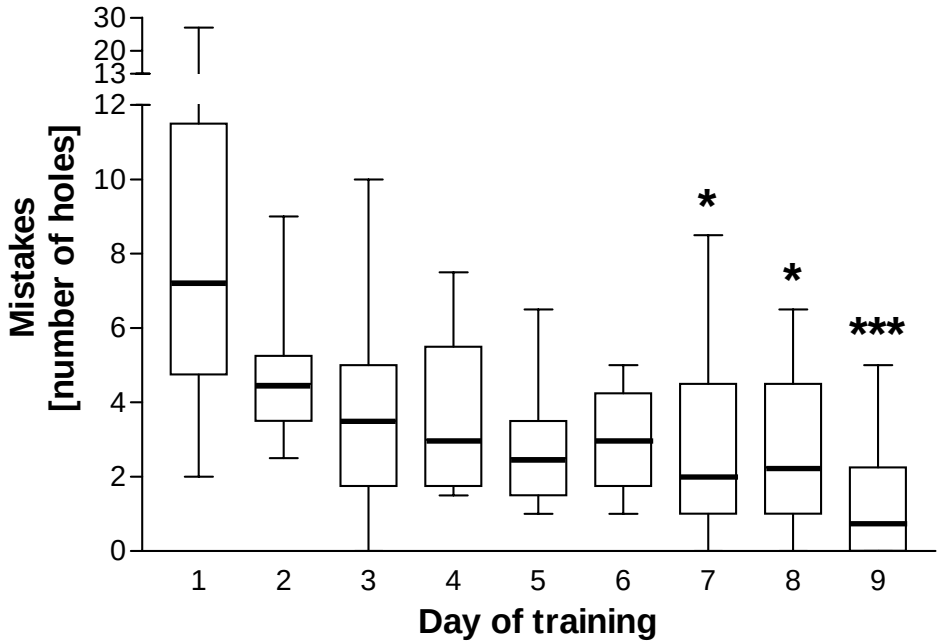


Fig.II.19: Performance of relearning 1 measured as mistakes
The number of mistakes is presented as box plots for all 9 days of training. By decreasing the number of mistakes the mice reveal an increased level of performance. *-p<0.5, **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

The relatively high value for the first day reflected the fact that several animals repeatedly revisited the old Target position. Since the mice were biased to visit the old Target on the first training day, the comparison in the Dunn's test was performed with the second day of training. Significant differences were found from day 7 on (Fig. II.19).

The acquisition curve for DTT resembles the acquisition curve found for mistakes (Fig. II.20). Starting at a median distance of just below 4 holes the performance reaches a level of below 1 hole on day 9. Again the Friedman test corroborates a significant effect within training days ($p < 0.001$). Since the old Target position laid in a distance of 4 holes the performance level of the first training day supports the expectation of a biased search on day 1. Therefore the post hoc comparison with the Dunn's test was performed with the second day of training and the differences became significant from day 5 on.

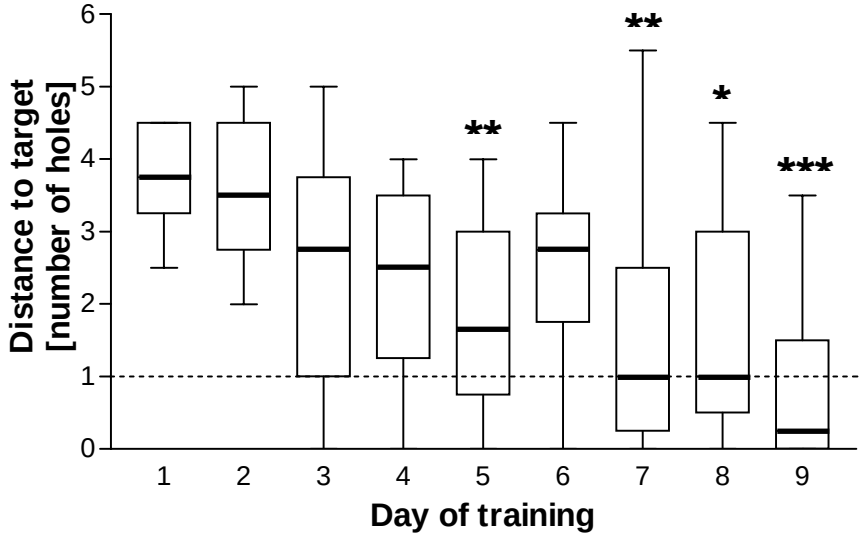


Fig. II.20: Performance of relearning 1 measured as distance to Target
Distance to Target, given as number of holes is presented as box plots. The mice perform with a decreasing distance to Target indicating an increased spatial orientation. The broken line indicates the distance matching the criterion. *-p<0.5, **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

As illustrated in Fig. II.21 the distribution of the first approaches for the 12 holes of the platform confirmed that the mice were focused to the old Target position (hole 2). The new Target was not approached at all. The mice were even more precise in preferring the old Target at ss 3. The approaches found for the new Target were still below chance level (8.3 % for 12 holes). A similar distribution pattern continued until ss 6. A different pattern was found in ss 7, in which the first approaches were almost evenly distributed between the holes 1-6, therefore covering exactly the both criterion areas of the old and new Target. In the last session the mice still visited the holes 2 and 3 of the old Target quadrant but displayed the strongest preference towards the new Target.

The distribution was tested with an Observed Versus Expected Frequencies test with the measured frequencies versus the expected frequency of chance level. Corresponding to the displayed preference for the old Target all sessions revealed a significant difference to chance level distribution (ss 1, 3, 9 $p<0.001$, ss 7 $p<0.001$).

The Kolgomorov-Smirnov Distribution Fitting test showed that the ss 1, ss 3 and ss 9 differed from a rectangular distribution that would correspond to a distribution around a chance level ($p < 0.01$ for all). Just the distribution of ss 7 was not significantly different from the rectangular distribution though it showed a strong bias to one half of the platform.

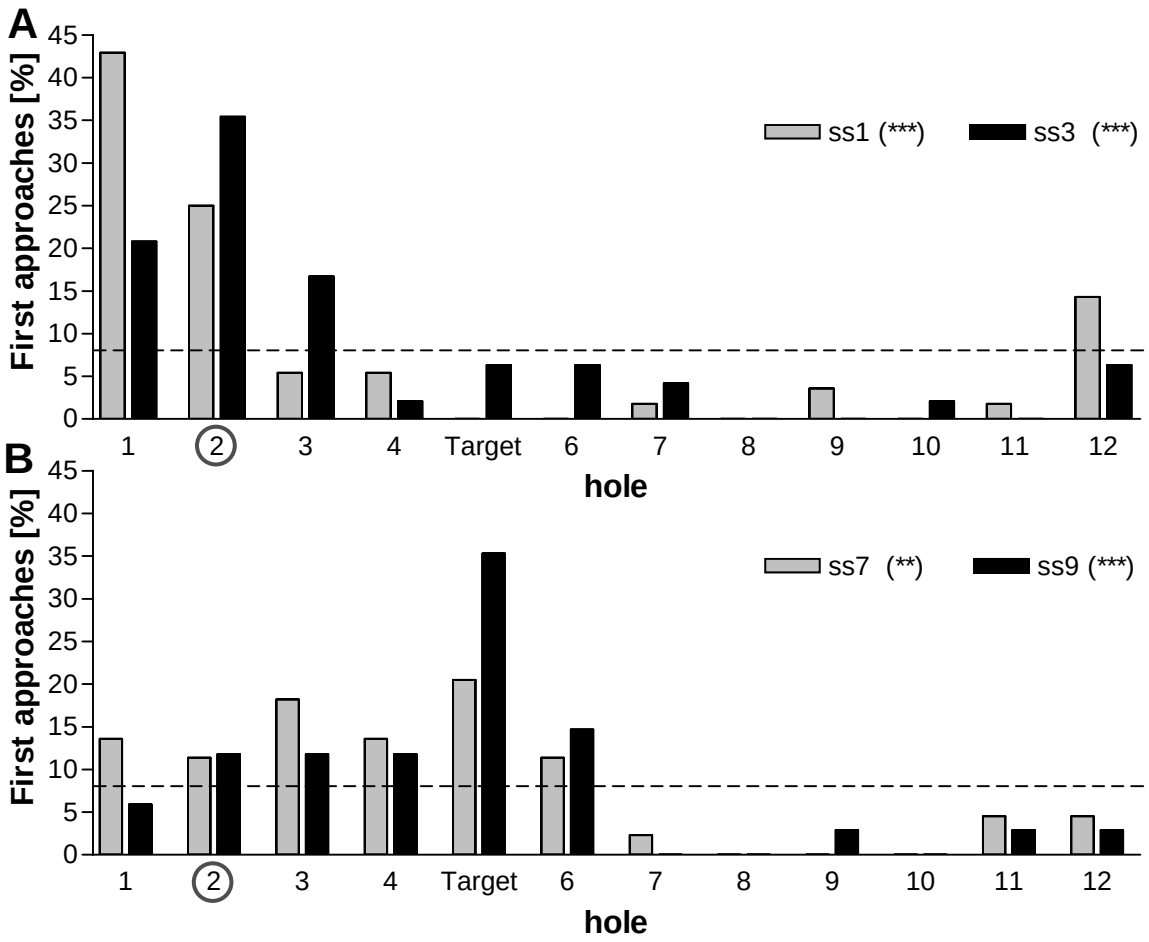


Fig.II.21: Distribution of first approaches for all holes of the platform in Relearning1
The frequency of first approaches given in percentage is presented for all holes. **(A)** The distribution for ss1 and ss3 reveals a strong preference for the old Target and the adjacent holes. **(B)** In ss7 the first approaches are relatively equal distributed between the old and new Target and their adjacent holes. The distribution for ss9 shows a strong preference for the new Target.
Chance level (=8.3%) is indicated as broken line. ○ = Target during Learning. Significance levels are given for the Observed Versus Expected Frequencies test. **-p<0.01; ***-p<0.001. Note: Kolgomorov-Smirnov Distribution Fitting test for rectangular distribution (ss1, 3 and 9 p<0.01; ss7 ns).

II.3.1.8 Relearning 2

The training in relearning 2 was performed to investigate, whether a fast new learning can also be achieved under more difficult conditions. Therefore, not only another new Target position was introduced but also the environment and thereby the landmark constellation was changed.

Regarding the parameter mistake no noticeable performance amelioration was found in the acquisition curve. The performance stayed at a level around 5.5 mistakes during the whole training. The Friedman test corroborated the missing effect of training days (Fig. II.22A).

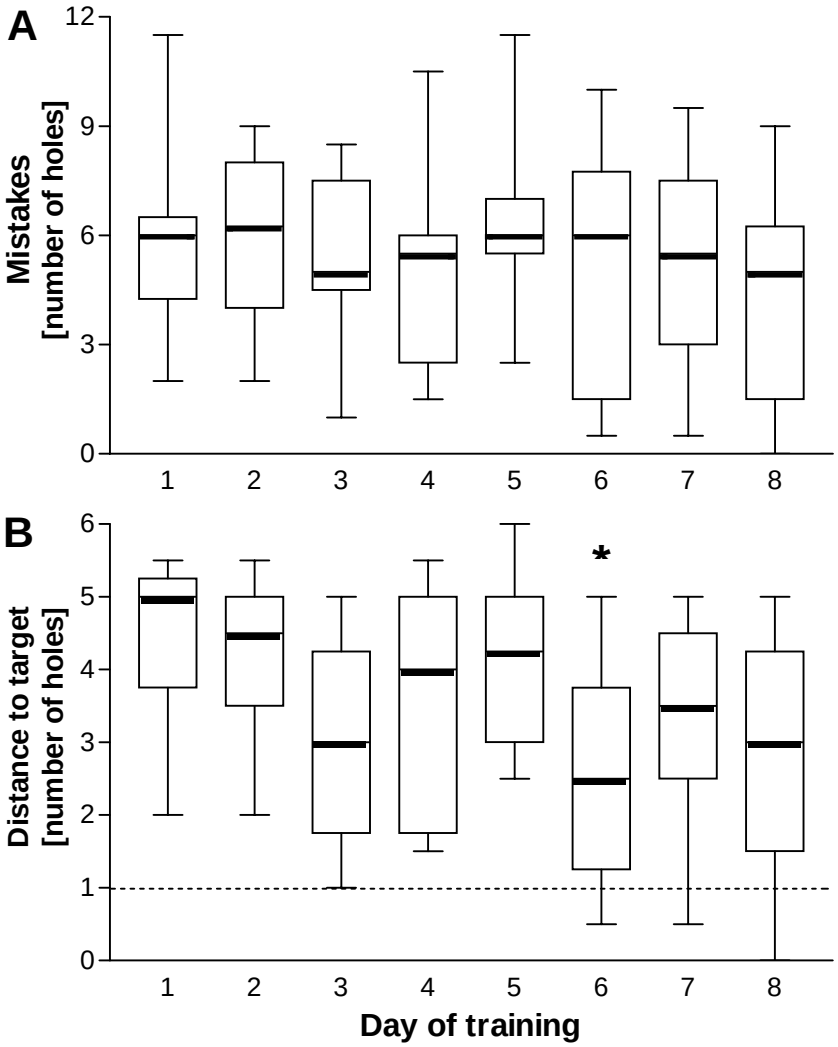


Fig. II.22: Performance of relearning 2 measured as mistakes and distance to Target
Mistakes **(A)** and distance to Target **(B)** are given as number of holes presented as box plots for all 8 days of training. In both parameters the mice show an inconsistent performance indicating that they cannot orientate successfully to find the Target. The broken line indicates the distance matching the criterion. *-p<0.5 (Dunn's test after Friedman test).

In analogy to these results, no decrease in the acquisition curve of DTT was found (Fig. II.22B). The lowest median value was observed on day 6. Still a difference was found in the Friedman test ($p < 0.001$) due to the significantly lower level on day 6 as revealed by the Dunn's post hoc comparison. As in Relearning 1 the comparison for the Dunn's test was performed with the second training day to avoid the potentially biased first training day.

The distribution of first approaches over the different holes showed a preference for hole 12 with a percentage of 40 % in ss 1 (Fig. II.23). This pattern was comparable to

ss 2. In ss 3 mice showed a preference for hole 6, the adjoining hole of the Target. This preference was not seen any more in ss 4 and 5, which showed a pattern more similar to the first ss. All preferences observed until then were vanishing from ss 6 to ss 8. This indicated that the low value for DTT found on day 6 (Fig. II.22B) was not due to a correct choice of Target.

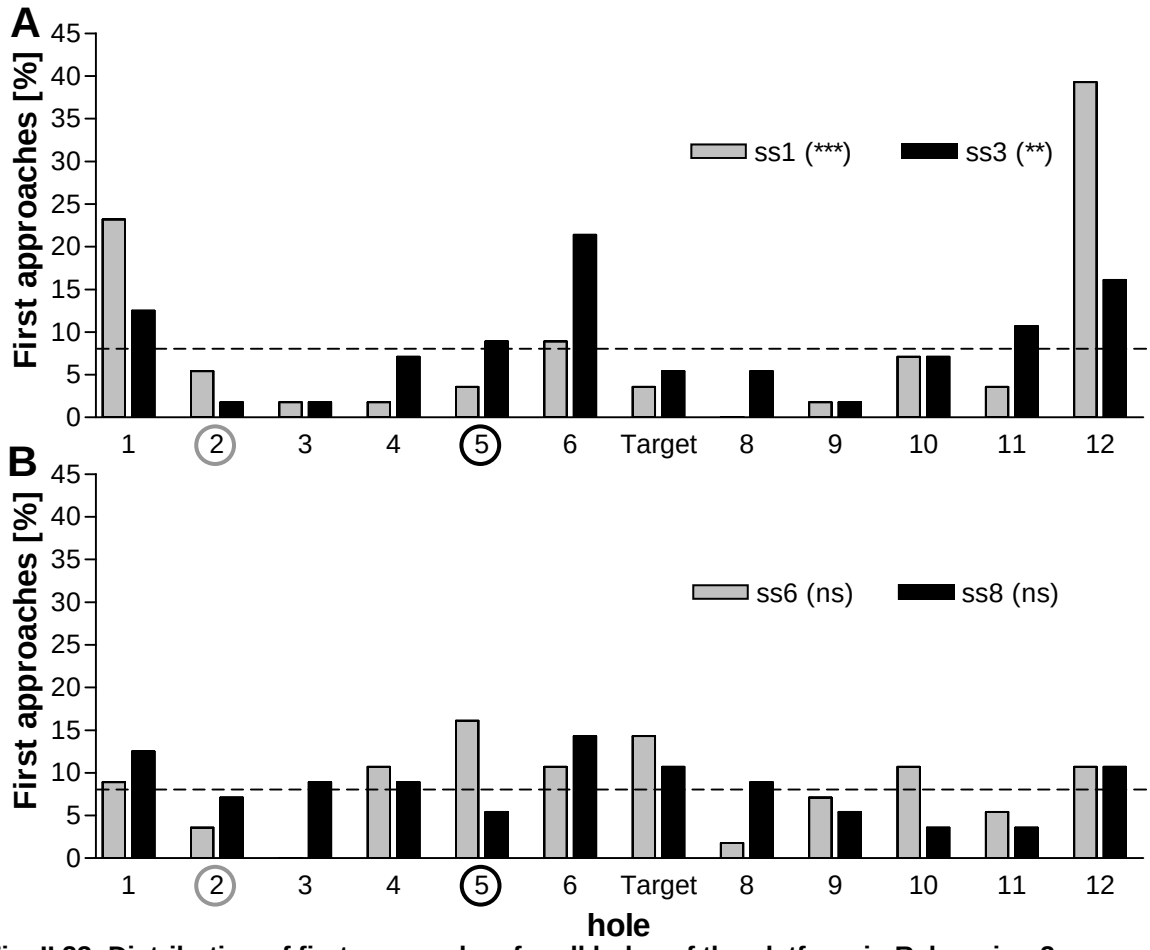


Fig. II.23: Distribution of first approaches for all holes of the platform in Relearning 2
The frequency of first approaches given in percentage is presented for all holes. **(A)** The distribution for ss1 and ss3 reveals an inconsistent preference to holes, which are neither actual nor old Targets. **(B)** In ss6 and ss8 the preferences vanished, revealing no specific distribution pattern. Chance level (=8.3%) is indicated as broken line. ○ = Target during Learning. ○ = Target during Relearning 1. Significance levels are given for the Observed Versus Expected Frequencies test. **-p<0.01; ***-p<0.001. Note: Kolgomorov-Smirnov Distribution Fitting test for rectangular distribution (ss1-2/ss11-12 ns; ss13-14/ss21-22 p<0.01).

The Observed Versus Expected Frequencies test showed a significant difference to the expected chance level for ss 1 ($p < 0.001$) and ss 3 ($p < 0.01$) but not for the ss 7 and ss 9. The Kolgomorov-Smirnov Distribution Fitting test revealed a significant difference from a rectangular distribution only for ss 1. In ss 3, ss 6 and ss 8 no difference to a distribution around a chance level was found.

II.3.1.9 Step Down Avoidance Task (SDA)

The Step Down Avoidance Task was performed after the CM in order to investigate a possible correlation between spatial learning and another learning paradigm seen as hippocampus dependent (Izquierdo et al., 1997).

The Step Down Avoidance examines hippocampus dependent memory in form of contextual memory in a one trial learning. The mice receive an electrical shock as soon as they step down a platform in a training session. This pairing of shock and stepping down is expected to result in a prolonged latency to step down in a recall session if the mice remember the situation and have learned the pairing.

Fig. II.24A shows that the latencies were significantly increased in the recall as compared to training session (Wilcoxon Matched Pairs test $p < 0.001$).

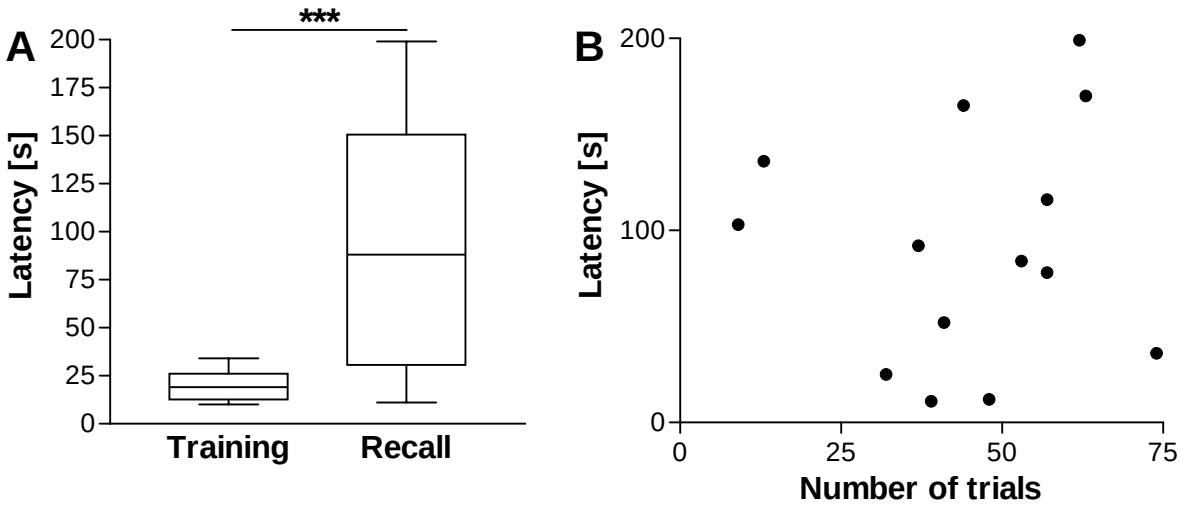


Fig. II.24: Step down latencies for training and recall and correlation to CM performances
(A) The latencies to step down in the training and recall session are shown as box plot. In the recall session the mice increased their latency to step down the platform dramatically indicating that they built up a contextual memory for the situation. *** =p<0.001 (Wilcoxon Matched Pairs test). **(B)** The recall latency and CM performance as number of trial needed to reach criterion is shown as x-y plot. No correlation was found between the performances in the different learning paradigms (Spearman r = 0.1914, ns).

By correlating the recall latencies with the number of trials needed to reach the criterion a possible connection to the learning in the CM was tested. No correlation was found between recall latency and number of trials to reach criterion (Fig. II.24B, Spearman r = 0.1914, ns).

II.3.2 Relearning Circular Maze (RCM)

In addition to the function as a learning paradigm for the c-fos study the RCM served as tool to investigate the modified design in more details. The differences in the protocol required small modifications in the form of analysis. Besides the parameters

analyzed in parallel to the ECM a detailed analysis of a probe trial performed at ss 7 provided more information about the learning process. In the Visible Cliff Task and Pre-training the mice were checked for their visual ability as well as their readiness to find and use the escape tunnel. Although a group of 9 young males was trained together with a group of 10 older females there were no differences found for any of the parameters presented in this chapter due to gender (respectively age). Therefore the data of all 19 animals were pooled for all parameters.

II.3.2.1 Escape Latency

As described for the ECM the escape latency is differentiated into the searching and the entering latency. The immediate reaction of the animal upon introduction into the CM arena was investigated by the latencies of the first trial per session. Considered were the sessions before the probe trial (session without escape tunnel) and the first session thereafter, which was ss 8. The following sessions were not taken into account since the number of animals was stepwise reduced by those mice that reached criterion and therefore underwent the relearning as second step of the experimental design.

As seen in Fig. II.25A the animals showed a high median searching latency of 81 s in ss 1 with a large variability that was similarly found just at the next day in ss 2. A rapid decrease was achieved down to a level of around 13 s for ss 5 and 6. Interestingly in ss 8 after the probe trial session the mice exhibited a similar latencies as in ss 3 and 4 with a median of 28 s.

The rapid decrease within training sessions was corroborated by a significant effect found in the Friedman test ($p < 0.001$). A post hoc comparison with ss 1 performed by the Dunn's test showed that significant lower latencies were found from ss 3 on.

Fig. II.25B illustrates the entering latency. This parameter started on a median value of 9 s and steadily decreased down to 3 s in ss 6. In this case the mice displayed the same entering latencies in ss 6 and ss 8 (the session immediately following the probe trail).

Again a significant effect of decrease within sessions was found in the Friedman test ($p < 0.001$). The post hoc analysis comparing ss 1 by the Dunn's test proved the latencies to be significantly lower from ss 4 on.

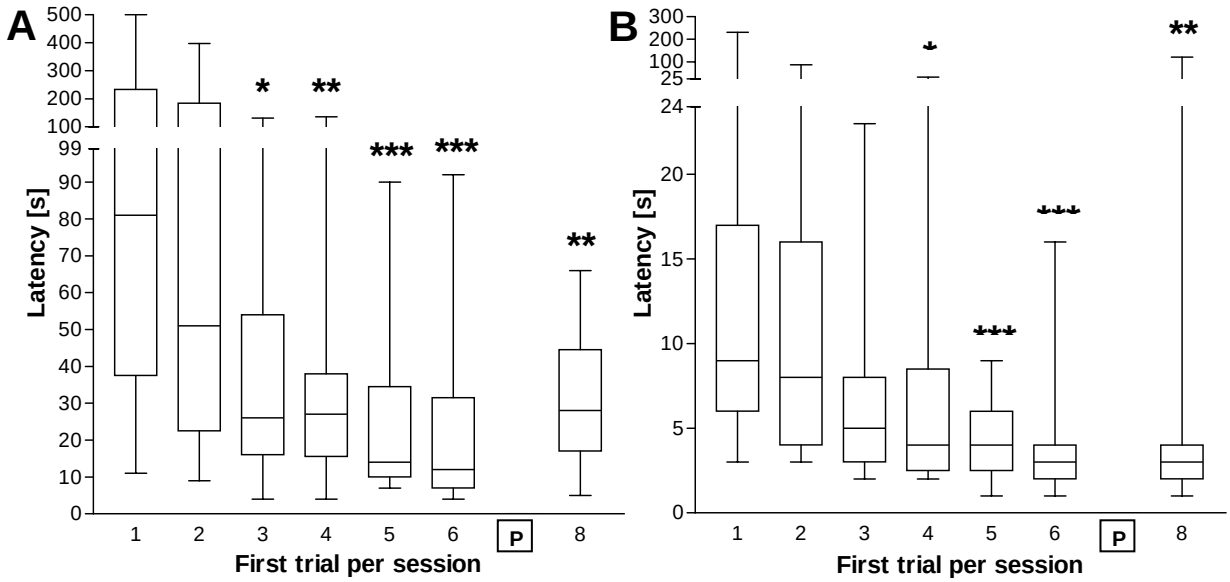
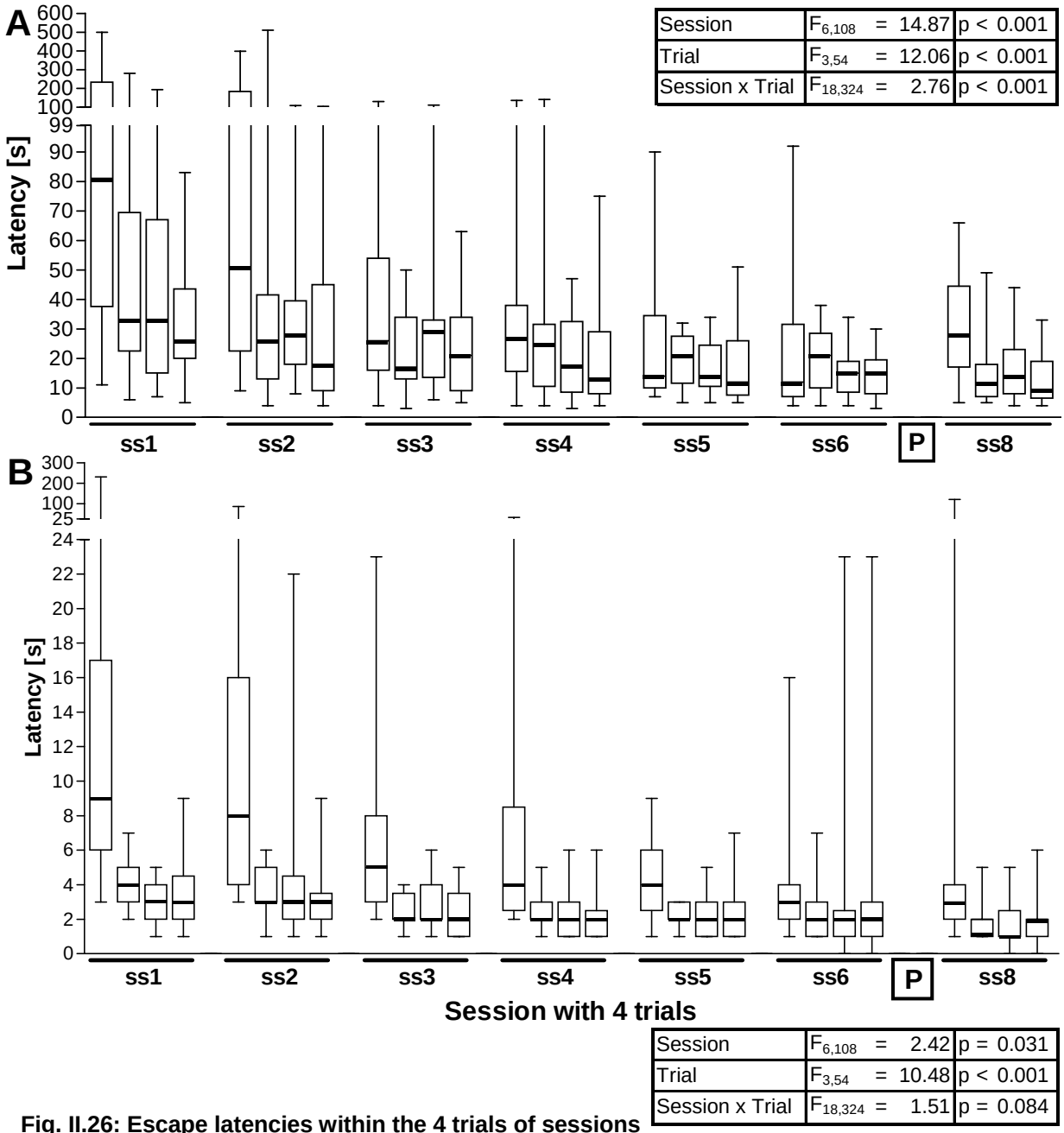


Fig. II.25: Escape latency
The escape latency distinguished for searching latency **(A)** and entering latency **(B)** is presented as box plot for the first 6 ss of training and ss 8, that followed a probe trial session -P.The following ss, in which the number of 19 mice were reduced stepwise are not presented. **(A)** The steady decrease in searching latency reveals that the mice increasingly familiarized to the CM arena and the protocol showing an effect of disturbance due to the probe trial session. **(B)** The entering latency shows an even faster decrease but no effect of the probe trial indicating that it did not affect the motivation to enter the tunnel immediately. *-p<0.5, **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

Since for the c-fos mapping study it was important that all animals received the same amount of training, the number of trials was fixed to 4 per session independent of success in contrast to the ECM. To see the influence of the repeated introduction due to the massed training providing no positive reward for success, the latency change within the 4 trials of a session was tested additionally by an ANOVA. Fig. II.26A shows the searching latencies of ss 1 to ss 8 for all 4 trials per session.

There was a significant effect of session ($F_{6,108} = 14.87$; $p < 0.001$), for which the post-hoc analysis revealed that the mice had higher searching latencies on ss 1 as compared to the other sessions. In addition there was a significant effect of trials ($F_{3,54} = 12.06$; $p < 0.001$). The post-hoc analysis showed that mice had higher searching latencies in trial 1 than in the following three trials. The post-hoc analysis of the significant interaction between sessions and trials ($F_{18,324} = 2.76$; $p < 0.001$) indicated that the trial effect was decreasing with session, resulting in indistinguishable latencies for all trials in ss 3. Therefore, from ss 3 on a significant decrease was neither found any more within sessions, nor within the 4 trials of a session.



Session	$F_{6,108}$ = 14.87	p < 0.001
Trial	$F_{3,54}$ = 12.06	p < 0.001
Session x Trial	$F_{18,324}$ = 2.76	p < 0.001

Session	$F_{6,108}$ = 2.42	p = 0.031
Trial	$F_{3,54}$ = 10.48	p < 0.001
Session x Trial	$F_{18,324}$ = 1.51	p = 0.084

Fig. II.26: Escape latencies within the 4 trials of sessions
The first 6 sessions of training and ss8, which followed a probe trial session - P are presented with their 4 trials as box plots. The following sessions, in which the number of 19 mice was reduced stepwise, are not presented. **(A)** In ss1 and ss2 the strongly reduced searching latencies of the second to fourth trials compared to the first trial indicate an effective familiarization within session due to repeated exposure to the CM arena in addition to the less strong familiarization observed from session to session, which is revealed by the first trials (see Fig II.25). **(B)** These effects are seen even stronger in the entering latencies. General effects of a 2-way ANOVA are shown in the textboxes.

