Structural plasticity of synaptic connectivity in the adult hippocampal mossy fiber projection

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THESIS SUMMARY

The brain has the lifelong ability to change itself based on new experiences such as learning and memory. This so called neuronal plasticity is reflected in the brain by structural and functional modifications which underlie our ability to learn, remember and adapt our behavior. The present thesis focuses on how learning reshapes structural connectivity between identified elements of neuronal circuits and what is functional role of structural changes in synaptic connectivity in learning and memory?

To address these questions, I investigated the relation between structural changes and learning and memory in the hippocampus, a brain region that plays a crucial role in our ability to recall everyday facts and events. I studied the synaptic connectivity of hippocampal mossy fibers upon defined learning events such as spatial navigation in a maze and contextual memory formation upon fear conditioning. In addition, I addressed the behavioral function of these learning-related structural changes at this synaptic site in relation to defined aspects of learning and memory. This study revealed growth of filopodial synapses, specific structural elements of the feedforward inhibitory connectivity at hippocampal mossy fiber terminals upon learning. Furthermore, this study provides causal evidence that learning-related growth of filopodial synapses plays a critical role for the precision of memories but not for associative memory per se. In addition, I also identified spatial and temporal rules for this learning-related plasticity in the different domains along the dorso-ventral axis of the hippocampus and during different forms of learning.

In summary, the present thesis provides causal evidence for a selective contribution of presynaptic structural plasticity in defined aspects of learning-related processes. Moreover this study establishes that learning of specific subtasks in complex trial-and-error learning is orchestrated by distinct regional domains along the dorso-ventral axis of the hippocampus. Together, these findings shed a new light on the role of structural plasticity in learning and memory and add to our understanding of the mechanisms underlying learning disabilities and memory impairments.

PREFACE

Over the course of life, our brain accumulates a record of experiences, and we constantly make use of this record in our daily life in a dynamic world. The brain's capacity to compile experiences and adapt to a perpetually fluctuating environment plays a central role in adaptive behaviors that form the basis for increased chances of survival and reproduction. Like behavior can be flexible, the brain itself is also far from being static and hard-wired. As we acquire new experiences or learn new skills our brain cells exhibit functional and structural changes. Thus, the term plasticity refers to this general ability of the brain to change as a result of experience and is thought to form the basis of learning and memory.

In this thesis I focus on the following general aspects of experience dependent plasticity: How do particular experiences such as learning and memory modify brain structures and what is the relation of defined structural changes with behavior? The following thesis centers on the plasticity in the nervous system with an emphasis on the functional role of structural changes in the adult hippocampus in relation to learning and memory. The hippocampus is a key system in learning and memory and has a critical role in our capacity to recall facts and events. I will begin this thesis with a general short introduction to the field of plasticity, which is followed by a paragraph elaborating on the specific neuronal circuitry of the hippocampus. Then, I will briefly outline the behavioral paradigms used in this study, and describe corresponding elemental circuit computations that can be revealed by these behavioral paradigms. Finally, I will close the main introduction by stating the general aims and topic of this thesis and the rationale for the experimental approaches.

In the first results part of this thesis, I investigated how structural plasticity relates to different types of hippocampal-dependent learning and memory. To study structural plasticity upon learning and memory formation, I used a combination of behavioral approaches and ex vivo structural analysis. In addition, I addressed the functional role of structural plasticity in the dorsal hippocampus in specific aspects of learning and memory. This work establishes the first evidence for a causal relationship between learning-related structural plasticity and the functional role of this kind of plasticity for behavior. This study demonstrates a critical role of structural plasticity in the precision

of learning and memory as revealed by a selective growth of defined synapses which provide feedforward inhibition in the adult brain. In addition, I uncovered a relationship between feedforward inhibition growth and plasticity at hippocampal mossy fibers with the precision of hippocampus-dependent memories.

In the second part of the thesis, I examined how structural plasticity in the ventral hippocampus relates to fear conditioning and incremental spatial learning in the Morris water maze. While many studies have implied the dorsal hippocampus in spatial learning and memory, the role of the ventral hippocampus is still not well understood. For example, it is not known to what extent dorsal and ventral hippocampus function as independent domains in hippocampal dependent learning and whether this might be reflected on a structural level in the different hippocampal regions. Water maze learning elicits structural plasticity at mossy fiber terminals which exhibits distinct temporal characteristics along the dorso-ventral axis of the hippocampus. In the dorsal hippocampus structural plasticity is expressed at late stages of navigation learning, when the spatial memory is most strong. In contrast, plasticity in ventral hippocampus reaches plateau already at early stages of goal-oriented learning. Interestingly, we found structural plasticity in the dorsal hippocampus to occur independently of the ventral hippocampus by performing lesion experiments of the ventral hippocampus. Altogether, this second study suggests a functional role of the ventral hippocampus during goal-oriented learning. Moreover, these results provide evidence for a sequential recruitment of ventral, intermediate and dorsal hippocampus during acquisition of defined behavioral stages during complex trial-and-error learning in a maze navigation task. In summary, the behavioral expression of specific search strategies for spatial navigation is associated with region-specific, local growth of feedforward inhibitory connectivity at hippocampal mossy fiber terminals along the dorso-ventral axis of the hippocampus.

In the third part of this thesis I will briefly summarize a series of unpublished results on the effect of acute innate fear on the structural connectivity along the hippocampal mossy fiber projection. Innate fear can be triggered by exposure to predator odors which induce an acute fear response but do not cause contextual memory formation. Using such a paradigm, I found that acute fear initiates rapid morphological and numerical alterations of postsynaptic structures, namely the thorny excrescences, of

CA3 pyramidal cells as well as presynaptic structural changes along the hippocampal mossy fiber projection. These transient alterations in synaptic connectivity relate to performance at the behavioral level as revealed by a deficit in pattern separation upon the experience of acute fear.

1. INTRODUCTION

The brain is plastic and has the lifelong ability to change itself based on new experiences. This capacity to act and react in a constantly changing world is a general feature found across many brain structures and can be expressed by various physical and chemical events in the brain. A prominent example of this so called neuronal plasticity occurs during the learning of new skills or the acquisition and memorization of new knowledge, which in turn is reflected by functional and structural changes in the neural pathways underlying these mnemonic processes. However, plasticity does not only occur in the processes of learning and adaption, but also early in development during the formation of neuronal circuits. Moreover, neuronal pathways are also reorganized in response to injuries and in diseases of the nervous system which cause the loss of proper neuronal functioning.

1.1 Experience dependent plasticity

We constantly receive information about our external world through our senses. The brain filters this sensory information depending on our internal states which in turn leads to the generation of our perceptions and subjective experiences. In principle every individual bit of perceptual information has the potential to affect the functioning of our brain and thereby may ultimately also influence our future behavior. Experience-dependent plasticity occurs throughout the whole brain and underlies our capacity to adapt our behavior in order to assure optimal chances for survival. Plasticity can be observed at various levels in the brain, ranging from the level of individual cells to neuronal networks up to the systems level. Traces of neuronal plasticity can be expressed by physiological, molecular and structural changes in the brain.

Initial studies on plasticity were based on brain lesions and sensory deprivation experiments to study the resulting adaptations of the brain to drastic modifications of sensory experience. For example, early work on neuronal plasticity Michael Merzenich showed that peripheral nerve damage or selective lesions induce the reorganization of cortical maps of sensory representations such as the topographic maps in primary sensory cortex (Buonomano and Merzenich 1998). For example, the cortical areas

corresponding to the denervated areas of skin on the hand of a monkey were re-occupied by nerves from neighboring skin areas within a few months (Kaas, Merzenich et al. 1983; Merzenich, Kaas et al. 1983; Merzenich, Nelson et al. 1984). The full potential of brain plasticity has been demonstrated in a variety of pathological perturbations such as the amputation of digits in monkeys. These studies reported similar changes in the functional receptive field properties as well as structural rearrangements of neurites in the affected areas of somatosensory cortex (Kalaska and Pomeranz 1979; Kelahan and Doetsch 1984; Calford 1991; Calford and Tweedale 1991). A recent study in mice also showed restructuring of neuronal circuits during functional reorganization upon sensory deprivation in visual cortex (Keck, Mrsic-Flogel et al. 2008). In somatosensory cortex of mice defined patterns of structural plasticity of excitatory and inhibitory axons have been shown within deprived and non-deprived whiskers barrels upon whisker removal (Marik, Yamahachi et al. 2010). Therefore structural plasticity mechanisms are thought to underlie topographic remapping upon sensory deprivation (Gilbert and Wiesel 1992). Altogether, these long-term structural and functional modifications likely reflect compensatory changes in the neuronal networks of the brain in order to recover functionality upon peripheral injury (Dancause, Barbay et al. 2005; Brown, Li et al. 2007).

Plasticity does not only occur upon large-scale alterations of sensory input, but also underlies many physiological functions including learning of new skills and memory formation. Several examples for learning related plasticity have been provided by studies investigating perceptual learning (Karni and Bertini 1997; Seitz and Dinse 2007). Perceptual learning involves plasticity in sensory systems and improves the ability to respond to features in the sensory environment. In this kind of learning process the perceptual abilities can be sharpened as the ability to distinguish similar sensory stimuli is trained by repeated exposure, which ultimately enhances the discrimination of the sensory stimuli. The paradigm of perceptual learning was applied to study the cellular and molecular features of experience-dependent plasticity in several identified sensory brain regions, such as visual cortex for orientation discrimination and auditory cortex in relation with pitch discrimination (Gilbert 1998; Dan and Poo 2006; Han, Kover et al. 2007). In humans perceptual learning was shown to selectively increase brain activity in specific sensory brain areas by using functional magnetic resonance imaging (Furmanski, Schluppeck et al. 2004; Li,

Luxenberg et al. 2006; Zhou, Huang et al. 2006; Li, Howard et al. 2008). Moreover, some of the corresponding changes in neuronal properties underlying these broad network effects have been identified. Perceptual learning can be reflected in the potentiation of specific individual neurons involved in the processing of the trained sensory stimuli (Schoups, Vogels et al. 2001; Frenkel, Sawtell et al. 2006) or can be expressed as a general increase in the number of neurons forming the sensory representation of the trained stimulus (Recanzone, Schreiner et al. 1993).

Plasticity is implemented in the brain through a series of distinct mechanisms all resulting in coordinated changes in the neuronal activity of individual neurons or neuronal ensembles. On the cellular level, plasticity can be expressed by selective changes in the synaptic strength between neurons as well as by adaptations of the excitability of individual neurons (Kim and Linden 2007; Sjostrom, Rancz et al. 2008). On the structural level, plasticity can be expressed by morphological rearrangements of existing synaptic connectivity, and/or as net alterations in circuit connectivity (Holtmaat and Svoboda 2009). Experience dependent plasticity can be accompanied by all of these different modifications, and it is likely that these different processes are orchestrated in a coordinated manner. In the following sections of this introduction I will discuss the mechanisms underlying experience-dependent plasticity in more detail.

1.1.1 Potentiation and depression of synaptic strength

Excitatory and inhibitory synapses exhibit several forms of activity-dependent synaptic plasticity. This functional plasticity results from synaptic modifications and is results in a change in the response amplitude of the synaptic potential to a constant stimulus. In particular long lasting kinds of synaptic plasticity are thought to underlie the encoding and storage of information and experiences in neuronal networks. Long-term synaptic plasticity is induced upon repeated trains of synaptic activity or upon selective pairing of pre-and postsynaptic firing. Synaptic efficacy can be modified in a bidirectional manner and altogether these forms of synaptic plasticity are referred to as long-term potentiation (LTP) and long-term depression (LTD) depending on the sign of plasticity with respect to baseline activity. Originally the phenomenon of LTP was described by Bliss and his colleagues (Bliss and Gardnerm.Ar 1971; Bliss and Lomo 1973). LTP

has long been hypothesized to play a role in learning and memory (Bliss and Collingridge 1993; Malenka and Nicoll 1999; Bennett 2000) but only recently has first evidence been provided that LTP indeed is necessary for storing spatial information *in vivo* (Pastalkova, Serrano et al. 2006). LTD represents the weakening of synaptic strength and can be induced by prolonged low frequency stimulation (Mulkey and Malenka 1992; Dudek and Bear 1993; Goda and Stevens 1996).

In order to elicit synaptic plasticity, neuronal activity of the pre- and postsynaptic cells has to be closely correlated in time. In addition, the actual temporal order of the pre- and postsynaptic spiking affects the sign of synaptic plasticity. If presynaptic spiking is preceding postsynaptic spiking LTP is induced, whereas spiking in the reverse order result in the induction of LTD. This particular form of activity-dependent LTP/LTD is referred to as spike timing-dependent plasticity (STDP) (Levy and Steward 1983; Markram, Lubke et al. 1997). Evidence that such Hebbian plasticity plays a crucial role in experience-dependent plasticity *in vivo* comes from studies in sensory cortices showing that correlated neuronal firing can induce receptive field and map plasticity (Clark, Allard et al. 1988; Schuett, Bonhoeffer et al. 2001; Fu, Djupsund et al. 2002; Allen, Celikel et al. 2003; Dan and Poo 2006).

1.1.2 Structural plasticity

Besides modifications of synaptic efficacy, structural alterations of synaptic connectivity represent an alternate and complementary mechanism to encode information in the brain. The properties of neuronal networks can change as a result of a specific gain and loss of synapses or the rearrangement of pre-existing synaptic connections. The dynamics of synaptic structures can be affected by neurotransmitters (De Paola, Arber et al. 2003; Brunig, Kaech et al. 2004) as well as by alterations in calcium concentrations (Korkotian and Segal 1999; Bonhoeffer and Yuste 2002; Tashiro and Yuste 2003; Brunig, Kaech et al. 2004; Segal 2005). For example, the synaptic release of the neurotransmitter glutamate has been shown to trigger spine growth via NMDA (N-Methyl-D-aspartic acid) receptor activation (Engert and Bonhoeffer 1999). Moreover a correlation between the turnover of dendritic spines as well as the size of individual dendritic spines in relation to synaptic strength has been demonstrated (Sala, Piech et al. 2001; Yuste and Bonhoeffer 2001; Yuste

and Bonhoeffer 2004). *In vitro*, induction of LTP by electrical stimulation, results in dendritic spine formation or elimination, respectively (Toni, Buchs et al. 1999; Nagerl, Eberhorn et al. 2004).

A large body of work suggests that activity-dependent structural changes in synaptic connectivity play an important role in experience-dependent plasticity (Lamprecht and LeDoux 2004; Holtmaat and Svoboda 2009). Several *ex vivo* studies demonstrated alterations in postsynaptic characteristics such as dendritic spine density and spine morphology upon sensory deprivation, sensory stimulation, stress, enriched environment as well as upon learning (Globus and Scheibel 1967; Parnavel.Jg, Globus et al. 1973; Moser, Trommald et al. 1994; Kozorovitskiy, Gross et al. 2005; Stewart, Medvedev et al. 2005). Recent studies using *in vivo* imaging of dendrites in mice have provided further evidence for structural plasticity of dendritic spines in the adult brain (Holtmaat, Trachtenberg et al. 2005; De Paola, Holtmaat et al. 2006; Majewska, Newton et al. 2006). In addition, presynaptic axonal structures such as presynaptic boutons have been shown to exhibit morphological rearrangements *in vivo* (De Paola, Holtmaat et al. 2006; Majewska, Newton et al. 2006; Marik, Yamahachi et al. 2010). Furthermore, it has been demonstrated that experience indeed affects spine growth and loss *in vivo* (Holtmaat, Wilbrecht et al. 2006).

Studies on the relation of structural plasticity with learning provided evidence of structural plasticity upon learning in specific areas of the brain that are specifically involved in this learning (Xu, Yu et al. 2009; Roberts, Tschida et al. 2010; Wang, Conner et al. 2011). For example, motor skill learning induced rapid spine formation and spine elimination in specific areas of motor cortex (Xu, Yu et al. 2009). Moreover, it was shown that the extent of spine remodeling correlated with performance improvement after learning. This led to the hypothesis that structural plasticity might have a critical function in learning and memory by leaving long lasting structural traces in the brain. In addition, a recent study provides evidence that learning with opposite behavioral outcome, such as fear conditioning and extinction, exhibit opposite effect on the remodeling of dendritic spines along the same dendrite (Lai, Franke et al. 2012). This result further supports the hypothesis of a specific relation between structural changes in dendritic spines with defined processes of the learning and memory.