The same analysis was performed for the entering latency, which is illustrated for all trials in ss 1 to 8 in Fig. II.26B. Here the general decrease within sessions showed a significant effect ($F_{6,108} = 2.42$; $p = 0.031$) as well as the decrease within the 4 trials of a session ($F_{3,54} = 10.48$; $p < 0.001$). Although no effective interaction was found between session and trial ($F_{18,324} = 1.51$; $p = 0.084$).

II.3.2.2 Probe trial

According to the two purposes of the RCM an additional probe trial session was performed during the acquisition phase. Since the mice cannot vanish into the escape tunnel within few seconds this allows a more extensive analysis of the behavior. Additionally the animals can be observed in a situation where the expected Target is missing. On the one hand this can prove that a preference for the Target is not led by the Target or escape tunnel itself. On the other hand the reaction to the missing Target can lead to valuable conclusions for principle processes occurring in case of a mismatching reward expectations. This is important for the comparison with the situation of relearning, where the reward is also not matching reward expectations.

Fig. II.27A shows the mean distance to a zone created for the Target hole and the opposite hole, a parameter calculated by the EthoVision system. Mice were found in a mean distance of 45 cm to the Target, which was highly significant less than the mean distance to the opposite hole with a median of 57.5 cm (Wilcoxon Signed Rank test $p < 0.001$).

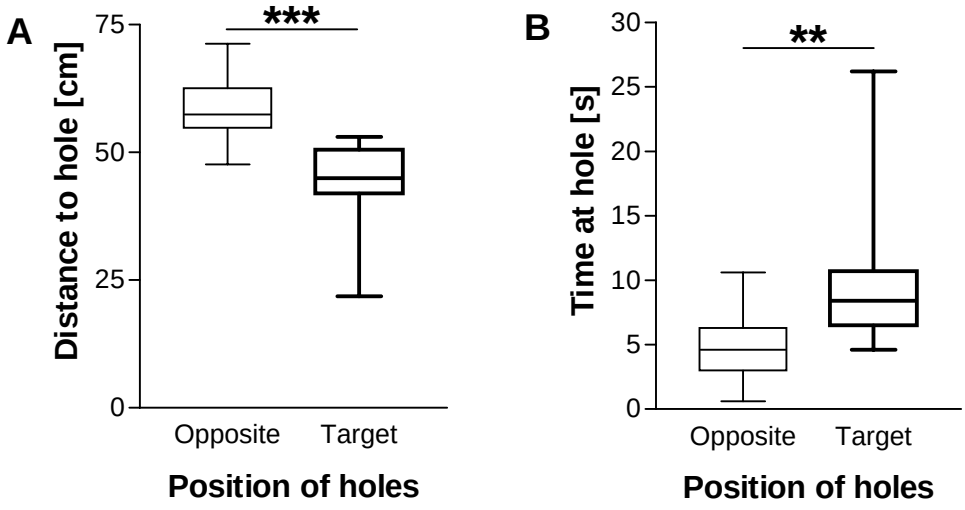


Fig. II.27: Localization as distance to hole and time at hole
(A) The mean distance to a zone defined for the Target and the opposite hole is shown as box plot. The mice are found closer to Target than to the opposite hole. **(B)** The time the mice stayed in a zone defined for the Target and the opposite hole during the 60s probe trial is presented as box plot revealing that the mice stay more time at the Target zone than in the zone of the opposite hole. ** =p<0.01, *** =p<0.001 (Wilcoxon Matched Pairs test).

Fig II.27B presents the time mice spent during the 60 s probe trail directly at the Target or the opposite hole. This corroborated significantly that the mice spent almost the double length of time at the Target with 8.5 s compared to 4.5 s at the opposite hole.

As a function combining way and time the localization of the mice can be calculated for every point of the CM arena as x-y function spatial histogram. Fig. II.28A presents the likewise calculated localization of the animals as a matrix of s / 5 cm^2 for the CM platform with a color-scale indicating the different time levels per area going from blue for low to red for high levels. This illustrated that the only positions of prolonged localization were found at the different holes. Within these the mice were seen mainly in the area of the Target and to a lower degree at the adjoining hole at each side.

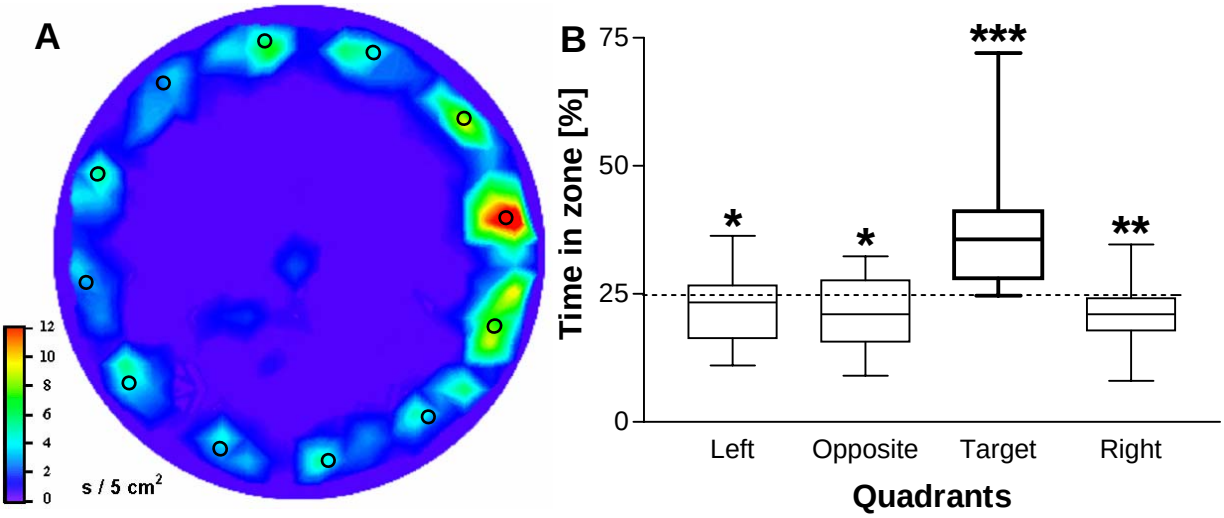
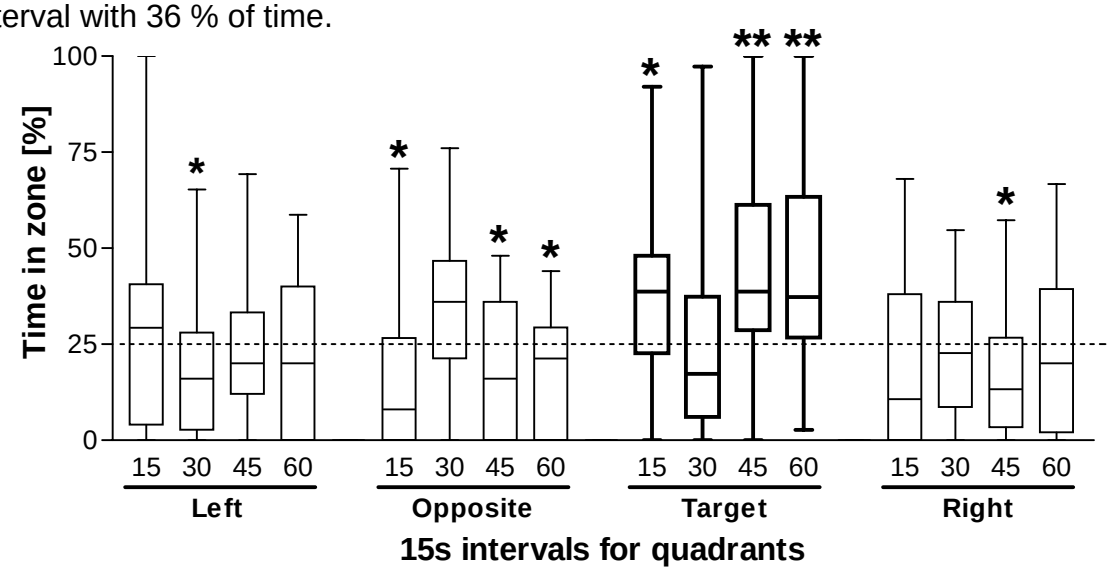


Fig. II.28: Localization as time per area-matrix and time in quadrant
(A) The length of stay for every point of the CM arena as x-y matrix is presented in s/5cm². The time is depicted by a color-scale reaching from blue for low to red for high levels of time. The black rings indicate the position of the holes (not exactly true to scale). The mice are found longest in the area around the Target and the adjacent holes at each side seen as red and yellow spots. **(B)** The time the mice stayed in the different quadrants of the CM given as percentage of the 60s probe trial is presented as box plot. The mice stay more time in the Target quadrant compared to the other quadrants as well as compared to the chance level of 25 % (indicated as broken line).
* =p<0.05; ** =p<0.01; *** =p<0.001 (Wilcoxon Signed Rank test comparison to chance level). Note: Dunn's test comparison Target/Left p<0.01; Target/Opposite p<0.01; Target/Right p<0.001).

The area covering the holes still matching the criterion corresponds to a quarter of the platform. Therefore another analysis of time spent was performed separately for the 25 % areas of the Target quadrant (covering the area of the Target +/-1 hole), left, opposite and right quadrant. The mice stayed significantly longer in the Target quadrant compared to the other three quadrants (Friedman test, $p < 0.001$; Dunn's test Target/Left p<0.01; Target/Opposite p<0.01; Target/Right p<0.001). Additionally the Wilcoxon Signed Rank test revealed significant differences to the chance level of 25 % for the Target laying above (median 35.7 %) and the other quadrants below (left: median 23.3 %; opposite and right: median 21.0 %, Fig. II.28B).
During the 60 s of the probe trial the mice can repeatedly visit every hole on the platform. The former analysis demonstrated where the animals search over the whole

time of the probe trial. However, it was to expect that the animals show variations in their searching behavior in between. Therefore the probe trial was divided into 4 intervals of 15 s for a more detailed investigation.

Fig. II.29 presents the time spent in the different quadrants comparing the 4 intervals. The most interesting fact was that the mice performed a rather constant search within the Target quadrant throughout the probe trial. There they were found around 38 % of time in each time interval beside the second, in which they were 17 % of time in the Target quadrant. This pattern corresponded to the time spent in the opposite quadrant, where they were always found even below chance level beside the second interval with 36 % of time.



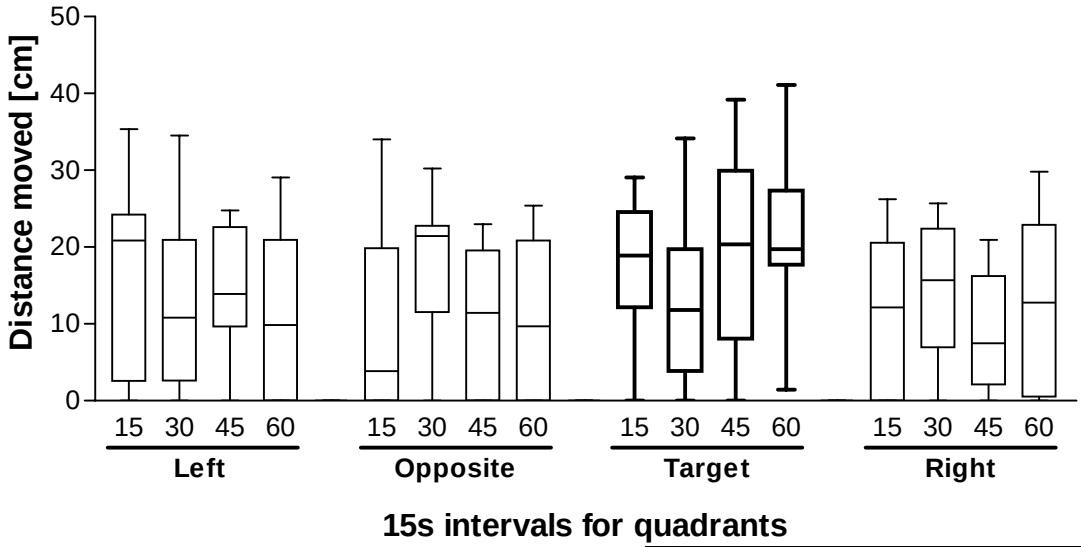
Interval	$F_{3,54}$ = 0.96	p = 0.419
Quadrant	$F_{3,54}$ = 13.89	p < 0.001
Interval x Quadrant	$F_{9,162}$ = 1.36	p = 0.211

Fig. II.29: Time spent in quadrants for 15 s intervals
The time the mice stayed within the different quadrants of the CM given as percentage of each 15 s interval is presented as box plot. The mice are found more often in the Target quadrant and less often in the opposite quadrant compared to the chance level of 25 % (broken line) in all intervals except the second. * =p<0.05; ** =p<0.01; *** =p<0.001(Wilcoxon Signed Rank test). This preference for the Target quadrant is supported by a 2-way ANOVA (see textbox). Note: Post hoc comparison of the Target quadrant with the Duncan's test for interval 45 (T/L p < 0.05; T/O p < 0.01; T/R p < 0.01) and interval 60 (T/L p < 0.05; T/O p < 0.01; T/R p < 0.05).

The Wilcoxon Signed Rank test corroborated this pattern with significant differences from chance level for all intervals beside the second in both the Target and the opposite quadrant (Fig. II.29).

In a 2-way ANOVA an effect of the factor quadrant ($F_{3,54}$ = 13.89; p = 0.001) was found with the Duncan's test as difference between the Target and other quadrants in interval 45 (T/L p < 0.05; T/O p < 0.01; T/R p < 0.01) and interval 60 (T/L p < 0.05; T/O p < 0.01; T/R p < 0.05). No effect was seen for the factor interval ($F_{3,54}$ = 0.96) and the interaction of interval and quadrant ($F_{9,162}$ = 1.36).

A very similar observation was seen for the parameter distance moved, which confirmed that the animals were really moving and searching in the visited quadrant instead of hesitating or waiting at one spot. As well the mice showed the longest distances moved of around 20 cm in the Target quadrant beside the second interval with just 12 cm opposed the distances moved in the opposite quadrant where the second interval staked out with a distance of 21 cm of the same level as the Target quadrant (Fig. II.30).



Interval	$F_{3,54}$ = 0.08	p = 0.970
Quadrant	$F_{3,54}$ = 10.42	p < 0.001
Interval x Quadrant	$F_{9,162}$ = 1.78	p = 0.076

Fig.II.30: Distance moved in quadrants for 15 s intervals
The length of way the mice moved in the different quadrants within each 15 s interval is presented as box plot. The mice showed a preference for the Target quadrant seen as a longer distance moved than in the other quadrants. General effects of a 2-way ANOVA are shown in the textbox. Note: Post hoc comparison of the Target quadrant with the Duncan's test for interval 45 (T/L ns; T/O $p < 0.05$; T/R $p < 0.05$) and interval 60 (T/L $p < 0.05$; T/O $p < 0.05$; T/R $p = 0.051$).

As seen in the former parameter the 2-way ANOVA showed an effect of the factor quadrant ($F_{3,54} = 10.42$). With Duncan Test the difference was found between the Target and the other quadrants in interval 45 (T/L ns; T/O $p < 0.05$; T/R $p < 0.05$) and interval 60 (T/L $p < 0.05$; T/O $p < 0.05$; T/R $p = 0.051$). No effect was seen for the factors interval ($F_{3,54} = 0.08$) and interval by quadrant ($F_{9,162} = 1.78$).

II.3.2.3 Learning performance

The RCM was performed as first part of a c-fos mapping study. To achieve a comparable successful performance as precondition for the following induction of c-fos expression the mice were trained in a first training phase until they reached the criterion corresponding to that defined in the ECM (see chapter II.3.1.5). As

soon as the mice matched the criterion to approach the Target +/ 1 hole in three consecutive trials they finished this first phase of training. The next day they underwent a day of training in the relearning phase.

The acquisition curve was created out of the days before the probe trial was performed, when all animals were still taking part in training, plus the two last training sessions of each animal. In this way each training day represented all 19 animals.

The animals started the training with a rather low number of mistakes of 5.3 on day 1 so that no strong decrease could be observed until day 6 with 4.5 mistakes.

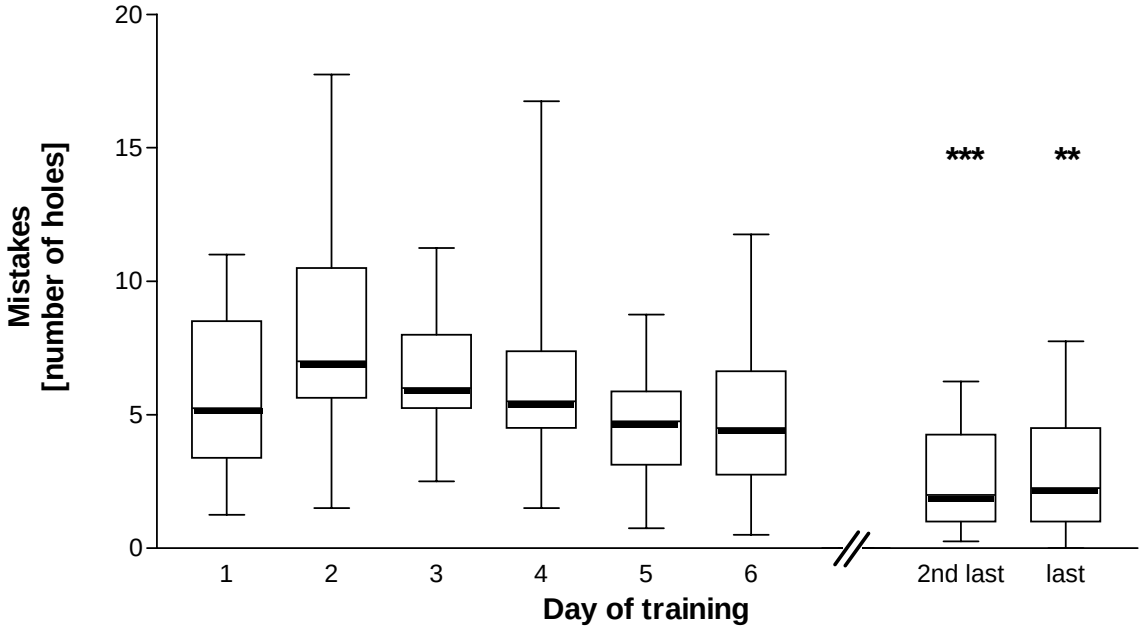


Fig. II.31: Learning performance measured as mistakes
The numbers of mistakes are presented as box plots for the days 1-6 and the two last days of training representing thereby all 19 mice. The mice decrease the number of mistakes to a stable level on the last two days indicating a completed learning process. **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

Accordingly the effect found in the Friedman test ($p < 0.001$) revealed in the post hoc comparison with the Dunn's test to day 1 the only significant differences on the 2nd last and last day with medians around 2 mistakes (Fig. II.31).

The presentation and analysis for the parameter DTT was performed in the same way. It revealed a steady decrease from a distance of 3 holes at day 1 down to 1 hole on the second last and last day of training. The Friedman test ($p < 0.001$) followed by a Dunn's post hoc comparison to day 1 corroborates the significant lower values for the second last and last day (Fig. II.32).

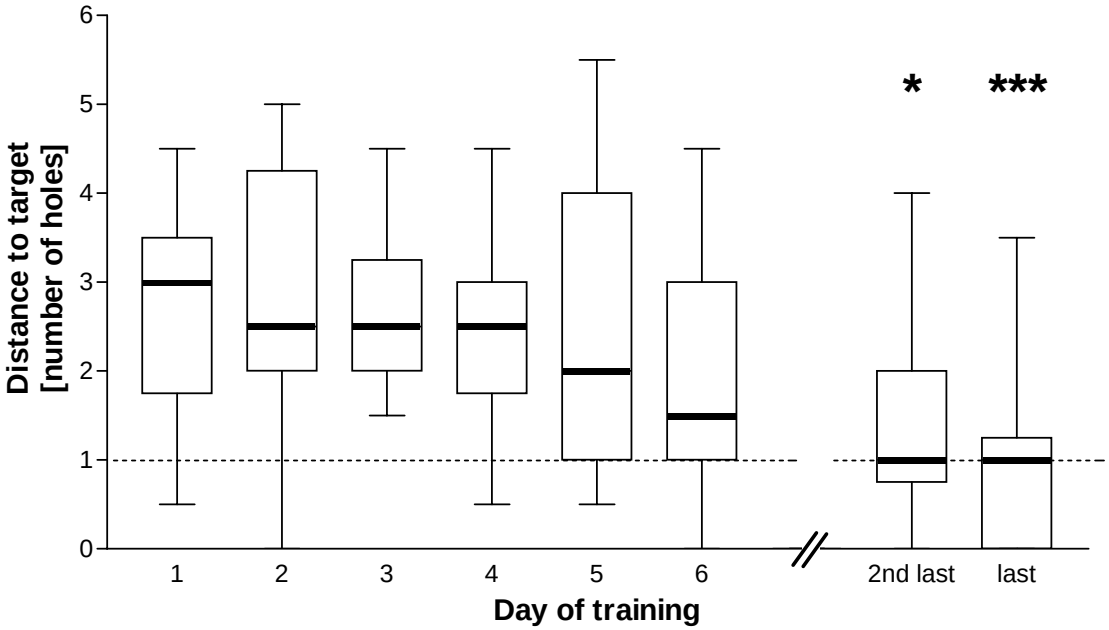


Fig. II.32: Learning performance measured as distance to Target
The distance to Target, given as number of holes is presented in box plots for the days 1-6 and the two last days of training representing thereby all 19 mice. The mice decrease the distance to Target to a stable level on the last two days that matches the criterion level. This shows that the mice orientate spatially when completing the learning process. The broken line indicates the distance matching the criterion. *-p<0.5, ***-p<0.001 (Dunn's test after Friedman test).

II.3.2.4 First approaches

The development of the spatial quality of the learning can be observed by the distribution of first approaches to the 12 holes of the platform. As illustrated in Fig. II.33 the mice showed no obvious preference to a specific hole in ss 1 and ss 3. In ss 6 a slight focus towards the Target and the adjoining hole 5 was noticeable with Target approaches reaching 22 % compared to a chance level of 8.3 %. A strong preference was found in the second last and last session towards the Target and the adjoining holes, in which the Target was visited with levels of 31 % and 39 % of approaches, respectively. This development of a preference was corroborated by the Observed Versus Expected Frequencies test revealing significant differences to the frequency corresponding a chance level of 8.3 % for the ss 6 ($p < 0.001$), the second last and last session (both $p < 0.001$). Correspondingly the Kolgomorov-Smirnov Distribution Fitting test for a rectangular distribution showed that ss 6 ($p < 0.05$), the second last ($p < 0.05$) and last session ($p < 0.01$) differed from a rectangular distribution that would correspond to a distribution around chance level.

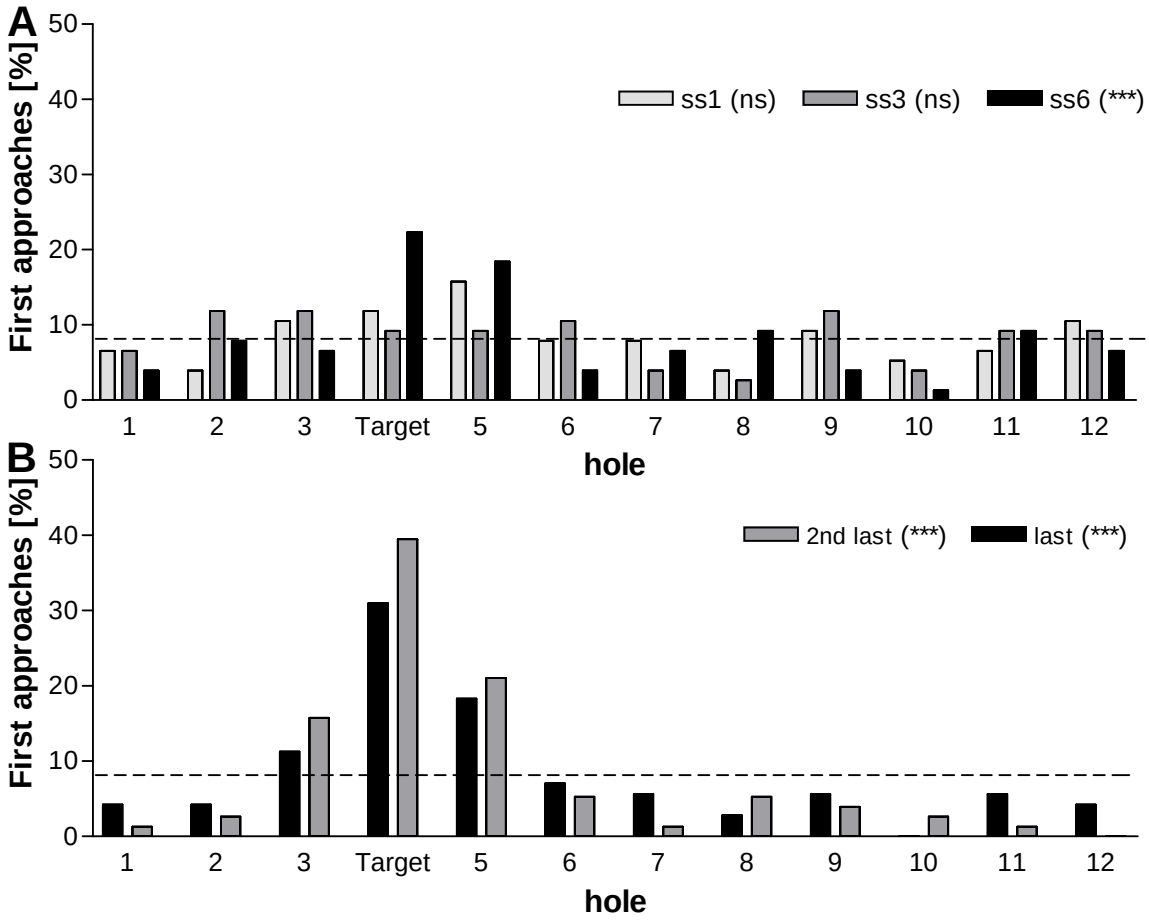


Fig. II.33: Distribution of first approaches for all holes of the platform.
The frequency of first approaches given in percentage is presented for all holes. **(A)** The distribution of ss1, ss3 and ss6 reveals an increasing focus of the mice towards the Target. **(B)** In the 2nd last and last ss the mice revealed a strong preference towards the Target and the adjacent hole at each side. Chance level (=8.3%) is indicated as broken line. Significance levels are given for the Observed Versus Expected Frequencies test. ***-p<0.0001. Note: Kolgomorov-Smirnov Distribution Fitting test for rectangular distribution (ns for ss1 and ss3; p<0.01 for ss6, 2nd last and last ss).

II.3.3 Reinforced Relearning Circular Maze (RRCM)

Corresponding to the RCM the behavioral data of the first training of the RRCM were analyzed for the parameters escape latency, mistakes, distance to hole and distribution of the first approaches. Due to the missing probe trial session and the fact that animals exceeding the maximal training duration of 10 days for reaching the criterion were excluded, the training progressed faster than in the RCM. Altogether 33 mice were included into the behavioral analysis presented below.

II.3.3.1 Escape Latency

The escape latency divided into searching and entering latency was analyzed for the first 3 ss. In this period the most changes were occurring as seen in former experiments.

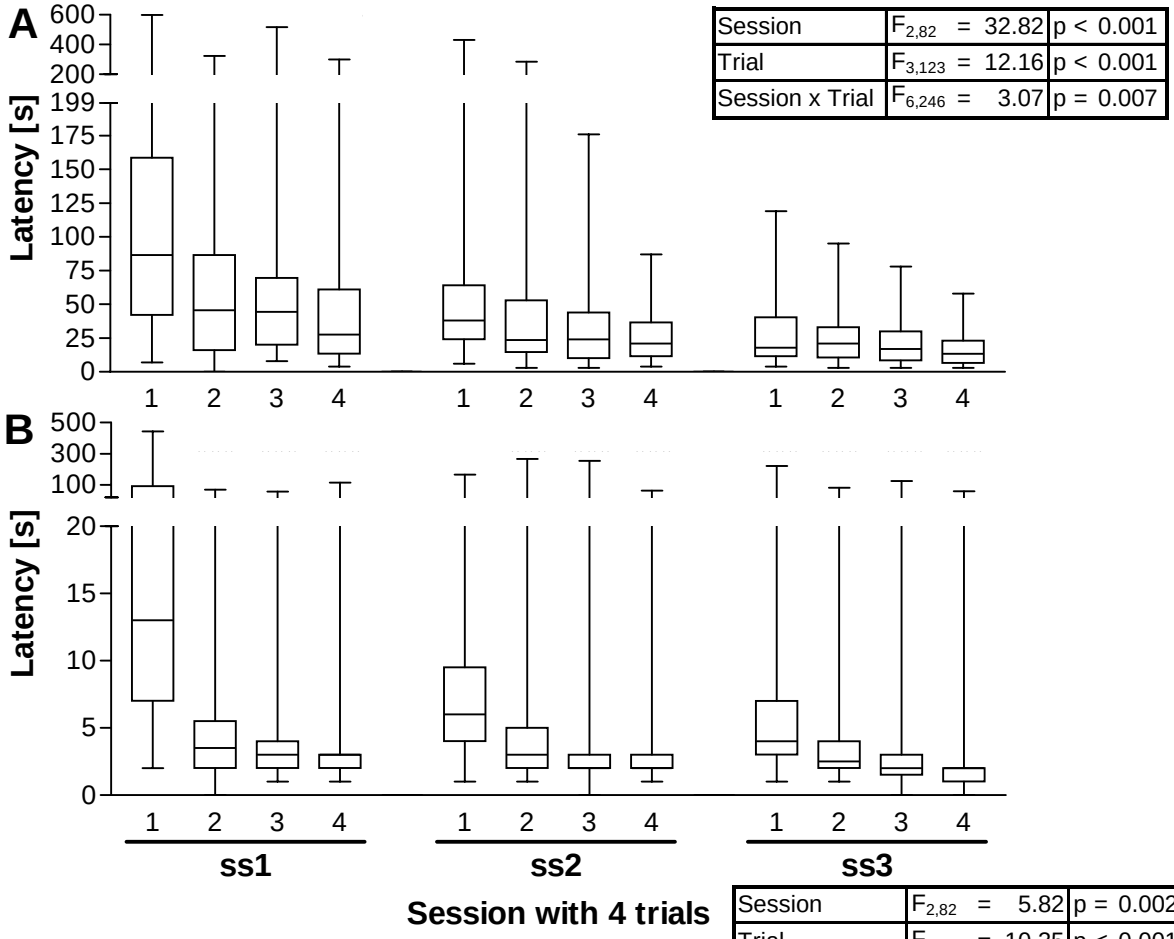


Fig. II.34: Escape latencies within the 4 trials of sessions
The first 3 ss of training are presented with their 4 trials as box plots for searching latencies **(A)** and entering latencies **(B).** In both cases decreases are found within and between sessions, indicating an effective familiarization within session due to repeated exposure to the CM arena in addition to the less strong familiarization observed from session to session. General effects of a 2-way ANOVA are shown in the textboxes.

The course of change was similar to that found in the ECM and RCM although even more stable due to the high number of 33 mice present. In ss 1 the mice started with a searching latency of 87 s decreasing to 28 s from trial 1-4 (Fig. II.34A). In ss 2 the range was already reduced to a level of 38 s going down to 21 s. The ss 3 was the only session, in which the first trial was with 18 s even lower than that of the second trial, indicating that a rather stable level was reached even for the first trials.

This course in latencies was corroborated by a 2-way ANOVA testing the factors session, trial and their interaction. Significant effects confirmed the decrease within sessions ($F_{2,82} = 32.82$; $p < 0.001$) and within trials ($F_{3,123} = 12.16$; $p < 0.001$) as well as the interaction of session and trial ($F_{6,246} = 3.07$; $p = 0.007$).

The entering latency as presented in Fig. II.34B revealed a very similar pattern on lower levels starting in ss 1 with 13 s and going down to 2 s in the end of ss 3. Striking was that the latencies of the second to forth trials reach the minimal levels of 3-2 s already from ss 2 on. The first trials of all sessions revealed a higher value although decreasing

(ss 1 = 13 s; ss 2 = 6 s; ss 3 = 4 s). Correspondingly the ANOVA revealed in addition to the significant decrease within sessions ($F_{2,82} = 5.82$; $p = 0.002$) and within trials ($F_{3,123} = 10.35$; $p < 0.001$) a highly significant interaction of session and trial ($F_{6,246} = 3.07$; $p < 0.001$).

II.3.3.2 Learning performance

As in the RCM the experimental design of the RRCM intended the elimination of mice from the first training as soon as they reach the criterion of Target +/-1 hole. Therefore paralleling the behavioral analysis of the RCM the parameters mistakes and DTT were analyzed for the first 4 sessions and the second last and last training session so that always all 33 animals were represented.

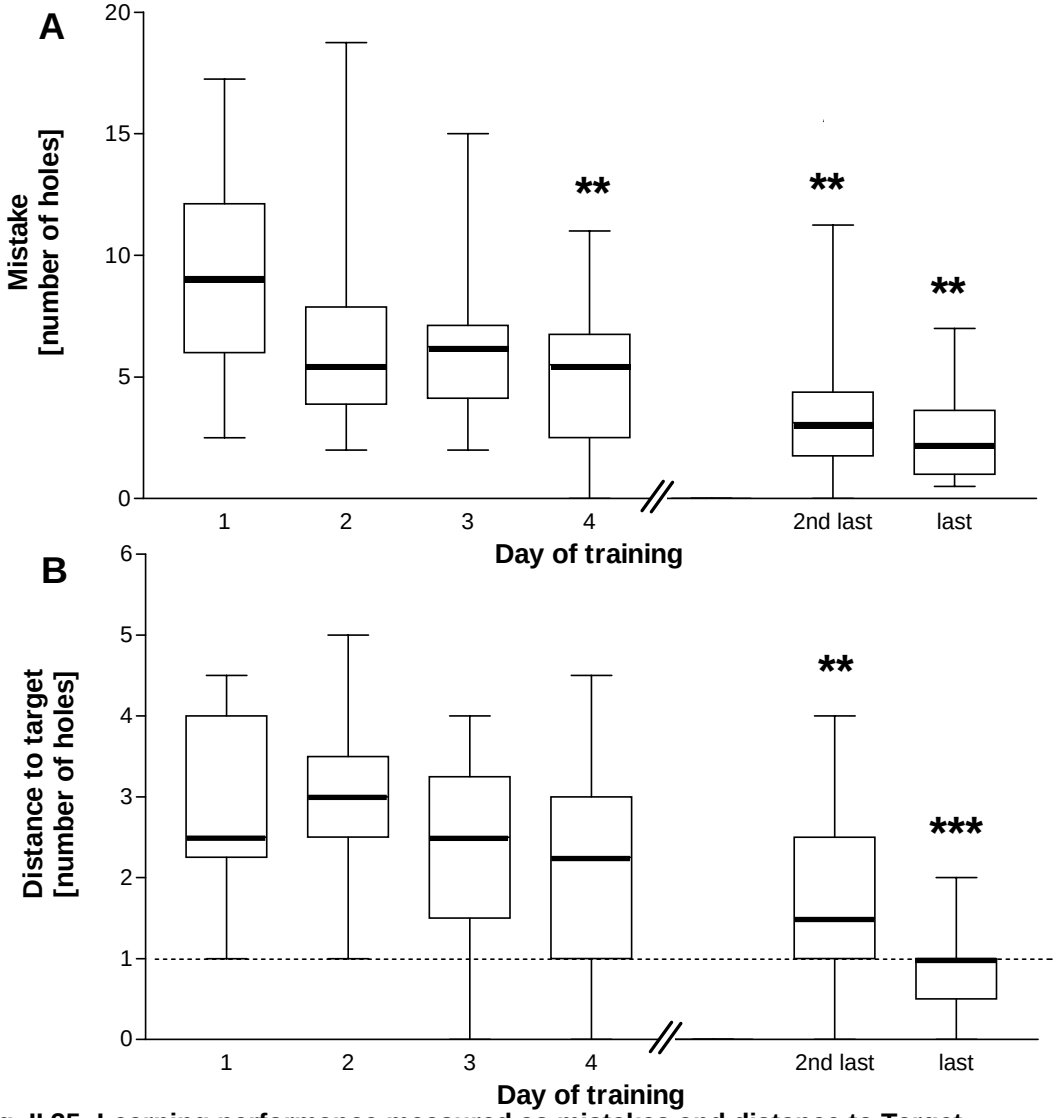


Fig. II.35: Learning performance measured as mistakes and distance to Target
Numbers of mistakes **(A)** and distance to Target **(B)** are presented as box plots for the days 1-4 and the two last days of training representing thereby all 33 mice. The decreases found in both parameters indicate that the mice learned successfully to orientate within the CM arena to find the Target. The broken line indicates the distance matching the criterion. *-p<0.5, **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

The mice performed the day 1 of training with 9 mistakes. This value decreased to 5.5 mistakes on day 4 and further down to 3.1 and 2.3 mistakes in the last two sessions, respectively. Significance reached the decrease from day 4 on compared to the first day (Friedman test $p < 0.001$, Fig. II.35A).

In the performance measured as DTT the mice displayed a low median value of 2.5 for day 1. Therefore no obvious decrease was found until day 4 with 2.3 holes distance. Accordingly the found effect of decrease with days (Friedman test $p < 0.001$) was found in the post hoc comparison to day 1 with the Dunn's test to be due to the low levels of the second last (median 1.5 holes) and last training day (median 1 hole, Fig. II.35B).

II.3.3.3 First approaches

The distribution of first approaches within all holes of the platform can give information about the spatial quality of learning and its development within training. Fig. II.36 illustrates that in ss 1 no obvious preference was observed.

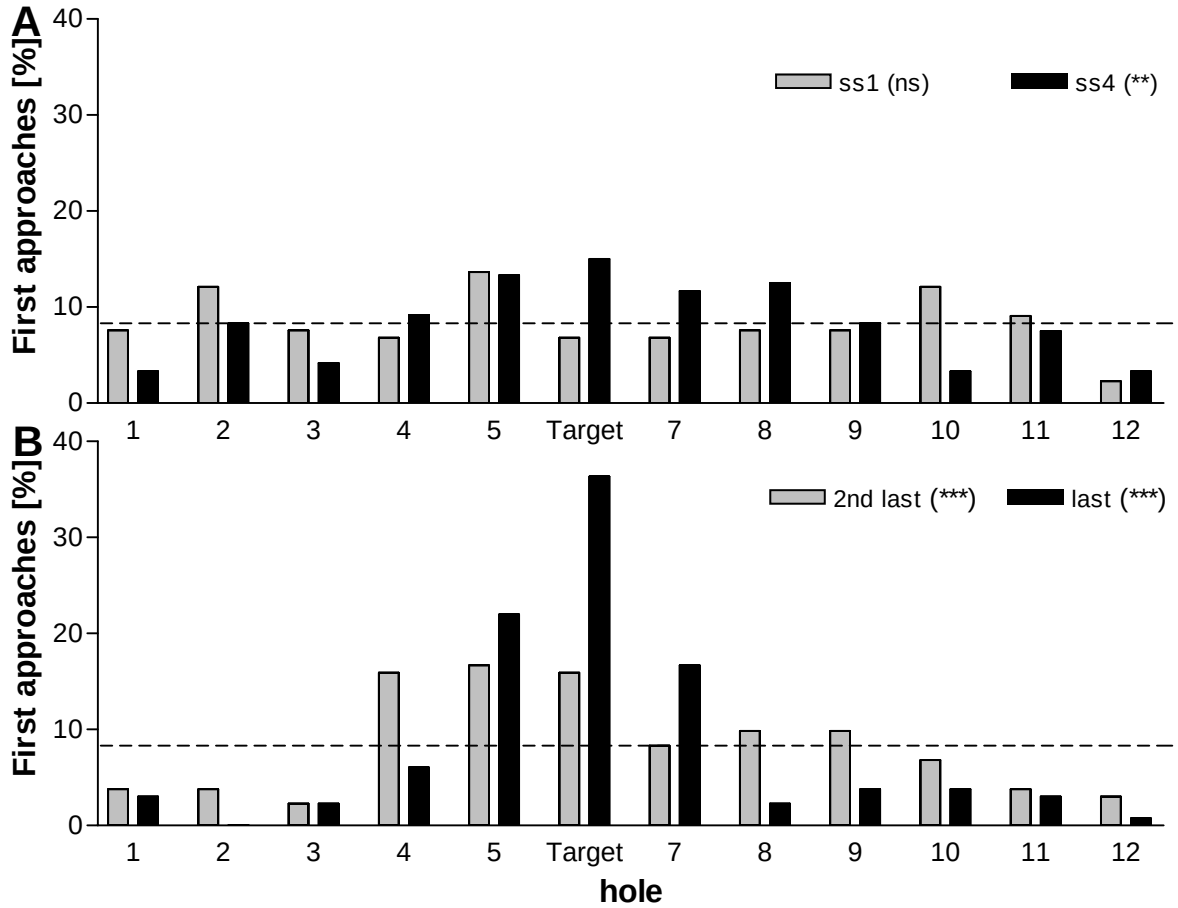


Fig. II.36: Distribution of first approaches for all holes of the platform.
The frequency of first approaches given in percentage is presented for all holes. **(A)** In ss1 the mice revealed an inconsistent distribution of approaches. In ss4 the mice started to focus towards the Target and 1-2 neighboring holes at each side. **(B)** In the 2nd last ss the mice showed a slight preference, in the last ss a strong one towards the Target and the adjacent hole at each side. Chance level (=8.3%) indicated as broken line. Significance levels are given for the Observed Versus Expected Frequencies test. **-p<0.01, ***-p<0.0001. Note: Kolgomorov-Smirnov Distribution Fitting test for rectangular distribution (ns for ss1 and ss3; p<0.01 for ss6, 2nd last and last ss).

In ss 4 a slight focus towards the Target region and away from the opposite side (hole 12) could be noticed (15 % and 3 %, respectively compared to a chance level of 8.3 %). The second last session revealed this pattern more obviously but a strong preference to the Target with 36 % of the approaches was only found in the last session. The Observed Versus Expected Frequencies test revealed significant differences to the frequency corresponding to a chance level of 8.3 % for the ss 4 ($p = 0.004$), the second last ($p < 0.001$) and last session ($p < 0.001$). However, the Kolgomorov-Smirnov Distribution Fitting test for a rectangular distribution corroborated the only significant difference from a rectangular distribution for the last session ($p < 0.01$).

II.4 Discussion

Within the current study, a learning paradigm was established and a protocol designed, that was optimized for the requirements of an investigation of c-fos expression pattern: The detailed behavioral analysis proved reliable spatial learning in the CM and provided a parameter specific for spatial selective performance. A criterion for individual learning success allowed to achieve highly similar preconditions for all animals. The induction of a relearning process in a familiar situation served for conditions with minimized side stimuli and offered control groups with maximal comparability to the experimental groups.

II.4.1 Motivational state

The mice were trained in the CM under low physical demands and without any penalty that they have to expect for making errors. Therefore, the pressure to perform correctly was low compared to paradigms as MWM or SDA. In many paradigms positive reinforcement as food reward is used but that requires a food deprivation for the mice. In the classical Barnes Maze design additional aversive stimuli such as fan or buzzer are necessary, since the reward is an unfamiliar small escape box (Barnes, 1979). The CM paradigm was designed to avoid these stressors and therefore a stronger, more 'natural' reward was used, namely the predictable escape into the familiar and safe home territory.

The searching and entering latencies during training have been investigated to prove that the home-cage by itself was an effective and strong reward causing a high

motivation to perform well. Searching and entering latency revealed the same course in the establishing (ECM), relearning (RCM) and reinforced relearning (RRCM) experiments. Whereas searching latency is influenced both by the ability to find the Target and the motivation to enter the tunnel, entering latency is independent from searching abilities and therefore better represents the motivation to escape from the platform. Both latencies were higher during the first trial compared to the following three trials in the beginning of the training. After 3-4 days, mice showed comparable latencies in all trials of a session. Most probably, the higher latencies in the first trial were due to the reaction of the mice to the novel situation, to which it was exposed. Therefore the first trials were most indicative for the direct reaction of mice to the introduction into the CM arena. Indeed, mice habituated to it within the first and second day. The low latencies showed by the mice throughout the training are indicative of the high motivation to enter the Target tunnel. The results show that the home-cage itself is a strong and effective reward for learning in the CM. Neither additional aversive stimuli, high pressure of physical demands or punishment, nor reinforcement going along with deprivations are necessary. All of these treatments can be seen as stress releasing factors, which are excluded in the CM. Since mice return to the home-cage via the escape tunnel, the reward is also independent of a handling, which is as well aversively and can elicit stress responses (Gerlai, 2001; Gerlai and Clayton, 1999). In addition, due to the predictability of a return to a safe and familiar territory the home-cage itself functions as stress reducing.

Therefore, it is assumed that this experimental design allows to test spatial learning in mice under lower anxiogenic and stressful conditions as compared to other paradigms commonly used to test cognition in rodents. Indeed, a low stress component is important to reduce side stimuli that can influence gene expression of learning linked genes such as c-fos in a mapping study (Kaczmarek and Robertson, 2002; Tischmeyer and Grimm, 1999).

II.4.2 Spatial learning

The Barnes Maze, which supplied the conceptual basis for the design of the CM described here, serves as spatial learning paradigm. The feature of spatial learning is important since it provides a cognitively demanding form of learning, which requires the use of multiple relationships among extra-maze landmarks (Inman-Wood et al., 2000). It is often seen as model for declarative learning in mice and as hippocampus

dependent form of learning. An important aim of this study is therefore to show that spatial orientation is a major component of the learning performance in the CM. Since regarding the c-fos mapping study the hippocampus is the area of main interest and the design requires training until a certain learning performance, the further aim is to find a reliable judgment for learning performance that specifically considers the spatial component.

Mice can use three different strategies to find the escape tunnel with different degrees of efficiency and cognitive demands. The shift of searching strategies used during training demonstrates how mice change behavior in adaptation to the situation, trying to escape the platform most efficiently.

The random search strategy with several platform crossings and change of directions is the least efficient way of searching, but goes along with no knowledge or ability requirements and is typical for the beginning of training. The serial strategy, in which the holes are visited sequentially, is already a more efficient form of systematic searching. The behavioral competence for the serial strategy is not very demanding since it only requires the mouse to remember to search each consecutive hole (Mansuy et al., 1998). Correspondingly, it can be performed already in early phases of the training (Barnes, 1979). The serial strategy is found as maximal performance level in experimental animals with spatial memory impairments and corresponds to the response strategy in other paradigms (Bach et al., 1995). The spatial strategy, in which mice directly orientate towards the Target, is the most efficient way to search but is also cognitively most demanding (Inman-Wood et al., 2000). It requires the hippocampus, as revealed by investigations in mice with hippocampus impairments, thus mice need to create a spatial representation for it (Mansuy et al., 1998).

The highest form of spatial cognition supposes that the mice navigate with a cognitive map. That would allow a correct movement between two completely new positions independent of direct cues. This requires a high degree of abstraction so that the mice can navigate from and to every point of the spatial environment. Though it is still under debate whether mice are able at all to create cognitive maps in a strict sense or not (for overview see Bennett, 1996). Disagreement concerns the abilities that are required for a true cognitive map as well as the involved learning mechanisms (for overview see Chamizo, 2002, Holscher, 2003). In the CM such a high abstraction of space is not necessary since the paradigm does not allow starts from completely new positions. As in the MWM (where mice know all different positions after some

training) and most other mazes the learning situations are very much simplified in their environmental features thereby limiting the possibilities to force the use of cognitive maps.

Still, the minimal prerequisite is that mice recognize their own position in relation to the Target position, in order to choose the shortest connection. For this the mice have to integrate information of different external and internal sources (Holscher et al., 2004).

Since the spatial strategy allows judging that mice have achieved a spatial representation, the use of this strategy is an important proof that the CM serves as spatial learning paradigm. Also it demonstrates when and how mice are reaching a successful spatial learning performance in the course of training.

The experimental data of the ECM match perfectly to the expectation for a spatial learning paradigm (Bach et al., 1995; Barnes, 1979; Inman-Wood et al., 2000; Mansuy et al., 1998). The random search serves as main strategy only at the first day of training. A closer look even reveals that the mice start shifting to the serial strategy already within this first session. An interesting observation is that in the first 3 days of training a noticeable high number of animals uses the random strategy on the first trial as compared to the other 3 trials. This corresponds to the higher latencies found in the first trials of these training days and may reflect enhanced vigilance behaviors due to the introduction into the not jet familiar situation in the CM arena.

The serial strategy is achieved very quickly and represents the mainly used search strategy between day 2 and day 13 of training. Mice do not use the spatial strategy at all during the first session and increase its use starting from day 12. From day 14 on, mice mainly used the spatial strategy.

The low level of random search strategy found until the end of the training can in part be explained by animals that approach one of the neighboring holes at first, start running in the wrong direction along few holes, but stop and re-orientate backwards to the Target soon. Although they chose the correct direction, the change in running direction assigns them to a random search strategy, due to the very strict definition for the strategies. In other cases, mice approached a neighboring hole but started searching in the wrong direction and performed a complete serial search for the rest of the way. These mice are as well not counted as performing a spatial search, although their first directional choice suggests a correct spatial orientation. Both cases most probably result from the very short distance between the holes. The fact

that the next hole is almost reached by just turning the head reinforces the drive to explore the next by hole.

In conclusion, the analysis of the searching strategies demonstrates that mice already within the first session start to adapt their behavior to the new situation of the CM. In addition it shows that this first learning effect can be separated from the higher cognitive function of spatial learning, which requires a spatial representation and results in reliance on the spatial strategy.

II.4.3 Learning performance

Mistakes and distance to Target (DTT) were used as parameters for learning performance. The acquisition curves of both parameters reveal a very similar course, indicating that the performance ameliorated over days.

The DTT provides a good performance level when mice directly approach the Target or one of the neighboring holes, independent of the following way of searching. If these mice turn to the wrong direction, they perform the trial with a rather high number of mistakes, and therefore with a bad performance level herein. Since the choice of direction supports a rather successful spatial orientation, the parameter DTT is seen as a more reliable and meaningful measurement for spatial orientation. It measures the orientation directly, not taking into account the behavioral strategy, chosen if the animal meets a dead end tunnel. This is an important aspect since mice have a high drive to explore holes adjacent to the first approached ones, as mentioned before. This behavior, which is performed if the mouse does not immediately find the escape tunnel, is not the main concern of learning performance.

A strong support for DTT as reliable parameter of spatial orientation is that it parallels the course of spatial search strategy during training, with a common step-like performance increase between the 12^{th} and 14^{th} day, in contrast to the parameter mistake. The superior role of DTT as spatial indicator is additionally stressed by the analysis of the first approaches that indicates that mice display a preference to the Target starting from session days 13-14.

Noteworthy, all these observations show an abrupt step of change between day 11/12 and day 13/14. Such a course is rarely seen in acquisition curves of learning. Though Gallistel et al. (2004) argue that the typically seen course of gradual performance increase is an artifact of group averaging. The individual subjects

normally show an abrupt transition from a bad to a good performance. This is flattened out by group averages by the different time points, when the transitions take place in the individuals. In line with this argumentation the steep increase in the ECM performance would suggests a very similar time point for transition in all subjects of this experiment. This could be due to the very uniform and simplified surrounding, which allows just a similar processing of information to reach a reliable spatial representation.
Although the last session of the training was performed after a break of 6 days, no changes in searching and entering latencies, mistakes and DTT was observed, suggesting that the learned information could be retrieved from LTM.

In conclusion, the CM serves efficiently as spatial learning paradigm. The training in the CM achieves a spatial knowledge, which enables mice to guide themselves correctly towards the Target. All parameters indicate increasing learning performance but differentially stress the multiple components of learning, with DTT giving the most reliable information for spatial learning abilities.

II.4.4 Learning criterion

The investigation of c-fos gene expression requires mice on the same stage of learning for a maximal comparability. Therefore, an important aim of this study was to reliably judge, when mice reach a successful performance, and thereby determine, when the learning process is completed. When learning is completed and a stable state is reached, a new learning process can be started, which serves the induction of gene expression. A learning process is completed when no behavioral change is seen anymore and an asymptotic level of performance is achieved. This shows, what is the maximal level of performance that an animal can reach by learning.
As mentioned before, classical acquisition curves represent group averages. Mice can show strong individual variances in learning speed. Thus each sample point consists of a value combining more and less successful mice, which are on different stages of the learning process. Therefore acquisition curves are unsuitable tools to find the time points of learning success and thereby judge the stage of process.
The same stage of learning for a maximal comparability can therefore just be achieved by judging the mice individually. The DTT has proven to be a reliable parameter for measuring the success in spatial orientation. It is therefore used as parameter to define a criterion that allows an individual judgment.

The maximal and thereby successful level of performance (in this case the minimal level of DTT) can vary between individuals as well as the time that individuals need to reach a successful performance. As Gallistel et al. (2004) review for several examples, acquisition time (indicating learning speed) and maximal performance do not even co-vary in many cases. Therefore the mice have to be allowed individual time spans for learning as well as delimited differences in the performance level that is defined to be successful by the criterion.

One factor influencing maximal performance (performance at asymptote) is the discrimination ability of the mice, which leads to differences in precision. For instance, mice can know the position of the Target without being able to discriminate between adjacent holes. Due to this they may never reach a certain level of performance, although they learned to use the spatial representation to locate the Target. The purpose of this paradigm is neither to investigate discrimination abilities of mice nor to distinguish mice due to their precision. Therefore the choice of the correct direction reveals sufficiently that mice learned the task. In consequence, the criterion has to be defined allowing a delimited variance in performance that is depending on precision abilities.

The analysis of first approaches demonstrates that at asymptote mice show a preference that includes the neighboring 3-4 holes of the Target. The gaussian-like distribution suggests that the spreading on neighboring holes is due to discrimination and precision abilities. This reveals that the constant median of 2-3 holes DTT in the end of training is mainly a precision effect.

In order to consider the varying discrimination abilities of the subjects, the level for the criterion is chosen allowing precision-related mistakes. Corresponding to the distribution found in first approaches the criterion level includes the 4 neighboring holes at each side additionally to the Target. This corresponds to an allowed area of 25 % of the CM arena, paralleling herein the spatial demands of the MWM, a commonly used spatial learning paradigm.

Enlarging the Target area leads to an increase in the probability that correct choices could be made by chance. To reduce the probability of chance to insignificance, the mice have to choose correctly in repetition. Therefore the criterion has been defined as first approach of Target +/-4 holes (correct choice) in three consecutive trials. This lowers the probability of chance level below 1.6 %.

In order to validate the defined criterion, it was applied to the ECM. The comparison of the number of trials with correct performance (DTT of 4 holes or below) before and after criterion day indicates a highly significant better performance after criterion day. This comparison also reveals that even after criterion day trials with bad performance still can be found.

It is important to say that an overly made observation in learning paradigms is that subjects keep making post-acquisition errors (Gallistel et al., 2004; Wilkie et al., 1999). Wilkie et al. (1999) argue that these errors cannot be taken as memory failures. Although the source of these errors is not clear, they are certainly influenced by perception and attention as well as uncontrolled, superstitious or emotional behaviors. An example for such attention driven errors in the post acquisition-training phase of a Barnes Maze is reported by Bach et al. (1995). Therefore a 100 % correct post-acquisition performance was not expected as indicator for a successful spatial orientation.

The criterion, as applied for the 36-hole learning training, is defined in a way that suggests fewer holes as sufficient for spatial training. The level found for successful performance hints that this high number of holes could overburden some of the mice. This can result in false negative cases, in which mice start serial searching if they reach one of the neighboring holes as described above. To minimize this problem the number of holes on the platform was reduced to 12 holes. Accordingly the discrimination of a specific hole is easier, since the direction to reach it noticeably differs to the other holes. In addition, the increased distance between the holes reduces the influence of the explorative drive, since the next hole is not reachable any more by a small head turning but requires a directed run towards it.

The 12-hole platform provides the same area as correct Target quadrant but with 3 possible holes instead of 9. Therefore the criterion level is defined as first approach to the Target plus one adjacent hole at each side.

After being trained on the 36-hole platform, mice were trained with the 12-hole platform for additional 6 days to accustom them to the new platform and to test the effects on performance. As expected, the number of mistakes was reduced during training on the 12-hole platform as compared to the 36-hole one. All other parameters did not differ between training on the two platform versions. Though the extremely low level together with a closer look to the performance revealed that serial search

around the whole platform following an approach to one of the Target-neighboring holes, as seen on the 36-hole platform, has vanished almost completely.
The fact that DTT remains unchanged supports the hypothesis that a first approach to one of the holes within the Target quadrant corresponds to the maximal level of precision that can be reached by the mice in this paradigm.

II.4.5 Relearning

In order to investigate the course of a new learning process under different conditions, the mice continued to be trained with two different forms of relearning. During the first relearning the Target was located in a new position whereas the spatial surrounding remained unchanged. Thus mice did not need to create a new spatial representation but just to adapt their orientation to the new Target position within a well-known spatial surrounding. Since this was a less demanding request, it was expected that mice could quickly reach a successful performance. Mice were trained for 9 days, reaching a performance of criterion level on day 7. A detailed analysis of the distribution of first approaches demonstrates that the mice constantly chose between old and new Target area in 80-90 % of all cases throughout the complete training. This indicates that they strongly stuck to the old Target and gradually shifted to the new Target avoiding the other parts of the maze completely. Analysis of the tracks showed that the mice on regular base first visited the three to four holes around the old Target position and then moved strait on towards the new Target position. Possibly, the mice visit the well-known old Target position in case they cannot decide for sure the direction of the new Target. This may be due to predictability of outcome. From this position it would then be a simple left-right decision to reach the new Target, a strategy that is easier than a direct choice from the center. Though this would be a kind of procedural learning. Packard and McGaugh (1996) showed that mice initially acquire place information depending on hippocampal activity. With extended training mice shift to caudate-dependent response learning, which is typical for habit learning. However, the relearning presents a new situation, in which mice should employ the faster and more flexible hippocampus dependent learning (O'Reilly and Rudy, 2000).
Alternatively, the mice are driven by a strong reward expectation to the old Target position. Missing reward even increase the appetitive behavior, where or when the reward is expected. Like this a strong appetitive behavior would be the main reason

for the frequent visits of the old Target position. This would mean that the spatial re-orientation is reached much faster but is not noticeable since mice do not even try to go directly towards the new Target. The almost complete missing of visits at the other quadrants, as mislead try, strongly contradicts that mice cannot discriminate the new Target position correctly. In addition the typical search behavior around the old Target before the mice move towards the new Target position strongly supports that mice first ensure that the Target is not in the old position before visiting the new one. The low pressure to avoid mistakes in the CM paradigm, since these are not penalized, may herein support a relatively long persistence to approach the old Target.

In the second relearning training in addition to another new Target position the spatial surrounding was changed profoundly. The known landmarks are covered by dark curtains and replaced by new and differently positioned landmarks. In this way mice must build a new spatial representation of the environment. Due to these higher demands a slower acquisition was expected.

As expected, the analysis of the first approaches indicates that mice showed no preference to the last Target position or that of the learning, indicating that mice were disorientated by the new surrounding. Noteworthy, until the end of training the mice do not show a relevant increase in performance. The missing decrease of number of mistakes and DTT, together with no preference for any hole at the end of training, indicates that the mice were not able to create a sufficient new spatial representation within the 8 days of training.

An interesting phenomenon was the preference for holes 1 and 12 found in the first session of training. It is possible that information of unchanged sources, like the sounds of the monitors in the adjacent room, or the direction the experimenter approached the platform, guided the choice of direction. The absence of any performance increase beyond day 8 could show that a relearning in a new surrounding takes as long as learning, when the animals are first introduced to the task, although in the relearning they already know situation and task demands. Alternatively, an interference of new and old information could retard the learning process in this situation. Another difficulty could be the total symmetry of room-shape, which is achieved by the dark curtains, while the room is asymmetric within its normal shape. Own experiments, starting from the beginning with curtains, revealed the difficulty that mice have to learn in the symmetric and as well minimally contoured

environment due to the dark curtains instead of light walls (unpublished observation). This observation corresponds to results in MWM experiments performed in the same room, revealing a poorer performance in training with the symmetric surrounding (Morellini, personal communication). Several experiments showed that room shape is a major component for orientation of mice and that hippocampal place cells respond to the shape of the experimental surrounding as well (Cheng, 1986; Holscher, 2003; Margules and Gallistel, 1988; O'Keefe and Burgess, 1996; Ramos, 2000).

Summarizing the observations of the two relearning trainings, one can say that a relatively fast relearning process is possible in a situation of unchanged spatial surroundings. This is not possible in a totally new spatial surrounding where other environmental stimuli might lead to uncontrollable interference. As well the influence of novelty for the latter relearning situation is high compared to a relearning in the familiar situation. This is an important factor in consideration of side stimuli and comparability for a mapping study of c-fos expression.

II.4.6 Step Down Avoidance Task

The Step Down Avoidance (SDA) Task is a passive avoidance conditioning task. Receiving a foot-shock paired to step down a platform, mice should avoid stepping down in a recall trial as sign for contextual memory. This is measured by the latency to step down, which is prolonged, if the mice learned to recognize the place of the conditioning situation.

In general, the mice learned the task as shown by the increased latency to step down. Nevertheless, it is important to underline that at recall all mice stepped down within the maximal trial duration of 5 min. Therefore even for the mice that indicate memory the step down latency just reveals the time point, at which the drive to step down is stronger than the fear of a shock. This means that the latency is influenced by the contribution of conflicting behaviors and how their balance is shifting with time, instead of a direct measure for memory abilities. Thus the measurement of memory abilities in the SDA is rather rough. Mice, which do not remember should reveal no behavioral change. In contrast, mice indicating contextual memory should reveal a change in behavior, which can be seen as prolonged latency of any intensity.

No correlation was found between the performance in the CM and in the SDA task. In the CM, all mice repeatedly showed a spatial learning ability, whereas three were classified as non-learners in the SDA, as they did not increase their step down

latencies during recall. Disagreements between a contextual conditioning and a spatial learning paradigm have been described by several authors (D'Hooge and De Deyn, 2001). For instance, Bach et al. (1995) found same spatial ability deficits in the Barnes Maze in two lines of mice with genetically modified CaMKII expression. When tested in the context conditioning paradigm, one transgenic line revealed no differences to the controls, whereas the other line showed lower levels of freezing but higher levels of active defense responses such as tail ratting and jumping.

Gerlai and Clayton (1999) point out the danger of misinterpretations in tasks, which miss out to consider alternative behavioral responses of mice. For them, the decrease in a passive avoidance behavior, which is usually interpreted as a loss of memory of the place, where rodents received a shock, could simply reflect that animals respond with behavioral reactions other than passive avoidance (Gerlai and Clayton, 1999).

These examples are in line with own observations that mice in the recall session e.g. start to move backwards down the platform, which was tried to avoid by positioning the platform in one corner of the chamber. Other behavioral responses found in the recall but never or very rarely in the conditioning session is tail rattling or jumping out of the introduction cylinder. These behaviors can be a sign for an active coping strategy instead of a passive (Brandewiede et al., 2004). Measured learning deficits could than be due to different behavioral traits (Gerlai and Clayton, 1999; McNish et al., 1997).

In addition to the potential false negative learners described above, the SDA treatment can also lead to false positive cases. An increase of latency that is due to other forms of learning (as cue learning) or a generally increased level of anxiety because of the received shock is not to distinguish from a behavioral change due to contextual learning (Crawley, 2000; Gerlai, 1998; Gerlai, 2001).

It is often argued that opposed performance in these different classes of learning paradigms may be due to different forms of synaptic plasticity involving different synaptic mechanisms (Bach et al., 1995). However, one should keep in mind that another source of difference could be the limited and therefore inflexible and rough estimation of learning abilities in the conditioning tasks (Kaczmarek and Robertson, 2002). This may especially be a problem working with C57Bl/6 mice, which are known for their poor performance of passive avoidance, maybe due to their unusually low level of anxiety-like behaviors (Crawley, 1996) in combination with high rates of

exploratory behaviors (Van Abeelen et al., 1975). In opposite to the fear-conditioning paradigm, a quantitative analysis of alternative behaviors in the SDA is hardly feasible, since the time span for such evaluations is very short, especially in the potential false negative cases as described before. For evaluations that exceed a mere qualitative description, a profound change in set-up and protocol would be necessary.

In general the outcome of the passive avoidance conditioning is influenced strongly by non-mnemonic factors such as exploratory drive and anxiety (Brandewiede et al., 2004). The missing agreement between performances in CM and SDA therefore mainly indicates that they are affected differently by factors like exploratory drive, anxiety or active and passive coping strategies.