In summary, several lines of evidence indicate that the brain is plastic, and that experience reshapes circuits in the brain. Structural plasticity has been observed *in vivo* in pathological conditions, under baseline conditions, and during learning of new skills. However, the functional role of this structural plasticity in relation to learning and memory has not yet been identified. Structural plasticity might reflect physical memory traces or so called memory engrams in the brain (Hubener and Bonhoeffer 2010). Alternatively, structural plasticity might alter network functions, thereby affecting information flow within and across brain systems (Kullmann, Moreau et al. 2012).

1.2 HIPPOCAMPUS

The hippocampus has a critical role in learning and memory, and is thus well suited to investigate how synaptic circuit elements relate to mnemonic brain function (Squire 1992). Hippocampal damage in humans leads to anterograde amnesia, which is the loss of the capacity to form new declarative memories, i.e. memories of everyday facts and events (Milner, Squire et al. 1998). Bilateral hippocampal damage further leads to a temporally graded retrograde amnesia in form of a selective impairment to retrieve recent memories, while remote memories are not affected (Zolamorgan and Squire 1990; Zolamorgan, Squire et al. 1995; Teng and Squire 1999). Perceptual and cognitive abilities are usually not affected upon hippocampal damage in humans (Scoville 1954). Working memory is maintained upon hippocampal damage. Together, these findings lead to the hypothesis that the hippocampal formation plays only a time-limited role in memory storage, and is not necessary for the ultimate storage or retrieval of remote memories (Zolamorgan and Squire 1990).

Similar to the described effects of hippocampal damage in humans, hippocampal lesions in monkeys and rodents have been shown to affect specific and similar aspects of learning and memory (Alvarezroyo, Zolamorgan et al. 1992). In particular, the temporal involvement of the hippocampus in learning and memory has been further investigated in rodents. Neural activity in the rodent brain has been demonstrated to shift in a time-dependent manner from the hippocampus to cortical regions, thereby suggesting that the memory was indeed being transferred from hippocampus to cortex over a prolonged time (Bontempi, Laurent-Demir et al. 1999). These findings have further supported the hypothesis that the hippocampus plays a key role in the process of memory consolidation in which short-term memories are converted into long-lasting memories (Polster, Nadel et al. 1991).

The hippocampal formation plays also a critical role in spatial information processing and is thought to function in the formation of a cognitive map of the spatial environment and locations within space (Okeefe 1979; Moser, Kropff et al. 2008). The hippocampal function in spatial encoding is based on the identification of ‘place cells’ in the hippocampus, which are neurons with a location-specific firing activity (Okeefe 1979). The discovery of place cells led to the cognitive map theory which suggests a

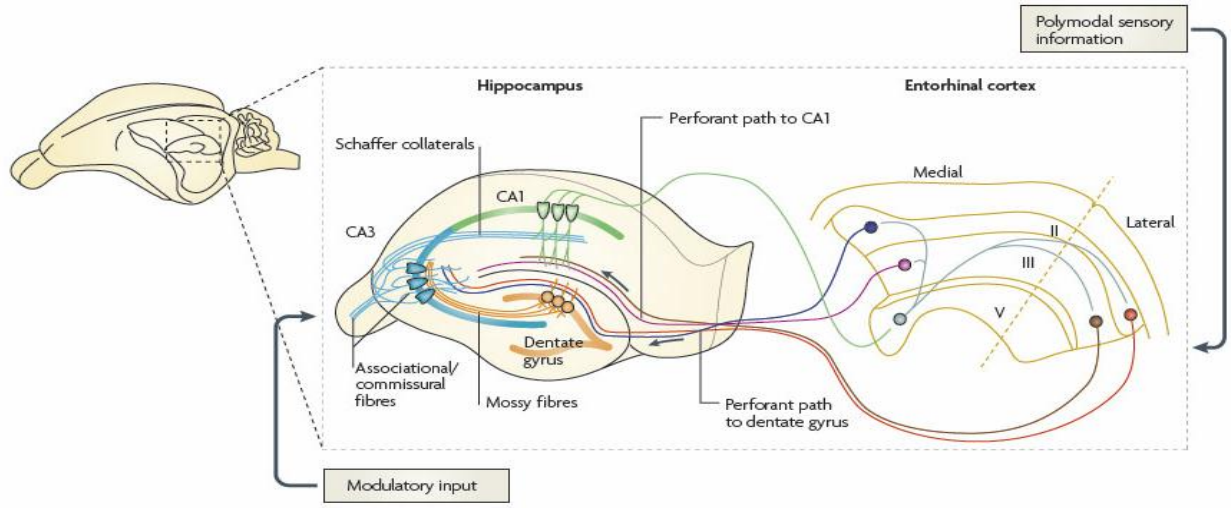
role of hippocampus in the formation of a mental representation of the external world and its spatial organization (Okeefe and Conway 1978). The cognitive map theory argues that the hippocampus provides a spatial map which serves as a spatial reference system to organize and remember items and events in a coherent manner. In contrast, the relation theory of hippocampal function argues that the hippocampus processes all kinds of associations and sequences of events. Moreover this theory argues that the main function of the hippocampus is to link these episodes into general relational frameworks (Eichenbaum 2004), which may underlie episodic memories. According to this theory, spatial navigation represents only a particular example of relational processing in the hippocampus (Eichenbaum, Dudchenko et al. 1999).

Experimental evidence for both concepts has been provided. For example, *in vivo* recordings lead to the identification of hippocampal cells that solely encode spatial or non-spatial information (Okeefe and Dostrovs.J 1971; Hampson, Simeral et al. 1999; Lee, Griffin et al. 2006; Royer, Sirota et al. 2010). In addition, a population of hippocampal neurons has been reported to encode both non-spatial as well as spatial information (Hampson, Simeral et al. 1999; Wood, Dudchenko et al. 2000; Lee, Griffin et al. 2006).

In summary, the experimental data supports both theories of hippocampal function (Okeefe and Conway 1978; Wood, Dudchenko et al. 1999; Knierim 2003; Moita, Rosis et al. 2003). According to the definition of Endel Tulving, episodic memory is engaged during recall of past events in relation to space and time (Tulving 1985; Tulving 1987). In line with this concept both described theories on the hippocampal function may coexist in the hippocampus. Place cells may contribute to episodic memory such as a component of contextual representations (Smith and Mizumori 2006; Smith and Mizumori 2006) and relational learning may provide the basis for events and episodes (Burgess, Maguire et al. 2002; Davachi and Wagner 2002; Davachi 2006).

1.2.1 Basic circuitry of the hippocampus

The main input of the hippocampus arises from axons of the perforant path, which originate from the entorhinal cortex (EC). The perforant path conveys multimodal sensory information to the hippocampus, via two distinct projections from entorhinal cortex (Schema 1). Layer II afferents of the lateral and medial entorhinal cortex send excitatory input to the dentate gyrus layer from where the trisynaptic loop is activated. In addition, EC layer exhibits two additional direct projections to the hippocampus. First of all, layer II afferents from lateral entorhinal cortex projects directly to the CA3 region of the trisynaptic loop. Secondly, Layer III afferents from medial and lateral entorhinal cortex project to area CA1 and the subiculum. Finally, the hippocampus also contains a large array of inhibitory interneurons which play a central role in local information processing and rhythm generation in the hippocampus (Lavenex, Lavenex et al. 2007; Mann and Paulsen 2007).



Schema 1 – Basic Wiring diagram of the hippocampus and the Entorhinal cortex from (Neves, Cooke et al. 2008)

The cellular organization of hippocampus is subdivided into three main layered subfields (DG, CA3 and CA1), that exhibit laminar organization. The three main subfields build a relay station, referred to as trisynaptic loop. Information flows from EC to DG to CA3 to CA1, with the loop being closed via subiculum and EC (Schema 1). Granule cell neurons of the DG initiate the trisynaptic loop by projecting their axons along the mossy fiber pathway onto the proximal apical dendrites of pyramidal

neurons in the CA3 region. In turn, CA3 pyramidal cells project their axons along the Schaffer collateral pathway to CA1 pyramidal cells on the ipsilateral side of the hippocampus. In addition, CA3 pyramidal neurons make recurrent connections with other CA3 cells. The output of the trisynaptic loop arises from afferents of CA1 pyramidal cells which project to subiculum and eventually back to EC layer V neurons from where the information is send to polymodal association cortices (Suzuki and Amaral 1994; Lavenex, Lavenex et al. 2007).

1.2.2 The mossy fiber projection

The granule cells of dentate gyrus give rise to unmyelinated axons which Ramón y Cajal termed mossy fibers in analogy to similarly structured fibers in the cerebellum. Mossy fibers exhibit a characteristic morphology due to the formation of large presynaptic boutons called large mossy fiber terminals (LMT). Mossy fibers project through the hilar region of the DG and along the CA3 area. LMTs of mossy fibers are large presynaptic boutons which target glutamatergic pyramidal neurons in CA3 (Acsady, Kamondi et al. 1998; Henze, Urban et al. 2000). In addition, mossy fiber axons form small en-passant varicosities targeting GABAergic interneurons along the entire CA3 region. In addition, LMTs exhibit filopodial extensions that also contact inhibitory interneurons in CA3. Along the CA3 region a single mossy fiber establishes about 10 to 15 evenly spaced LMTs. At the distal end of CA3, mossy fibers lose the lamellar organization and project longitudinally toward the ventral pole of the hippocampus (Amaral and Dent 1981; Lavenex, Lavenex et al. 2007).

LMTs form synaptic contacts with CA3 pyramidal neurons by specialized postsynaptic structures termed thorny excrescences. Thorny excrescences are large spines with a complex morphology consisting of a single neck connecting to about 1 to 15 spines heads (Hamlyn 1962; Amaral 1978; Stirling and Bliss 1978; Amaral and Dent 1981; Fitch, Juraska et al. 1989; Chicurel and Harris 1992). Typically, a thorny excrescence is contacted by a single LMT, however individual LMTs can contact several thorny excrescences (Chicurel and Harris 1992; Acsady, Kamondi et al. 1998).

The mossy fiber projection represents a particularly advantageous system to study the relation of plasticity with learning and memory. From the structural point of view, the

mossy fiber provides the possibility to study excitatory as well as inhibitory circuit components by structural elements of LMTs. This is due the distinct structural features of LMTs which provide monosynaptic feed forward excitation onto CA3 via the terminal core as well as di-synaptically mediated feedforward inhibition (FFI) via en passant varicosities and filopodial extensions. On the behavioral level the dentate gyrus and the CA3 region are contributing to learning and memory by performing pattern separation and pattern completion (Amaral, Ishizuka et al. 1990; Scharfman, Witter et al. 2000; Leutgeb, Leutgeb et al. 2007; McHugh, Jones et al. 2007).

Pattern separation is the formation of discrete representations of similar inputs. The process of pattern separation by granule cells in the DG is achieved by computations in the hilar region of the DG and is thought to distinguish similar but distinct events and memory representations (Oreilly and Mcclelland 1994; Leutgeb, Leutgeb et al. 2007). In addition, efficient pattern separation is thought to depend on the strong and sparse feed-forward excitatory input arising from the mossy fibers to CA3 pyramidal cells and feed-forward GABAergic interneurons (Treves and Rolls 1992; Henze, Wittner et al. 2002; Leutgeb, Leutgeb et al. 2007). Furthermore, the CA3 circuitry has been suggested to set up a spatial map of the environment in which pattern completion and pattern separation can be directly dependent on each other by using shared or entirely different sections of the spatial map (Leutgeb and Leutgeb 2007).

1.2.3 Mossy fiber LTP

Pyramidal cell in the CA3 region of the hippocampus receive three distinct sets of excitatory input, mossy fiber (MF) synapses of granule cell in the DG, associational/commissural afferents from other CA3 cells (Amaral and Witter 1989) and direct input from the perforant path. As described earlier LMTs exhibit untypical structural features of presynaptic boutons such as a large terminal size and multiple release sites per terminals. This lead to the hypothesis, that these terminals exhibit special functional properties exceptional to other glutamatergic synapses in the hippocampus. The earliest indication that LMTs have distinct physiological properties originated from the finding that, NMDA receptor binding was much lower in stratum lucidum than in other areas of the hippocampus (Monaghan and Cotman 1985). This

evidence was further strengthened by the finding that the induction of LTP at LMTs was independent of NMDA receptor activation (Harris and Cotman 1986). Since MF-LTP was induced independently of activity on the postsynaptic site, it was suggested that this LTP was solely triggered by the presynaptic terminal itself (Zalutsky and Nicoll 1990). This was also in line with the notion, that the induction of MF-LTP has exhibits a strongly reduced sensitivity to buffering of postsynaptic calcium (Williams and Johnston 1989). Furthermore, MF-LTP expression was shown to interact with paired-pulse facilitation, which is another presynaptic process (Zalutsky and Nicoll 1990). NMDA-receptor-independent LTP at mossy fiber synapses can be induced by long trains of high frequency stimulation (Urban and Barrionuevo 1996) and causes the rise of cAMP in LMTs, which in turn leads to the persistent enhancement of transmitter release probability at LMTs (Nicoll and Malenka 1995). Finally, the presynaptic vesicle protein Rab3a and the active zone protein Rim1α have been shown to be required molecular components of the presynaptic mechanism underlying the maintenance and expression of MF-LTP in the hippocampus (Castillo, Janz et al. 1997; Lonart, Janz et al. 1998; Castillo, Schoch et al. 2002)

1.2.4 Pattern separation and Pattern completion

The hippocampal formation plays a critical role for the generation of memories of facts and episodes of events (Scoville and Milner 1957; Burgess, Maguire et al. 2002; Squire, Stark et al. 2004). Pattern separation is the rapid formation of distinct representations of similar inputs which enables the distinction of similar stimuli and episodes. In the hippocampus, the dentate gyrus has long been thought to function as a pattern separator by generating non-overlapping representations of the spatial and temporal relationships of similar events, and indeed several lines of experimental data have confirmed this hypothesis (Treves and Rolls 1992; Kesner, Lee et al. 2004; Leutgeb, Leutgeb et al. 2007; McHugh, Jones et al. 2007; Bakker, Kirwan et al. 2008). Specific events and episodes do typically not reoccur in the exact same way in real life. Therefore the hippocampus should also be able to recall memory based on partial cues, which is a process called pattern completion. Pattern completion is performed by the recurrent network in the CA3 region in the hippocampus (Nakazawa, Quirk et

al. 2002; Vazdarjanova and Guzowski 2004; Gold and Kesner 2005; Leutgeb, Leutgeb et al. 2007).

On the neural circuit level, the DG provides sparse but strong feedforward excitation (FFE) onto CA3 pyramidal cells via the mossy fiber pathway. In addition, mossy fibers activate feedforward inhibitory interneurons, which generate feedforward inhibition (FFI) onto CA3 pyramidal neurons (Lawrence and McBain 2003; Lawrence, Grinspan et al. 2004). A mossy fiber innervates more inhibitory interneurons than CA3 pyramidal cells (Acsady, Kamondi et al. 1998) and although the filopodial extensions of mossy fibers onto inhibitory interneurons only establish one or two active zones (Acsady, Kamondi et al. 1998), the activation of interneurons via the mossy fibers is highly effective (Henze, Wittner et al. 2002). In particular the interplay of FFE and FFI arising from mossy fibers may control the timing of spike generation in individual CA3 cells (Pouille and Scanziani 2001; Lawrence, Grinspan et al. 2004; Mori, Abegg et al. 2004) and may also increase the sparseness of the activity patterns in CA3 via surround inhibition (Mori, Abegg et al. 2004).

The mossy fibers are considered to duplicate the information which CA3 receives already via its direct input from entorhinal cortex since EC layer II cells project to both DG and CA3. Computational models have suggested that the function of mossy fibers is the enforcement of strongly separated activity patterns onto the CA3 region, which would present a new memory that can overcome the interference by older memory traces which were already stored in the CA3 recurrent network (Mcnaughton and Morris 1987; Treves, Tashiro et al. 2008). On the behavioral level, pattern separation in the hippocampus is thought to facilitate specific storage of distinct yet similar episodic memories and to reduce errors in memory recall (McHugh, Jones et al. 2007; Nakashiba, Buhl et al. 2009).