II.4.7 Consequences for the CM design

This study shows that mice perform the CM with high motivation. Indeed, the home-cage is a sufficient reward, so that no aversive stimuli are necessary and the paradigm can be used under low anxiety and stress conditions for the mice. The learning situation in a dry maze, where the animal searches for a way back to the home-cage via a tunnel certainly comes closer to a natural situation as compared to swimming to find a submerged platform or avoiding a foot-shock by staying on a small platform. Therefore, this paradigm, despite the artificial laboratory situation, tests spatial learning under ethologically relevant conditions requiring a species-specific behavioral repertoire, which is an important requirement in behavioral studies (Gerlai and Clayton, 1999; Lipp and Wolfer, 1998).

In fact, strongly artificial laboratory situations can result in misled performance such as floating in the MWM or jumping in passive avoidance paradigms. Even more crucial are those situations, in which the behavior of mice is misinterpreted due to a lack of ethological perspective. Animals can respond to stimuli that seem irrelevant to the experimenter or might not attend to those judged as crucial for solving the task (for review see Gerlai and Clayton, 1999).

The low stress component and reliable performance of learning are important for a study on gene expression. Since the CM task offers several parameters for analysis, it allows a detailed investigation of different behavioral components that can be used to estimate learning abilities. The analysis of searching strategies and first approaches of holes proves that spatial learning is achieved in this paradigm as well

as it shows that DTT is an exact and reliable measurement specific for the spatial learning. Therefore DTT can be used to define a learning criterion that allows judging the performance of individual mice, which is important for the c-fos mapping study since all mice can be trained to the same level of performance. This ensures successful performance without over-training.

The results from the relearning experiments showed that a new Target position in a familiar unchanged spatial surrounding allows a fast learning with a very low novelty component and offers the possibility to create highly comparable control groups.

In consideration to the ECM results few modifications are introduced in the protocol for the CM used in the mapping study:

- A uniform massed training with 4 trials per session can be performed since the mice reveal a continuously stable and high motivation independent of the number of trials per session. An additional rewarding effect by aborting the session after successful performance is not necessary.
- Training with the 12-hole platform to the criterion of Target +/- 1 hole in three consecutive trials achieves specific spatial learning but allows a limited variance in performance due to precision abilities. Compared to training with the 36-hole platform mice are more consistent in using the spatial search strategy and rarely switch back to the serial strategy.
- The learning training finishes with the session in which the mouse reaches the criterion and is followed by a short training phase serving for induction of gene expression, in which the mice are divided in experimental groups with different treatments.

II.4.8 Learning in the Relearning Circular Maze (RCM)

For the analysis of spatial learning in the slightly modified training of the RCM, a probe trial session is performed on day 7. The probe trial indicates that already at day 7 of training mice on average develop a preference for the Target.

Interestingly, the preference for the Target, which is seen in the first 15 s interval, decreases strongly in the second interval and then increases again in the third and fourth interval. Most probably, after the mice noticed that the Target is not in the expected position and moved away, they return to the Target position again. They seem to enhance the search in the position matching expectation after they inspected other areas, indicating that they know the correct position. The behavior of

the mice during the probe trial confirms the observation that during acquisition mice easily switch to serial searching if they hit a hole nearby the Target, although the correct directional choice proved their spatial orientation. Moreover, the enhanced preference at the end of the probe trial supports the hypothesis that mice increase their appetitive behavior and searching at the old Target position when the Target is displaced, as seen during relearning 1 of the ECM experiment.
Consistently, after the probe trial session mice slightly increased the performance in the first trial on day 8 (data not shown). That the probe trial session affects the following performance is shown even more pronounced in the increased searching latency of the first trial thereafter.
The results achieved in the probe trial are an important completion for the results seen during training, where the analysis of DTT and first approaches only represents the first choice made by mice, which is most indicative for spatial orientation. In contrast, the analysis of the probe trial indicates a preference for the Target area that goes beyond the first approach and extends over a period of 60 sec. Indeed, it is particularly impressive that, though a repeated expectation mismatch, mice continue to inspect the Target.

As group average a learning success is already noticeable on the day of the probe trial. This performance cannot be taken as overall success since averaging evens out mice that perform well with those performing badly (Gallistel et al., 2004). This is especially important for cumulative results as that of the probe trial, where good performance adds up over time. Correspondingly, the analysis of individual mice indicates some variance (from a minimum of 8 days to a maximum of 15 days) in the time mice need to reach criterion. Indeed, an averaged group performance is not a good indicator to achieve a homogeneous and comparable level of performance between mice, whereas the use of a learning criterion allows judging the individual performance so that mice are trained until they reach the same level of performance.
An uniform and thereby comparable performance, as revealed by the acquisition curves and first approaches, is an important precondition before mice are divided into experimental groups and treated for induction of c-fos expression.

II.4.9 Learning in the Reinforced Relearning Circular Maze (RRCM)

As in the RCM, the training to reach criterion results in equally successful performance at the final day, which is a precondition for the following c-fos induction.

The acquisition curve for DTT is even steeper than that of the RCM, suggesting a low variability within the group, probably due to the fact that slow learners were excluded by the maximal number of 10 days to reach criterion used in this experiment. The low variability suggested by the behavioral parameters is an additional advantage for the mapping study, which was the purpose of this experiment. It reveals that the CM task is first of all capable to detect this subtle variability, and thereby also offers the possibility for limiting this variability, if necessary.

II.4.10 Conclusion for the CM study

Aim of this study was to design a spatial learning task to allow analysis of neuronal activity induced by learning under conditions of reduced novelty, stress and aversion for the mice. The behavior of the mice during acquisition and the probe trials clearly shows that the circular maze established in this study can be used to test specifically spatial learning in the mouse. This is achieved in a learning task with a certainly higher ethological relevance and a lower stress component as compared to other learning paradigms commonly used. Indeed, under ethological conditions the performance of the mice is less influenced by factors not related to cognitive abilities such as stress, anxiety, locomotion and motivation, allowing a better interpretation of the behavioral response of the mice. Moreover, through a detailed behavioral analysis it was possible to dissect those parameters that better represent the learning abilities of the mice and that could be used to evaluate the performance of individual animals. In this way mice could be trained until they equally learned the task to achieve highly similar preconditions before being trained in a relearning protocol that minimized any new stimulus beside the spatial displacement of the Target.

An advantage of the CM is that it offers an array of different behavioral parameters, which allows a far more detailed analysis of learning as well as of different behavioral components, than many learning tasks. This is especially important for comparison of mice that are expected to vary in more than one trait or ability, which is typical for transgenic or physically manipulated mice models. The CM allows the investigation of 'if', 'when' and 'how' mice solve the maze demands and therefore accounts for the natural variance of behavioral responses much better than tasks allowing just a 'yes' or 'no' response. This is not just important with respect to detecting even subtle differences between mouse lines or manipulated mice. A more detailed analysis as well allows judging whether possible alterations are due to mnemonic or other factors

that contribute to a complex behavior. Finally, it is also important for a better control and insight concerning influencing factors, a major advantage for mapping studies.
In addition to these main aspects of the investigation, the detailed behavioral analysis revealed several interesting insights into mechanisms in the spatial learning process. The CM trains mice under low-pressure conditions, namely with low physical demands and no error penalty. Under these low-pressure conditions it is possible to see, that mice can use different strategies to solve the task and that these strategies do not exclude each other. In fact, mice used different strategies in a hierarchical order, preferring the most efficient method, but switching to the next efficient in case of failure. The use of different strategies controlled by different memory systems finds support in many studies that indicate that these memory systems work in concert (Floresco et al., 1997; O'Reilly and Rudy, 2000; Packard and McGaugh, 1996). This interplay of different systems allows the animal to react flexibly with a complex behavioral response that changes in adaptation to situational variations.

Within the training the mice seem to develop an extremely strong expectation drive for the Target. As the probe trial in the RCM and the relearning 1 in the ECM indicate, the appetitive behavior in the before learned Target area is even enhanced, if the Target is missing in the expected position. On the one hand this stresses the strength of the reward for the home-cage, as already indicated by the escape latencies. Even more important, it shows that a mismatch in expectation elicits enhanced appetitive behavior within a longer trial period as well as over several sessions. This phenomenon is important to consider, especially for investigation of relearning performance. The performance in learning a new Target position or something adequate in other tasks can be influenced strongly by the expectation drive and thereby mask or hinder potential relearning abilities.

Finally, although this study focused on the spatial learning components of the task, the CM is suitable to test the influence of anxiety and motivation on the performance of the mice by varying the positive and negative reinforcement used in the task.

III INVESTIGATION OF NEURONAL ACTIVATION PATTERN

III.1 Introduction

Understanding the structural organization is the basis to understand the functionality in any biological system. The nervous system is an example for interrelationships of structure and function in a complex network. However, its complex connectivity complicates the assignment of a single structure to a function, since multiple systems work in parallel, cooperative or coordinated with each other.

In the case of spatial learning and memory there are various factors that guide the animal to its goal. The use of different spatial information as allocentric (e.g. geometric arrangement of landmarks) or idiothetic (e.g. proprioception and vestibular input), cause the interaction of multiple brain areas (Aggleton et al., 2000; Compton et al., 1997; Goodridge and Taube, 1997). The processes that lead to a transformation of environmental information into memory contents involve long-term plastic changes in neuronal cells. This form of plasticity depends on permanent functional alterations that require the reprogramming of gene expression (Tischmeyer and Grimm, 1999).

A prerequisite for a change in gene expression is the connection of environmental stimuli with intracellular messengers that control the expression pattern. Inducible transcription factors (ITF) encoded by immediate-early genes (IEG) are assumed to accomplish the coupling of short-term neuronal activity due to external stimuli and responses on the transcriptional level (Tischmeyer and Grimm, 1999). The investigation of their expression offers an effective method to map the pattern of postsynaptic stimulation within an intact working brain even in single cell resolution (Morgan and Curran, 1991; Zhu et al., 1997). They can be used to visualize the connection of external stimuli with internal information storage as activation pattern, thereby indicating the contribution of different brain areas or sub-regions (Aggleton et al., 2000; Gall et al., 1998). In this study, the IEG c-fos is used to investigate the involvement of sub-regions of the hippocampus and NAc in spatial learning and the detection of spatial novelty.

III.1.1 The immediate-early gene c-fos

The c-fos gene was first discovered as a proto-oncogene and thereby discerned to function in several aspects of signal-transduction processes. It encodes a nuclear

protein of 62 kDa that undergoes extensive post-translational modifications. As other members of the Fos-family (Fos B, ΔFos B, Fra-a, Fra-2), c-Fos reveals several structural and expressional similarities with proteins of strongly related genes of the Jun-family (Morgan and Curran, 1991). Together with these, c-fos is part of a class of genes called immediate-early genes (IEG), which belong to the first genes that are regulated in their gene expression after stimulation. Most of them encode for inducible transcription factors (ITF) that are responsible for the regulation of 'downstream' effector genes (Guzowski, 2002).

The group of IEG shares several characteristics. Their basal expression in quiescent cells is low or undetectable but is induced within minutes after stimulation. This transcription is independent of prior de novo protein synthesis (Sheng and Greenberg, 1990). Both mRNA upregulation and the following protein accumulation show a typical rapid but transient expression pattern.

The time course of induction for c-fos shows a transcriptional activation within 5 min and continues for 15 to 20 min. The mRNA accumulation declines 30 to 45 min after induction with a half-life for the mRNA of 10 to 15 min (Morgan and Curran, 1991). The protein is accumulated within 30 to 90 min and has a half-life from less then 2 h (Kaczmarek and Robertson, 2002).

The rapid decline of expression is due to the short half-lives of the gene products in combination with a shut-off mechanism in which Fos is involved as a negative regulator of its own expression. The following refractory period for re-induction can last for several hours in some circumstances (Morgan and Curran, 1991).

Many IEG encode for ITF that control the expression of effector genes thereby mediating the coupling of external stimuli to long-term changes as cellular response.

The c-Fos protein must dimerize with one of the members of the Jun-family (c-Jun, JunB, JunD, which can also dimerize with themselves) to produce a functional transcription factor that allows DNA-binding (Chaudhuri, 1997). Like that a variety of combinations are possible for a transcription factor with diverse transactivational abilities (Tischmeyer and Grimm, 1999).

The proteins interact via a conserved dimerization domain in form of a leucine zipper. The structure consists of a α-helix with leucine side chains spaced seven amino acids apart resulting with an alignment of leucine residues along one face of the helix. Via the parallel association of the α-helices, adjacent highly basic stretch of amino acids in each protein partner come close together. Both contact DNA as

necessity for binding. The c-Fos/c-Jun heterodimer binds DNA with a high affinity and specificity at the consensus sequence TGACTCA, also known as AP-1 binding site, and activates nearby promotor regions.

Jun and Fos were shown to be the major constituents of AP-1. The different combinations of dimers reveal distinct variations in their binding efficiency and their regulatory specificity. Jun reveals a relatively broad DNA binding specificity that, in the presence of Fos and Fos-related proteins, achieves higher specificity for AP-1 sites due to an increasing stability of the protein-DNA interaction.

The regulatory specificity reaches from activation as in c-Fos/c-Jun to repression like in c-Fos/JunB complexes seen under some circumstances (for more details see Morgan and Curran, 1991; Sheng and Greenberg, 1990).

Very little is known about AP-1 Target genes in the brain. Genes that are possibly driven by c-fos/AP-1 in nerve cells include NGF, preencephalin, prodynophin, endorphin, neurotensin, tyrosine hydroxylase, neuropeptide Y, TIMP-1 (tissue inhibitor of metalloproteinases-1) and others (Kaczmarek and Robertson, 2002).

III.1.2 Regulation of the IEG response

As mediators between external stimuli and the reprogramming of gene expression pattern, ITF are induced in the nervous system by a variety of stimuli, e.g. growth factors, neurotransmitters, peptides, depolarization, seizure, ischemia, brain injury or sensory stimuli (Tischmeyer and Grimm, 1999). This works via an induction cascade in which multiple signaling pathways trigger rapid phosphorylation and dephosphorylation events. They modulate the activity of constitutively expressed transcription factors (including CREB, SRF, TCF) that in turn regulate the transcription of several IEG (Tischmeyer and Grimm, 1999). The second messenger systems known to be able to activate c-fos are the DAG, cAMP and Ca^{2+}-calmodulin pathways (for comprehensive overview see Morgan and Curran, 1991; Sheng and Greenberg, 1990). An important coordinator in the ITF induction is the Ca^{2+} influx into the cell occurring either through the NMDA-receptor Ca^{2+}-ionophore complex after glutamate binding or through voltage sensitive calcium channels (VSCC) following membrane depolarization (Chaudhuri, 1997).

Since ITF are implicated in various processes from growth and differentiation to processes supporting neuronal plasticity as LTP, kindling, regeneration, learning and memory (Tischmeyer and Grimm, 1999), they have to serve a huge variety of

functions and therefore need to be able to lead to reprogramming of distinct sets of effector genes.

In consequence, the IEG response is a highly coordinated direct and indirect interaction among an assembly of different ITF, whose specific effects are depending on the nature of the stimulus as well as the differentiation state and type of the cell involved (Morgan and Curran, 1991).

To serve this sophisticated coupling between environmental stimuli and cellular changes, the IEG response is organized by a network of regulation steps, where several in parallel induced pathways influence each other in converging as well as dispersing events and by variation of interaction partner combination within the AP-1 complex and the subset of ITFs expressed in the IEG response with differential effects. For instance, it was shown that the nature of the external stimulus is capable to exert a differential influence on c-fos regulation via the preferential triggering of a pathway. The efficiency to couple second messengers with the responsive elements seems to be not equal in the various IEG and therefore account for phenotypic differences in the sensitivities of particular IEG induction via certain pathways. These findings are in concordance with the observation that the nature of the external stimulus affects the composition of IEG expressed in the constituents as well as in their ratio (for more details see Morgan and Curran, 1991; Sheng and Greenberg, 1990).

As another level of differential regulation serves the repression of IEG expression. In addition to a negative feedback loop that Fos exerts to its own expression, the refractory period for re-induction of expression can last for hours, suggesting a repressor influence of Fos related proteins with a protracted abundance. Fra-1 is one example to mediate its repressive function via SRE (Morgan and Curran, 1991).

The repression seems to result from a concerted interplay among several IEG proteins and SRE, probably including an interaction between Fos related proteins and modifying enzymes as protein kinases and phosphatases that regulate the function of SRF. These events stress the significance of the composition of expressed IEG for the response (Morgan and Curran, 1991).

Beside the backward also the forward regulation is influenced by the set of IEG expressed. So the combination of the protein partners in the dimeric AP-1 complex determines the specificity for the regulation of the late-response genes in binding efficiency as well as in transactivational abilities.

The diversity of affecting DNA binding and gene expression of AP-1 receives additional complexity by its ability to interact with other transcription factors as glucocorticoid receptors, NFAT, ETS, Zif268, capable to modify the AP-1 activity (Kaczmarek and Robertson, 2002).
In addition, it has been suggested that post-translational modifications of the Fos/Jun complex are determined by the nature of the stimulus (Sheng and Greenberg, 1990) and the involved signal cascade (Morgan and Curran, 1991).
The magnitude of variability in regulative influences suggests for AP-1 a role as integrator of inputs mediated by diverse signaling systems coming from a variety of environmental stimuli.

Altogether the IEG response involves several different signal cascades that diverge and converge on different steps within the cascade. This net-like interplay of regulation indicates that the system is capable to achieve a certain cell response as reaction to the integration of diverse stimuli that characterize an external situation. Therefore, the expression pattern of c-fos, as an important player within this response, is a valuable tool to bridge between external conditions and internal processes, although it cannot be an indicator for a specific following gene expression (Kaczmarek and Robertson, 2002).

III.1.3 The c-fos expression in learning and memory

Learning reflects a behavioral change in response to a new or changed external situation. Gene expression of c-fos is induced under conditions of learning during acquisition and early phases of consolidation (Tischmeyer and Grimm, 1999). In addition, diverse blocking experiments that parallel the suppression of c-fos expression and memory formation strongly suggest that c-fos is involved in the transition from short- to long-term memory (Guzowski, 2002; He et al., 2002; Morrow et al., 1999; Swank et al., 1996, Tischmeyer and Grimm, 1999). In several studies with unilateral sensory occlusion or lateral impairments the lateralization of the c-fos response in downstream brain regions ruled out a widespread unspecificity of the signals (Jenkins et al., 2002; Kaczmarek and Robertson, 2002). Mapping expression pattern gave many insights into the not unitary but rather complex process of learning, consisting of several components provided by different brain areas and circuits (for comprehensive overview see Kaczmarek and Robertson, 2002).

This study addresses different time-points and the influence of novelty and spatial learning using the regional and temporal pattern of c-fos expression. The study of Wan et al. (1999) indicates that it is possible to distinguish c-fos expression patterns for different forms of novelty, namely novel items or novel arrangements of familiar items. A differential involvement of brain areas in the different stages of a learning process via c-fos was investigated by Bertaina-Anglade et al. (2000) in an appetitive operant conditioning task. Also cooperative interactions in which simultaneously activated memory systems seem to guide the learned behavior in a timeline manner switching within extended training (Packard and McGaugh, 1996), or competitive interactions that result in correlative de- and increase of different memory systems, as suggested by Poldrack and Packard (2003), can be addressed via expression patterns in intact working brain.

However, in addition to the activation of c-fos expression in several types of learning (for a review see Kaczmarek and Robertson, 2002), c-fos is also expressed following concomitant factors as sensory stimulation, motor activity, stress, arousal and novelty (Badiani et al., 1998; Fichtel and Ehret, 1999; Montag-Sallaz et al., 1999; Papa et al., 1993; Shim et al., 2001; Titze-de-Almeida et al., 1994; Wan et al., 2001). Although investigations often design experimental conditions to discriminate these effects from learning and memory, many show the expression pattern to be influenced by unsolicited stimuli. A striking example shows that c-fos expression in the *nucleus tractus solitarius* during conditioned taste aversion, which is often seen as molecular correlate of taste aversion learning, is also sufficiently evoked by placing an animal into a novel environment (Swank, 2000). Furthermore, if the conditioning and testing occur in the home-cage instead of a novel environment, the expression in the *nucleus tractus solitarius* even decreases below control levels despite animals show the aversion reaction. A typical problem of conditioning paradigms is that the exposure situation can elicit gene expression governed by a diverse range of phenomena including recall, behavioral execution, relearning or extinction and a retention test in a even minor changed situation can lead to variances in the expression pattern (Kaczmarek and Robertson, 2002; Radulovic et al., 1998; Tischmeyer and Grimm, 1999). Especially the extinction processes contain a new learning condition within the change of experience that engages different regions than the acquisition (Kaczmarek and Robertson, 2002; Morrow et al., 1999).

Moreover, the use of pseudo-conditioning, such as shock application unpaired to the neutral stimulus, need to be critically observed. They are valuable for behavioral assessment, e.g. that no conditioning to the neutral stimulus was achieved. However, they cannot preclude other forms of learning (Kaczmarek and Robertson, 2002). The application of identical but unpaired stimuli often reveals similar activation as for the conditioned animals (Grimm and Tischmeyer, 1997; Milanovic et al., 1998). Already Rescorla (1967) showed that a control situation not allowing an association of stimuli could lead to learning the lack of association. That another form of learning occurred is seen by the fact that it is more difficult to learn new associations after being pseudo-conditioned (Rescorla, 1967). Often animals in these shock groups show behavioral changes indicating excitement as tail rattling, increased locomotion or jumping, suggesting that other associations than those in the conditioned group are formed and can serve for induction (Gerlai and Clayton, 1999; Tischmeyer and Grimm, 1999).

In aversively driven paradigms a critical factor concerns induction by stress, which is known to change the c-fos expression in several brain areas (Titze-de-Almeida et al., 1994). Although probably any form of learning is accompanied by some sort of stress, which can even support learning to some extent (Akirav et al., 2004; Sapolsky, 2003). Increasing stress often enhances motivational pressure and thereby performance. For example, the highly aversive passive and active avoidance paradigms are often learned sufficiently after one trial. On the other hand such fast acquisition disables a more detailed investigation of activation pattern in correspondence to behavioral performance. Controversy findings revealing positive correlations between performance and upregulation (Bertaina-Anglade et al., 2000; Guzowski et al., 2001) as well as negative correlations (Nikolaev et al., 1992) are due to different acquisition times. A long phase of exponentially rising performance (increase of learning parallels increase of performance) may allow a positive correlation, while in fast acquisitions a asymptotic phase is reached immediately, showing negative correlations.

The decreasing activity in a later stage of learning mirrors a widely observable property of c-fos expression pattern. Several examples show that an over-trained behavior leads to reduced c-fos expression, although alterations in the experimental conditions as well as a relearning process still can increase expression (Chaudhuri, 1997; Gall et al., 1998; Kaczmarek and Robertson, 2002; Vann et al., 2000). Also the

stress response gets attenuated with repeated exposure, but a new stressor will lead to new induction (Kaczmarek and Robertson, 2002).

Generally the novel stimuli provide the strongest activation, which is therefore found in initial training sessions, after sensory deprivation or with introduction of a new component into a repeated exposure (Chaudhuri, 1997). With increasing familiarity to the stimuli and their contingencies, learning reaches an asymptotic level being strongly diminished and therefore the induction effect attenuates or totally disappears. The attenuation effect is observed in learning situations, sensory stimulation, novelty or stressor representation (Montag-Sallaz and Buonviso, 2002; Papa et al., 1993; Papa et al., 1995; Tischmeyer and Grimm, 1999; Titze-de-Almeida et al., 1994). These findings suggest that less the sort of stimulus or stressor but more its novel character arises the inductive moment. Papa et al. (1993) argue that reduced induction itself proves a role of learning since otherwise unchanged sensory stimuli should reveal unchanged expression pattern.

Many investigations of gene expression pattern after learning use the presentation of novel environments or profound changes in the spatial environment. Learning is in general connected to a novel stimulus and therefore the mere effect of novelty is hard to separate from learning. However, these experimental designs pronounce the influence of novelty and increase the possibility of interference of novelty- and learning-induced gene expression. As advantage for the c-fos expression study the here established CM offers the possibility of investigating a relearning process under conditions, which minimizes the influence of novelty. However, novelty detection occurs as inseparable precondition of learning and is suggested to have a distinct contribution in the sub-regions of the hippocampus paralleling a functional distinction (Jenkins et al., 2004). The discrimination of purely novelty-induced pattern could elucidate the novelty-specific contribution to expression pattern, which are stimulated in a learning process within the hippocampus. Such a novelty-specific influence may be even more sensitively noticeable in a region connected with arousal- and novelty-induced behavior as the NAc (Hooks and Kalivas, 1995). Since both regions are involved in spatial learning processes and in the response to novelty, this study tries to dissect the different contributions of learning and novelty for their sub-regions. Due to the fact that different forms of novelty, as e.g. object or spatial novelty, affect activation pattern differently (Jenkins et al., 2004; Wan et al., 1999; Zhu et al., 1997)

a comparing paradigm should present a spatial novelty with high similarity to the situation in the spatial learning task.

III.1.4 Aims of the study

For this study, a relearning event in the CM is used to induce c-fos expression. This design allows analyzing neuronal activity induced by learning under conditions of reduced novelty, stress and aversion for the mice, therefore providing a situation with minimized stimuli other than learning. The extensive Pre-training should attenuate c-fos induction by motor and sensory inputs or stress. Exposing the animals to relearning in a familiarized situation should minimize the novelty component. A first training phase, which trains them to equal performance levels, provides that a first learning process is completed and equal preconditions are achieved, before the relearning event induces a new learning process. A pronounced and fast learning is provided since the mice know the task demands. At the same time this design offers an ideal control group with mice presented to the same situation, providing exactly the same stimuli for induction beside the novel relearning situation. The involvement of the hippocampal sub-regions, namely DG, CA3 and CA1, in this relearning process is investigated via their differential c-fos expression pattern with regard to a functional segmentation. Beside two time-points in the early stages of learning, a later stage is addressed by reinforcing the relearning on a second day.

In addition to the hippocampus the NAc is analyzed to investigate the contribution of a region that is often assigned to an independent circuit (Milner et al., 1998). Mainly seen to be involved in the motivation and novelty concerning aspects of behavior (Bassareo et al., 2002; Hooks and Kalivas, 1995; Papa et al., 1993; Rebec et al., 1997), it was as well recently shown to play a role in spatial learning (Annett et al., 1989; Ploeger et al., 1994; Sargolini et al., 2003; Smith-Roe et al., 1999). As this it could be involved in the spatial learning process by an alternative learning strategy (Floresco et al., 1997), as well as by supplying the influence of motivational or novelty components (Arleo and Gerstner, 2000; Floresco et al., 1996) or by a direct participation in spatial processing.

The novelty-induced c-fos expression is investigated in an exploration task without learning incentive but providing an environmental situation almost identical to that of the CM task. An extensive habituation serves for an attenuation of gene expression as in the first experiment. Thereafter a changed the environmental surrounding with

no direct consequence for the exploration behavior and therefore with no incentive for learning evokes c-fos expression purely governed by spatial novelty.

III.2 Animals & Methods

III.2.1 Animals and husbandry

For details see chapter II.2.1. Three separate experiments were conducted with three different batches of animals.

Experiment 1 - Relearning CM: A group of 19 FLZ (fos-lacZ transgenic) mice consisting of 10 females 6 - 10 months and 9 males 4 - 6 months old was transferred to the animal facility for training in a circular maze (cf. chapter II.2.1).

Experiment 2 - Novelty Exploration Task: A group of 16 FLZ mice with an age of 2 - 3 months was transferred to the animal facility. 12 animals were trained on a circular exploration platform, the other 4 animals remained naïve serving as cage controls.

Experiment 3 - Reinforced Relearning CM: 49 C57bl/6 mice aged 2 ½ - 3 months were transferred to the animal facility in three batches. 42 animals were trained in three batches in a circular maze, the additional 5 mice served as cage controls and therefore remained naïve (cf. chapter II.2.1).

The FLZ originate from a breeding line of transgenic mice carrying an additional fos-lacZ reporter gene expressing β-Galactosidase governed by the regulator region of the c-fos gene (Smeyne et al., 1992). The animals were at least 4 generations backcrossed into the C57Bl/6J breeding line.

III.2.2 Relearning Circular Maze (RCM)

The RCM was performed as described in chapter II.2.7.

III.2.3 Novelty Exploration Task (NET) on a Circular Platform

Novelty is another possible factor that could induce gene expression during learning or relearning events. The Novelty Exploration Task (NET) on a circular platform was performed to investigate c-fos expression induced by novelty, thereby dissecting novelty versus learning induced gene expression. The NET was therefore designed

to expose the mice to a new environmental stimulus without requiring or inducing learning. To allow a better comparison with the RCM and RRCM, the NET was performed with conditions and protocol as similar as possible.

To receive an inescapable circular platform serving exploration, the apparatus as described in chapter II.2.2 was equipped with a size matching 1 mm thin PVC sheet without holes covering the platform (see Fig.II.1). During the NET the room was equipped with black stripes in the corners to stress the asymmetry of the room as described in the RRCM. Prior to the NET the animals performed the Visible Cliff Task and the Pre-training as described previously.

The protocol paralleled that of the CM to allow better comparison. The daily sessions consisted of two trials with an inter-trial interval (ITI) of 1 min. The mice were introduced via a cylinder and allowed to explore for 5 min in the first session and for 2 min in all following sessions. At the end of the trial the animals were picked up, placed back into the home-cage and left undisturbed for the ITI. During the session the home-cage was placed on the board beneath the platform. Between the trials the platform was rotated in a similar manner to the CM. In addition to the analysis with the video tracking system EthoVision, fecal boli and urine were noted.

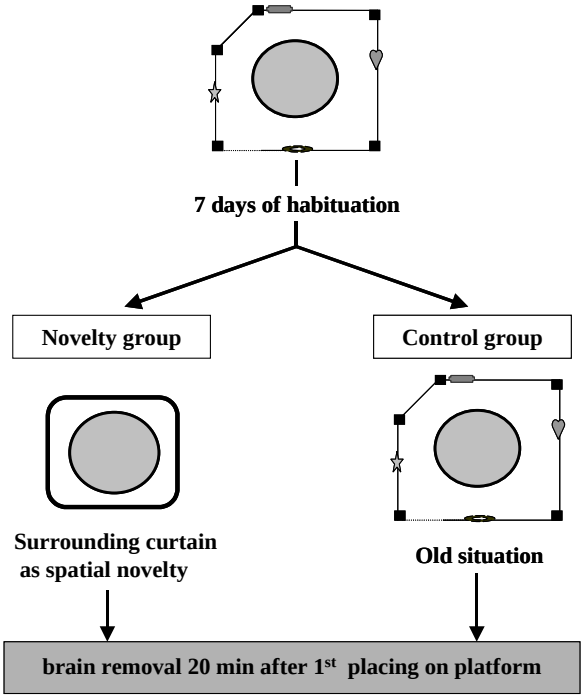


Fig. III.1: Schematic schedule for the NET
The NET starts with 7 days of habituation training allowing the mice to explore the arena in two consecutive trials per day. On the 8th day the animals were split into two experimental groups with different treatment. For the novelty group an induction of neuronal activity was achieved by a spatial novelty, consisting of a curtain surrounding the platform. The controls performed an unaltered training with the old spatial situation. Brain removal was performed 20 min after session start.

In a habituation phase the animals performed 7 days of exploration as described before. At day 8, the animals were divided pseudo-randomly into two groups. The control group performed another exploration session under the same conditions as in the habituation phase. For the novelty-group a dark-brown colored curtain

surrounded the platform. All other conditions were unchanged. The animals were killed 20 min after they were introduced into the NET arena for brain removal (Fig. III.1). Additionally to the animals that were trained in the NET, a group of 4 animals served as cage controls. The animals only performed the Visual Cliff Task and stayed afterwards undisturbed in the animal facility for a time-span paralleling the NET training until brain removal.

III.2.4 Reinforced Relearning Circular Maze (RRCM)

The RRCM was performed as described in chapter II.2.8. Animals not reaching the criterion within 10 days were excluded from further investigation. Thereby 9 animals were excluded resulting in 11 animals in each of the three groups. An additional group of 5 animals served as cage controls. After performing the Visual Cliff Task they were left undisturbed in the animal facility for a time-span paralleling that of the CM training before brain removal.

III.2.5 Brain removal and preparation

The brain removal was carried out within 3 min. The animal was killed by cervical dislocation followed by a decapitation. After removal of the skull cap and detach of the optic nerve the brain was removed including the olfactory bulbs. It was embedded into Tissue Tek® filled foil caps and frozen in an isopropanol bath in liquid nitrogen. The brains were stored until cutting at –80°C. The fresh-frozen tissue was cut on a cryostat at –20°C into 14 µm sections and mounted on glass slides (SuperFrost®Plus, Menzel-Gläser/Roth). The sections were continuously kept frozen and stored at –80°C until used for RNA *in situ* hybridization.

The coronal sections were taken for the region of the dorsal hippocampus at the site ranging from –2.30 to –1.82 mm according to bregma and for the region of the NAc from 0.74 to 1.70 mm according to brema (Franklin and Paxinos, 1997). The cutting was performed considering orientation of the rostral to caudal location in the horizontal plane as well as the dorso-ventral and lateral localization within the coronal plane to ensure the comparability between and within cuts of the different brains.

In order to ensure comparability between groups every slide contained sections of at least one member of each experimental group. Moreover, the different experimental groups were located on varying positions within the different slides.

III.2.6 Molecular biological methods

If not indicated otherwise, standard biological techniques were carried out as described by Sambrook et al., 1989). Methods of the *in situ* hybridization are described in more detail.

III.2.6.1 Production of competent bacteria

E. coli DH5α bacteria (Invitrogen) were streaked on LB-agar dishes and grown overnight at 37°C. 50 ml of LB-medium was inoculated with 5 colonies and grown at 37°C under constant shaking (>200 rpm) until the culture reached an optical density (OD_{600}) of 0.35-0.45. Growth of bacteria was stopped by a 5 min incubation step on ice. Cells were pelleted at 1000 x g for 15 min (4°C) and – after removal of the supernatant – resuspended in 17 ml pre-chilled RF 1 (4°C). Following 15 min incubation on ice, the centrifugation was repeated. The cell pellet was resuspended in 4 ml pre-chilled RF 2 (4°C) and incubated again for 15 min on ice. 100 µl aliquots were frozen in liquid nitrogen and stored at -80°C. Transformation efficiency of cells was tested by transformation with a distinct quantity (pg-ng) of purified supercoiled plasmid DNA.

RF 1: 100 mM RbCl
50 mM $MnCl_2$
30 mM KOAc
10 mM $CaCl_2$

→ pH 5.8 (with 0.2 M acetic acid)

RF 2: 10 mM MOPS (pH 6.8)
10 mM RbCl
75 mM $CaCl_2$
150 g/l glycerol

III.2.6.2 Transformation of DNA into bacteria

10 ng of plasmid DNA were added to 100 µl of competent DH5α bacteria and incubated for 20 min on ice. A heat-shock was performed at 42°C for 2 min and a recovery on ice for 2 min. 900 µl of LB-medium were added and the bacteria were cultivated at 37°C for 1 h under constant agitation. Then cells were centrifuged (10 000 x g, 1 min, RT), after removal of the supernatant resuspended in 100 µl LB-medium and plated on selective agar plates (20 g/l agar in LB-medium with ampicillin). Colonies formed after incubation at 37°C for 12-16 h.

III.2.6.3 Maintenance of bacterial strains

Selected bacterial strains, which contain plasmids of interest, were stored as glycerol stocks (LB-medium, 25 % (v/v) glycerol) at -80°C for up to one year or at -20°C for up to 2 months. Bacteria grown on agar plates containing antibiotics were stored up to 6 weeks at 4°C.

III.2.6.4 Small scale plasmid isolation (Miniprep)

In order to purify 10-20 µg of plasmid DNA for further restriction analysis and sequencing reactions, minipreps were carried out. 4 ml LB-amp-medium (100 µg/ml ampicillin) were inoculated with a bacteria colony and incubated over night at 37°C with constant agitation. Cultures were transferred into 2 ml Eppendorf tubes and cells were pelleted by centrifugation (12 000 rpm, 1 min, RT). The procedure was repeated for amounts of more than 2 ml. Plasmids were isolated from the bacteria using the GFX *micro* plasmid prep system (APB), according to the manufacturer's protocol. The DNA was eluted from the columns by addition of 50 µl Tris-HCl (10 mM, pH 8.0) with subsequent centrifugation (12 000 rpm, 2 min, RT). Plasmid DNA was stored at -20°C.

III.2.6.5 Large scale plasmid isolation (Maxiprep)

For preparation of large quantities of DNA, the Qiagen Maxiprep kit was used. 100 ml LB-amp-medium (100 µg/ml ampicillin) was inoculated with 5 µl bacteria suspension of the glycerol stocks. This culture was incubated at 37°C with constant agitation overnight. Cells were pelleted in a Sorvall centrifuge (6 000 g, 15 min, 4°C) and DNA was isolated as described in the manufacture's protocol. At the end the DNA pellet was resuspended in 150 µl of pre-warmed (70°C) Tris (10 mM, pH 8.0) and the DNA concentration was determined.

III.2.6.6 Determination of DNA concentration and purity

DNA and RNA molecules absorb UV light of a wavelength of 260 nm, whereas proteins absorb strongest at λ = 280 nm. Thus, the absorption at 260 nm of purified nucleic acid solutions can be converted into concentrations via a factor. The absorption of 1 OD (A) is equivalent to approximately 50 g/ml dsDNA and 40 g/ml RNA. Additionally interference by protein contamination was recognized by the

calculation of the ratio of A260/A280. Pure DNA should have a ratio of 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at λ = 320 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the ratio A260/320 should be approximately 2.2. Concentration and purity was determined spectrometrically using an Amersham-Pharmacia spectrometer. Absorbance at 260 nm needs to be between 0.1 and 0.6 for reliable results.

III.2.6.7 Endonuclease restriction analysis

Endonuclease restriction enzymes were used to digest and analyze the sequence of plasmids or DNA fragments. Digestions were performed by incubating dsDNA molecules with an appropriate amount of restriction enzyme, the respective buffer as recommended by the supplier (New England Biolabs), and at the optimal temperature for the specific enzyme, usually at 37°C. In general, 20 µl digestions were planned. For preparative restriction digestions the reaction volume was scaled up to 50 µl. Digestions were composed of DNA, 1 x restriction buffer, the appropriate number of units of the respective enzyme (due to glycerol content the volume of the enzyme added should not exceed 1/10 of the digestion volume), and the sufficient nuclease-free H_2O to bring the mix to the calculated volume. After incubation at the optimal temperature for a reasonable time period (mostly 2-3 h or overnight), digestion was terminated by addition of sample buffer and applied on an agarose gel.

III.2.6.8 DNA agarose gel electrophoresis

To analyze restriction digestions and quality of nucleic acid preparations horizontal agarose gel electrophoreses were performed. Gels were prepared by heating 0.8-2 % (w/v) agarose (Gibco) in Tris-acetate buffer (TAE), depending on the size of fragments to be separated. DNA samples were adjusted to 1 x DNA sample buffer and were subjected to electrophoresis at 10 V/cm in BIO-Rad gel chambers in 1 x TAE running buffer. Afterwards, gels were stained in 0.5 µg/ml ethidium bromide in 1 x TAE solution for approximately 20 min. Thermo-photographs of transilluminated gels were taken, or bands were made visible on an UV-screen (λ = 360 nm).

III.2.6.9 DNA fragment extraction from agarose gels

For isolation and purification of DNA fragments from agarose gels, ethidium bromide stained gels were transilluminated with UV-light and the appropriate DNA band was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment was isolated using the silica matrix-based QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol. The fragment was eluted from the column by addition of 50 µl pre-warmed (70°C) Tris-HCl (10 mM, pH 8.0) and the DNA concentration was determined.

III.2.6.10 Precipitation of DNA

The salt concentration of an aqueous DNA solution was adjusted by adding 1/10 volume of 7.5 M sodium acetate pH 5.2. Afterwards 2.5 volumes of cold ethanol, -20°C was added and the sample was mixed well. Following incubation at -20°C for 30 min, samples were centrifuged for 30 min at 4°C (16 000 x g). Supernatants were removed carefully and the pellets were washed by adding 1 ml cold 75% (v/v) ethanol, -20°C, and centrifuged for 5 min at 4°C (16 000 x g). After removal of the supernatant the DNA pellet was air dried to evaporate residual ethanol (approximately 5 min at RT). DNA was resuspended in an appropriate volume of pre-warmed nuclease-free water.

III.2.6.11 Sequencing of DNA

Sequence determination of dsDNA was performed by the sequencing facility of the ZMNH (Dr. W. Kullmann, M. Daeumigen). Fluorescent-dye labeled chain-termination products (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Wellesly, MA, USA) were analyzed with an ABI Prism 377 DNA Sequencer (Perkin Elmer). For preparation, 0.8-1 µg of DNA was diluted in 7 µl ddH_20 and 1 µl of the appropriate sequencing primer (10 pM) was added.

III.2.6.12 Generating RNA by *in-vitro* transcription

To generate *in-vitro* transcribed RNA, 5-10 µg of plasmid DNA containing the insert and a SP6 polymerase promotor was digested with restriction endonucleases (BamH I, NEB) overnight, at a position that was located 5' of the RNA polymerase promoter and 3' of the desired strand of DNA. By doing this, the DNA polymerase

transcribed only the strand of interest and no vector-specific sequences. Linearized DNA was purified using the MiniElute PCR purification kit according to manufacturer's instructions (Qiagen), or by precipitation (see chapter III.2.6.13). In order to obtain Digoxigenin (DIG) labeled RNA probes for *in situ* hybridization, transcription of the desired templates was performed with Ambion's Megascript system. For the generation of DIG labeled RNAs, the DIG-UTP mix shown below was used instead of NTPs provided by the manufacturer.

DIG-UTP mix (10x)	10 mM	ATP
	10 mM	CTP
	10 mM	GTP
	6.5 mM	UTP
	3.5 mM	DIG-11-dUTP (Roche)

20 µl *in-vitro* transcriptions were essentially performed as recommended by the manufacturer. Generated mRNA was purified by LiCl precipitation, analyzed on a denaturating agarose/formaldehyde gel and stored at –80 °C.

III.2.6.13 Precipitation of RNA

To remove unincorporated nucleotides and proteins, a LiCl precipitation was applied. To a 20 µl transcription sample 2.5 µl LiCl (4 M) and 75 µl cold ethanol, -20°C, was added. After incubation for 2 h at -20°C, the samples were centrifuged for 20 min at 4°C (16 000 x g). Supernatants were removed carefully and the pellets washed by adding 100 µl 70 % ethanol and repeating the centrifugation step. After removal of the supernatant the RNA pellet was air-dried and resuspended in an appropriate volume of pre-warmed nuclease free water.

III.2.6.14 RNA agarose gel electrophoresis

The integrity of the RNA probes was checked by gel electrophoresis under denaturating conditions with an agarose/formaldehyde gel. 1 % (w/v) agarose gels were prepared with MOPS running buffer containing formaldehyde (2.2 M). Before loading to the gel, RNA samples were denaturated for 15 min at 65°C in a mix of MOPS, formaldehyde and formamide. After cooling on ice the RNA samples were adjusted to 1 x sample buffer and subjected to electrophoresis at 5 V/cm in 1 x MOPS running buffer. Afterwards the gel was stained in 0.5 µg/ml ethidium bromide for 1 h and washed for at least 2 h in DEPC H_2O. Thermo-photographs of transilluminated gels were taken under UV-illumination.

III.2.6.15 Dot Blot

In order to test the marker efficiency of the generated DIG labeled RNA probes, the semi-quantitative method of a dot blot was performed. Descending dilution series for the generated probes and a labeled control RNA were dotted (1µl) onto a nylon membrane (Hybond-N+, Amersham) and fixed by crosslinking with UV radiation (0.07 J/cm2). Membranes were equilibrated in DIG buffer 1 and blocked for 30 min in DIG blocking solution. This blocking solution was poured off and membranes were incubated with the Anti-DIG AP-conjugated antibody (Roche) diluted 1:5 000 in DIG blocking solution at RT. After incubation for 30 min with gentle agitation, the antibody solution was discarded and membranes were washed twice for 15 min with DIG washing buffer and equilibrated in detection buffer.

The detection was done either by incubation with CSPD (Roche, 1:100 in detection buffer), where dephosphorylation leads to chemiluminescence. Therefore the membrane was exposed to BioMax Light-1 films (Kodak, Rochester, NY) for evaluation. Alternatively the incubation with BCIP and NTB (BCIP/NBT tablet, Sigma-Aldrich, solved in 10 ml ddH_20) lead to blue precipitation products due to a redox-reaction. The membrane could directly be used for estimation.

Independently of the detection system the marker efficiency was estimated by comparing the intensities of labeled control RNA ad the generated probes.

DIG buffer 1 (2 X)	0.2	M	maleic acid
	0.3	M	NaCl
		→ pH 7.5 (solid NaOH)	
DIG washing buffer	3	g/l	Tween in DIG buffer 1
DIG blocking stock	10	g	Blocking reagent (Roche)
	100	ml	DIG buffer 1
DIG blocking solution	10	%	(v/v) DIG blocking stock in DIG buffer 1
Detection buffer	0.1	M	Tris-HCl (pH 9.5)
	0.1	M	NaCl

III.2.6.16 RNA *in situ* hybridization (ISH)

Digoxigenin (DIG)-labeled RNA sense and antisense probes for c-fos were generated using the Megascript™ system (Ambion) according to the manufacturer's instructions (see chapter III.2.6.12). To perform non-radioactive detection of mRNAs, 14 µm

sections were cut from fresh-frozen tissue on a cryostat and mounted on glass slides (SuperFrost®Plus, Menzel-Gläser/Roth). The sections were fixed in 4 % paraformaldehyde in PBS (pH 7.3) at 4°C overnight. The next day, sections were washed three times in 1 x PBS, treated with 70 % (v/v) ethanol for 10 min and washed twice with H_2O. Then they were treated with 0.1 M HCl for 10 min and washed again twice with PBS. An acetylation step was performed in 0.1 M triethanolamine containing 0.25 % acetic anhydride for 20 min. After washing twice in PBS the sections were dehydrated in an ascending ethanol series (70%, 80%, 95%).

Finally, sections were air dried and pre-hybridized for 3 h at 37°C with hybridization buffer. Hybridization with the DIG labeled probes occurred at 55°C overnight in humid chambers. DIG labeled probes were diluted to a concentration of 30 ng RNA/100 µl hybridization buffer. After hybridization, sections were washed twice in 0.2 x SSC at 55°C, followed by three washing steps in 0.2 x SSC containing 50 % formamide (for each 90 min at 55°C).

The immunological detection of the digoxigenin started with equilibration in DIG buffer 1. To prevent unspecific binding, sections were incubated in blocking buffer for 30 min before anti-DIG AP-conjugated antibodies (Roche Diagnostics), diluted 1:500 in blocking buffer, were applied overnight at 4°C. To remove unbound antibody, sections were washed twice in DIG buffer 1 for 15 min. The washing solution was poured off and the sections were equilibrated for 5 min with DIG buffer 3. The signal was developed in the dark with DIG buffer 3 containing 0.35 g/l 4-nitroblue tetrazolium chloride (NBT, Roche Diagnostics), 0.175 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche Diagnostics) and 0.25 g/l levamisole (Sigma-Aldrich) until signals became visible under a stereomicroscope. Sense probes were developed in parallel under the same conditions as the anti-sense probes. The developing was terminated by a washing step in PBS. For counterstaining a blue fluorescence nuclear staining with bis-Benzimide (Sigma-Aldrich) was applied and after extensive washing the slides were coverslipped.

Hybridization buffer	**10 x “base stock”**
25 ml deion. formamide	2 ml 1 M Tris pH 7.5
5 ml 10x “base stock”	200 µl 0.5 M EDTA
3.3 ml 5 M NaCl	2 ml 50 x Denhardt´s solution
2.5 ml 2 M Dtt	2 ml tRNA (25 mg/ml)
4.7 ml DEPC-H_2O	1 ml poly A^+-RNA (10 mg/ml)
10 ml dextransulfate	2.8 ml DEPC- H_2O

DIG buffer 1

100 mM Tris
150 mM NaCl
→pH 7.5

Blocking buffer

1 % (w/v) Blocking reagent (Roche Diagnostics)
0.5 % (w/v) BSA
in DIG-buffer 1

DIG buffer 3

10 mM $MgCl_2$
100 mM Tris
100 mM NaCl → pH 9.5

III.2.7 Quantification of c-fos positive signals

The quantification of c-fos positive signals was performed with the computer-assisted light microscope system Neurolucida. With the Neurolucida system a computer generated image can be projected by the Lucivid into the visual field of the microscope. The overlaid image can be used to surround structures and mark signals that can then be analyzed with the Neurolucida software. In this way the size of structures was measured together with the signal evaluation and allowed a normalization of signals according to area (Fig. III.2). For the hippocampal region the signals were counted independently for complete areas of CA1, CA2/3 and DG and distinguished between the principal and the adjacent cell layers. The analysis was performed with brightfield illumination. For the better distinction of structures the visualization of a fluorescent counterstaining under Hg-light conditions was used.

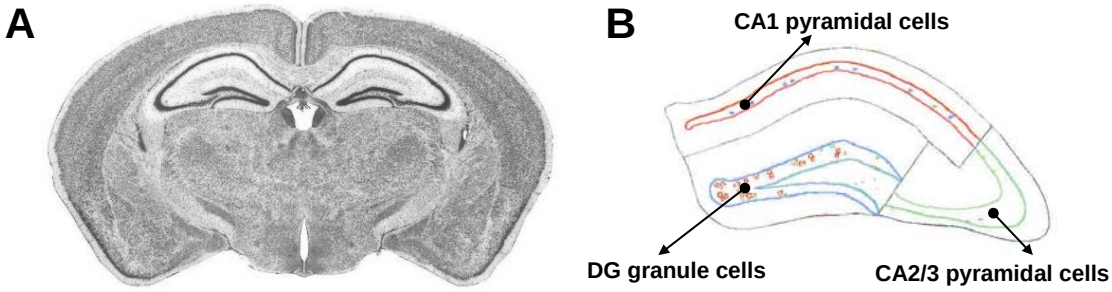


Fig. III.2: Signal evaluation in the hippocampus
Signals were evaluated in coronal sections of the dorsal hippocampus ranging from –2.30 to –1.82 mm according to bregma **(A)** producing a graphical overlay with the Neurolucida system **(B)** allowing to mark the signals and measure areas in order to normalize signals to the size of the analyzed cell layer.

The NAc was evaluated in a 1.1 mm^2 area within a mask that was orientated according to the anterior commissure. This area was subdivided to allow an approximation of functional distinction between core and shell area. Within the mask the lateral compartment mainly consisted of core area while the medial compartment included mainly shell area (Fig. III.3).

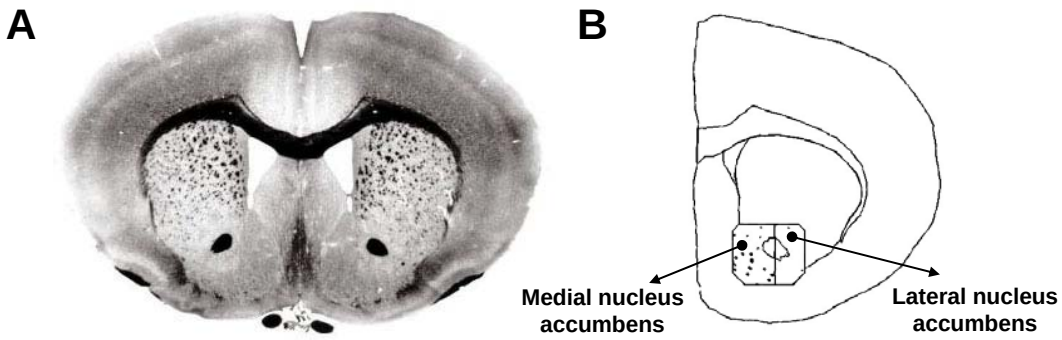


Fig. III.3: Signal evaluation in the nucleus accumbens
Signals were evaluated in coronal sections of the nucleus accumbens ranging from 0.74 to 1.70 mm according to bregma **(A)** within a mask created with the Neurolucida system **(B)** that allows normalizing signals to the size of the analyzed area.

The evaluation was performed after extensive training for signal regain/validation and blinded for experimental conditions of the section corresponding animals. For every animal three sections were evaluated and their mean was used for further analysis.

III.3 Results

The c-fos gene expression, which serves as a marker for neuronal activation, is not only induced by learning, but also by several other factors (see chapter III.1). An experimental design has to consider the effects of unwanted influencing factors such as novelty and stress. For this reason, the setup of the experiments created a familiar situation for the animals, in which a relearning process was induced. Additionally, it presented a situation to the control groups, which is as close as possible to that presented to the induction groups. In the first phase of training of all experiments, all animals were treated equally until reaching an identical stable situation as precondition. Only in a second phase were the mice split into the control and induction groups. In the CM experiment, one group of mice was trained with the Target in the same position as during the previous phase (control), whereas another group was trained with the Target in a new position (relearner). In the Novelty Exploration Task (NET) experiment, one group was exposed to the same environment as during the previous phase (control), while another group was exposed to a new environment (novelty).

III.3.1 Relearning Circular Maze (RCM)

The behavioral part of this experiment consisted of a CM training split into the two phases mentioned before. The second phase, which comprised one session of four consecutive trials, ended with the removal of the brain 20 min after the start of the

session. The brains were morphologically analyzed using RNA *in situ* hybridization with a c-fos antisense probe to investigate upregulation of expression due to learning.

III.3.1.1 Behavioral analysis

The mice were trained in a 12-hole CM to find a Target by spatial orientation. The first phase of training lasted until they reached the criterion that was established to be an adequate indicator of success at spatial learning. As soon as the mice matched the criterion of approaching the Target +/-1 hole in three consecutive trials the first training phase ended. The next day the animals were trained in one session of 4 trials using a protocol that was determined by the experimental group they belonged to. This protocol was either identical to the former training for controls or presented a new Target position for the relearner group. The main aspects of the behavioral analysis of the first learning phase of the CM are already described in the previous study. Therefore, the current presentation is restricted to the parameter DTT that was the decisive discriminator for the criterion and thereby the termination of the first training phase. An additional behavioral analysis was performed on the second phase of training, which defined the experimental groups for the investigation of c-fos expression.

Fig. III.4 shows the DTT the mice revealed in the course of training. The length of the first training period was dependent on the time they needed to reach criterion.

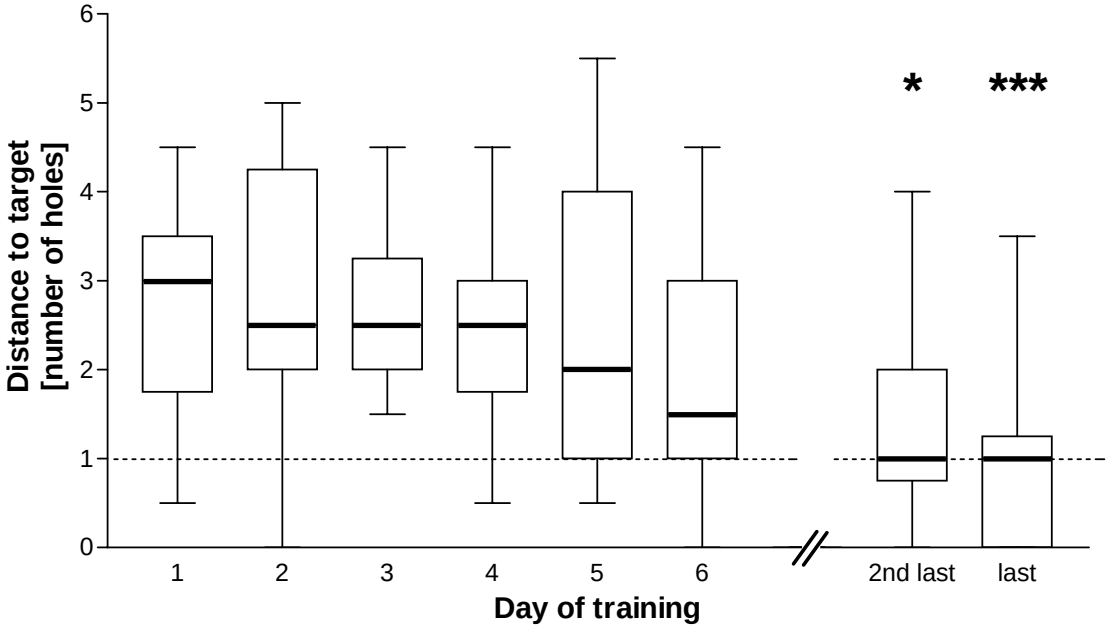


Fig. III.4: Learning performance measured as distance to Target
The distance to Target, given as number of holes is presented in box plots for the days 1-6 and the two last days of training representing thereby all 19 mice. The mice decrease the distance to Target to a stable level on the last two days that matches the criterion level. This shows that the mice orientate spatially when completing the learning process. The broken line indicates the distance matching the criterion. *-p<0.5, ***-p<0.001 (Dunn's test after Friedman test).

Therefore, only the first 6 days and the last 2 days of training were analyzed in order to represent all 19 mice. On day 1, the DTT was 3 holes, decreasing significantly to 1 hole on the last two training days ($p < 0.001$, Friedman test). Post hoc comparison to day 1 demonstrated significant lower values on the last two days.

The localization of the animals within the CM arena is a good indicator of whether or not they show the expected place preference for the Target position. To investigate, in addition, if a change of behavior was found within the one day of training, the localization of the relearner group was compared between the first and last trial of the first 5 s. The localization is shown in a spatial histogram depicting time per area as x-y matrix for the CM arena indicating the length of time in a color-scale (Fig. III.5A).

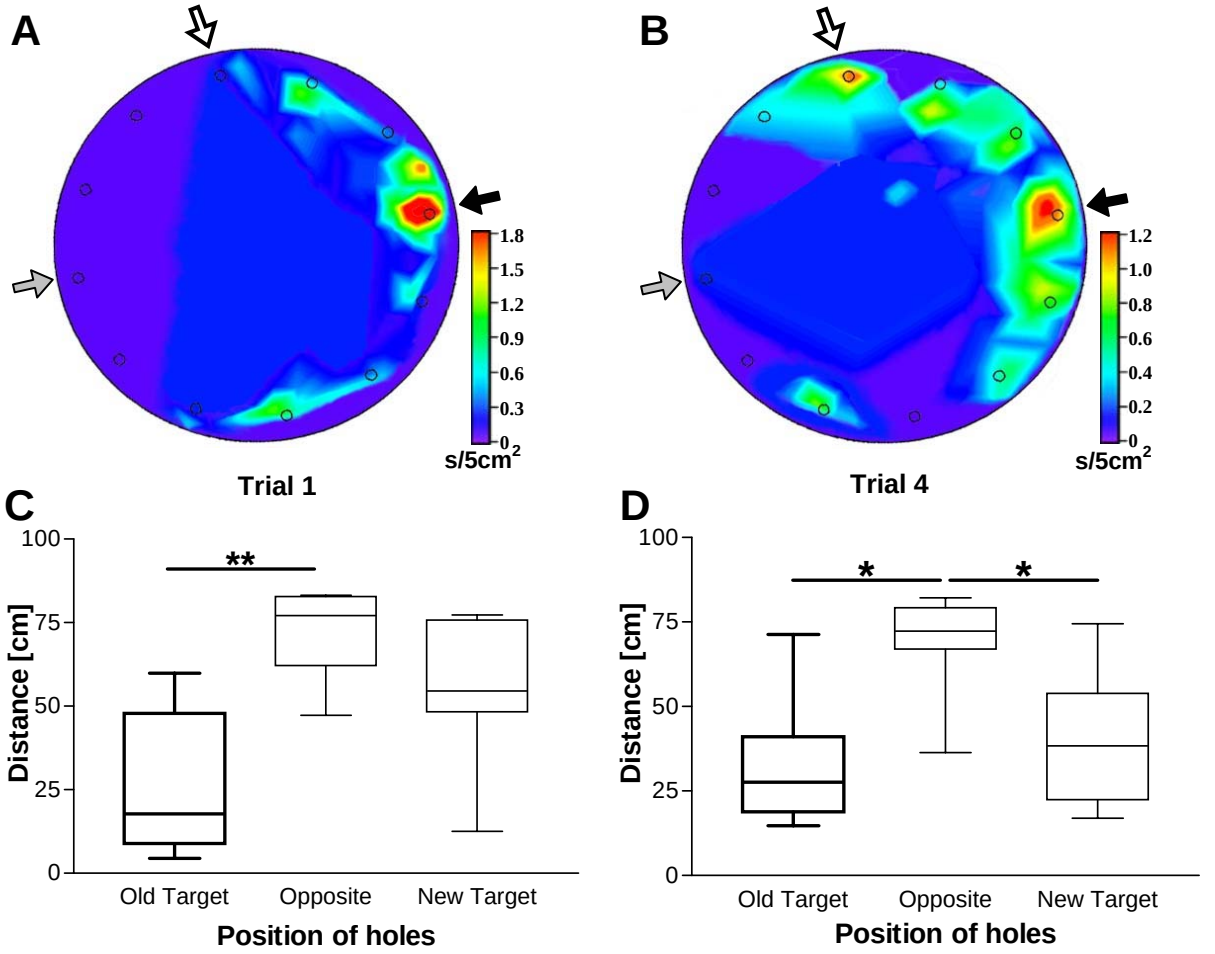


Fig. III.5: Localization as time per area-matrix and time in quadrant

(A), (B) The spent time for every point of the CM arena as x-y matrix is presented in s/5cm². The time is depicted by a color-scale reaching from blue for low to red for high levels of time. The black rings indicate the position of the holes (not true to scale). ⬅ old Target, ⇦ opposite hole, ⇦ new Target

(A) The mice show a strong preference for the old Target area seen as a red spot in the first trail. **(B)** In the last trial the mice indicate a more spread distribution with a slightly enhanced time spent at the new Target seen as a second red spot in addition to the preference found for the old Target.

(C) In the first trial the parameter mean distance to zone reveals that the mice are found closer to the old Target than to its opposite hole, indicating that they were successfully trained to this Target in the previous training. **(D)** In the last trial mice are found at a similarly short distance from both Target positions compared to the opposite hole indicating a change of behavior already within the one session of relearning. *-p<0.5, **-p<0.01, (Dunn's test after Friedman test). Note: Comparison of the distance to new Target in trial 1 and 4 (p=0.019, Wilcoxon Matched Pairs test)

This presentation revealed that the relearner mice had a strong preference for the old Target area in the first trial. The parameter distance to zone, comparing the mean distance to the old Target, its opposite hole and the new Target, corroborated this localization pattern (Fig. III.5C). The mice are found in a significantly shorter distance to the old Target than to the opposite hole (Friedman test $p = 0.003$). The distance to the new Target lay between these levels.

Since several of the control mice vanished into the escape tunnel in less than 5 s, no comparable data were obtained for this group. However, results showed that these animals had the same Target preference as seen in the first trial of the relearner mice regarding the available few seconds only (data not shown).

The strong preference for the old Target in the first trial revealed that the mice indeed learned to find the Target in the position of the former training. A comparison to the fourth trial shows that the mice already changed their localization within this session of relearning. In the fourth trial the mice are more spread within the CM arena (Fig. III.5B). Although they still reveal a strong preference to the old Target, their localization to the area of the new Target is also slightly enhanced. Correspondingly the mice are found in a similar distance to the old and new Target, which both are significantly lower than the distance to the opposite hole (Fig. III.5D, Friedman test $p = 0.006$). In comparison to the first trial the distance to the new Target became significantly lower in the fourth trial ($p = 0.019$, Wilcoxon Matched Pairs test). This shows that the mice had already started reorientation towards the new Target during one day of training.

III.3.1.2 Analysis of c-fos expression in the Hippocampus

The *in situ* hybridization of c-fos visualizes the expression of c-fos RNA by labeling it with a digoxigenin marked antisense RNA probe. The c-fos probe was tested for correct length and sequence and the hybridization specificity and signal sensitivity was controlled with dot blots and *in situ* hybridizations together with a known working alternative probe (kindly provided by Dr. Marius Ader). The signal specificity was confirmed by comparing the signal pattern of the *in situ* hybridization with that of a β-galactosidase staining of sections of the FLZ mice with a lacZ reporter gene expressing β-galactosidase under control of the c-fos promotor. As control within an *in situ* hybridization served an additional section that was treated with a sense instead of the

antisense probe of c-fos, therefore revealing the purely unspecific binding of the hybridization.

Fig. III.6 shows examples for the *in situ* hybridization in hippocampal sections of a relearner (A, B) and a control mouse (C, D). The sense treated sections showed no staining at all thereby indicating a very low unspecific binding due to the hybridization procedure (E, F). Comparison to the sense probe treated section revealed specific signals in both antisense treated mice. The signals in the relearner and control mice were mainly found in the principal cell layers of the CA regions (pyramidal cells) and the DG (granule cells) (A, C). The higher magnification of the DG area showed more signals in the relearner mice (B) than in the control mice (D).