1.3 HIPPOCAMPUS-DEPENDENT LEARNING PARADIGMS

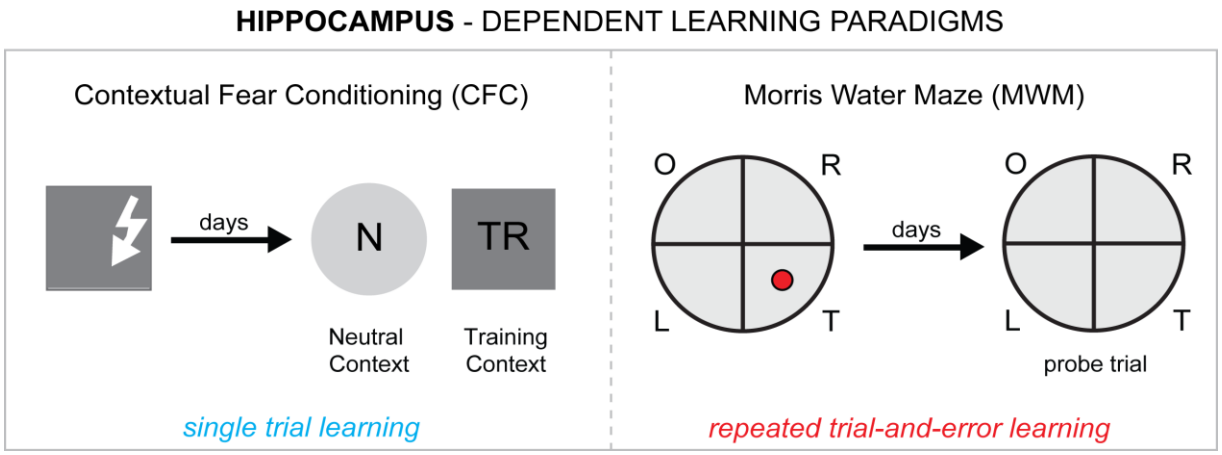
1.3.1 Fear conditioning

Classical fear conditioning is a specific form of Pavlovian learning which involves the formation of a robust association between a stimulus and an aversive effect. In fear conditioning, the subject is typically exposed to a noxious unconditioned stimulus (US), such as a foot-shock, in conjunction with a neutral conditioned stimulus (CS) such as an acoustic or visual stimulus (Maren 2001). Contextual fear conditioning is a hippocampal-dependent version of fear learning in which the subject learns the association of the training context (CS) with the US. Contextual fear conditioning has been shown to be hippocampus- dependent because hippocampal lesions interfere with contextual fear memory acquisition and recall (Phillips and Ledoux 1992). Upon training, the context acquires aversive properties and subsequent exposure to the paired context will trigger a recall of the fear memory and elicit an array of characteristic fear responses (Schema 2). In rodents, these fear responses include freezing behavior, a reduction of exploratory behavior, and the facilitation of reflexes as well as changes in the activity of the autonomous nervous system and the release of stress hormones (Iwata and LeDoux 1988; Davis 1992; LeDoux 2000).

Subsequent to fear condition the associated memory can be modified or even behaviorally suppressed after secondary experiences such as extinction and reconsolidation. For example, the fear memory expression can be behaviorally suppressed when the conditioned stimulus, such as the tone or context, is repeatedly represented to the subject. This prolonged stimulus representation initiates a new learning process called fear extinction (Myers and Davis 2002; Phelps, Delgado et al. 2004; Myers and Davis 2007). Alternative, memory recall can trigger a second memory consolidation cascade, called reconsolidation, which requires new protein synthesis (Duvarci and Nader 2004; Suzuki, Josselyn et al. 2004; Monfils, Cowansage et al. 2009).

1.3.2 Morris water maze

The Morris water maze (MWM) can be used to assess spatial learning and memory in rodents (Morris 1984; D'Hooge and De Deyn 2001). This navigational task was developed by Richard Morris who initially showed that hippocampal lesions impact on spatial learning in rodents (Morris, Garrud et al. 1982). In the classical water maze task an animal has to search for a hidden platform in a pool filled with opaque water. The swimming pool is surrounded by external landmarks which serve as orientation cues to find the hidden platform upon repeated training (Schema 2). Over several days of trainings the animals learn to efficiently find the hidden platform and form a reference memory of the platform position based on external landmarks (Vorhees and Williams 2006). The learning process is monitored by measuring the escape latency, the time it takes to locate the hidden platform, across trials and over consecutive training days. Memory performance in the water maze is typically assessed by a probe trial in which the platform is removed and the relative search behavior of the animal is monitored over this single trial in the absence of the rescue platform. Typical measures to assess probe trial performance include the quadrant occupancy as well as the number of platform crossings (Vorhees and Williams 2006; Maei, Zaslavsky et al. 2009).



Schema 2. Hippocampus dependent learning paradigms

1.3.3 Novel object recognition

In rodents, the novel object recognition test (NOR) is a standard procedure for assessing recognition memory. NOR was initially introduced to assess the ability of rats to recognize a newly presented object in a familiar environment (Ennaceur and Delacour 1988). In the first phase of the NOR test, the animal is exposed to an environment containing two identical objects in order to familiarize with the environment and the objects. In the second NOR phase, the animal is then confronted with two dissimilar objects placed in the same environment: one familiar object, which was already used in the first phase, and a second novel object. In this test phase, the exploration time spent with each of the two objects is measured and the object discrimination is determined.

Humans with hippocampal damage exhibit marked deficits in the NOR task due to a temporally graded retrograde amnesia (Mckee and Squire 1993; Pascalis, Hunkin et al. 2004). Similarly hippocampal lesions in monkeys also result in performance impairments in the NOR task (Pascalis and Bachevalier 1999; Clark, Zola et al. 2000; Zola and Squire 2001; Nemanic, Alvarado et al. 2004). Finally, studies in rodents have demonstrated deficits in NOR recognition upon hippocampal damage (Clark, Zola et al. 2000; Broadbent, Squire et al. 2004). Taken together, these findings have established that recognition memory in the NOR task depends on the hippocampus.

1.3.4 Non-spatial mnemonic functions of the hippocampus

A variety of studies using a non-spatial memory tasks suggest that the mnemonic operations of the hippocampus are not only limited to spatial contents. For example, functional imaging in humans has demonstrated hippocampal activation during encoding of visual and verbal information (Squire, Ojemann et al. 1992; Nyberg, McIntosh et al. 1996; Rombouts, Machielsen et al. 1997; Fernandez, Weyerts et al. 1998; Kelley, Miezin et al. 1998). In rodents, social transmission of food preference, which is an olfactory based association, has been shown to depend on the hippocampus (Alvarez, Lipton et al. 2001; Alvarez, Wendelken et al. 2002). In addition, memory tasks which present associated choice stimuli at random spatial locations also depend on the hippocampus (Wood, Dudchenko et al. 1999; Fortin,

Wright et al. 2004). Moreover, olfactory discrimination learning increases spine density along apical dendrites of CA1 pyramidal neurons (Knafo, Ariav et al. 2004), indicating that non-spatial forms of learning can trigger structural plasticity in the hippocampus. Finally, several studies showed hippocampal cell firing in response to task-relevant but non-spatial cues such as goal presence (Kuperstein and Eichenbaum 1985; Eichenbaum, Kuperstein et al. 1987; Tamura, Ono et al. 1992; Otto and Poon 2006). Together, these findings indicate an involvement of the hippocampus in the formation of general relations of the nature of a task rather than exclusive encoding of spatial attributes.

As described earlier, many studies strongly support the role of the hippocampus in spatial learning (Bures, Fenton et al. 1997; Maguire, Burgess et al. 1999; Holscher, Jacob et al. 2003). However it is important to note that hippocampal damage produces a range of spatial learning and memory deficits. The behavioral impairments depend on the extent of hippocampal damage as well as the kind of training protocol, and the nature of the spatial task (Morris, Schenk et al. 1990; Martinez, Quirarte et al. 2002). These discrepancies are even more pronounced in respect to the hippocampal involvement in non-spatial tasks. For example, a series of olfactory tasks have been shown to be independent of hippocampus (Mair, Burk et al. 1998; Burton, Murphy et al. 2000; Kaut, Bunsey et al. 2003; Jonasson, Ballantyne et al. 2004; Wood, Agster et al. 2004; Goddyn, Leo et al. 2006), while other studies have shown olfactory paradigms that depend on the hippocampus (Agster, Fortin et al. 2002; Kesner, Gilbert et al. 2002).

A key question to potentially solve these discrepancies is whether the hippocampus serves as a unitary structure in learning and memory or whether multiple kinds of hippocampal-dependent memories might depend on distinct intra-hippocampal modules. While the intrinsic connectivity of hippocampus mainly consists of the trisynaptic loop, which is basically repeated along the dorso-ventral axis of the hippocampus, the pattern of afferent and efferent connections of the hippocampus changes along this longitudinal axis (Moser and Moser 1998; Fanselow and Dong 2010). Such an anatomical division in hippocampal subdomains along the dorso-ventral axis may be central for an orchestrated encoding, storage and retrieval of different kinds of hippocampal-dependent memories.

1.4 PARCELLATION OF THE HIPPOCAMPUS ALONG THE LONGITUDINAL AXIS

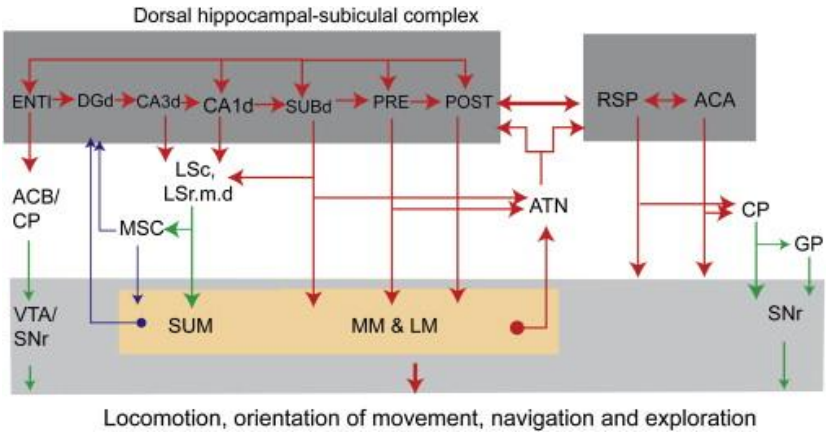
1.4.1 Segregation of the hippocampus along the dorso-ventral axis

The hippocampus is mainly connected to the retrohippocampal cortices which comprise the subiculum, presubiculum, parasubiculum, and entorhinal cortex. In addition, the hippocampus is also connected to several subcortical forebrain areas such as the amygdala. The main input of the hippocampus arises from entorhinal cortex (EC). The EC can be divided into three band-like zones which project topographically along the dorso-ventral axis to distinct and mostly separate portions of the dentate gyrus (Ruth, Collier et al. 1982; Witter, Vanhoesen et al. 1989; Dolorfo and Amaral 1998; Dolorfo and Amaral 1998). This specific projection pattern creates three main divisions of the hippocampus which are referred to as dorsal, intermediate and ventral hippocampus in this study (Moser and Moser 1998). Each entorhinal cortex band receives a specific combination of cortical and subcortical input thereby further channeling information flow to the defined hippocampal modules (Deacon, Eichenbaum et al. 1983; Naber, CaballeroBleda et al. 1997; Burwell 2000). This parcellation of the hippocampus along the dorso-ventral axis is also preserved in respect of the efferent connectivity. For example, the axons of CA1 pyramidal cells and subiculum project back to entorhinal cortex along the lateral to medial axis of entorhinal cortex (Vangroen and Wyss 1990; Witter 1993; Moser and Moser 1998; Witter, Wouterlood et al. 2000). In turn, entorhinal cortex projects to perirhinal cortical areas that subsequently form projections back to the original cortical areas which provided the initial input to the system (Insausti, Herrero et al. 1997). In addition, subcortical connectivity of the hippocampus is topographically organized (Moser and Moser 1998; Fanselow and Dong 2010). For example, the ventral CA1 region of the hippocampus forms a direct projection to the amygdala (Pitkanen, Pikkarainen et al. 2000). The subiculum of the dorsal and intermediate hippocampus projects to the mamillary complex whereas the ventral subiculum projects selectively to the amygdala, nucleus accumbens and the rostral hypothalamus (Moser and Moser 1998).

In summary, the overall input and output connectivity of the dorsal and ventral pole of the hippocampus strongly differs (Schema 3, Schema4). In addition, the hippocampal subcircuits in different portions along the dorso-ventral axis exhibit differences in their

circuit constitution. For example, the density of neuromodulatory terminals from noradrenergic, dopaminergic and serotonergic fiber is higher in the ventral hippocampus of the rat (Gage and Thompson 1980; Verney, Baulac et al. 1985; Moser and Moser 1998). On the functional level, the number of place cells in the dorsal hippocampus is higher than in ventral hippocampus and the respective place fields are smaller and more sharply tuned in dorsal hippocampus (Jung, Wiener et al. 1994; Poucet, Thinus-Blanc et al. 1994). The scale of this spatial representation increases almost linearly towards the ventral pole of the hippocampus resulting in place cells with very large place fields of more than 10 meters in size (Kjelstrup, Solstad et al. 2008).

Taken together, the selective input and output connectivity of the hippocampus led to the hypothesis, that the hippocampus may be composed of several independent circuit modules. If distinct kinds of information are processed in separate domains along the dorso-ventral axis of the hippocampus, it should be feasible to attribute specific functions to these hippocampal subdomains by performing targeted lesion experiments. In the following section, I will briefly summarize the behavioral data providing evidence for this concept of segregated hippocampal subdomains along the longitudinal axis.



Schema 3. Illustration of the Organization of the Dorsal Hippocampal Network. (Fanselow and Dong 2010) Abbreviations: ACA, anterior cingulated area; ACB, nucleus accumbens; ATN, anterior thalamic complex; CP, caudoputamen; DGd, dorsal domain of the dentate gyrus; ENTl, the caudolateral band of the entorhinal cortex; GP, globus pallidus; LM, lateral mammilary nucleus; LSc, the caudal part of the lateral septal nucleus; MM, medial mammilary nucleus; MSC, medial septal complex; PRE, presubiculum; POST, postsubiculum; RSP, retrosplenial cortex; SNr, reticular part of the substantial nigra; SUBd, dorsal subiculum; SUM, supramammillary nucleus; VTA, ventral tegmental area.

1.4.2 Role of the dorsal hippocampus in spatial learning

Several lesion studies in the rat suggested a differential role of the dorsal and the ventral hippocampus in behavior (Hughes 1965; Nadel 1968; Stevens and Cowey 1973). Most consistently these initial studies identified learning deficits in maze solving tasks upon lesion of the dorsal hippocampus whereas ventral hippocampus lesions did not impair this kind of spatial learning. Following studies have further supported the function of the dorsal hippocampus in spatial learning. Hippocampus lesion in the dorsal proportion markedly impaired spatial memory formation in the water maze task while ventral lesions of equal size do not have a strong impact in spatial memory formation (Moser, Moser et al. 1993; Moser, Moser et al. 1995). Notably, the deficit in spatial learning upon dorsal hippocampal lesions was proportional to size of lesion of in this domain. Interestingly, spatial learning was possible with only 20-30% of remaining tissue in the dorsal hippocampus (Moser, Moser et al. 1995). In conclusion the spatial navigation has been shown to mainly rely on the dorsal hippocampus. This is in line with reports indicating a higher proportion of place cells in the dorsal hippocampus. In addition, place fields in the ventral hippocampus have been shown to be less selective and more wide which is also indicative a different kind of spatial processing in the ventral hippocampus (Jung, Wiener et al. 1994).