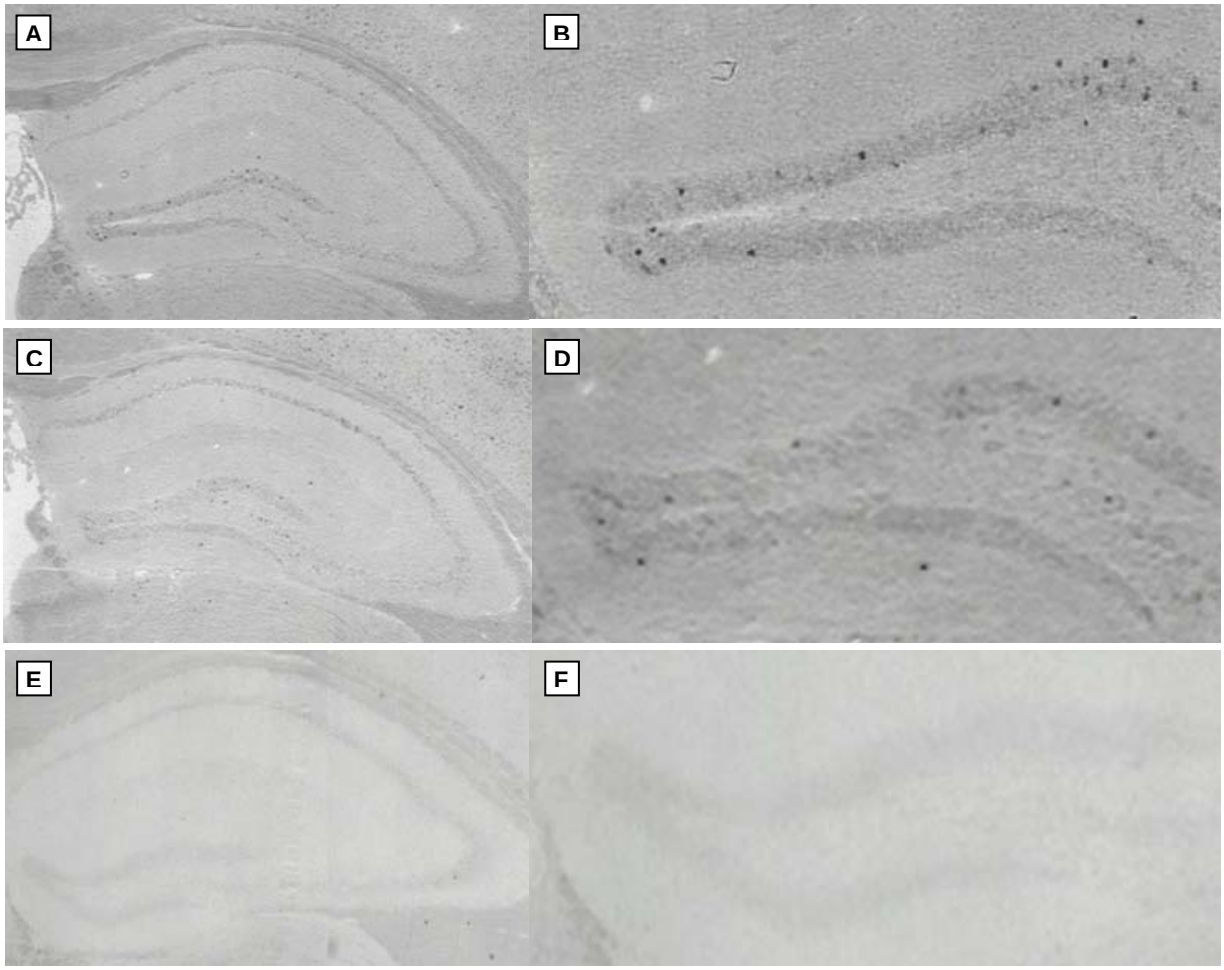


Fig. III.6: C-fos in situ hybridization in the dorsal Hippocampus
The *in situ* hybridization visualized a c-fos upregulation as distinct signals mainly seen in the principal cell layers of the relearner **(A)** and the control mice **(C)**, while no signals are found in the sense probe treated section **(E, F)**. The higher magnification of the DG region demonstrates that more signals are found in the relearner **(B)** than the control mice **(D)**, indicating a stronger c-fos upregulation for relearner mice.

The signals were counted separately for the different hippocampal regions and normalized to the corresponding area. For the granule cell layer of the DG significantly more signals were found in the relearner than in the control group ($p = 0.044$, Mann Whitney-U test). This indicates an increased upregulation of the c-fos expression as an

effect of the relearning training in the CM. No significant effect was seen regarding the pyramidal cells of the CA1 and CA3 region (Fig. III.7).

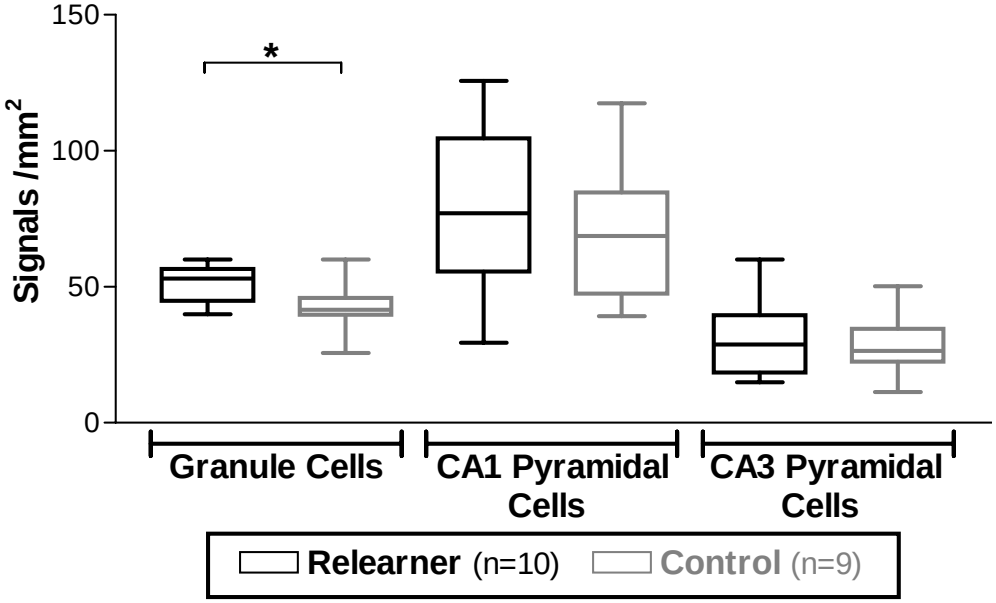


Fig. III.7: C-fos signals in the dorsal Hippocampus
The c-fos signals of the relearner (black) and control group (gray) are presented in box plots separate for the different sub-areas of the hippocampus. More signals are found in the granule cells of the DG, indicating a stronger upregulation due to learning in this region. * -$p<0.05$ (Mann Whitney-U test).

III.3.1.3 Analysis of c-fos expression in the Nucleus Accumbens

In parallel to the hippocampus the same morphological analysis was performed for the area of the nucleus accumbens. The section treated with the sense probe revealed a very low unspecific binding. The specific c-fos expression patterns were seen as distinct signals spread over the NAc region.

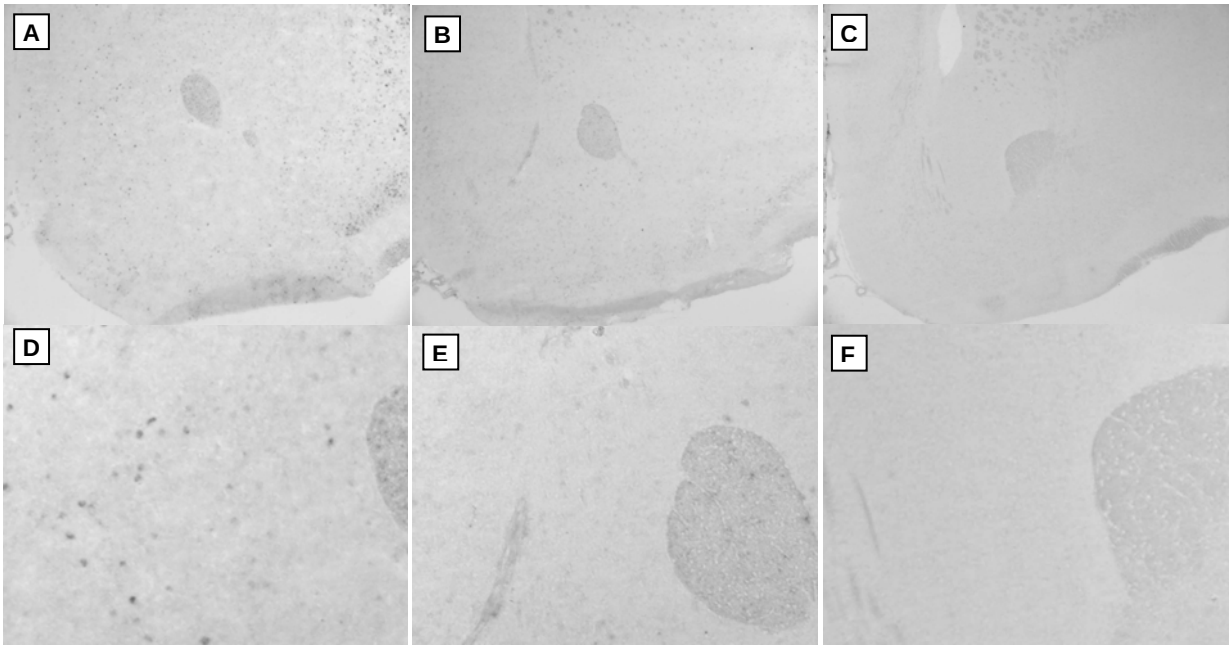


Fig. III.8: C-fos *in situ* hybridization in the Nucleus Accumbens
The *in situ* hybridization visualized a c-fos expression in relearner **(A, D)** and control mice **(B, E)** and the staining of unspecific binding in a sense probe treated section **(C, F)**. The higher magnification reveals that a c-fos expression is seen as distinct signals spread over the area of the nucleus accumbens of both groups **(D, E)**, while no signals are found in the sense probe treated section **(F)**.

Fig. III.8 shows examples for c-fos *in situ* hybridization of the relearner group (A, D), the control group (B, E) and in addition a sense probe treated section (C, F).

The signals were counted in a 1.1 mm² area surrounding the anterior commissure and normalized as signals per area separately for the lateral and medial nucleus accumbens.

Relearning training in the CM caused a weak upregulation of c-fos expression in the relearner group in the region of the medial nucleus accumbens compared to the control group (p = 0.054, Mann Whitney-U test, Fig. III.9).

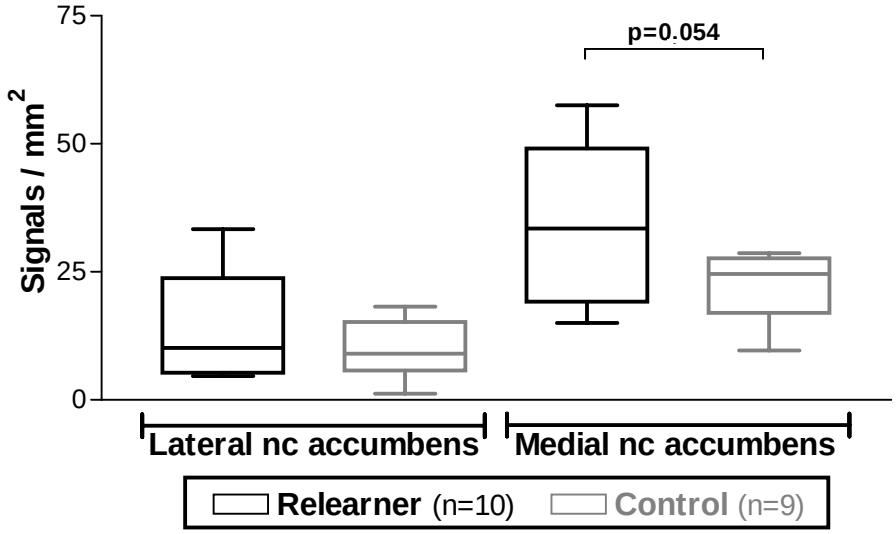


Fig. III.9: C-fos signals in the Nucleus Accumbens
The c-fos signals of the relearner (black) and control group (gray) are presented in box plots for separate areas defined as lateral and medial nucleus accumbens. In the medial nucleus accumbens a slightly higher c-fos expression is found for the relearner mice (Mann Whitney-U test).

III.3.2 Novelty Exploration Task (NET) on a Circular Platform

The NET was performed to investigate the influence of spatial novelty, which is not connected to an incentive to learn, on c-fos expression. Animals were allowed to explore an environment that corresponded to that of the CM to maximize the comparability, but on a modified platform without holes.

Paralleling the conditions of the RCM, the brain was removed 20 min after session start on day 8.

III.3.2.1 Behavioral analysis

The training phase of the NET lasted 7 days allowing extensive habituation of all 12 mice to ensure a stable behavioral response. On day 8 the mice of the control group were exposed to the same environment as in the previous habituation phase.

In contrast, the novelty group was exposed to a new environment, namely a dark curtain surrounding the platform.

Fig. III.10 presents the parameter distance moved as indicator for the exploration of the animals. Both groups showed the strongest effect of habituation from day 1 to day 2, thereby reducing the distance moved to around 50 % (novelty: 998 +/-58 cm to 469 +/-85 cm; control: 976 +/-97 cm to 561 +/-152 cm). Both stayed at a similarly low level with an identical small increase on day 5 due to a two-day break followed by further decrease until day 7.

On day 8, the control group stayed on the same level as the previous days. In contrast, the novelty group covered longer distances as compared to the control group ($p = 0.037$, Mann Whitney-U test) and increased the distance moved by 62 % as compared to the previous day, though this did not reach significance ($p = 0.075$, Wilcoxon Matched Pairs test).

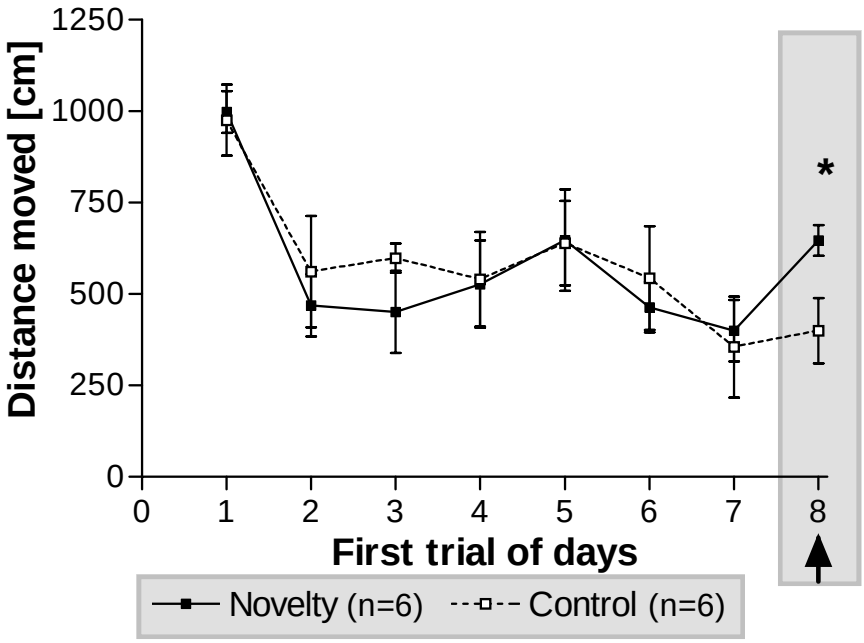


Fig. III.10: Distance moved during habituation and spatial novelty in the NET
Distance moved is presented as median +/- SEM. For the 7 days of habituation the training was the same for both groups. The decrease in distance moved indicates that the strongest habituation effect is found after the first day for both groups and reveals no difference in exploration between them for the 7 days of habituation. On day 8 the training of the controls was identical to the habituation while the novelty group received a new spatial surrounding in form of a dark curtain (←). On this day mice of the novelty group reach a longer distance moved indicating an enhanced exploration due to the change in the surroundings. * -$p<0.05$ (Mann Whitney-U test). Note: Comparison of day 7 and 8 of the novelty group ($p=0.075$, Wilcoxon Matched Pairs Test).

III.3.2.2 Analysis of c-fos expression in the Hippocampus

The analysis was performed as described for the RCM. In addition to the two trained groups another group of 4 naïve cage controls animals was included into the analysis

to measure the basal c-fos expression. Fig. III.11 shows examples of c-fos hybridized sections of novelty (A, B), control (C, D) and cage control animals (E, F). For the basal expression revealed by the cage controls distinct signals were rarely found. In contrast, in both trained groups distinct signals indicating a c-fos upregulation were found in the principal cell layers.

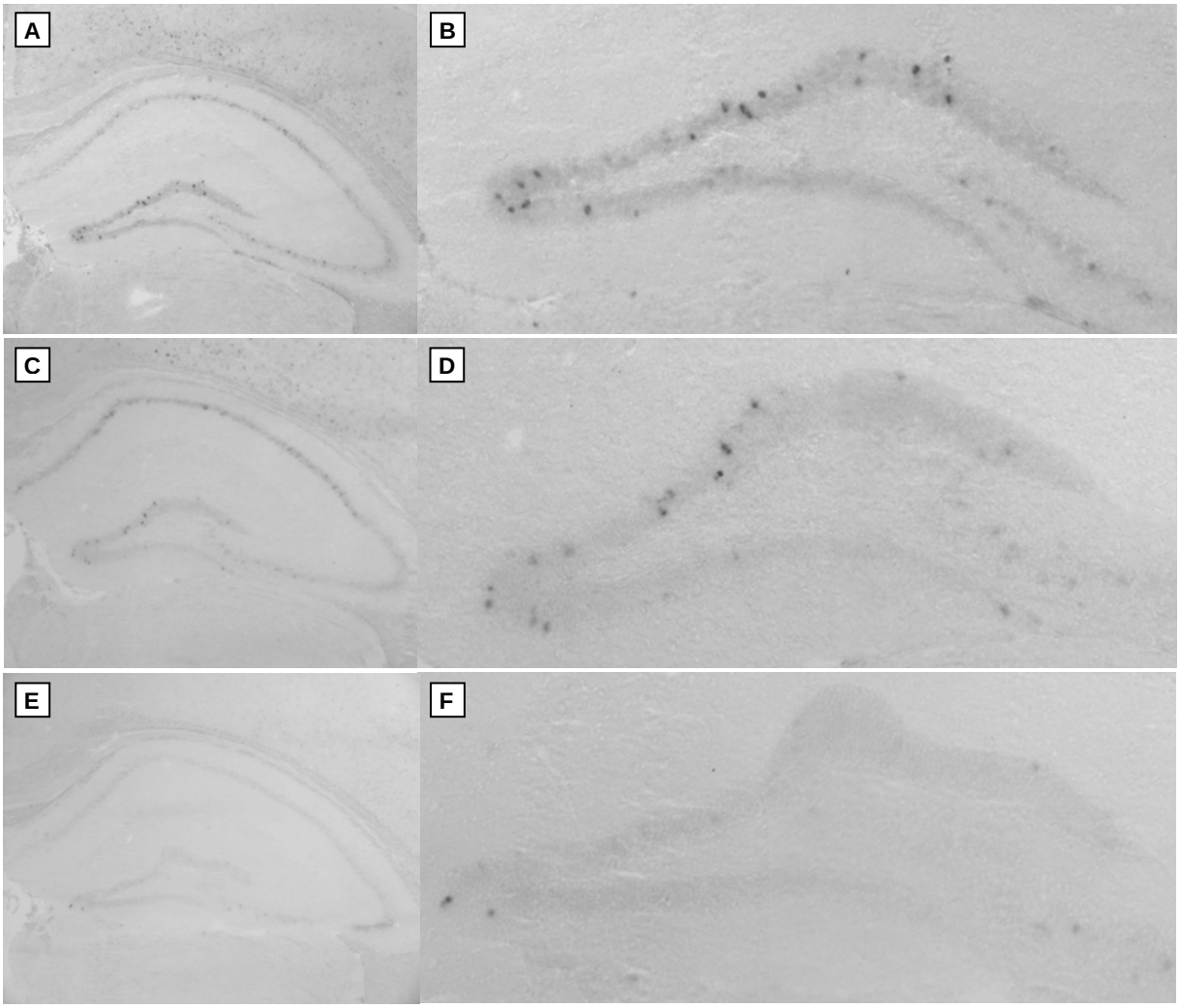
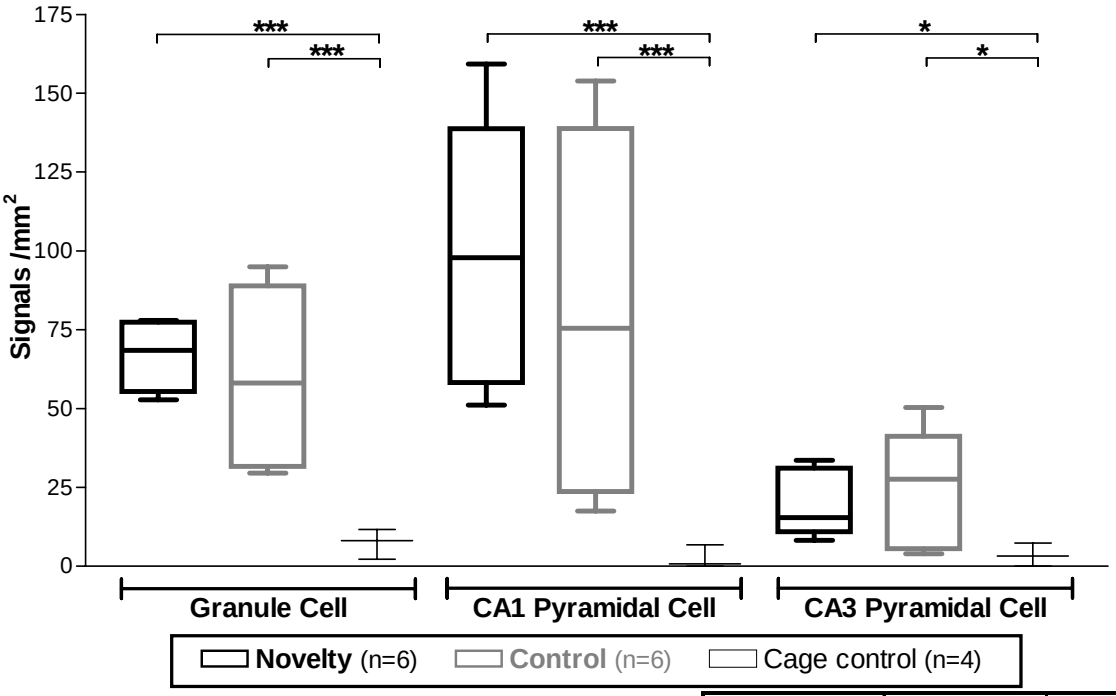


Fig. III.11: C-fos *in situ* hybridization in the dorsal Hippocampus
The *in situ* hybridization visualized a c-fos upregulation as distinct signals mainly seen in the principal cell layers of the novelty **(A)** and the control mice **(C)**, while they are rarely found in cage controls **(E)**. The higher magnification of the DG region demonstrates that many signals are found in the novelty **(B)** and the control mice **(D)**, indicating upregulation of the c-fos expression due to the training in the exploration task. **(F)** Almost no signals are found in the naïve cage controls, which reveal the basal expression of c-fos.

Signals of all three groups were counted separately for the different hippocampal regions and normalized to the according area. A 2-way ANOVA was performed revealing a general effect of group ($F_{2,12} = 0.31$, $p = 0.026$) and region ($F_{2,24} = 81.33$, $p < 0.001$) as well as an interaction of both ($F_{4,24} = 1.39$, $p < 0.01$). The comparison to the basal expression of the cage controls revealed an upregulation of c-fos in both groups for all three regions although to a lesser degree for the CA3 pyramidal cells

(Duncan's test, Fig. III.12). This indicates an effect of the training in the NET on the c-fos expression. However, except for a higher variability within the control group no difference was found as result of the spatial novelty.



Group	$F_{2,12}$ = 0.31	p = 0.026
Region	$F_{2,24}$ = 81.33	p < 0.001
Group x Region	$F_{4,24}$ = 1.39	p < 0.01

Fig. III.12: C-fos signals in the dorsal Hippocampus
The c-fos signals of the novelty (black), control (dark gray) and cage control group (light gray) are presented in box plots separate for the different sub-regions of the hippocampus. The high number of signals in novelty and control mice indicates that both are upregulated in their c-fos expression compared to the basal expression revealed by the cage control animals. This indicates a general effect of the training in the exploration task on the c-fos expression. * =p<0,05; ** =p<0,01; *** =p<0,001 (Duncan test after ANOVA). General effects of a 2-way ANOVA are shown in the textbox.

III.3.2.3 Analysis of c-fos expression in the Nucleus Accumbens

Examples for *in situ* hybridizations of all three groups are given in Fig III.13. For the basal c-fos expression, revealed by cage control mice, even less signals were found than in the hippocampal regions (C, F). The signals in both trained groups were found in a spread pattern similar to that seen in the RCM experiment (A, D and B, E). The counted signals normalized for area in the lateral and medial nucleus accumbens are presented in Fig. III.14. Differences in signal strength were seen as general effect of group ($F_{2,13} = 3.86$, $p = 0.048$, 2-way ANOVA) and region ($F_{1,13} = 25.38$, $p < 0.001$) as well as an interaction of both ($F_{2,13} = 5.99$, $p = 0.018$).

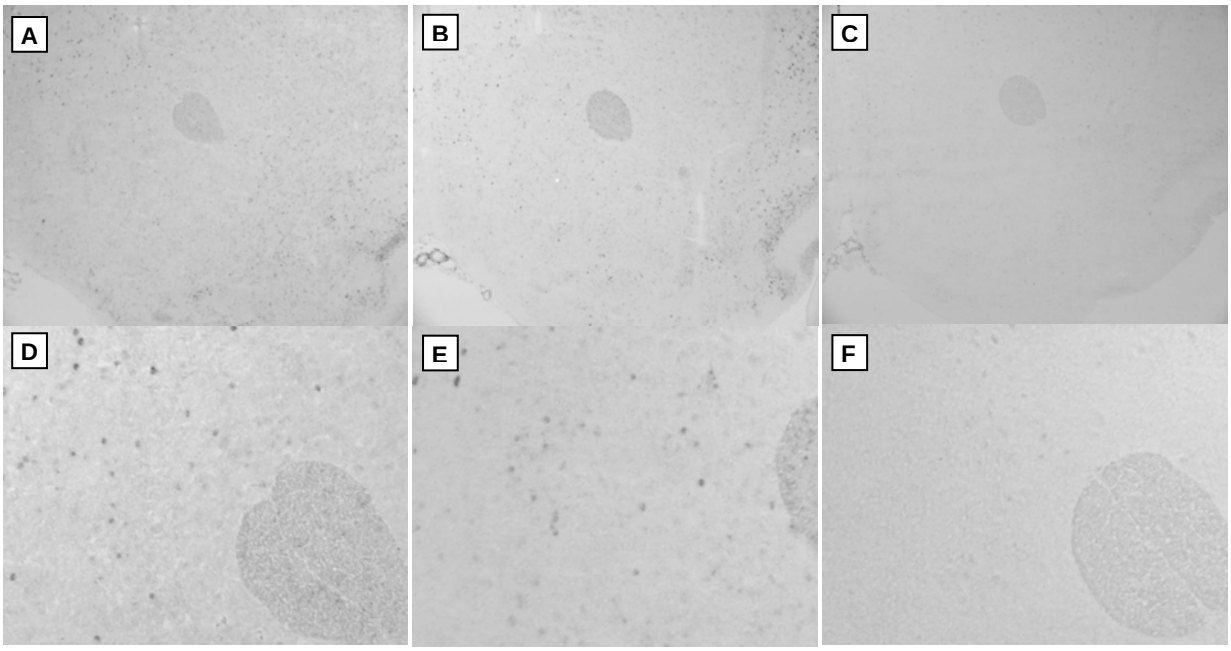
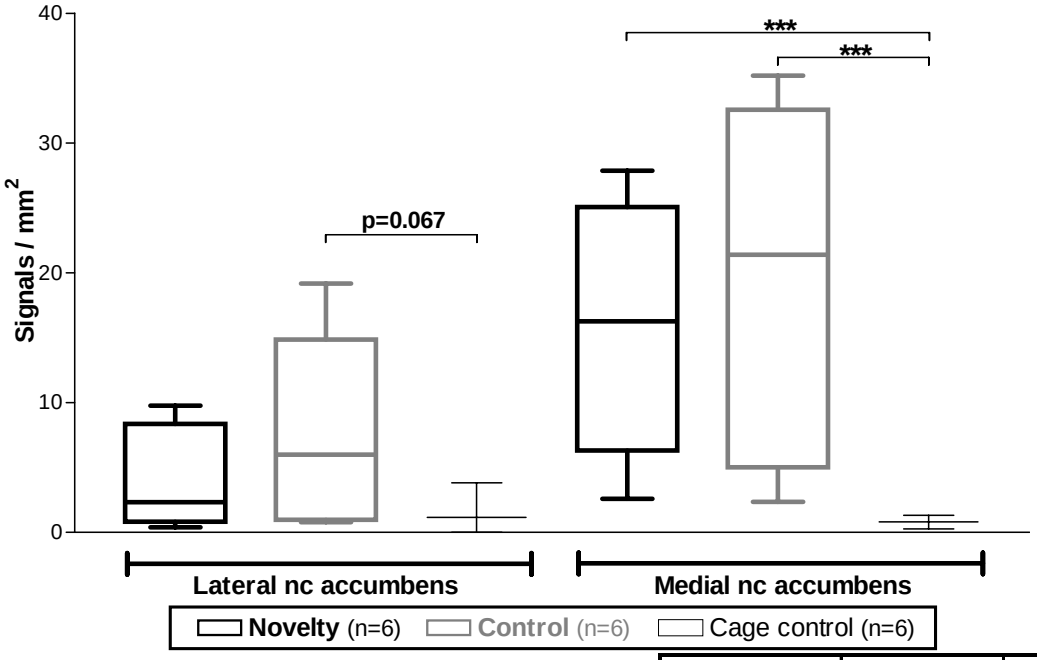


Fig. III.13: C-fos *in situ* hybridization in the Nucleus Accumbens
The *in situ* hybridization visualizes a c-fos upregulation in novelty **(A, D)** and control mice **(B, E)** compared to the basal expression in cage control mice **(C, F)**. The higher magnification reveals that a c-fos upregulation is seen as distinct signals spread over the area of the nucleus accumbens of both groups **(D, E)**, while almost no signals are found for the basal expression in the cage controls **(F)**.



Group	$F_{2,13}$ = 3.86	p = 0.048
Region	$F_{1,13}$ = 25.38	p < 0.001
Group x Region	$F_{2,13}$ = 5.59	p = 0.018

Fig. III.14: C-fos signals in the Nucleus Accumbens
The c-fos signals of the novelty (black), control (dark gray) and cage control group (light gray) are presented in box plots separate for lateral and medial nucleus accumbens. A strong upregulation is found in the medial nucleus accumbens of both trained groups compared to the basal expression of the cage controls, indicating a general effect of the exploration task. ** =p<0,01 (Duncan test after ANOVA). General effects of a 2-way ANOVA are shown in the textbox.

For the medial nucleus accumbens an upregulation due to the training in the NET was found in both groups compared to the basal expression of the cage controls (Duncan's test, Fig. III.14). In the region of the lateral nucleus accumbens only the control group revealed a weak upregulation as compared to the cage control group. No difference between control and novelty group was found. As for the hippocampus, the control group showed a high variability of c-fos signals in the nucleus accumbens.

III.3.3 Reinforced Relearning Circular Maze (RRCM)

The RRCM paralleled the protocol of the RCM, with an additional reinforcement of the relearning for one group on a second post-criterion training day. This allowed the investigation of c-fos expression in different stages of a learning process.

III.3.3.1 Behavioral analysis

On day 1 the mice performed with a median distance of 2,5 holes to Target. This is followed by a relative increase on day 2 and then a steady decrease down to a distance of 1 hole at the last training day. A significant effect of days was found (Friedman test $p < 0{,}001$) and post hoc comparisons showed a difference between the last two days to day 1 (Dunn's test, Fig. III.15).

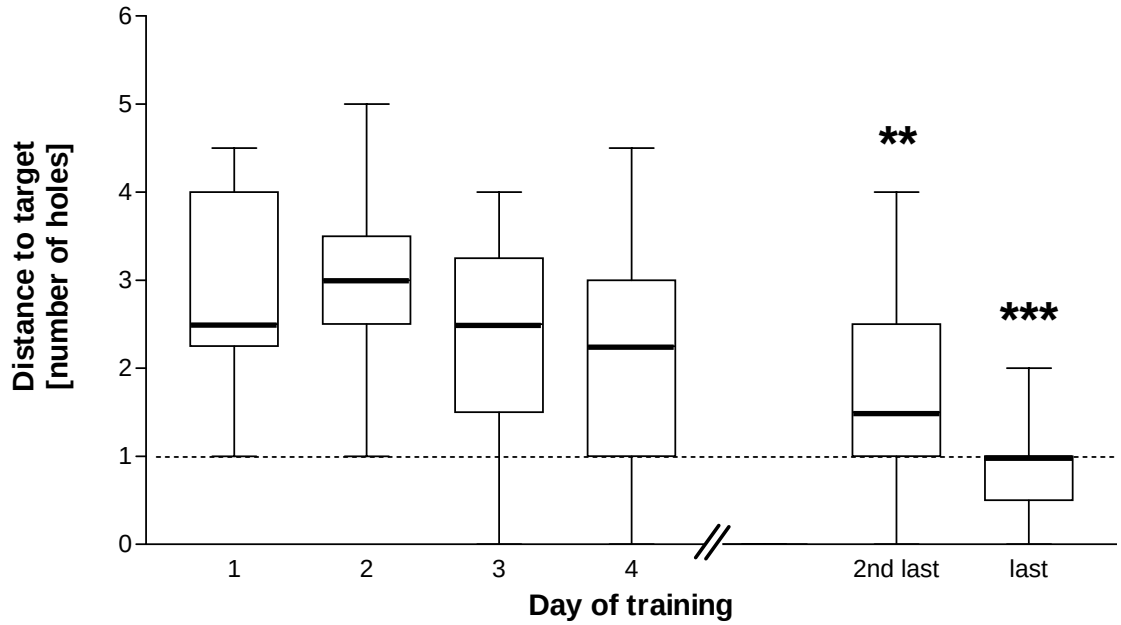


Fig. III.15: Learning performance measured as distance to Target
Distance to Target, given as number of holes is presented as box plots for days 1-4 and the two last days of training representing thereby all 33 mice. The decrease of distance shows that the mice learned successfully to orientate within the CM arena to find the Target. The broken line indicates the distance matching the criterion. **-p<0.01, ***-p<0.001 (Dunn's test after Friedman test).

A behavioral analysis of the second phase of training was performed to investigate, whether the previous training was successful and if the mice changed their behavior within the relearning training. Therefore the localization of the animals within the first 5 s was analyzed for the relearner and reinforced relearner mice in the first and fourth trial of day 1 of the relearning event and the reinforced relearner mice in the fifth and last trial of day 2. A spatial histogram with time per area depicted as x-y matrix for the CM arena indicates the length of time in a color-scale in Fig III.16.

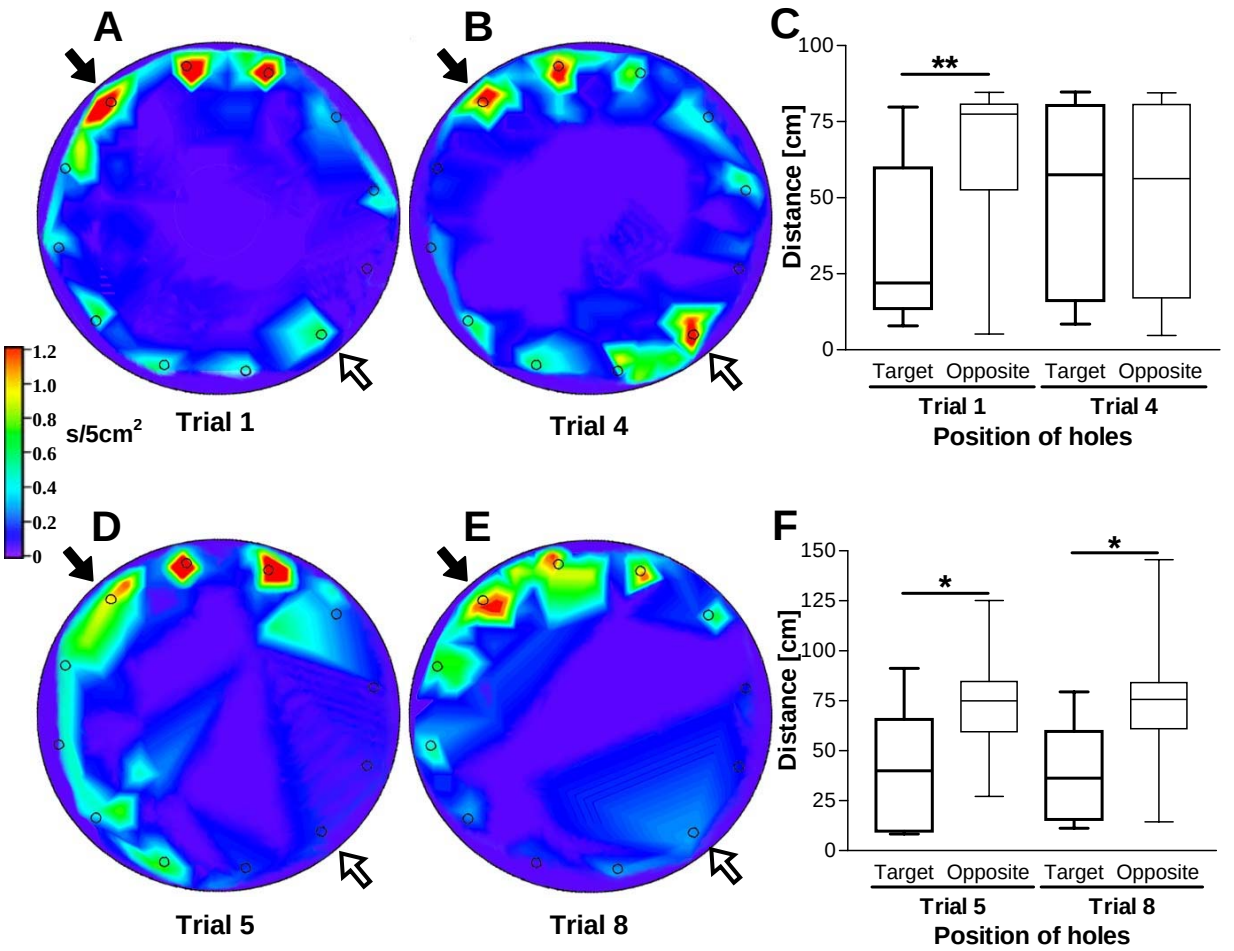


Fig. III.16: Localization as time per area-matrix and time in quadrant
(A, B, D, E) The spent time for every point of the CM arena as x-y matrix is presented in s/5cm² for the first 5s. Time is depicted by a color-scale reaching from blue for low to red for high levels of time. The black rings indicate the position of the holes (not exactly true to scale). ⬅ old Target, ⇦ new Target
(A) The mice show a strong preference for the old Target area and two neighboring holes seen as red spots in the first trail. **(B)** In the fourth trial the mice spent a similar time span at the old Target and the opposite hole, which was the new Target for the relearning. In the fifth **(D)** and last trial **(E)** of relearning on the next day the mice are just found at the old Target and the neighboring holes but not at the opposite hole, which was the new Target.
(C) The parameter mean distance to zone reveals that the mice are found closer to the old Target than to the opposite hole in the first trial, indicating that they were successfully trained to this Target in the training before. In the fourth trial mice are found in a similar distance at the old Target and the opposite hole, which was the new Target. This indicates a change of behavior already within the first session of relearning. **(F)** On the second day of relearning the mice are found closer to the old Target than to the opposite hole in both the fifth and last trial. This indicates that the mice even enhance the search at the old Target on the reinforcement day. *-p<0.5, **-p<0.01, (Wilcoxon Signed Rank test).

These results illustrate that the mice had a strong preference for the Target and two neighboring holes in the first trial (A). They enhanced the time spent at the opposite hole, which was the new Target position, in trial 4, indicating a change in their searching behavior (B). Surprisingly this change is not found any more in trial 5 (D) and trial 8 (E) on the next day, in which they reveal a similar distribution as in the first trial.

The parameter mean distance to zone (area of the Target or opposite hole) corroborates these localization patterns. While the mice are found in a significantly shorter distance to the old Target than to the opposite hole (new Target) in trial 1 (Wilcoxon Signed Rank test, $p = 0.009$), they stayed at the same distance to both destinations in trial 4 (Fig. III.16C). In trial 5 and 8 they are again found significantly closer to the old Target than to the opposite hole (Fig. III.16F, Wilcoxon Signed Rank test, trial 5: $p = 0.027$, trial 8: $p = 0.037$). This shows that on the second training day they enhanced again searching at the old Target position instead of reinforcing the switch to the new Target at the opposite hole.

III.3.3.2 Analysis of c-fos expression in the Hippocampus

As in the NET the analysis was performed for the three experimental groups of the learning paradigm plus an additional group of 5 naïve cage control mice. Fig. III.17 shows examples of c-fos hybridized sections of the hippocampus of all four groups. The cage control animals revealed the basal expression of c-fos, wherein distinct signals were very rarely found (G, H). In the three experimental groups the upregulation was mainly seen in the principal cell layers of the different hippocampal regions (A, C, E). The higher magnification of the DG demonstrates that most signals were found in the relearner group (B) compared to the control mice (F), while the reinforced relearner group (D) was on a level between these groups.

The evaluation of signals per area for the different regions indicated an upregulation of the c-fos expression for the three trained groups as compared to the cage control mice (Fig. III.18). A 2-way ANOVA was performed to compare the three trained groups, revealing a general effect of group ($F_{2,27} = 5.98$, $p = 0.007$) and region ($F_{2,54} = 115.67$, $p < 0.001$) as well as an interaction of both ($F_{4,54} = 2.70$, $p = 0.040$).

The strongest induction of c-fos expression was achieved in the first relearning training as indicated by a strong upregulation found in the granule cells of the DG

and the CA1 pyramidal cells in the relearner group compared to the cage control group (Duncan's test, Fig. III.18).

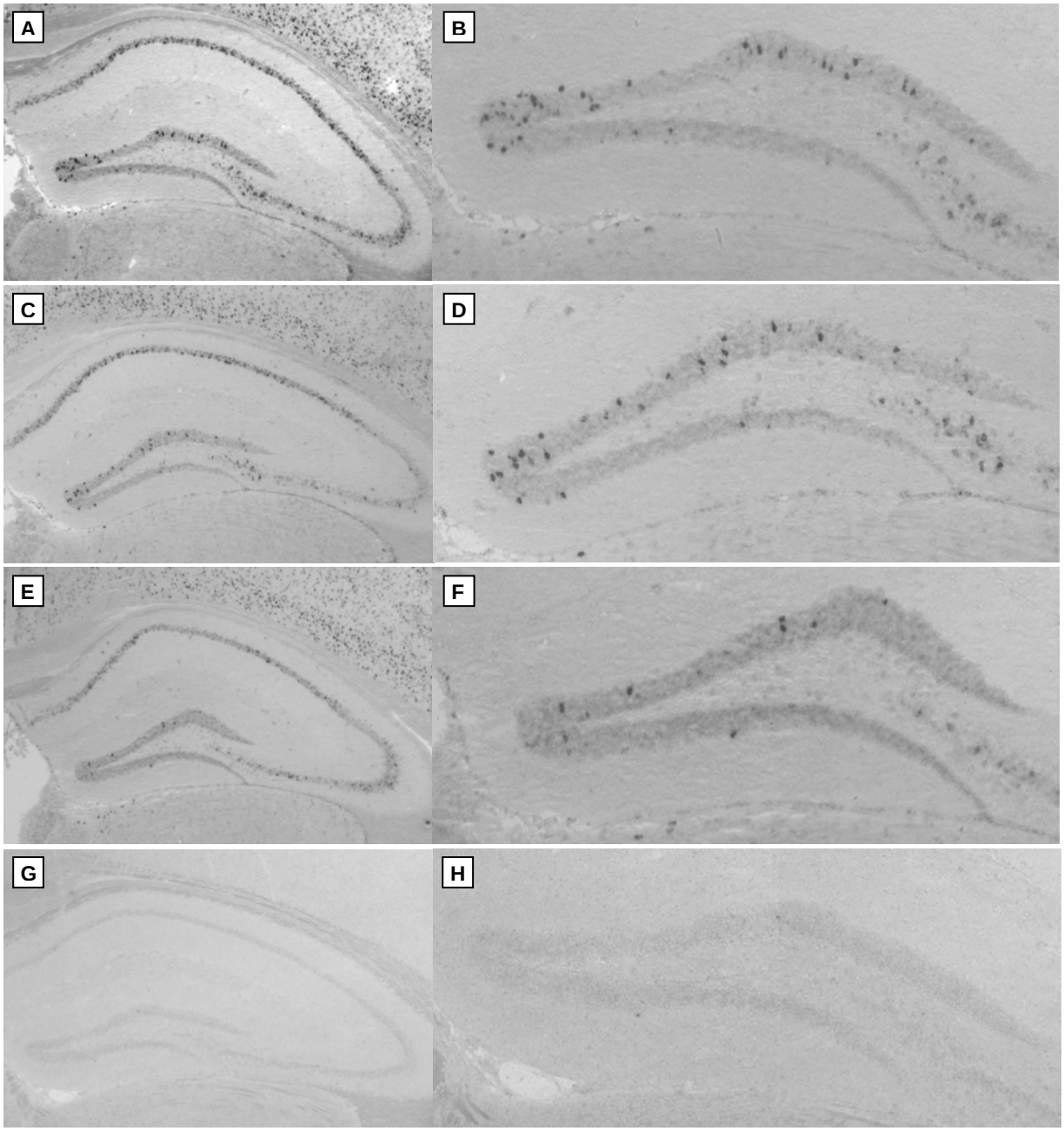
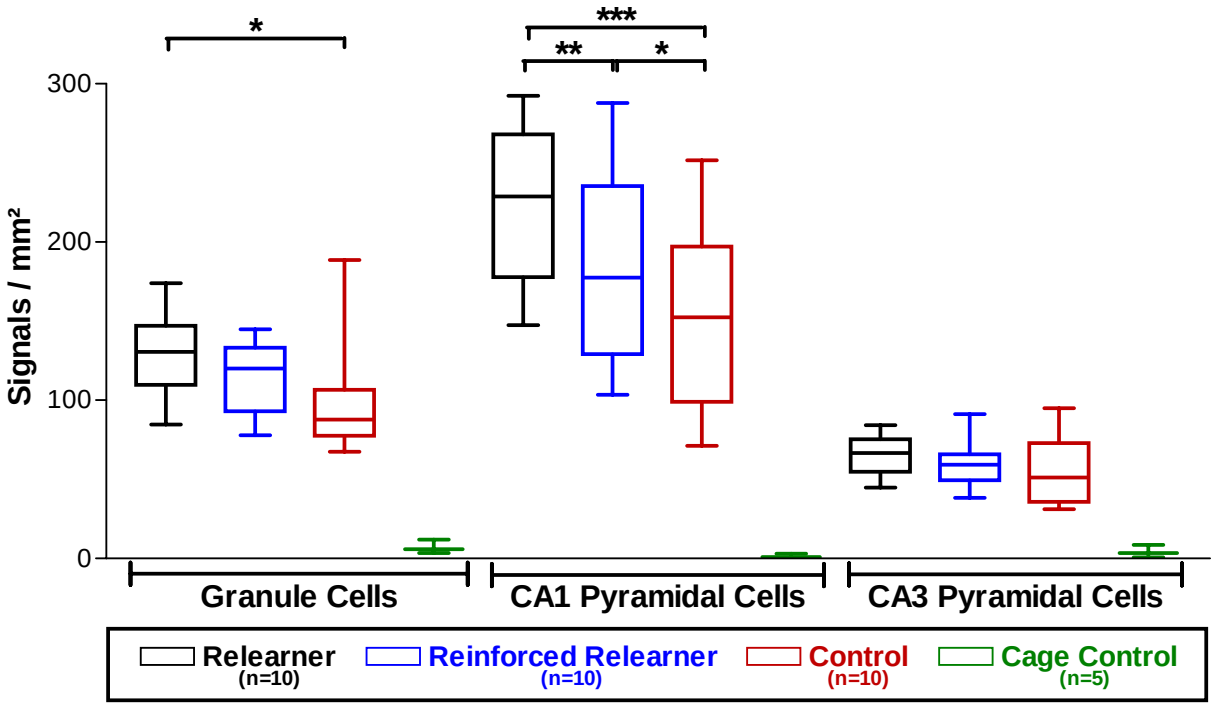


Fig. III.17: C-fos *in situ* hybridization in the dorsal Hippocampus
The *in situ* hybridization visualized a c-fos upregulation as distinct signals in the principal cell layers of relearner **(A, B)**, reinforced relearner **(C, D)** and control mice **(E, F)**, while signals are rarely found in cage control animals **(G, H)**. The higher magnification of the DG region demonstrates that the upregulation is strongest in relearner **(B)**, intermediate in reinforced relearner **(D)** and lowest in control mice **(F)**.

The reinforcement training on the second day was less effective at upregulating c-fos as indicated by the reinforced relearner group. They showed an intermediate level of upregulation, between relearner and control group. Still their c-fos upregulation was significantly higher than that of the control animals in the CA1 pyramidal cells.

No difference between the three groups was found in the pyramidal cells of the CA3 region, which at the same time revealed the weakest expression of the three regions analyzed.



Group	$F_{2,27}$ = 5.98	p = 0.007
Region	$F_{2,54}$ = 115.7	p < 0.001
Group x Region	$F_{4,54}$ = 2.70	p = 0.040

Fig. III.18: C-fos signals in the dorsal Hippocampus
The c-fos signals of the relearner (black), reinforced relearner (blue), control (red) and cage control group (green) are presented in box plots separate for the different sub-areas of the hippocampus. A general effect of the training is seen in the three CM trained groups. In addition the regions of the DG and CA1 pyramidal cells indicate a strong effect of the first relearning training. The reinforced training reveals to have a less strong effect on the upregulation of c-fos, as seen by the intermediate level of signals in the reinforced relearner group. * =p<0,05; ** =p<0,01; *** =p<0,001 (Duncan test after ANOVA). General effects of a 2-way ANOVA are shown in the textbox.

III.3.3.3 Analysis of c-fos expression in the Nucleus Accumbens

As shown in the examples in Fig. III.19 the basal c-fos expression of the cage control mice (D, H) was very low as compared to that of the relearner (A, E), reinforced relearner (B, F) and control group (C, G). Correspondingly, the evaluation of the signals per area for the lateral and medial nucleus accumbens indicated an upregulation in all three trained groups (Fig. III.20). A 2-way ANOVA on these three groups revealed an effect of region ($F_{1,27} = 81.33$, $p < 0.001$), while no effect was seen for group ($F_{2,27} = 0.39$) and the interaction of both ($F_{2,27} = 1.39$).

A regional difference can clearly be seen, since higher c-fos expression was detected in the medial compared to the lateral nucleus accumbens. The slight upregulation due to relearning that was found in the RCM (the relearner had higher c-fos

expression than the control) was also observed in this experiment, as relearner mice tended to have higher c-fos expression in the medial nucleus accumbens as compared to the control (p = 0.077, Duncan's test).

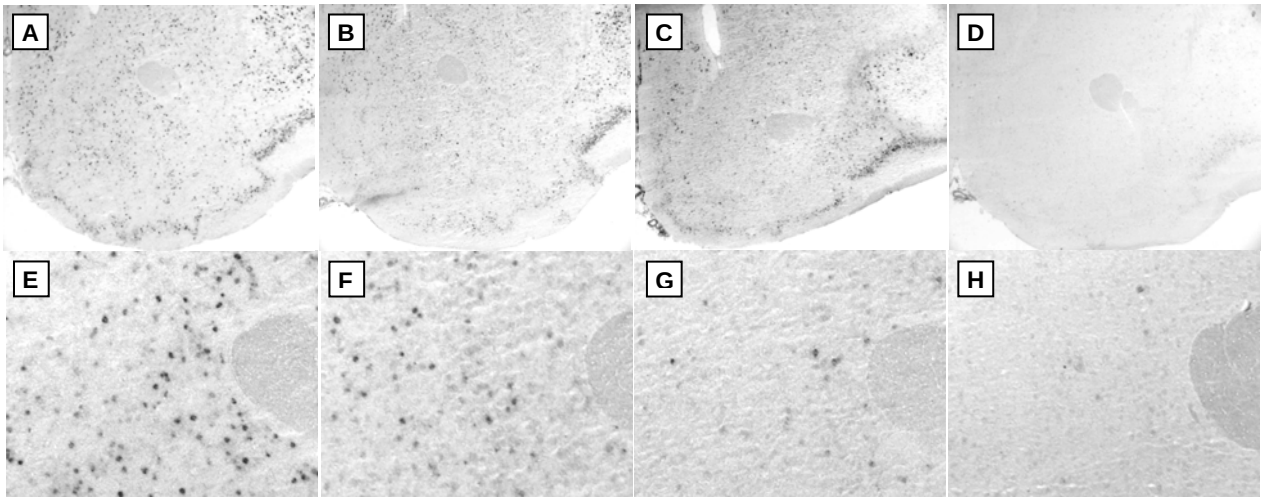
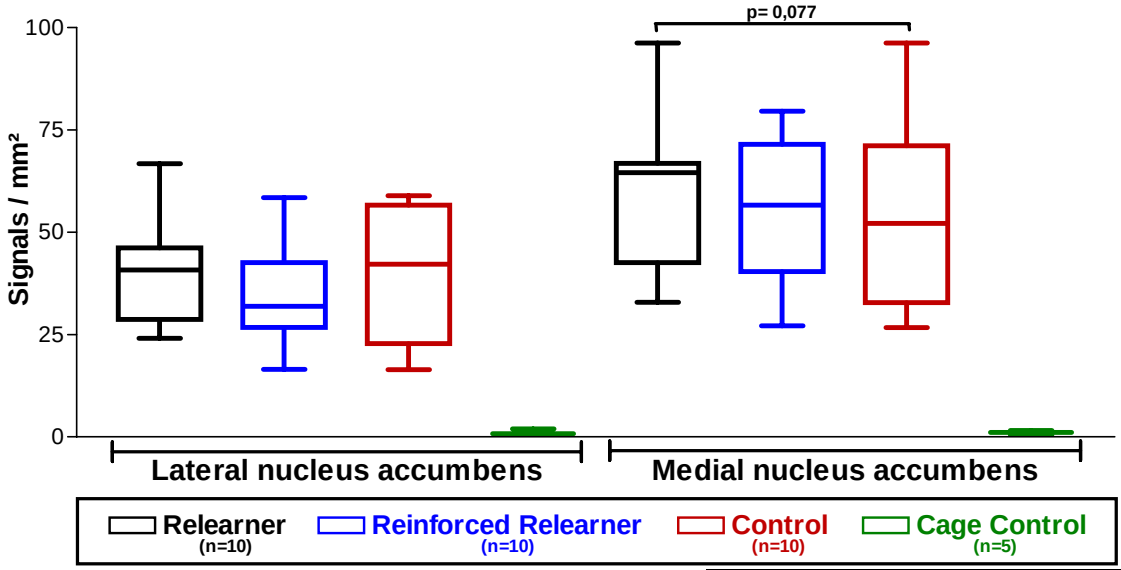


Fig. III.19: C-fos *in situ* hybridization in the Nucleus Accumbens
The *in situ* hybridization visualized a c-fos upregulation in relearner **(A, E)**, reinforced relearner **(B, F)** and control mice **(C, G)**, and the basal expression of cage control mice **(D, H)**. The higher magnification reveals a c-fos upregulation in all three groups **(E, F, G)** compared to the basal expression of the cage controls **(H)**.



Group	$F_{2,27}$ = 0.31	p = ns
Region	$F_{1,27}$ = 81.33	p < 0.001
Group x Region	$F_{2,27}$ = 1.39	p = ns

Fig. III.20: C-fos signals in the Nucleus Accumbens
The c-fos signals of the relearner (black), reinforced relearner (blue), control (red) and cage control group (green) are presented in box plots for separate areas defined as lateral and medial nucleus accumbens. The high number of signals in the three trained groups indicates that all three are upregulated in their c-fos expression due to the training revealing a slightly higher upregulation for the relearner mice (p-value for Duncan test after ANOVA). General effects of a 2-way ANOVA are shown in the textbox.

III.4 Discussion

Three experiments are performed to investigate the influence of spatial relearning and spatial novelty in neuronal activation measured as c-fos expression. This gene reveals learning induced activation allowing a parallel investigation of brain areas with a high resolution due to a cellular and temporally confined expression pattern. The differential activation pattern within the hippocampal sub-regions and the NAc indicate distinct roles of these brain areas in the processing of a relearning event and the detection of novelty.

III.4.1 Relearning Circular Maze (RCM)

In order to allow the investigation of the c-fos expression induced by a relearning event, it is necessary to achieve a homogeneous performance between mice at the end of the learning process. Therefore the mice are trained on the 12-hole platform in a learning phase until they reach the learning criterion (first approach of the Target +/- 1 hole in three consecutive trials). The acquisition curve of distance to Target (DTT) shows that all mice finish this first training phase at the same level of successful performance.

On the following day the mice perform one training session that serves for induction of c-fos expression. While the control mice perform a session identical to the previous training, the relearner group is trained with a new Target position as relearning event. In this relearning training the relearner mice reveal a significantly lower distance to the old Target than to its opposite hole, which indicates a strong preference to the formerly trained Target position. The comparison of the first and fourth trial of the relearning session indicates that the relearner mice start to develop a preference to the new Target already within this session. The mice significantly decrease the distance to the new Target from trial 1 to trial 4 and thereby reveal in trial 4 a comparable low distance to the old and new Target, which both are shorter as compared to the hole opposite of the old Target. This reveals a first learning success therefore proving that a relearning event is induced effectively.

The c-fos expression pattern that is elicited by this relearning process is indicated by comparison to the gene expression of control mice. The main difference between the c-fos expression of the two groups is found in the granule cell layer of the DG. The c-fos expression is enhanced by 28 % because of relearning. Although the general c-fos expression is even higher in the CA1 pyramidal cells compared to the DG, no

difference is found between the groups herein as well as in the CA3 pyramidal cells, which reveal the lowest expression of the three hippocampal sub-regions analyzed. Since the training protocol of the control group is identical to that of the relearner group beside the new Target position, many influences can be ruled out as inducing factors for the differences found. Locomotion as well as visual, auditory and olfactory stimuli are the same in both groups. In addition, the mice are used to hit a closed hole from time to time without any negative consequences, making an influence of stress in the new situation unlikely. Possible influencing stimuli can be delimited to the relearning process, the slight novelty of the new position and the mismatch of expectation for reward. Especially the novelty component is often discussed as important factor for hippocampal activity (Fyhn et al., 2002; Jenkins et al., 2004; Lee et al., 2004; Vann et al., 2000; Vinogradova, 2001; Wan et al., 1999; Wiebe and Staubli, 1999).

Novelty and especially reward related behaviors are suggested to involve the NAc (Arleo and Gerstner, 2000; Bassareo et al., 2002; Everitt et al., 1999; Rebec et al., 1997). Recent findings show a connection as well to spatial components, which is often explained by the integration of spatial knowledge with reward expectation to a reward guided behavior (Annett et al., 1989; Gal et al., 1997; Lavoie and Mizumori, 1994; Mogenson et al., 1980; Ploeger et al., 1994; Smith-Roe et al., 1999). The comparison of c-fos expression in the NAc of relearner and control mice revealed a strong tendency towards a higher c-fos expression in the medial NAc due to the relearning event. The low expression of c-fos in the lateral NAc was not distinguishable between the groups.

However, this experiment by itself does not allow to distinguish, whether the found expression pattern is more typical for the early phase of a spatial relearning process or more related to a mere novelty effect.

III.4.2 Novelty Exploration Task (NET) on a Circular Platform

The design of the CM experiment already minimizes the novelty component that is involved in the relearning event inducing the c-fos expression. Though a novelty component cannot be completely excluded during a learning process. However, the expression patterns were analyzed for a very early stage in the relearning process. Therefore, it would be interesting to see, whether it may be due to the detection of a spatial change rather than an actual learning process. In order to dissect the novelty

effect it is necessary to separate a spatial change from any learning incentive. This is achieved in a pure exploration task, in which the presented spatial situation cannot be used to achieve a reward or reach any kind of goal.

All mice are habituated in the exploration paradigm that presents the same spatial situation as the CM paradigm, besides the absence of holes and an escape tunnel. The strongest effect of habituation is achieved between day 1 and 2, in which the mice of both groups reduce the distance moved by 50 %. The sensitivity of the parameter distance moved is shown on day 5, where both groups respond to a re-exposure to the NET after a 2-day break with an identical amount of increased exploration activity. On day 8 the control mice perform a session identical to the previous habituation training. Correspondingly they move a similar distance as on the previous day. The novelty group is trained with the same protocol as before but with a change in the spatial surrounding. The platform is surrounded by a dark curtain, which masks the light walls with the well-known landmarks. The novelty experiencing mice respond to this change with a significantly higher distance moved compared to the control mice. The increased locomotion proves that the mice effectively noticed the change in the spatial surrounding. The strong increase in distance moved is impressive, since the change can neither be explored directly by the animals, nor has any consequence on their behavior within the NET.

The obvious behavioral reaction to spatial novelty is not reflected by a corresponding change in the c-fos expression pattern. A general activation due to the training in the NET is detectable in all areas of the hippocampus, since both trained groups reveal a significant higher c-fos expression compared to the basal expression of the cage control mice. However, no difference is found between the novelty and control group. Interestingly the control mice reveal a stronger variability compared to the novelty group. This may show the difficulty of achieving a homogeneous status, if no concrete incentive is connected to the situation, which the mice are exposed to. Such an indefinite situation may provoke more variable responses in the mice.

Similarly, in the NAc the c-fos expression of the novelty and the control group reveals no difference. The comparison to the cage control mice indicates general activation due to NET training only in the medial NAc. The expression in the lateral NAc not even differs effectively from the basal expression found in the cage control mice.

These results suggest that spatial novelty without incentive to learn or guide behavior does not activate c-fos expression in the investigated areas. This suggests further

that the expression patterns found in the RCM experiment cannot be explained by a mere effect of spatial novelty.

These results are in line with the hypothesis that the hippocampus serves as filter to detect relevant novelty (Fyhn et al., 2002; Mizumori et al., 1999; Vinogradova, 2001). In this context it is important to consider, that the spatial changes presented in the NET are more profound than that of the RCM, which consisted of a new Target position but no obvious change in the surrounding. Vinogradova (2001) suggests that not the extent of novelty is decisive for hippocampal activity, but the relative quality of novelty. Novelty detection is dependent on relevance of the stimulus, and past history of neural activity importantly influences perception (Mizumori et al., 1999). Perception of irrelevant stimuli can be processed differently depending on situation. E.g. unpredictable and therefore non-informative stimuli are shown to activate fewer cells in the hippocampus than relevant but less obvious changes (Fyhn et al., 2002). A differential activation by different forms of novelty has been suggested by Wan et al. (1999, 2001). Novel items strongly activated areas such as the perirhinal cortex in case of visual stimuli or the auditory association cortex in case of auditory stimuli. In contrast, the CA1 area revealed an increased activation, if familiar items were presented in a novel arrangement (Jenkins et al., 2004; Wan et al., 1999). These findings are in line with the argumentation of Vinogradova (2001) stating that the hippocampus and especially the CA1 region functions as comparator of stored information with changes in the environment. Such relevant configurationally changes in the familiar surrounding occur, when the Target position is changed in the CM experiments.

III.4.3 Reinforced Relearning Circular Maze (RRCM)

The RRCM experiment was designed to compare the early stages of a relearning process with processes occurring in reinforced relearning. The reinforced relearning consisted of an additional relearning session on the second post-criterion day. In opposite to the RCM, which approached the very first events measuring the c-fos expression 20 min after session start, the brain-removal in this experiment is delayed to 30 min after session start to comprise events in an enlarged time range.

As in the RCM the mice are trained until they reach the learning criterion to achieve the same stable successful level of performance. The acquisition curve for the

parameter DTT reveals that a uniform performance level is acquired by all animals at the end of the learning training as precondition for the following induction training.