1.4.3 Function of the ventral hippocampus learning and memory

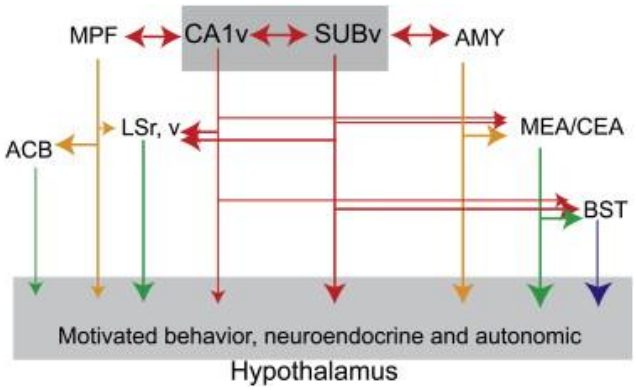
The ventral hippocampus has a direct efferent connectivity to subcortical forebrain structures including the amygdala and rostral hypothalamus and prefrontal cortex (Verwer, Meijer et al. 1997; Kishi, Tsumori et al. 2000; Pitkanen, Pikkarainen et al. 2000; Kishi, Tsumori et al. 2006; Fanselow and Dong 2010; Parent, Wang et al. 2010). The specific and direct connections of the ventral hippocampus suggest a functional role in various control systems ranging from autonomous and endocrine reactions to defensive and emotional responses (Schema 4). In line with this hypothesis, lesions of the ventral hippocampus have been shown to affect stress and emotional behaviors. For example, a reduction in anxiety has been shown upon lesion of the last quarter of the ventral hippocampus (Kjelstrup, Tuvnes et al. 2002; Pentkowski, Blanchard et al. 2006). In particular, an increase in the number of entries to open arms in the elevated plus maze was shown, which indicates a regulatory role

of the ventral hippocampus in anxiety-like behavior. In addition, animals with ventral hippocampus lesions exhibit a milder increase in corticosterone in response to stressful events as the exposure to brightly lit environments (Kjelstrup, Tuvnes et al. 2002). Ventral hippocampus has also been implied to contribute to emotional learning. In line with this hypothesis, infusions of NMDA receptor antagonists into ventral hippocampus interfere with the acquisition of contextual fear (Bast, Zhang et al. 2001; Zhang, Bast et al. 2001).

In summary, the ventral hippocampus has been shown to be involved in affective and emotional behaviors. Whether ventral hippocampus has absolutely no contribution to spatial learning is still not well established. This is mainly due to previous discrepancies in the definition of the size of the dorsal and the ventral domains and the frequent use of permanent lesions of ventral hippocampus before learning. In particular, permanent lesion before learning may result in compensatory learning mechanisms. It is still not well understood whether, and what kind of information ventral hippocampus is encoding during learning besides its regulatory function in emotional states like anxiety.

Recent evidence for a contribution of the ventral hippocampus in non-spatial learning is based on maze tasks in rodents (Trivedi and Coover 2004; Royer, Sirota et al. 2010). In a radial arm maze discrete place fields of several CA3 neurons in the dorsal hippocampus of mice encoded all positions in the maze. However, ventrally located CA3 cells exhibited distinct firing characteristics. For example, ventral CA3 neurons differentiated between open and closed arms of the radial arm maze. In addition ventral CA3 neurons fired selectively with respect to the goal presence on multiple arms (Royer, Sirota et al. 2010). The ventral hippocampus has also been suggested to function in a goal-related aspect of passive avoidance in an elevate T-maze task (Trivedi and Coover 2004). These findings indicate the ventral hippocampus may play a role in goal-related learning in rodents. This is in line with, recent human studies which also suggest a role of the human anterior hippocampus, which corresponds to ventral hippocampus in mice, in goal-directed spatial decision making (Viard, Doeller et al. 2011). Interestingly, an earlier study indicated a functional dissociation within the human hippocampus with respect to relative familiarity of study items (Strange, Fletcher et al. 1999). Neural response activity in the anterior hippocampus was related

with the novelty of items whereas activity in the posterior hippocampus was related with the familiarity of items with behavioral relevance. Together these findings highlight the potential role of ventral hippocampus in providing goal-related representations in learning and memory.



Schema 4 . Illustration of the Major Neuronal Connectivity of the Ventral. Hippocampus (Fanselow and Dong 2010); Abbreviations: ACB, nucleus accumbens; AMY, cortical-like amygdalar areas (nuclei); BST, bed nuclei of the stria terminalis; CEA, central amygdalar nucleus; LSr, v, the rostral and ventral parts of the lateral septal nucleus; MEA, medial amygdalar nucleus; MPF, medial prefrontal cortex; SUBv, the ventral subiculum.

1.5 AIM AND RATIONAL OF THIS THESIS

This thesis addresses the question of how structural plasticity of identified neuronal circuit elements in the brain relates to learning and memory at the behavioral level. In this thesis, I address this question by using hippocampal- dependent learning paradigms as an approach to study the interplay of structural plasticity and behavior. Classically, structural plasticity has been hypothesized to underlie the encoding of long-term memory traces and experiences in the brain. Yet, it is still largely unknown how morphological rearrangements contribute to changes in the input-output properties of individual neurons or neuronal ensembles and how structural traces encode memory. Moreover, it is not known whether structural plasticity has specific functional roles beyond the suggested storage of information.

In the first results section I show that hippocampal-dependent learning triggers structural plasticity at hippocampal mossy fiber terminals via an increase in the number of filopodial extensions of LMTs, which di-synaptically mediate feedforward inhibition onto the CA3 region. In addition, I show that filopodia formation depends on mossy fiber LTP and that this specific kind of structural plasticity is required for memory precision as revealed by behavior. Altogether these results establish a causal relationship of structural plasticity and an identified aspect of mnemonic behavior and provide the first evidence for a specific functional role of morphological plasticity in the adult brain in relation with behavior. In addition, we found learning related plasticity of filopodia extensions at cerebellar mossy fiber terminals specifically upon cerebellum-dependent learning tasks. Thereby these results suggest that this kind of presynaptic structural plasticity is generally associated with learning in a system specific manner.

In the second results part of this thesis, I examine in a joint effort with Dominique Spirig how distinct subdomains along the dorso-ventral axis of the hippocampus contribute to different phases and aspects of learning and memory. The work in the first part of this thesis has focused on the role of dorsal hippocampus in spatial learning and memory, which is considered to be the primary site for spatial learning in the hippocampus. In contrast, ventral hippocampus has been considered to predominately act in emotional and affective behaviors, which is mainly due to its direct connectivity to other brain systems such as the amygdala and prefrontal cortex.

However, recent *in vivo* electrophysiological studies support an additional role of ventral hippocampus in goal-oriented behavior. The precise functions of the ventral hippocampus in different stages of learning and memory have not yet been identified. In addition, the relative behavioral contribution of ventral hippocampus in relation with intermediate and dorsal hippocampus is still not well established.

We explored structural plasticity in ventral hippocampus in incremental spatial learning in the water maze and upon contextual fear conditioning as well as novel object recognition. Our results indicate that ventral hippocampus establishes structural plasticity of the feedforward inhibitory connectivity at mossy fiber terminals upon learning. However, structural plasticity exhibits a distinct time course than previously described in dorsal hippocampus. In addition, plasticity in ventral hippocampus is involved in goal-oriented learning during the earliest phase of learning in the water maze. Our results indicate that alterations in feedforward inhibitory connectivity selectively contribute to defined stages of hippocampus-dependent learning in complex trial-and-error learning of maze navigation. Our data indicates a sequential recruitment of distinct hippocampal sub regions along the dorso-ventral hippocampus as revealed by region specific excitotoxic lesion experiments. We also provide evidence for early goal-oriented learning via recruitment of the ventral hippocampus and local learning-related plasticity of feedforward inhibitory connectivity. In addition we found a sequential involvement of the intermediate and dorsal hippocampus in later stages of spatial learning which is also associated with local structural plasticity.

The third section of the results centers on the relation of structural plasticity in the hippocampus and innate behavior. We investigated the interplay of structural plasticity and behavior in an innate fear paradigm. I found that acute exposure to a predator odor induces morphological rearrangements of mossy fiber terminals as well as numerical and structural changes in the postsynaptic components. In particular, I found changes in the density and structure of thorny excrescences of CA3 pyramidal cells. In addition, we provide evidence that these structural changes are related with behavioral deficits in pattern separation. In summary, our data indicates that synaptic connectivity underlies and contributes to specific circuit functions such as pattern separation in the hippocampus.

2. RESULTS

2.1 LEARNING-RELATED GROWTH OF FEEDFORWARD INHIBITORY CONNECTIVITY REQUIRED FOR MEMORY PRECISION

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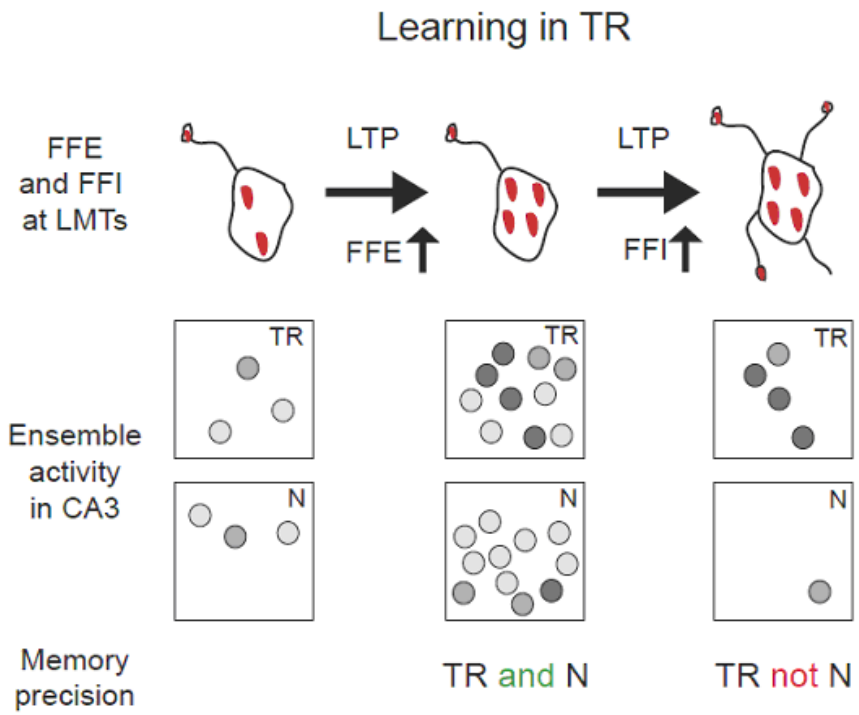
Abstract

In the adult brain, new synapses are formed, and pre-existing ones are lost, but the function of this structural plasticity has remained unclear[1-5]. Learning of new skills is correlated with formation of new synapses[6-8]. These may directly encode new memories, but they may also have more general roles in memory encoding and retrieval processes[2]. Here we investigated how mossy fiber terminal complexes at the entry of hippocampal and cerebellar circuits rearrange upon learning, and what is the functional role of the rearrangements. We show that one-trial and incremental learning both lead to robust, circuit-specific, long-lasting and reversible increases in the numbers of filopodial synapses onto fast-spiking interneurons that trigger feedforward inhibition. The increase in feedforward inhibition connectivity involved a majority of the presynaptic terminals in the circuit, restricted the numbers of c-Fos expressing postsynaptic neurons at memory retrieval, and correlated temporally with the quality of the memory. We then show that for contextual fear conditioning and Morris water maze learning, increased feedforward inhibition connectivity by hippocampal mossy fibers has a critical role for the precision of the memory and the learned behavior. In the absence of mossy fiber LTP in *Rab3a*$^{-/-}$ mice[9], c-Fos ensemble re-organization and feedforward inhibition growth were both absent in CA3 upon learning, and the

memory was imprecise. By contrast, in the absence of β-Adducin[10] c-Fos re-organization was normal, but feedforward inhibition growth was abolished. In parallel, c-Fos ensembles in CA3 were greatly enlarged, and the memory was imprecise. Feedforward inhibition growth and memory precision were both rescued by re-expression of b-Adducin before learning specifically in hippocampal mossy fibers. These results establish a causal relationship between learning-related increases in the numbers of defined synapses and the precision of learning and memory in the adult. The results further relate plasticity and feedforward inhibition growth at hippocampal mossy fibers, to the precision of hippocampus-dependent memories.

Introduction

The hippocampus accounts for the rapid generation and contextualization of episodic memories[11,12]. Within the main hippocampal circuit, the subregion CA3 establishes links between the modalities of individual episodes, and between related episodes through its auto-associational network[11,13]. The mossy fiber projection, which consists of the axons of glutamatergic dentate gyrus granule cells, conveys highly contextualized information from the dentate gyrus onto CA3 through its Large Mossy fiber Terminals (LMTs; Fig. 1a)[14,15]. These mediate powerful monosynaptic feedforward excitation onto pyramidal neurons through their core terminals, and di-synaptic feedforward inhibition through filopodia that emanate from the terminals. The filopodial synapses excite inhibitory interneurons, which in turn inhibit the pyramidal neurons in CA3 (Fig. 1a)[16,17]. For simplicity, we will designate the filopodia synapses as feedforward inhibition connectivity. The plasticity and connectivity properties of this feedforward excitation/feedforward inhibition arrangement are thought to ensure shunting of pyramidal neuron excitation at low activation levels of the mossy fibers, and recruitment of small ensembles of pyramidal neurons under conditions of high activation (Suppl. Fig. 1)[17-19].



Supplementary Figure 1 - Learning-related feedforward inhibition connectivity growth required for memory precision. The schematic summarizes the main finding of this study. Upper row: Learning-related feedforward excitation (FFE) and feedforward inhibition (FFI) growth at hippocampal mossy fiber terminals. Upon hippocampus-dependent learning, Rab3a-dependent mossy fiber LTP leads to enhanced numbers of active zones at core LMTs (enhanced FFE, center), and to b-Adducin-dependent higher numbers of filopodia (enhanced FFI connectivity, right). Red spots: synaptic sites at core LMTs and filopodial varicosities. Middle row: In the absence of learning, the different contexts TR and N elicit comparable numbers of c-Fos positive pyramidal neurons in CA3 (left). Learning produces mossy fiber LTP- and b-Adducin-dependent re-organization of activated ensembles in CA3, leading to substantially augmented recruitment of pyramidal neurons upon re-exposure to training context TR, and suppressed recruitment upon exposure to novel context N (right). In the absence of FFI growth, LTP and ensemble re-organization produce an excess of recruited pyramidal neurons in CA3 (center). Darker grey tones indicate higher levels of c-Fos accumulation in individual pyramidal neurons. Lower row: FFI growth

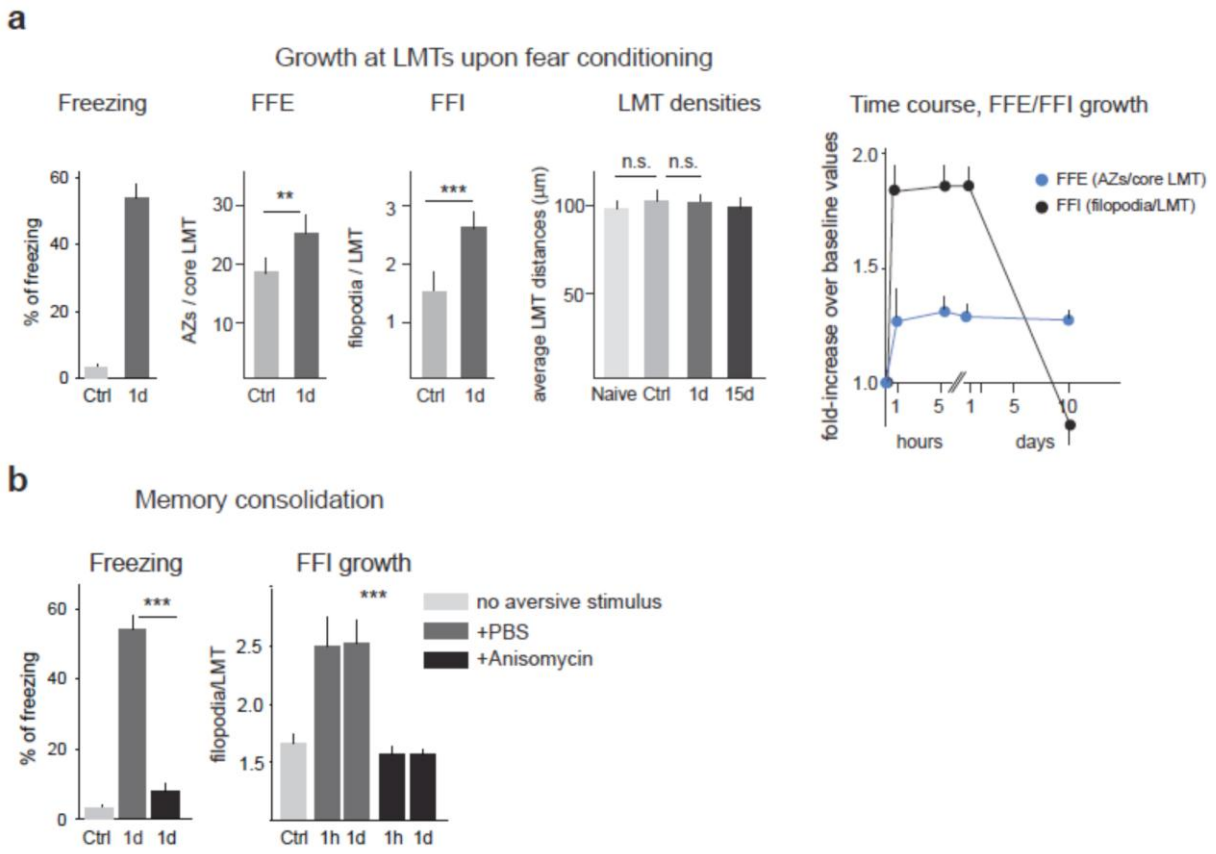
Results

To determine whether hippocampus-dependent learning may produce structural rearrangements in LMT components involved in feedforward excitation and/or feedforward inhibition in CA3, we analyzed GFP-positive LMTs in dorsal hippocampus of *Thy1-mGFP(Lsi1)* reporter mice[5] that had been subjected to contextual fear conditioning (Methods). In addition, the main findings of this study were confirmed in mice in which hippocampal mossy fibers were labeled upon transduction with an mGFP-lentivirus (not shown)[20]. Fear conditioning led to a robust increase in the average number of filopodia per LMT (1.82-fold, $p<0.001$; feedforward inhibition connectivity; Fig. 1b, c, Suppl. Fig. 2a), and to a less pronounced increase in the average numbers of Bassoon-positive putative release sites per core LMT[20] (1.31-fold, $p<0.01$; feedforward excitation connectivity; Suppl. Fig. 2a). By contrast, there was not change in the densities of LMTs in CA3b at any time upon fear conditioning (Suppl. Fig. 2a).

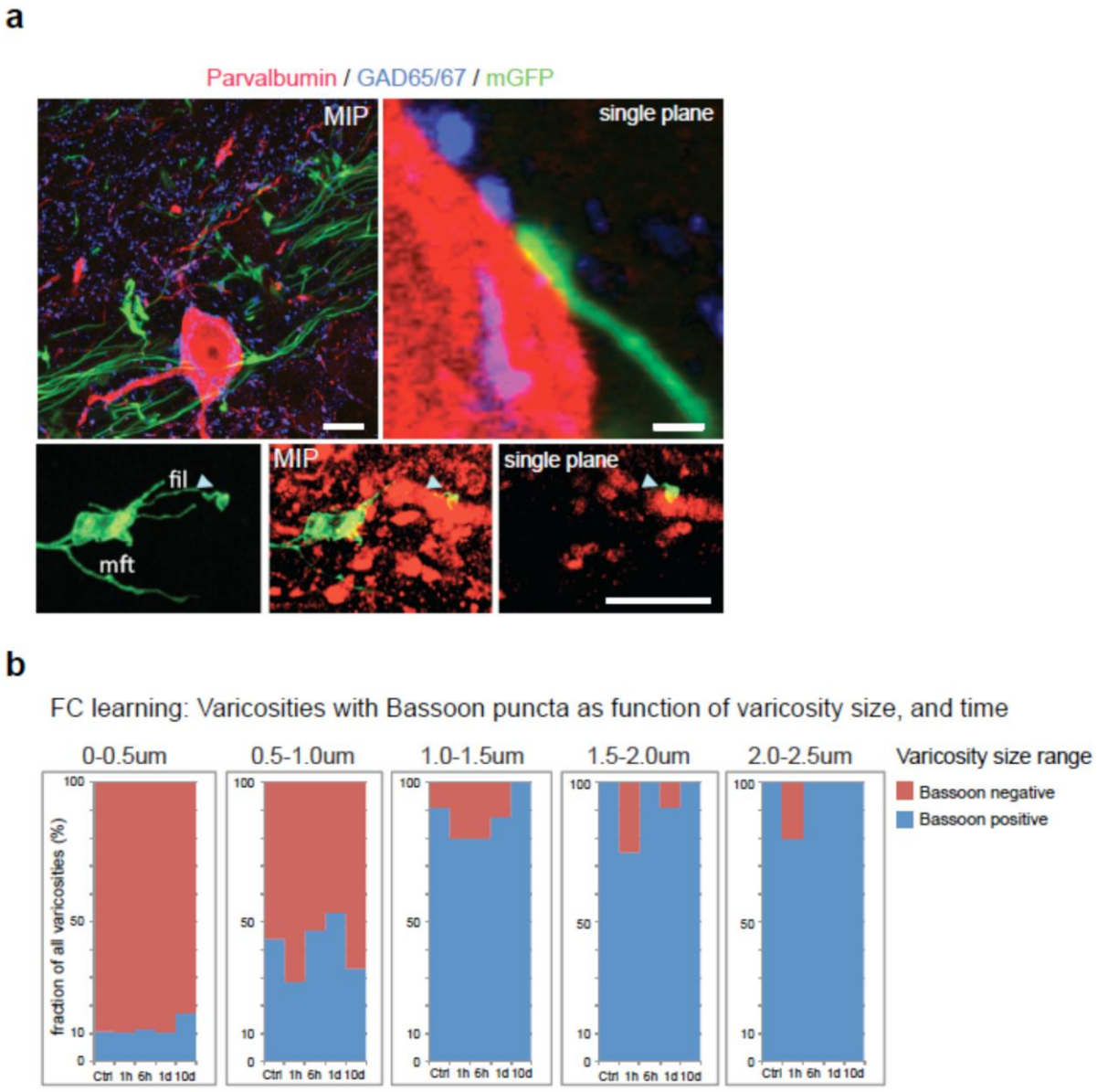
Upon fear conditioning, filopodial growth was rapid: it reached peak values at 1h, and these values were maintained during 1-1.5 days after fear conditioning (Fig. 1c). Subsequently, filopodial contents decreased to intermediate values (about 1.3-fold higher than baseline levels), and decreased again to reach naive values at 8-10 days (Fig. 1c, Suppl. Fig. 2a). In control mice and 1d after fear conditioning, about 50% of the filopodia exhibited varicosities larger than 1 mm in diameter, which in more than 95% of the cases were associated with Bassoon-positive active zone puncta (Fig. 1d, Suppl. Fig. 3b). The filopodia contacted spine-free dendrites of Parvalbumin-positive interneurons in CA3b (Figs. 1e, Suppl. Fig. 3a), suggesting that they induce feedforward inhibition through fast-spiking interneurons[16,18]. At 1h, and to a lesser extent at 6h after fear conditioning, slightly larger fractions of filopodia lacked varicosities with Bassoon-positive puncta, suggesting that the formation of new synapses went on during several hours after fear conditioning (Fig. 1d, Suppl. Fig. 3b). The more modest increase in feedforward excitation connectivity at LMTs also developed within 1h upon fear conditioning, but was more long lasting than the increase in feedforward inhibition connectivity (Suppl. Fig. 2a). When the protein synthesis inhibitor anisomycin was administered shortly before learning, it suppressed both consolidation of the fear memory and the increase in filopodia numbers (Suppl.

Fig. 2b), suggesting that the filopodial growth occurred as part of the memory consolidation process[12]. To estimate the fraction of LMTs in CA3b with altered contents of filopodia, we analyzed LMT/filopodia distributions in naïve, control and fear conditioning mice. Shifts in the fractions of LMTs with no filopodia, and with more than four filopodia revealed that, on average, at least 45% of the LMTs established increased numbers of filopodia as a consequence of fear conditioning (Fig. 1f).

To determine whether an increase in stratum lucidum feedforward inhibition connectivity may be generally associated with hippocampal learning, we analyzed mice that underwent a Morris water maze learning protocol. During this 8 days protocol, mice learn to locate a fixed hidden platform position, starting from any point at the edge of the pool. Like fear conditioning, the protocol involves hippocampus-dependent spatial learning, but unlike fear conditioning the learning process is incremental[12]. Latencies to find the hidden platform exhibited a bi-modal curve: mice improved rapidly, but exhibited a pronounced variability within the first 3-4 days, whereas further improvement during the next 3-4 days led to low variability plateau levels of performance at 7-8 days (Fig. 1g). In parallel, the mice established enhanced numbers of filopodia at LMTs (Fig. 1g). Notably, filopodial contents were only slightly increased over naive values during the first 3-4 days of training, whereas they increased markedly between days 4 and 8 (Fig. 1g). Again, we detected no changes in the densities of LMTs in CA3b upon Morris water maze learning (not shown). Testing mice for the memory of the platform position revealed that this reference memory only began to differ from chance after 3 days of training. The reference memory reached plateau values at day 8 (Fig. 1h), suggesting that filopodial growth correlated with the establishment of a precise spatial memory in the Morris water maze test. Like in the fear conditioning experiment, a large fraction of the LMTs exhibited higher filopodial contents at plateau values (Fig. 1i). The reference memory of the platform position persisted for at least 45 days after cessation of the training, and, unlike in the fear conditioning experiment, elevated filopodia per LMT values also persisted for at least 45 days (Fig. 1h, 53d values).



Supplementary Figure 2 - Properties of feedforward inhibition connectivity growth upon contextual fear conditioning. (**a)** Freezing: behavioral freezing upon fear conditioning, as shown in Fig. 3a. FFE and FFI: Extent of FFE (mean Bassoon puncta (i.e. AZs)/LMT values) and FFI (mean filopodia/LMT values) growth 1d after fear conditioning. N=5, 50 LMTs each. LMT densities: average distance of LMTs (swellings with largest diameter > 3□m) along individual mGFP positive axons in CA3b; N=3, 30 LMT each. Naïve: cage control; Control: exposed to training context without aversive stimulus; 1d, 15d: time after fear conditioning. Time course of FFI and FFE growth upon fear conditioning: note how the more modest increase in FFE release sites persisted much longer than that in FFI connectivity. N=5 mice; 100 LMTs each. (**b**) Protein synthesis blockade at learning prevents consolidation of fear memory and FFI growth. Anisomycin (75mg/kg body weight) or PBS were applied 20 min before, and 2h after (only for 1d time point) fear conditioning. N=5 mice, 100 LMTs each.



Supplementary Figure 3 - Growth of FFI connectivity upon fear conditioning. (**a**) LMT filopodia varicosities contact parvalbumin-positive interneurons in CA3. Examples of varicosities contacting soma (upper row) and dendrite (lower row, arrowheads) of parvalbumin-positive interneurons. MIP: maximum intensity projection; single plane: single confocal plane; fil: filopodium; mft: mossy fiber terminal. More than 95% of varicosities were in comparable contact with a parvalbumin-positive interneuron (N=600 varicosities, from 4 mice; not shown). (**b**) Varicosities with Bassoon puncta as a function of varicosity size and time upon fear conditioning. N=3, 100 LMT each. Note how most varicosities (filopodial swellings larger than 1 µm) exhibited Bassoon puncta, and how varicosities without Bassoon puncta were slightly more frequent at 1h and 6h after fear conditioning. Bars: 10 and 0.5 (top row, right panel) µm.

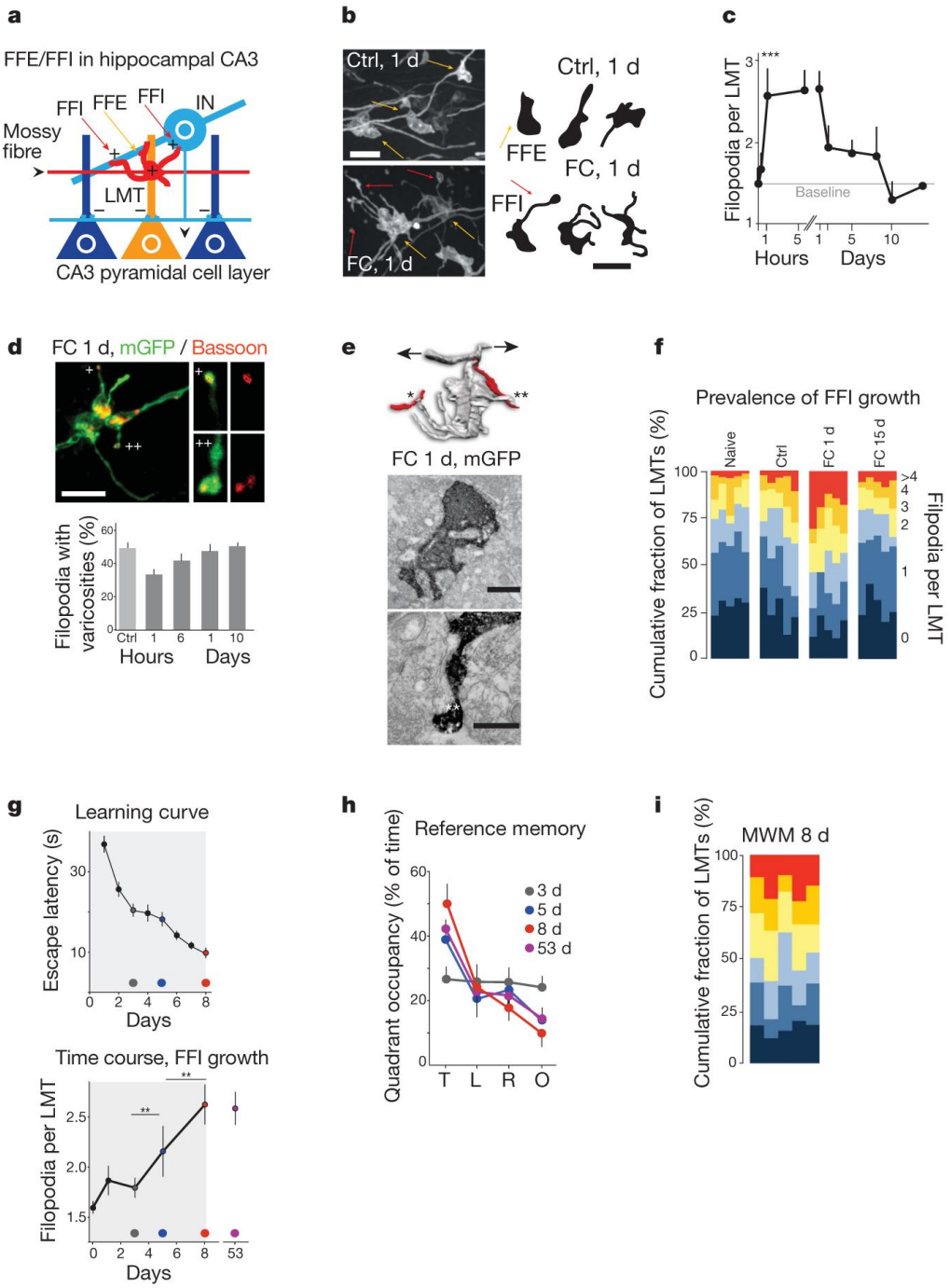
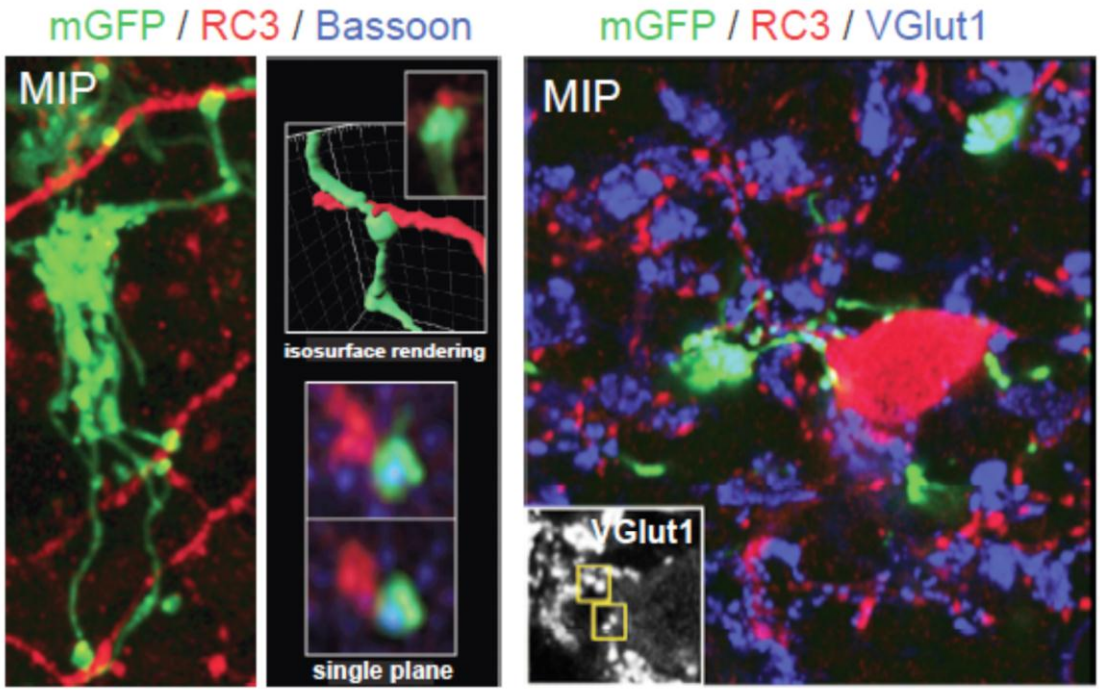