In accordance to the findings in the RCM the relearner and reinforced relearner reveal a strong preference to the old Target position in the first trial. As in the RCM the animals start to re-orientate within this first relearning session. Therefore they show the same distance to the old Target and the opposite hole, which serves as new Target. The spatial histogram for the CM arena indicates that the mice spent a similar prolonged time-span close to the old and new Target position.

Surprisingly, the beginning reorientation is not found any more on the second day of relearning training in the reinforced relearner mice. In trial 5 and 8 of the relearning mice reveal the same preference to the old Target as found in trial 1. This finding fits to the enhanced appetitive behavior after expectation mismatch that is seen in the relearning 1 of the ECM or the probe trial of the RCM. It supports the suggestion that mice are able to re-orientate very fast, if the Target position changes in a familiar spatial surrounding, but a successful performance is overlaid by an enhanced search in the expectation matching position.

The c-fos expression pattern in the granule cells of the DG resembles the findings of the RCM. The relearner mice reveal a significantly higher expression compared to the control group. In the reinforced relearner group the c-fos expression level lies between the relearner and control mice but reaching no significant difference to any of them. In addition to the differential activation found in the DG, all three groups differ significantly in their expression in the CA1 pyramidal cells. Even more pronounced as in the DG the relearner mice reach the highest c-fos expression, while the reinforced relearner mice reveal an intermediate level between the relearner and control group. In the CA3 area no differences are found between the three trained groups.

The expression pattern supports that the activation pattern found in the RCM is reflecting a neuronal activation specific for relearning. It is also obvious that the main activation occurs on the first day of relearning training, while the second day is less effective for the upregulation of c-fos expression. This corresponds well to findings of Lee et al. (2004). They show that the main stabilization of place fields takes place on the first day presenting the spatial novelty, if the presentation is long enough. Just in case of a short presentation, a strong activation can still occur on the second day of presentation. In the case of the RRCM the behavioral data prove that already the first

day is effective to achieve a re-orientation of mice within the session. In paradigms with fast acquisition it is therefore probable that good performers reveal already a decreasing activation in repeated training. In contrast, in bad performers that did not acquire the task within first presentation, the repeated learning is combined with strong activation. Corresponding expression patterns are reported by several authors (Kelly and Deadwyler, 2002b; Kelly and Deadwyler, 2002a; Nikolaev et al., 1992; Ryabinin, 1998).

The finding of Bertaina-Anglade et al. (2000) that better performer reveal a stronger activation, can as well be explained by the acquisition time. The positive correlation of activation and performance within first and second training day reveals that good performers reach the strong activation during steep acquisition increase first in paradigms with a longer acquisition time (in this case 5 training days, Bertaina-Anglade et al., 2000).

In the RRCM the main activation seems to occur during the first relearning session, fitting well to the behavioral data. On the second day the influence of relearning is less strong. This reveals at the same time, that the enhanced appetitive searching behavior due to expectation mismatch has a minor influence on the activation pattern in the hippocampal regions.

As in the RCM, no strong influence of the relearning event for the c-fos expression is found in the NAc. A general activation due to the CM training is found in all three trained groups compared to the basal expression revealed by the cage control mice. However, only a tendency towards a higher c-fos expression due to relearning is indicated in the medial NAc (shell) comparing the relearner and control mice, which resembles the pattern that is found in the RCM. This reproducible finding suggests that the differential expression pattern is a distinct though weak effect due to relearning.

III.4.4 Comparison of c-fos expression pattern over experiments

Both RCM and RRCM reveal no changes in c-fos expression due to of relearning in the CA3 region. Functional concepts for the CA3 region comprise the comparison of memorized and current spatial representation. Since in the CM the spatial surrounding is not changed, activation due to mismatches in the memorized and current spatial state is not to expect. A second role of the CA3 region is connected with the comparator function. The network of recurrents in this region is predestined

to allow a dynamic self-localization and routing within moving (Amaral and Witter, 1995; Redish and Touretzky, 1998). In line with this concept Lee and Kesner (2003) suggest a function for CA3 in auto-association of visual landmark, vestibular and kinesthetic stimuli. Following this argumentation, a stronger c-fos expression for the relearner group is not to expect, since the self-localization and routing is necessary in the same way for both the relearner and the control mice moving in the CM. As well the formation of a new spatial representation is not required for the relearning situation of the CM, which is another key role assigned to the CA3 region (Lee et al., 2004).

Comparable results for RCM and RRCM are also found in the DG granule cells. In both experiments the relearner group indicates an effective c-fos upregulation due to the relearning event. Amaral et al. (1990) showed that the DG afferents from the EC are widespread compared to the spatially and numerically limited projection efferents running towards the CA3 region suggesting a filtering function for this area. Electrophysiological recordings show that in this relay a secondary simplification of signals occurs (Vinogradova, 2001). The DG can probably be separated from the direct assembly line for spatial representation (Barnes et al., 1990). On the one hand, granule cells reveal no reliable spatial selectivity comparable to the CA3 and CA1 pyramidal cells, on the other hand DG lesions do not alter the place field in CA3 and CA1 (McNaughton et al., 1989), suggesting another role for the DG in spatial learning.

Roullet and Lassalle (1992) suggest a role for DG in detection and coping with novelty in familiar situations. This concept as well as the suggestion that computational processes such as pattern separation are performed by DG (Lee and Kesner, 2003; Mizumori et al., 1999) fits with the c-fos upregulation found in the DG of the relearning groups. Indeed, in this study the novelty consists of a mismatch pattern where to expect and find the Target with no changes in the spatial environment or the situation in general. Consistently, the reinforced relearner group showed a lower increased c-fos expression as compared to the relearner mice, due to the lower degree of novelty by repetition on the second day of relearning. Indeed, the behavioral data indicate that a coping with the new situation is already reached at the end of the first relearning day.

Whereas the patterns of c-fos expression in the DG and CA3 were consistent between the two CM experiments, dissimilar results were obtained for the CA1

region. In the RRCM an effect of the relearning event is indicated by an increased c-fos expression in the pyramidal cells of the CA1 region of the relearning groups, which is even stronger than in the DG region.

A very similar activity pattern is found regarding AMPA binding activity following SDA learning (Cammarota et al., 1995). At the time point 30 min post-training (which is the same as in the RRCM) the AMPA binding is increased in the CA1 and DG with strongest binding in CA1, but not in the CA3. Noteworthy, no increase in any of the hippocampal sub-regions was seen in mice that were freely exploring the training chamber, which is in line with the findings of the NET experiment, in which novelty alone did not alter c-fos expression. The comparability in pattern and time-range of AMPA binding and c-fos expression is interesting under the aspect that AMPA binding is also increased after induction of LTP in vivo (Cammarota et al., 1995), a stimulus that can also upregulate the IEG response (Tischmeyer and Grimm, 1999). An involvement of increased AMPA binding in memory consolidation is suggested by Bernabeu et al. (1997).

Electrophysiological recordings reveal that CA1 place cells show a more complex firing pattern with more multimodal discharging cells, in contrast to CA3 cells, encoding also task relevant or motivational information (Holscher, 2003). Therefore CA1 and DG may act in concert, in parallel with a minor contribution of the CA3 region, if information processing is less concerning the formation of spatial representation itself. Interestingly a parallel influence on CA1 and DG but not on CA3 are found in studies disconnecting the anterior thalamic nucleus (ATN) from the hippocampus, resulting in reduced Fos levels in the CA1 and DG but not CA3 region (Jenkins et al., 2002). Several studies reveal the importance of the ATN in processing head direction information (Goodridge and Taube, 1997; Taube, 1995; Taube and Muller, 1998; Warburton et al., 2001). A parallel of the found pattern in the CM experiments is suggestive since the relearning involves changes for head direction by changing the Target position far more than influences on the spatial representation. Leutgeb et al. (2000) found CA1 cells coding for directional heading independent of location information and suggest that head direction information participates in synaptic interactions when new location codes are formed.

A strong CA1 activation in general is found by several researchers in different contexts and learning situations (Bertaina-Anglade et al., 2000; Gall et al., 1998; Hess et al., 1995a; Hess et al., 1995b; Jenkins et al., 2004; Wan et al., 1999). The

discrepancy to the RCM is most probable due to the time-point of brain removal, which is the only profound difference between the experiments. For the RCM a very narrow time interval until removal of the brains was chosen to address the first stages of a relearning process and in addition avoid any stimulation post-training. The RRCM intended to investigate intermediate and later stages in the relearning process allowing a broader time interval before the brain removal and additional relearning events on the next day.

The narrow time range of the RCM may therefore revealed a pronounced effect of the first trial of the relearning session combined with a lower influence of the following trials. Lee et al. (2004) suggest that plasticity mechanisms in CA1 either are not activated on the first experience with a novel cue configuration or follow a slower time course, since the change in place fields in the CA1 region in contrast to the CA3 region was just seen on the second exposure on the next day. Frank et al. (2004) found that the largest changes in neuronal activity occur on day 2 of exposure to a novel place if an animal had just little experience (in their case < 4 min). In contrast, longer exposures on day 1 were associated with smaller changes on day 2. A different time-window for the CA1 region is suggested as well by other researchers and corresponds to the function of CA1 as output area integrating several inputs of other regional processing as mismatches found in the CA3 or changes in motivational state coming from the EC (Lee and Kesner, 2003; Minami et al., 2002; Mizumori et al., 1999; Redish and Touretzky, 1998).

In the CM, the mice receive a 4 times repeated exposure to the new Target and the behavioral data of the first relearning session in both experiments suggest strongly, that this exposure already lead to changes. In line with the before mentioned finding of Frank et al. (2004), sufficient exposure on the first day, associated with smaller changes in activity on the second day, would therefore explain the minor increase in c-fos expression found in the reinforced relearner group of the RRCM. If indeed the narrow time-range between start of session and brain removal (20 min) leads to a missing or reduced impact of the repetition of exposure over trials in the RCM, this can explain why the c-fos expression did not reach significant upregulation levels in the CA1 region in the relearner group. Since CA3 and DG are both regarded to react immediately on changes in spatial representation or novel situation, respectively (Lee et al., 2004; Roullet and Lassalle, 1992), the narrow time range is less probable to affect the activation in these regions, which explains that the c-fos expression is

significantly increased in the DG of the relearner group. In this contest it is also interesting to note that Lee and Kesner (2003) found in a hippocampal sub-region-specific lesion study, that CA1 lesions led to impairments of a delayed-non-matching-to-position task only in delays of 5 min, in opposite to lesions of CA3 and DG that as well resulted in impaired performances with a delay of 10 s.

A strong hint for an influence of the spatial relearning event on c-fos expression in the NAc is given by the fact that the tendency towards an increased c-fos expression in the medial NAc is found repeatedly in both CM experiments. However, if this increased pattern is not found by chance it reveals just a weak effect of the relearning event onto the neuronal activation of the shell area of the NAc. Lesions in the NAc, especially in the shell area, are often reported to induce impairments similar to those of hippocampal lesions (for overview see Gal et al., 1997). The contribution of the NAc to spatial learning is suggested to involve routing (Redish and Touretzky, 1998) and the integration of motivational and reward information (e.g. from the BLA) with spatial information to motor systems to guide behavior (Lavoie and Mizumori, 1994; Pratt and Mizumori, 2001). However, the relearning event presents a situation in which neither the form of reward and the motivation to reach it, nor the necessity of routing the way through the CM arena changed. Therefore the change that is achieved by the relearning event, which results from a mismatch in the expected position of the reward, may not be sufficient to produce a strong activation of this region. This supports that the involvement of the NAc in spatial learning, which is indicated by NAc-lesions (Annett et al., 1989; Gal et al., 1997; Smith-Roe et al., 1999), concerns the expression of spatial behavior by integrating spatial knowledge and locomotion (Lavoie and Mizumori, 1994) rather than the processing or storage of spatial information. This distinction is hard to address by lesion studies since these always interfere the behavioral output as multi-systemic cooperation, giving just indirect hints, which contribution is disturbed.

Several researchers showed that a novel environment evokes profound changes in the neuronal activity measured as well in gene expression (Jenkins et al., 2004; Vann et al., 2000; Wan et al., 1999) as with electrophysiological methods (Frank et al., 2004; Lee et al., 2004). However, in these experiments the change in the surroundings is of pronounced relevance for the animals, as in the radial maze, in which they use the spatial surrounding for orientation. Gall et al. (1998) reported that switching from mere presentation of a task environment to requiring a spatial

orientation as well leads to changes in activation of the hippocampus, especially in the CA3 region. In the case of the NET experiment a spatial and task environment comparable to the CM task is presented, however, without the requirement to use it for orientation. The spatial novelty, consisting of a complete change of room-shape, color and landmarks, is an obvious and profound environmental change compared to the moved Target position in the CM. In spite of this, no noticeable influence on c-fos expression is achieved by the spatial environmental novelty. The behavioral data rule out that the mice have paid no attention to the changes. In contrast, the small change within the CM relearning event, in terms of novelty, revealed profound changes in the c-fos expression pattern. These findings correspond to observations of Fyhn et al. (2002) that a new selective spatial firing pattern was found in the hippocampus, if a new platform position was presented differing from the constant position previously trained in a water maze. However, if the platform location varied randomly from trial to trial, thereby presenting novelty but preventing a particular expectation or memory, no new activity was elicited. As well the presentation of a completely novel but task-irrelevant stimulus in form of warm water current elicited no activation.

The results of the NET experiment as well certainly reveal that novelty per se is not sufficient to activate the NAc. In contrast to the CM experiments, no distinct routing and guiding towards any reward is required in the NET. Therefore changes comparable to the weak effect suggested for the relearning in CM were not to expect. The spatial novelty experienced in the NET neither has any relevance for the task, nor is explorable, which may be the main reason for the missing influence on a brain area that is seen as involved in novelty seeking behaviors (Rebec et al., 1997). Several researchers show a dissociation of different forms of novelty and the distinct effects they can have on the different brain regions (Fyhn et al., 2002; Vinogradova, 2001; Wan et al., 1999). Therefore the missing of a novelty influence in the NET experiment is not unexpected and stresses again that not the extent of novelty or the mere exposure to novelty experience but its relevance for the animal decides about the effectiveness. As Vinogradova (2001) suggests the detection of mismatch then even evokes a response by changes nearly equal to the differential threshold of sensitivity. The results of the NET experiment reveal that a strong novelty as the complete change of spatial surrounding is less effective as the much less obvious but relevant change just of the Target position as in the RCM. Thereby it is as well stressed that not a mere novelty effect can cause an increase of c-fos expression in

the hippocampus and NAc, but a relevance for spatial learning is required for the observed pattern.

III.4.5 Conclusion

The three experiments reveal several interesting insights in the influence of spatial relearning and spatial novelty on the neuronal activation measured as c-fos expression. A clear effect of relearning is found in the hippocampus with a differential response of the hippocampal sub-regions supporting the concept of functional distinction of the sub-regions suggested by many investigators.

For the DG the results indicate a distinct activation from the earliest phases of relearning on, corresponding to a role in receiving several different inputs as first stage in the hippocampus for filtering relevant stimuli and detecting novel pattern by computational processes as pattern separation (Lee and Kesner, 2003; Mizumori et al., 1999).

The CA1 region reveals an even stronger though little delayed activation (RRCM), suggesting a slightly slower time-course for its plasticity mechanisms (Lee et al., 2004). This fits well to its role as output structure (Lee and Kesner, 2003), which integrates information of several sources, e.g. sensory, expectational or motivational information from the EC, information about localization, spatial representation and discrepancies in current and expected situations from intra-hippocampal sources, before transmitting them to extra-hippocampal regions (Fyhn et al., 2002; Mizumori et al., 1999; Moser and Paulsen, 2001).

The CA3 region, which is more sensitive to changes in the spatial environment than the CA1 (Mizumori et al., 1999) and plays a role in routing and self-localization (Mizumori et al., 1999; Redish and Touretzky, 1998) and rapid formation of spatial representation including the comparison of the expected and current state (Lee et al., 2004; Mizumori et al., 1999; Vinogradova, 2001), shows no activation, reflecting that the relearning event in the CM presents no changes in the spatial environment.

Reinforcement of relearning on a second day elicits less activation suggesting that the repeated experience on a first relearning day induces a fast learning process, which is sufficient to elicit main plasticity changes already on the first day, resulting with smaller changes at the second day (Frank et al., 2004).

A weak effect of the relearning event is found in the c-fos expression of the medial NAc (shell region). This finding fits to the involvement of the NAc in spatial behaviors,

as supported by several lesion studies. As well it supports that its main role in self-localization and reward-guiding behavior, which are addressed to a minor degree by the changes of the relearning event, and less the storage of spatial information. This stresses in addition the dichotomy between the processing and storing of spatial information and the executing spatial behavior.

A pure novelty effect causing the activation pattern in the CM experiments can be excluded by the findings of the NET. This demonstrates that neither in the hippocampus nor in the NAc the mere experience of spatial novelty is sufficient to elicit an increased activation, even if it is more obvious and profound than the changes in the relearning event of the CM.

IV GENERAL COMMENT AND OUTLOOK

The current PhD project investigated neuronal activation induced by spatial learning measuring c-fos expression patterns. In a first study a spatial learning paradigm was established in order to address specifically the hippocampus, which is crucial for many cognitive functions and memory consolidation. A sophisticated behavioral analysis enabled to design a protocol optimized for the investigation of c-fos expression. It was possible to eliminate interfering side stimuli by familiarizing the animals to the maze situation in a first learning. The mice finished this first learning on an equal stable performance level to achieve highly comparable precondition, before they performed a relearning event. The behavioral analysis of all experiments revealed that the mice seem to develop a strong reward expectation that drives them to visit the previously trained Target position, although they indicated to be already able to locate the new Target position.

In the second study, this design was used for the investigation of learning induced c-fos expression. In a relearning event, which was sufficient to achieve a behavioral reorientation, gene expression was induced in a reliable and timely confined manner. In addition, as relearning event was presented a dislocation of the Target, thus the influence of novelty was minimized. The results of the RCM experiment revealed an increased c-fos expression of the DG granule cells in a very early phase of learning, probably indicating the detection of Target dislocation. The results of the RRCM reflected the c-fos expression that integrated detection and first learning success as increase in DG and CA1 principal cells in the relearner group. A similar but less strong activation was found in response to a reinforced relearning with a second

training on the next day. The results support a functional segmentation of the hippocampus with distinct functions of the sub-regions. A reproducible weak effect of relearning was found in the shell region of the nucleus accumbens. The results suggest an involvement of this region in the spatial relearning event to a different extent than the hippocampus, probably as part of an independent memory system with a distinct contribution for self-localization and reward guiding.

A novelty task was designed to investigate the c-fos expression induced by a spatial novelty without learning incentive in a situation comparable to the CM task. Both hippocampus and NAc showed no differential activation, thus indicating that a mere novelty is not sufficient to increases c-fos expression. Therefore the activation pattern found in the CM experiments can be assigned specifically to a spatial relearning process.

In general, the CM was shown to be capable to dissect distinct contributions in a learning process on a physiological and a behavioral level. It allows detecting subtle differences in learning performance of animals, which can be used to homogenize an experimental group or to indicate impaired or changed cognitive functions of different experimental groups. These features assign the CM task as valuable tool for diverse future applications.

The mechanisms of transferring external stimuli into memory could be addressed by following up the cellular response induced by the distinct and timely confined relearning event in the CM.

Other IEG that are supposed to be stronger related to learning than c-fos may provide more mechanistic insights in transition events. A certain subset of IEGs could serve as coincidence detector (Kaczmarek and Robertson, 2002) to prepare the cells for synaptic plasticity, e.g. by marking excited synapses with a synaptic tag as suggested for homer or by directly excitation-governed structural contributions as suggested for arc/arg 3.1 (Lanahan and Worley, 1998). Also the late response genes that are regulated by the learning induced IEG response are of interest regarding the processes in memory consolidation. It is possible to investigate the differential regulation of gene expression in a general approach performing gene microarray analysis to find candidate genes involved in learning (Leil et al., 2002). In contrast, genes that are involved in synaptic plasticity as cell adhesion molecules (Becker et al., 1996; Benson et al., 2000; Luthi et al., 1994) or pre- and postsynaptic proteins

(Cammarota et al., 1998; Helme-Guizon et al., 1998) could be linked to the IEG response by their regional expression pattern.

In addition the CM offers a more detailed investigation of a learning process by dissecting the distinct contributions of different stimuli correlating behavioral aspects and gene-expression similar as in the current project. Changes in aversive conditions, additional reward or effort to reach the Target could show the influence of stress and emotionality, exploration or reward value (Holscher et al., 2003; Pratt and Mizumori, 2001; Sapolsky, 2003). Changes in the protocol or external and internal stimuli could show the guiding mechanisms in spatial learning with the contributions of other cognitive functions such as procedural learning and path integration as well as pattern completion (Knierim, 2002; Leutgeb et al., 2000; Packard and McGaugh, 1996).

Since the CM allows a sophisticated behavioral analysis, it can be an appropriate tool to find subtle differences between animals that are manipulated either transgenically or pharmacologically. The low-pressure training conditions do not force compensation effects, since the Target can as well be reached without a spatial knowledge. The behavioral analysis enables to differentiate non-spatial strategies and would as well allow dissecting differences that are not due to cognitive functions. A more detailed investigation of the spatial representation could dissect factors as room shape or symmetry, internal and external cues of different types and the configuration relations between cues.

V SUMMARY

Studies that map neuronal activation enable to investigate the interplay of different memory systems and the functional segmentation of brain areas regarding their role in cognitive processes. Several methods can be used to investigate neuronal activation.

The current PhD project investigated neuronal activation that was induced by spatial learning via measuring c-fos expression. This immediate-early gene and its gene product is part of a cascade of events, following external stimulation, that lead to protein-biosynthesis, which is required for the stabilization of long-term memory. Expression of c-fos was shown to be inducible by several forms of learning. With its transient and ubiquitous expression pattern, c-fos enables a high temporal and regional resolution frame to investigate neuronal activation on cellular level for sub-regional distinction in multiple brain areas non-invasively. The feature that c-fos is induced by various stimulations offers to dissect the contributions of different stimuli, however, it also requires a careful handling of interfering side stimuli.

Therefore, in a first study a Circular Maze task was established to elicit spatial learning-induced neuronal activation with appropriate conditions for c-fos expression. A detailed behavioral analysis ensured spatial learning to address specifically the hippocampus as region with a key role in memory consolidation. In addition, a protocol was designed to achieve equal preconditions for all animals before they performed a relearning process. The relearning was performed in a familiar situation that minimized side-stimuli and served for a highly comparable situation for the control groups.

In a second study this design was used to induce c-fos expression. A differential activation due to relearning was found in the sub-regions of the hippocampus and nucleus accumbens. The distinct expression pattern of the sub-regions supported a functional segmentation of the hippocampus during the processing of spatial information. The CA3 region was not activated, corresponding to the familiar spatial surroundings, while the DG and CA1, which are assigned in pattern separation and novelty detection, showed an increased c-fos expression. The results concerning the nucleus accumbens shell suggested an involvement of this region in spatial relearning to a different extent. This supports that the nucleus accumbens belongs to another memory system, which works in concert with the hippocampus dependent memory system and reveals a distinct contribution to spatial learning. For both regions a mere novelty effect was excluded by an investigation of c-fos expression in a novelty-task that did not show a c-fos expression increase due to mere novelty.

VI ZUSAMMENFASSUNG

Kognitive Prozesse erfordern das Zusammenspiel verschiedener Gedächtnis-Systeme und damit unterschiedlicher Gehirn-Areale. Deren Interaktion und funktionelle Segmentierung läßt sich anhand regional unterschiedlicher neuronaler Aktivierung untersuchen.

Im Rahmen der vorliegenden Dissertation sollten neuronale Aktivierungen, die durch räumliches Lernen induziert wurden, über die Messung der c-fos Genexpression untersucht werden. Das 'immediate-early gene' c-fos und dessen Genprodukte sind Teil einer Signalkaskade, die aufgrund externer Stimulation zur Proteinbiosynthese und damit zur Stabilisierung von Langzeit-Gedächtnis führt. Die Expression von c-fos ist ubiquitär durch verschiedene Formen von Lernen transient induzierbar. Dadurch ermöglicht c-fos eine nicht-invasive Analyse neuronaler Aktivierungen in verschiedenen Gehirn-Arealen mit einer hohen zeitlichen und räumlichen Auflösung auf zellulärem Niveau. Da c-fos durch diverse Stimuli induziert werden kann, ermöglicht es die Untersuchung der Auswirkung dieser Stimuli auf das Lernen, erfordert jedoch gleichzeitig einen kontrollierten Umgang mit interferierenden Neben-Stimuli.

Zunächst sollte daher ein 'Circular Maze Task' etabliert werden, der eine neuronale Aktivierung durch räumliches Lernen unter Bedingungen induziert, die für eine Untersuchung der c-fos Expression optimiert sind. Durch eine detaillierte Verhaltensanalyse wurde deutlich, daß die Mäuse räumliches Lernverhalten zeigten. In diese Art des Lernens ist speziell der Hippocampus involviert, der eine bedeutende Rolle bei der Gedächtnis-Konsolidierung spielt. Zusätzlich schaffte das Design des Versuchsablaufs identische Voraussetzungen für alle Tiere, bevor sie einem neuen Lernprozess in Form eines Umlernens unterzogen wurden. Das Umlernen erfolgte in einer bekannten Situation, so daß Neben-Stimuli minimiert wurden und gleichzeitig eine hohe Vergleichbarkeit mit der Situation der Kontroll-Gruppen gewährleistet war.

In einer zweiten Studie wurde dieses Versuchsdesign verwendet, um die Expressionen von c-fos zu untersuchen. Die Aktivierung der c-fos Expression in den verschiedenen Regionen innerhalb des Hippocampus und Nucleus Accumbens, ausgelöst durch das Umlernen, verdeutlicht die funktionelle Untergliederung der Gehirn-Areale beim Prozessieren räumlicher Information. Die für räumliche Repräsentationen zuständige CA3 Region zeigte, aufgrund der bekannten räumlichen Umgebung, keine Aktivierung, während im *gyrus dentatus* und der CA1 Region, die

der Muster-Unterscheidung und Novelty-Erkennung zugeordnet werden, ein Anstieg der c-fos Expression zu verzeichnen war. Des weitern sprechen die Ergebnisse dafür, daß der Nucleus Accumbens zwar in räumliches Lernen involviert ist, allerdings in einer anderen Weise als der Hippocampus. Sie unterstützen die Annahme, daß der Nucleus Accumbens einem anderen Gedächtnis-System zugeordnet ist, das im Zusammenspiel mit dem hippocampalen Gedächtnis-System einen eigenen Beitrag zum räumlichen Lernen leistet.

Für beide Gehirn-Areale konnte ein reiner Novelty-Effekt ausgeschlossen werden, da die c-fos Expression in Mäusen nach einem 'Novelty-Task' keine Veränderungen zeigte.

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VIII APPENDIX

Instruments

Equipment not listed in the table below were of common laboratory standard. Particular devices are referenced throughout the respective protocols.

Axiophot microscope	Zeiss	Göttingen, D
Bench-top centrifuges 5417R / 5403	Eppendorf	Hamburg, D
Bench-top centrifuge CR422	Jouan, Inc	Winchester, VA, USA
Centrifuge RC50*plus* with HB-6 SLA3000, SLA 1500, SA600-rotors	Sorvall, Kendro	Hanau, D
Cryostat CM3050	Leica	Bensheim, D
E.A.S.Y. UV-light documentation	Herolab	Wiesloh, D
EthoVision video tracking system	Noldus	Wageningen, NL
Microcentrifuge 5415D	Eppendorf	Hamburg, D
Neurolucida image software	MicroBrightfield	Magdeburg, D
Phosphimager BAS-1000	Fujifilm, Raytest	Straubenhardt, D
Power supplies Power Pac series	Bio-Rad	München, D
Spectrophotometer Ultrospec 3000	APB	Freiburg, D
UV crosslinker	APB	Freiburg, D

Reagents, disposables, etc

All chemicals were obtained from the following companies in *pro analysis* quality: Amersham Pharmacia Biotech (APB, Freiburg, D), Bio-Rad (München, D), Invitrogen (Karlsruhe, D), Carl Roth (Karlsruhe, D), Merck (Darmstadt, D), Serva (Heidelberg, D) and Sigma-Aldrich (Deisenhofen, D). Molecular cloning reagents were obtained from Ambion (Cambridge, UK), APB (Freiburg, D), BD Biosciences Clontech (Heidelberg, D), Promega (Mannheim, D), Qiagen (Hilden, D) and Statagene (Amsterdam, NL). DNA and RNA purification kits and reaction kits were purchased from Ambion (Cambridge, UK), APB (Freiburg, D) and Qiagen (Hilden, D). Nucleic acid molecular weight markers were purchased from Roche (Mannheim, D) and New England Biolabs (NEB, Frankfurt a. M., D).

Buffers and stock solutions

Buffers and stock solutions are listed below. All more method-specific solutions are specified in the accompanying sections.

DAB-stock solution	1	% (w/v)	diaminobenzidine
DEPC H_2O	0.1	% (w/v)	diethylpyrocarbonate → autoclave after stirring overnight

DNA sample buffer (5x)	20	% (w/v)	glycerol in TAE buffer
	0.025	% (w/v)	orange G
EDTA stock solution	0.5	M	EDTA
		→ pH 8.0	
Ethidiumbromide-staining solution	10	µg/ml	ethidiumbromide in 1xTAE
LB-agar (per liter)	10	g	NaCl
	10	g	tryptone or peptone
	5	g	yeast extract
	20	g	agar
		→ pH 7.0 with 5 N NaOH (optional)	
Antibiotics were added when needed (LB-amp, 100 mg/l ampicillin).			
LB-medium (per liter)	10	g	NaCl
(Luria broth)	10	g	tryptone or peptone
	5	g	yeast extract
		→ pH 7.0 with 5 N NaOH (optional)	
Antibiotics were added when needed (LB-amp, 100 mg/l ampicillin).			
MOPS running buffer	0.1	M	MOPS pH7.0
	40	mM	sodium acetate
	5	mM	EDTA
4 % Paraformaldehyde	4	% (w/v)	paraformaldehyde
dissolved at 60 °C under stirring in 1 x PBS			
PBS (10 x)	1.36	M	NaCl
(Phosphate buffered saline)	0.1	M	Na_2HPO_4
	27	mM	KCl
	18	mM	KH_2PO_4
		→ pH 7.4	
RNA sample buffer (5x)	50	% (v/v)	glycerol
	1	mM	EDTA
	0.25	% (w/v)	bromphenol blue
	0.25	% (w/v)	xylene cyanol
SSC (20x)	0.3	M	*tri*-sodium citrate
(citrate buffer)		→ pH 7.4	
TAE (50x)	2	M	Tris-Acetate, pH 8.0
	100	mM	EDTA
TE (10x)	0.1	M	Tris-HCl, pH 7.5
	10	mM	EDTA

Abbreviations

× g	g-force
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
AP-1	Activatory protein 1
ATN	Anterior thalamic nucleus
ATP	Adenosine triphosphate
BLA	Basolateral amygdala
BSA	Bovine serum albumine
CA	*Cornus ammoni*
cAMP	Cyclic adenosine monophosphate
CM	Circular maze
CREB	CRE binding protein
CTP	Cytosine triphosphate
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DTT	Distance to Target
Dtt	Dithiothreitol
E. coli	*Escherichia coli*
EC	Entorhinal cortex
ECM	Establishing Circular Maze
EDTA	Ethylendiamintetraacetic acid
ETS	Transcription factor
fMRI	Functional magentic resonance imaging
Fra-1	Fos related antigen-1
GABA	Gamma amino butyric acid
IEG	Immediate-early gene
ITF	Inducible transcription factor
ITI	Inter-trail interval
KDa	Kilo-Dalton
LB	Luria Bertani
LTM	Long-term memory
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
MOPS	(4-(N-morpholino)-propan)-sulfonic acid
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NET	Novelty Exploration Task
NFAT	Nuclear factor of activated T-cells
NGF	Neuronal growth factor
NMDA	N-methyl-D-aspartate
OD	Optic density
PET	Positron emission tomography
PBS	Phosphate buffered saline
PFC	Prefrontal cortex
RCM	Relearning Circular Maze
RNA	Ribonucleic acid
rpm	Rounds per minute
RRCM	Reinforced Relearning Circular Maze
RT	Room temperature
SDA	Step Down Avoidance
SRF	Serum response factor
TCF	Ternary complex factor
TGF	Transforming growth factor
TIMP-1	Tissue inhibitor of metalloproteinases-1
Tris	Tris(-hydroxymethyl)-aminomethane
U	Unit (emzymatic)
v/v	Volume per volume
VSCC	Voltage sensitive calcium channel
VTA	Ventral tegmental area
w/v	Weight per volume
Zif268	Transcription factor

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