Figure 1. Learning-related feedforward inhibition connectivity growth in the hippocampus. (**a**) Schematic of hippocampal feedforward excitation (FFE) and feedforward inhibition (FFI) circuit in stratum lucidum of CA3. Left: general FFE/FFI circuit diagram. Right: FFE (core LMT synapses onto CA3 pyramidal neurons; yellow arrow) and FFI (filopodial synapses onto inhibitory interneurons; red arrows) connectivity by LMTs in stratum lucidum of CA3. (**b-f**) FFI growth at hippocampal mossy fiber LMTs upon contextual fear conditioning. **b**: Micrographs and representative camera lucidas of mGFP-labeled mossy fibers and LMTs in hippocampal stratum lucidum (CA3b). Yellow arrows: core LMTs (FFE); red arrows: filopodia (FFI). **c**: Average filopodia/LMT values upon fear conditioning. N=5 mice (100 LMTs each). ***: p<0.001. **d**: Filopodial synapses upon fear conditioning

(FC). Bassoon-positive varicosities at LMT filopodia 1d upon fear conditioning learning (overview panel: maximal intensity projection of mGFP-positive LMT with 4 filopodia; detail panels: single confocal planes of 2 of the filopodia (+ and ++); Bassoon signal outside of LMT was masked); bar diagram: fraction of LMT filopodia with varicosities as a function of time upon fear conditioning (N=3, 100 LMT). **e**: Filopodia upon FC learning contact spine-free dendrites. Immuno-electron microscopy of mGFP-positive LMT with 4 filopodia, 1d after fear conditioning; left, 3D reconstruction of immuno-labeled LMT (red: spine-free dendrites contacted by two of the filopodia in the example (* and **); center, immuno-labeled LMT; right, immuno-labeled filopodium with ** contact. **f**: Distributions of filopodia/LMT contents for individual mice. N=100 LMTs. The plots display the relative contents of LMTs with 0, 1, 2, 3, 4, >4 filopodia as a fraction of the total LMT population. Vertical rows represent individual mice. (**g-i**) FFI growth at hippocampal mossy fiber LMTs upon Morris water maze (MWM) training. **g**: learning curve and time course of FFI growth. N=5 mice (100 LMTs each). Grey area: daily training period. The circles highlight the positions on the curves as compared to reference memory (right). **h**: Reference memory at 3, 5, 8, and 53 days. The graphs plot the percentage of time spent by the mice in target (T), left (L), right (C) and opposite (O) quadrant. N=5 mice. Representative camera lucida examples at 53d (45d without training) are shown on the right. **i**: filopodia/LMT distributions after 8d of training, as described in (b). Bars: 5 (b, d top, g), 1 (d, bottom center) and 0.5 (d, bottom right) µm.

To determine whether learning-related induction of FFI connectivity growth might be a general phenomenon not restricted to spatial learning in the hippocampus, we analyzed mossy fiber terminals in cerebellar cortex, which also consist of powerful large core structures associated with filopodia[21]. Cued fear conditioning, in which animals learn that a tone predicts an aversive stimulus, involves plasticity in cerebellar cortex lobule 5 (multiple sensory inputs), but not lobule 9 (major vestibular inputs)[22]. In parallel, cued fear conditioning led to a robust and reversible increase of filopodial numbers per mossy fiber terminal in lobule 5, but not lobule 9 (Figs. 2a, d). In a second set of experiments, we trained mice to balance on an accelerating rotating rod (rotarod). This cerebellum-dependent motor skill task involved incremental learning over 4-6 days, which was accompanied by a parallel increase in the filopodial contents of mossy fiber terminals in lobule 9, but not lobule 5 (Figs. 2b, d). Upon termination of the training at day 7, the motor skill was gradually lost over a period of 15-20 days, and so were the excess filopodia (Fig. 2b). Since the possible targets of mossy fiber terminal filopodia in cerebellar cortex had not been identified, we investigated the possibility that these filopodia may make synaptic contacts with Golgi cells, i.e. the fast-spiking interneurons that convey feedforward inhibition to cerebellar granule cells[21,23]. At least for the Golgi cells that could be visualized with the marker RC3,

mossy fiber terminal filopodia extended along their dendrites, and established numerous varicosities, where synaptic markers co-distributed (Fig. 2c, Suppl. Fig. 4). More than 95% of the filopodial varicosities within a granule cell layer volume exhibiting an RC3-positive Golgi cell made putative synaptic contacts with that Golgi cell. Therefore, learning is specifically correlated with the growth of feedforward inhibition connectivity in both hippocampal and cerebellar circuits.

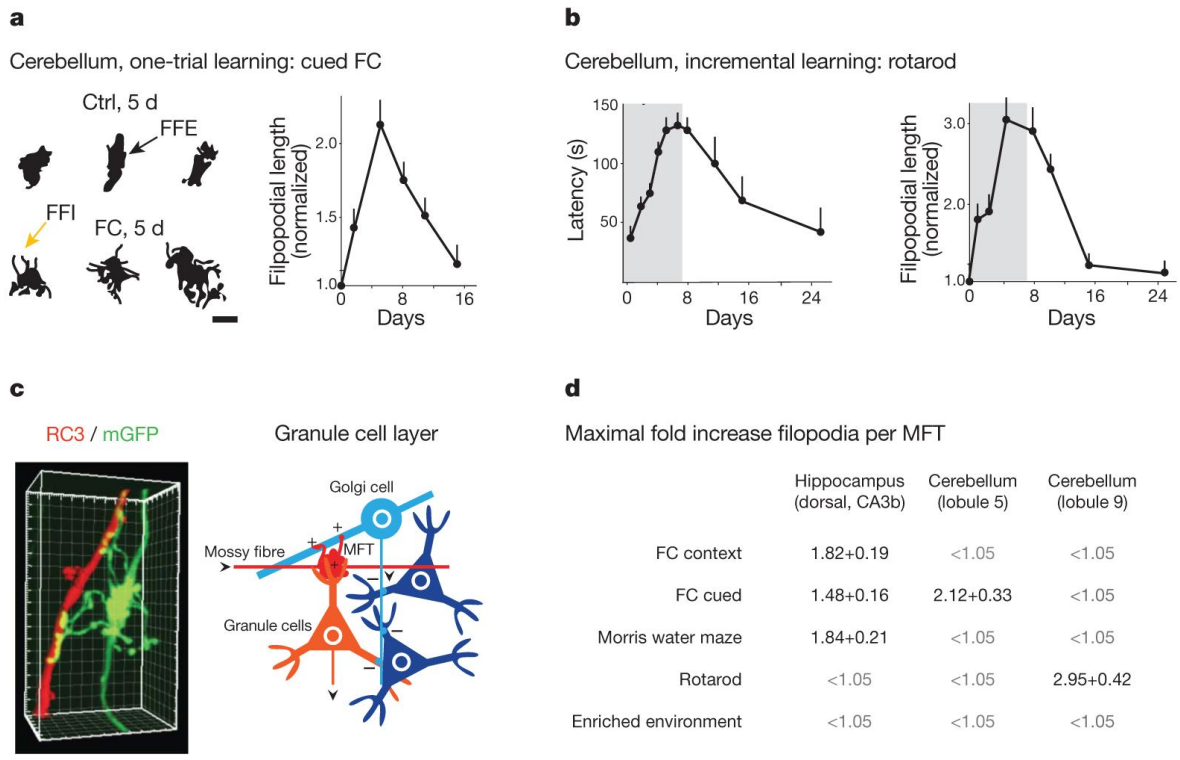


Supplementary Figure 4 - Cerebellar mossy fiber terminal filopodia synapse onto Golgi cells. Examples of synaptic contacts onto RC3-positive Golgi cells are shown in the panels. Within granule cell layer volumes with an RC3-positive Golgi cell more than 95% of varicosities were in comparable contact with that RC3-positive Golgi cell (N=300 varicosities, from 3 mice).

To further investigate the specificity relating learning to feedforward inhibition connectivity growth, we analyzed hippocampal CA3 and the cerebellar lobules 5 and 9 in mice that had been subjected to different behavioral experiences. Consistent with the existence of contextual components in the cued fear conditioning experiment, this protocol led to filopodial growth both in dorsal hippocampus and in cerebellar lobule 5 (Fig. 2d). By contrast, contextual fear conditioning or Morris water maze learning did not produce filopodial growth in cerebellar lobules 5 or 9 (Fig. 2d). In addition, rotarod training affected filopodial numbers in cerebellar lobule 9, but not in dorsal

hippocampus (Fig. 2d). Finally, housing mice under environmentally enriched conditions, which leads to enhanced motor activity and improved potential for learning, but does not by itself involve any specific skill learning, did not affect filopodial numbers in dorsal hippocampus nor in the cerebellum (Fig. 2d). Therefore, feedforward inhibition connectivity growth is specifically correlated to learning, and is confined to brain structures involved in that learning paradigm.

What may be the function of the learning-related growth in feedforward inhibition connectivity? In the fear conditioning experiments, the excess filopodia were lost within 8-10 days after learning, at a time when the fear memory is strong, indicating that the excess filopodia are not a requirement for expression of the fear memory[24,25]. To further investigate how filopodial growth relates to behavioral expression of the fear memory, we elicited extinction of cued and contextual fear conditioning by repeatedly re-exposing mice to training context (TR) and tone (20 times) without the aversive stimulus, 5h and 24h after fear conditioning. This protocol produced behavioral extinction of the fear response, but instead of reducing filopodial numbers at hippocampal LMTs, it prolonged peak filopodial responses from 1 to 4-6 days (Fig. 3a). Re-exposing mice briefly to TR, and to one tone instead of twenty at 5h and at 24h did not extinguish the fear response, but also extended peak filopodial response values to 4-6 days (Fig. 3a), suggesting that the temporal extension of excess filopodia was induced by recall of the fear-associated memory.



	Hippocampus (dorsal, CA3b)	Cerebellum (lobule 5)	Cerebellum (lobule 9)
FC context	1.82+0.19	<1.05	<1.05
FC cued	1.48+0.16	2.12+0.33	<1.05
Morris water maze	1.84+0.21	<1.05	<1.05
Rotarod	<1.05	<1.05	2.95+0.42
Enriched environment	<1.05	<1.05	<1.05

Figure 2. Specificity of learning-related feedforward inhibition growth.

(**a-b**) Learning-related FFI connectivity growth in cerebellar cortex. **(a)** FFI growth at lobule 5 cerebellar cortex mossy fiber terminals upon cued fear conditioning (FC). Labeling as in Fig. 1b. **(b)** FFI growth at lobule 9 cerebellar cortex mossy fiber terminals upon rotarod learning. Labeling as in Fig. 1c. **(c)** In cerebellar cortex, mossy fiber terminal (MFT) filopodia contact inhibitory Golgi cells. Left: 3D rendering of contacts by MFT filopodia onto RC3-positive Golgi cell dendrite. Right: FFE/FFI circuit in granule cell layer of cerebellar cortex. **(d)** Specific relationship between learning and feedforward inhibition growth. Average fold increase values at peak response (FC hippocampus: 1d; FC cerebellum: 2d; Morris water maze (MWM): 8d; Rotarod: 5d). N=5, 100 LMTs or mossy fiber terminals each. Bar: 10 µm.

Feedforward inhibition can restrict and temporally sharpen the spread of excitatory activity in CA3[20] and in the granule cell layer of cerebellar cortex[21,23], suggesting that the feedforward inhibition growth might support precision in the behavioral expression of the memory[19,21]. Consistent with this notion, testing of individual mice during the Morris water maze training protocol revealed a strong correlation between the reference memory of the platform position and mean filopodial contents per LMT for individual mice (Fig. 3b), suggesting that the extent of filopodial growth was correlated to the precision of the learning.

To investigate a possible role of feedforward inhibition connectivity growth in memory precision, we monitored generalization, i.e. decreased behavioral precision of the fear memory in the contextual fear conditioning experiment. In agreement with previous reports[26,27], generalization of the memory for context in fear conditioning was not detectable during the first 5-7 days after learning, but was detected at longer intervals after fear conditioning as an enhanced freezing response and reduced exploratory activity in a novel context (N) (Fig. 3c). A brief re-exposure of mice to training context TR in the absence of the aversive stimulus at 15d after learning produced a suppression of generalization at retest, which lasted 8-12 days (Fig. 3d). In parallel, TR re-exposure induced a pronounced re-induction of the filopodial response, which again lasted for 7-10 days (Fig. 3d). By contrast, exposure to a novel context affected neither generalization nor filopodial growth (Fig. 3d), suggesting that retrieval of the specific memory was necessary to re-induce feedforward inhibition connectivity growth in hippocampal CA3, and to suppress generalization. With our fear conditioning protocol, recall at 40 days only partially reduced generalization during 2-3 days[12], and did not induce feedforward inhibition growth (Fig. 3d). Time-dependent generalization is typically most pronounced upon fear conditioning protocols like the one used in this study, which did not involve previous familiarization of the mice with TR[26,27]. Indeed, when mice were pre-exposed to TR for 5 min on the day before learning, the decline in learning-induced filopodial growth was delayed, and so was the loss of memory precision (Suppl. Fig. 5). Taken together, the results of these behavioral experiments provide correlative evidence that learning-related feedforward inhibition growth at the mossy fiber projection in CA3 might support the precision of hippocampus-dependent memories[28].

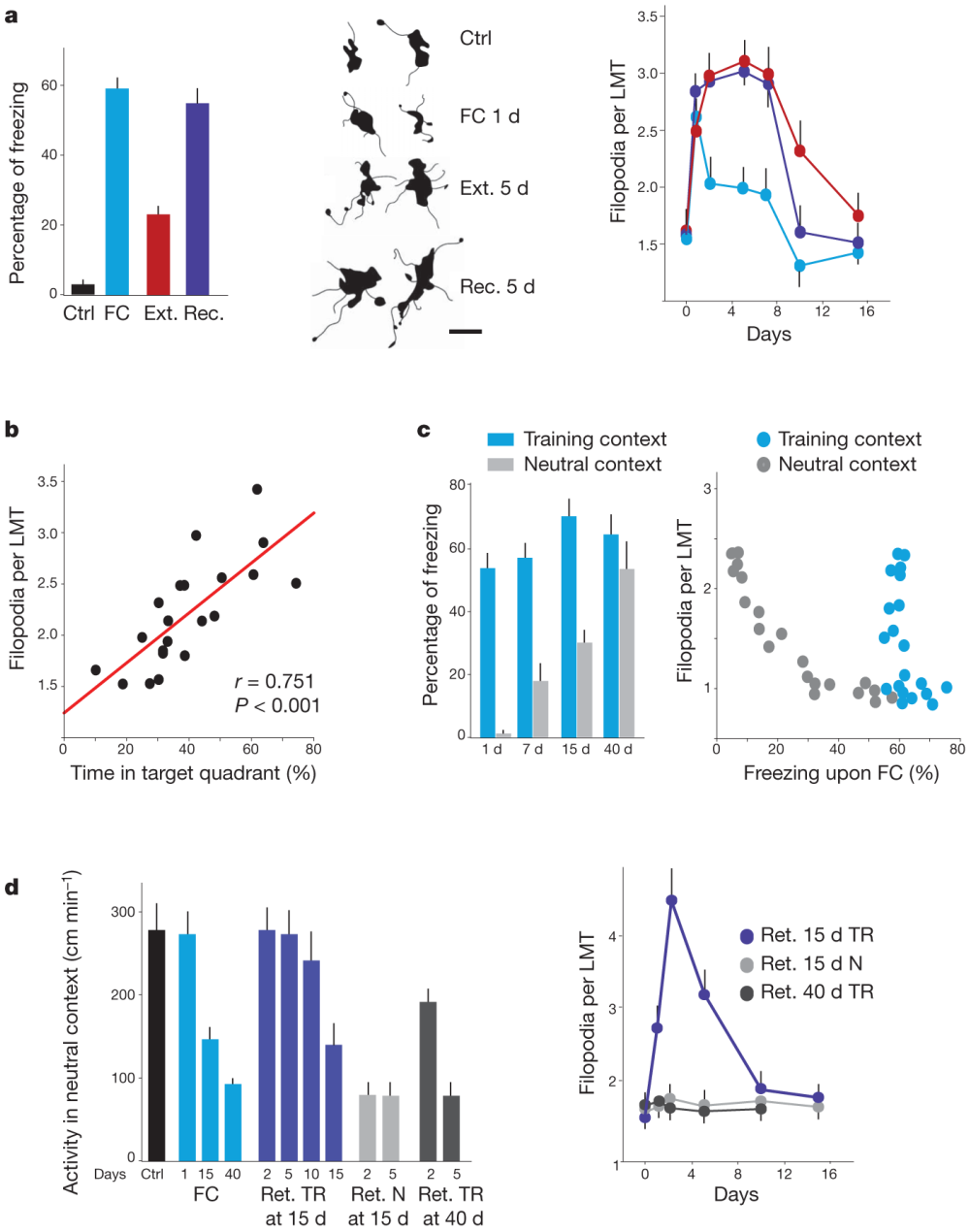
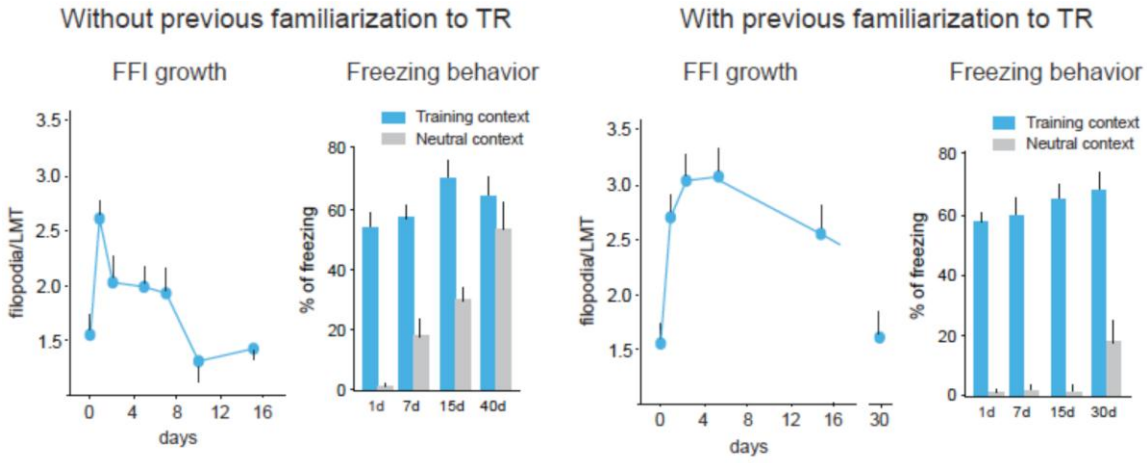


Figure 3. Correlation between feedforward inhibition growth and quality of hippocampal learning and memory. Memory retrieval prolongs peak levels of FFI growth upon cued fear conditioning (FC). Pale blue: FC, no recall (at 1d); red: FC followed by extinction at 5h and 24h (at 5d); violet: FC followed by recall at 5h and 24h (at 5d). N=5 mice (100 LMTs each). **(b)** Correlation between reference memory precision and average filopodial contents per LMT in Morris water maze (MWM) task. Dots: individual mice analyzed between day 1 and day 8 of the training procedure (100 LMTs each). **(c)** Time-dependent generalization upon contextual FC learning. Right: dots represent average values for individual mice at different times after FC learning (100 LMTs each). **(d)** Re-growth of filopodia and re-contextualization upon retrieval of training context memory. Left: exploratory activity in novel context as a function of days after last manipulation. Bars: 5 µm.



Supplementary Figure 5. Prolonged feedforward inhibition growth and delayed generalization upon previous familiarization with training context in contextual fear conditioning experiment. Experimental details as in Fig. 3c. N=5, 100 LMTs each.

What may be the functional correlate of feedforward inhibition connectivity growth for the CA3 pyramidal neuron ensemble in behaving adult mice? Since upregulation of the immediate early gene c-Fos has been correlated with activity-related plasticity in neurons[29], we analyzed c-Fos-positive pyramidal neurons in CA3b in the contextual fear conditioning experiment. On day 0, mice were exposed to TR without or with aversive stimulus. In the absence of aversive conditioning, re-exposure on day 1 to either TR or a novel context N produced closely comparable increases in the fractions of pyramidal neurons with high and intermediate c-Fos signals when compared to naïve cage control mice (Fig. 4a). In stark contrast, association of TR with aversive stimulus led to a specific and robust relative increase in the number of pyramidal neurons expressing high c-Fos signals upon recall of the memory in TR, and to a marked reduction of the high and medium c-Fos signals upon exposure to the novel context N (Fig. 4a). Therefore, and consistent with robust behavioral precision, association of the training context TR with the aversive stimulus led to an organization of the cell ensemble in CA3 upon memory recall that was very different from the ensemble in the conditioning control group (TR alone): fear memory formation to TR resulted in strong c-Fos responses specifically to fear-related context (TR), and in suppressed responses to a novel context (N). As shown in Fig. 3c, exposure to TR elicited comparable freezing responses at 1d and 15d after fear conditioning, but at 15d filopodia levels had returned to control values and discrimination against a novel context N had decreased. In parallel, recall in TR at 15d led to decreased high-signal c-Fos neurons, whereas exposure to N at 15d led to dramatically increased low-signal

c-Fos neurons (Fig. 4b). Notably, in parallel to increased filopodial numbers and the re-establishment of memory precision, memory recall in TR at 15d after fear conditioning suppressed excess responses upon subsequent exposure to a novel context N (Fig. 4b). Taken together, these results are consistent with the hypothesis that decreased LMT filopodial contents paired to persistent hippocampal memory lead to high numbers of c-Fos positive neurons recruited by a novel context N in CA3 and a loss of fear memory precision. The results further suggest that retrieval-induced feedforward inhibition growth restricts the ensemble of CA3 pyramidal neurons recruited by unrelated context, thus supporting precision of the learned fear.

What may be the cellular mechanism underlying learning-related feedforward inhibition growth and memory precision? To address the role of mossy fibers and their plasticity, we carried out fear conditioning experiments in *Rab3a*$^{-/-}$ mice, which specifically lack LTP at mossy fibers, but not at other synapses in the hippocampus[9]. We found that in the absence of Rab3a mice learned the relationship between the training context TR and the aversive stimulus, but already generalized 1d after fear conditioning (Fig. 4c). In parallel, *Rab3a*$^{-/-}$ mice lacked any learning-related increase in putative release sites at core LMTs (feedforward excitation connectivity; *Rab3a*$^{-/-}$: 5.61$\pm$0.08 (control) versus 5.62$\pm$0.07 (fear conditioning, 1d) Bassoon positive puncta per 10 mm^3 of LMT volume; wildtype: 5.48$\pm$0.08 (control) versus 7.18$\pm$0.11(fear conditioning, 1d); N=60 LMTs from 3 mice each), or any learning-related increase in filopodia numbers at LMTs in CA3 (feedforward inhibition connectivity; Fig. 4c). LMT densities in *Rab3a*$^{-/-}$ mice were identical to those in wildtype mice (average LMT distances in CA3b were 98.2$\pm$2.1 mm (wildtype) and 100.4$\pm$2.3 mm (*Rab3a*$^{-/-}$); N=60 terminal pairs, from 3 mice each).

Furthermore, analysis of c-Fos positive neurons upon recall 1d after learning revealed a complete absence of ensemble activity rearrangements in CA3 upon fear conditioning, leading to comparable contents of c-Fos positive neurons upon re-exposure to TR or exposure to unrelated N, regardless of associative learning through aversive pairing (Fig. 4d). These results suggest that synaptic plasticity at LMTs in CA3 is required to re-organize pyramidal neuron ensemble activity in CA3 upon fear conditioning learning, to establish a precise memory of context in the hippocampus, and to induce learning-related FFI growth.

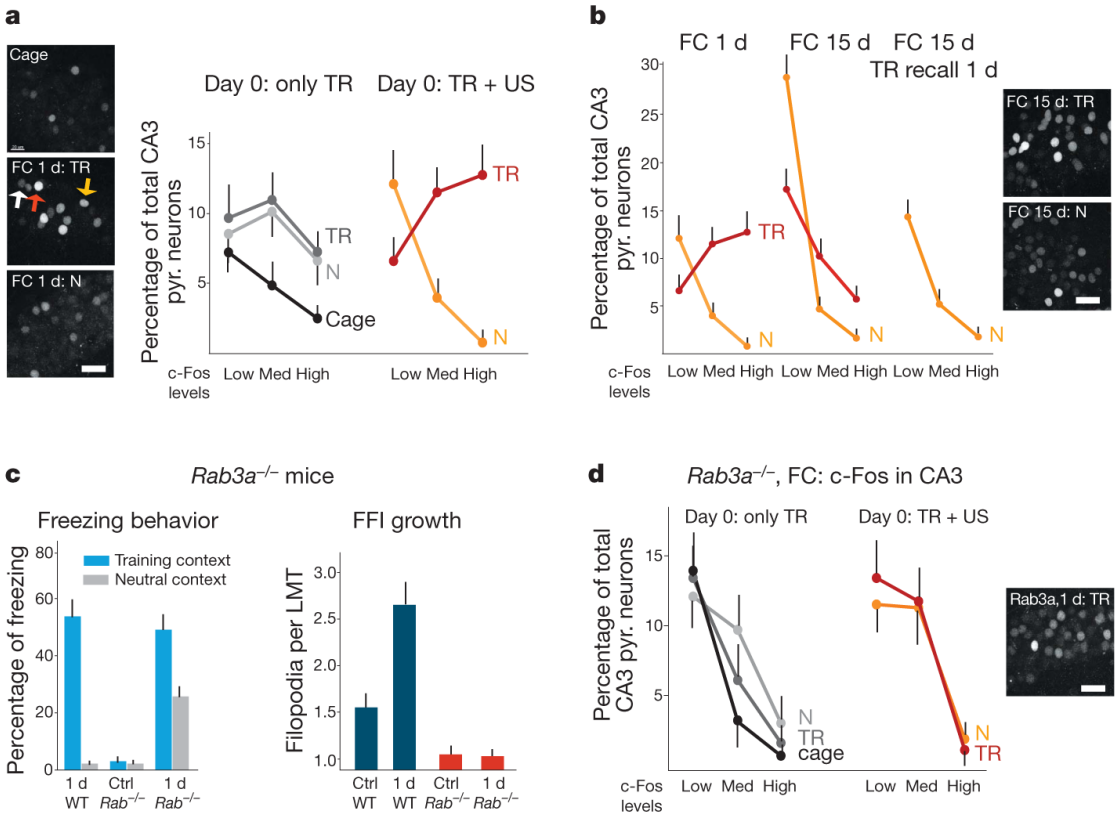


Figure 4 -Relationship between induction of c-Fos in CA3 pyramidal neurons and behavioral memory precision upon contextual fear conditioning. (**a**) c-Fos immunoreactivity in CA3 pyramidal neurons upon TR or N, with or without aversive association. Panels: representative examples of c-Fos immunoreactivity in CA3b. c-Fos neurons classified as weak (white arrow), medium (yellow arrow), strong (red arrow). N=3, 500 pyramidal neurons each. (**b**) c-Fos immunoreactivity in CA3 pyramidal neurons 15d after FC: effect of recall with TR. Details as in (a). N=1000-1500 pyramidal neurons, from 3 mice each. (**c**) Generalization and absence of learning-induced FFI growth in *Rab3a*$^{-/-}$ mice. N=5 mice (100 LMTs each). (**d**) c-Fos immunoreactivity in CA3 pyramidal neurons of *Rab3a*$^{-/-}$ mice upon FC. Details as in (a). N=1000-1500 pyramidal neurons, from 3 mice each. Bars: 20 µm.

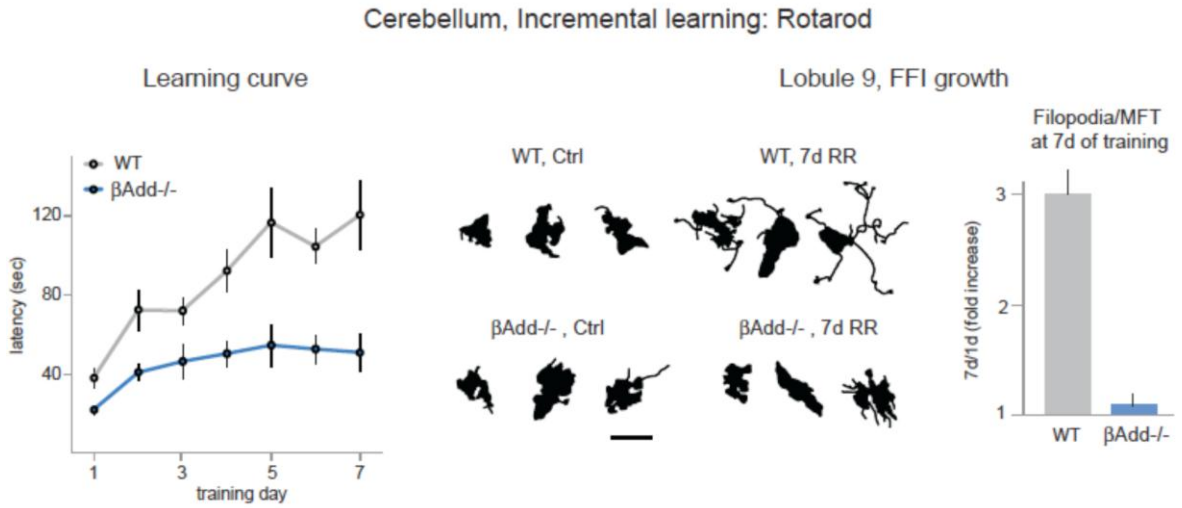
To test the notion that learning-related feedforward inhibition growth is necessary for memory precision, we then carried out learning experiments in β-*Adducin*$^{-/-}$ mice[10], which exhibit early LTP, but have a defect in synapse stabilization due to impaired linkage between the cell membrane cortex and the actin cytoskeleton (Bednarek and Caroni 2011). In naive *β-Adducin*$^{-/-}$ mice, average values of filopodia per LMT were closely comparable to those in wild type mice. Unlike *Rab3a*$^{-/-}$ mice, *β-Adducin*$^{-/-}$ mice did exhibit enhanced putative release sites per core LMT upon fear conditioning

(feedforward excitation connectivity; 5.64±0.11 (control) versus 6.62±0.15 (fear conditioning 1d) Bassoon positive puncta per 10 mm^3 of LMT volume; N=60 LMTs from 3 mice each). However, *β-Adducin*$^{-/-}$ mice completely failed to establish higher numbers of filopodia upon fear conditioning (feedforward inhibition connectivity; Fig. 5a). LMT densities in *β-Adducin*$^{-/-}$ mice were identical to those in wildtype mice (average LMT distances in CA3b were 98.2±2.1 mm (wildtype) and 98.1±1.8 mm (*β-Adducin*$^{-/-}$); N=60 terminal pairs, from 3 mice each). In parallel, and like *Rab3a*$^{-/-}$ mice, *β-Adducin*$^{-/-}$ mice learned to associate fear with context, but the memory was imprecise and mice already generalized 1d after fear conditioning (Fig. 5a).

In a second set of experiments we carried out Morris water maze spatial learning experiments in *β-Adducin*$^{-/-}$ mice. Again, *β-Adducin*$^{-/-}$ mice exhibited no learning-related increase in the content of filopodia per LMT (Fig. 5b). Mutant mice learned to locate the platform and established a reference memory of the correct quadrant (Fig. 5b). However, the characteristic improvements beyond 3-4 days were absent, and when the precision of the reference spatial memory was assessed by a quantitative analysis of swimming behavior as a function of platform position, mice lacking b-Adducin were clearly impaired (Fig. 5b). In a third set of experiments we determined whether *β-Adducin*$^{-/-}$ mice may also be impaired in learning-related filopodial growth at cerebellar mossy fiber terminals, and if so how that might affect cerebellar learning. We found that *βAdducin*$^{-/-}$ mice failed to establish extra filopodia at lobule 9 mossy fiber terminals upon rotarod training (Suppl. Fig. 6). In parallel, mice lacking b-Adducin were greatly impaired in learning the rotarod task (Supp. Fig. 6). Therefore, absence of learning-related feedforward inhibition connectivity growth in β-*Adducin*$^{-/-}$ mice is correlated with poor precision of the learned memory in the fear conditioning and Morris water maze paradigms, and with a near to complete failure to learn the rotarod task.

We then investigated c-Fos positive CA3 pyramidal neuron ensembles in response to fear conditioning in the *βAdducin*$^{-/-}$ mice. In stark contrast to *Rab3a*$^{-/-}$ mice lacking mossy fiber LTP, and consistent with increased feedforward excitation connectivity, *βAdducin*$^{-/-}$ mice exhibited c-Fos ensemble re-organization responses in CA3 that were qualitatively closely comparable to those in wild-type mice (Fig. 5c). Remarkably, however, net total numbers of c-Fos positive neurons were more than 2.5 times higher

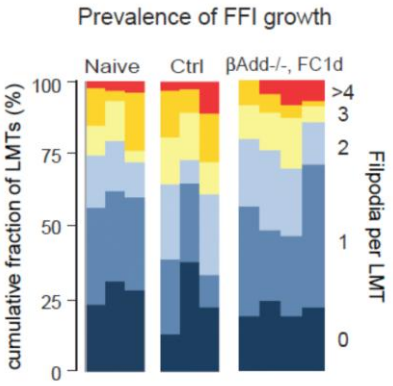
for each experimental condition in *β-Adducin*$^{-/-}$ mice compared to wild-type mice (Fig. 5c). By contrast, numbers of c-Fos positive pyramidal neurons in naive *β-Adducin*$^{-/-}$ mice were not higher than those in naive wild-type mice, indicating that the mutant mice did not just exhibit elevated levels of c-Fos in CA3 neurons (Fig. 5c). Consistent with the notion that the absence of feedforward inhibition connectivity growth at LMTs of *β-Adducin*$^{-/-}$ mice may account for the excess of c-Fos positive neurons under our experimental conditions, filopodia/LMT distributions in the mutant mice after learning were comparable to naïve, and not to TR control (no aversive stimulus) wild-type mice (Suppl. Fig. 7). In experiments aimed at further investigating how feedforward excitation and feedforward inhibition connectivity ratios at LMTs influence CA3 pyramidal neuron recruitment, we analyzed mice that had been housed under environmental enrichment conditions, a procedure leading to markedly enhanced contents of Bassoon puncta at core LMTs[30], but not to higher filopodial contents at LMTs (Fig. 2d). Consistent with the notion that enhanced levels of Bassoon puncta at core LMTs in the absence of a corresponding increase in filopodial synapses can produce elevate levels of c-Fos positive neurons in CA3, enriched mice exhibited c-Fos positive ensembles in CA3 that were markedly higher than those of mice housed under control conditions (Suppl. Fig. 8).



Supplementary Figure 6

Mice lacking βAdducin have a major deficit in learning the rotarod task, and fail to establish extra filopodia in cerebellar cortex lobule 9 upon rotarod training. Experimental details as in Fig. 1f. N=3 mice, 100 LMT each. Bar: 10 µm.

If the absence of feedforward inhibition connectivity growth at LMTs accounts for the excess of c-Fos positive CA3 pyramidal neurons upon fear conditioning in *β-Adducin*$^{-/-}$ mice, then the activation excess may be absent in the neurons of origin of the LMTs, i.e. dentate gyrus granule cells. Accordingly, to determine whether it was c-Fos positive neuronal ensembles in CA3 that were specifically enlarged in β-*Adducin*$^{-/-}$ mice, we also analyzed c-Fos positive dentate gyrus granule cells in the fear conditioning experiments. In wild-type mice, a re-organization of TR/N ensembles upon fear conditioning was also detected in granule cells, but it was much less dramatic than in CA3 (Fig. 5d). Notably, however, and in stark contrast to CA3, distributions and numbers of c-Fos positive granule cells in *β-Adducin*$^{-/-}$ mice were not different from those in wild-type mice for all experimental conditions tested (Fig. 5d). Therefore, *β-Adducin*$^{-/-}$ mice re-organized their CA3 pyramidal neuron ensembles like wild-type mice, but, consistent with a complete absence of feedforward inhibition connectivity growth at LMTs, they failed to restrict the numbers of activated pyramidal neurons in CA3 upon stimuli. Furthermore, c-Fos activation patterns in CA3 correlated with memory precision, whereas those in dentate gyrus did not, suggesting that the absence of *β-Adducin* in mossy fibers and their LMTs may account for the impaired memory precision in *β-Adducin*$^{-/-}$ mice.

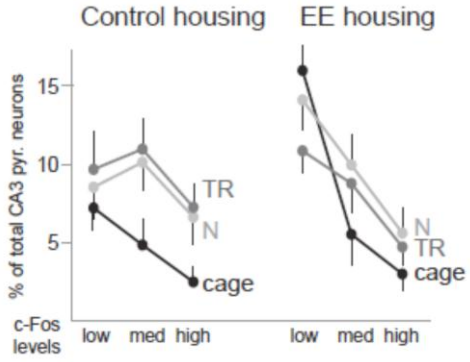


Supplementary Figure 7

Comparison of filopodia/LMT content distributions for individual wild-type (naïve; control, 1d) and *βAdducin*$^{-/-}$ (FC, 1d) mice. N=100 LMTs. Details as in Fig. 1e.

To establish a causal link between learning-related feedforward inhibition growth at LMTs and memory precision, we determined whether re-expression of β-Adducin specifically in granule cells and their mossy fibers was sufficient to rescue filopodial growth and memory precision upon fear conditioning. To achieve specific re-

expression in the adult, we expressed b-Adducin selectively in the dentate gyrus[20] of *β-Adducin*$^{-/-}$ mice using a lentiviral construct. One month after viral transduction, 15-22% of granule cells throughout the entire hippocampus exhibited virus-driven gene expression, whereas expression outside the dentate gyrus was extremely rare (Fig. 5e). The re-introduction of β-Adducin in mossy fibers was sufficient to rescue filopodial growth at LMTs of transduced granule cells in response to fear conditioning (Fig. 5f). Most notably, and in parallel to restored feedforward inhibition growth, re-expression of b-Adducin in granule cells rescued behavioral contextualization upon fear conditioning (Fig. 5g)



Supplementary Figure 8

Elevated recruitment of c-Fos in CA3 pyramidal neurons of mice housed under environmental enrichment (EE) conditions. Experimental conditions as in Fig. 4a. N=3, 400 pyramidal neurons each. EE housing was for 4 weeks. Control values are reproduced from Fig. 4a.

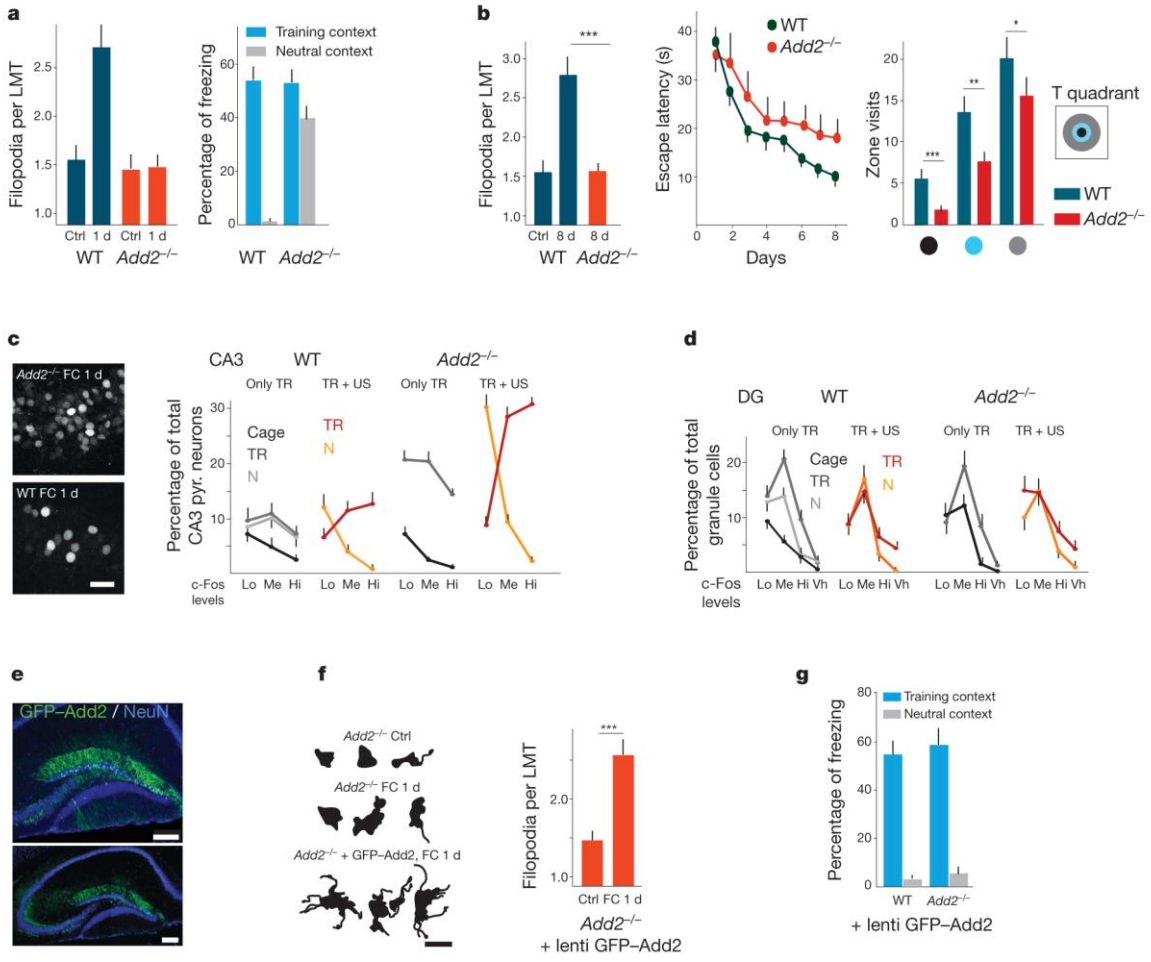


Figure 5 - Critical role of mossy fiber β-Adducin for feedforward inhibition growth at LMTs and hippocampal memory precision. (**a**) Absence of FFI growth upon contextual FC, and generalization in *β-Adducin*$^{-/-}$ mice. Conditions as in Fig. 4c. (**b**) Absence of FFI growth upon Morris water maze learning, and imprecise spatial memory in *β-Adducin*$^{-/-}$ mice. Conditions as in Fig. 1c. (**c, d**) c-Fos immunoreactivity in CA3 pyramidal neurons (**c**) and in DG granule cells (**d**) of wildtype and *β-Adducin*$^{-/-}$ mice upon TR or N, with or without aversive conditioning. Conditions as in Fig. 4a. (**e-g**) Rescue of FFI growth and contextualization, upon re-expression of β-Adducin in granule cells of adult *βAdducin*$^{-/-}$ mice. **e**: examples of transduced hippocampus (dorsal third of hippocampus). **f**: Rescue of feedforward inhibition growth; lucidas: transgene expression visualized by the GFP-β-Adducin construct in the absence of mGFP reporter. **g**: Behavioral rescue of contextualization. Conditions as in (a). Bars: 5 (f), 20 (c) and 200 (e) µm.

Discussion

Our results establish a causal relationship between learning-associated structural alterations in identified circuit connectivity and a specific behavioral output. We provide evidence that increased feedforward inhibition connectivity upon learning by mossy fiber LMTs in CA3 is critically important for the behavioral precision of learning-related hippocampal spatial memories. We further show that, upon learning, the increased feedforward inhibition connectivity is brought about through structural plasticity at a substantial fraction of LMTs in CA3, leading to about a doubling in the numbers of excitatory synapses onto Parvalbumin-positive inhibitory interneurons.

Our findings suggest that the absence of mossy fiber LTP in *Rab3a*$^{-/-}$ mice may compromise memory precision by failing to induce learning-induced re-organization of CA3 ensemble activity. This interpretation is consistent with the strikingly low contents of high- and medium-signal c-Fos positive neurons in the *Rab3a*$^{-/-}$ mice, and suggests that mossy fiber LTP has an important role for the behavioral precision of learning-related hippocampal memories. By contrast, in β-*Adducin*$^{-/-}$ mice ensemble re-organization was normal in CA3, but the sizes of c-Fos positive neuronal ensembles were greatly enlarged, and memory precision was again impaired. The absence of increased feedforward inhibition connectivity in *β-Adducin*$^{-/-}$ mice may thus compromise memory precision by failing to globally restrict network activity upon learning, thereby allowing otherwise sub-threshold partial stimuli to produce memory retrieval[19,31] (Suppl. Fig. 1). Consistent with this interpretation, elevated core LMT levels of Bassoon positive puncta persisted upon fear conditioning in wildtype mice after the extra filopodia had vanished (Suppl. Fig. 2a), time-dependent loss of the excess filopodial synapses coincided with behavioral generalization of the fear memory (Fig. 3c), and retrieval of the specific training context memory restored the excess filopodia and memory precision (Fig. 3c). This latter finding further suggests that upon filopodial retraction the specific memory did persist, within and/or outside the hippocampus, but activity patterns in CA3 elicited by a novel context were not sufficiently filtered to prevent behavioral generalization. A plausible interpretation of our findings is that retrieval and reconsolidation of the training memory through the hippocampal circuit re-activates plasticity sufficient to re-induce feedforward inhibition

growth and a high-threshold for subsequent activity flow[19,32]. The extent to which this process is intrinsic to the hippocampus remains to be determined.

Our results introduce a distinction between spatial learning, which is present in *β-Adducin*$^{-/-}$ mice, and the behavioral precision of the learning, which is compromised in these mutant mice. The distinction is consistent with the notion that the hippocampus is critically important for the precision of contextual memories[28]. Within the hippocampal circuit, the dentate gyrus establishes fine-grained representations of experience, which it transmits to CA3. Upon learning-induced potentiation, this high-resolution information may augment the detection of similarities among unrelated events through the associational network in CA3. Accordingly, filtering of the mossy fiber output through feedforward inhibition connectivity upon learning may support memory precision by restricting the extraction of relational representations in CA3. This interpretation is in line with the results of recent physiological studies, which have provided evidence that feedforward inhibition mainly acts to globally reduce network activity, without affecting relative activity patterns[32]. The interpretation is further reminiscent of early theoretical proposals suggesting that low activation thresholds upon LTP may prevent “perfect recall” within hippocampal networks through saturation processes, and that the recruitment of feedforward inhibition circuitry may be well suited to keep activation thresholds within sub-saturation ranges[33]. The increase in feedforward inhibition connectivity through structural plasticity discovered in this study may thus have important roles to ensure the precision of behaviorally relevant memories upon learning, under normal and pathological conditions.

Material and Methods

Rab3a$^{-/-}$ and *β-Adducin*$^{-/-}$ mice[9,10] were from Jackson laboratories, Bar Harbor, Maine; the reporter line *Thy1-mGFP(Lsi1)* was as shown before[5]. The mGFP lentivirus to trace mossy fiber projections was as described[20]; the GFP-b-Adducin construct was cloned into a lentivirus vector, and dentate gyrus infections were as described[20].

For anatomical analysis, mice were perfused with ice-chilled 4% paraformaldehyde in 0.1M PBS, and brains were post-fixed overnight. Hippocampi were mounted in 3% agarose blocks, and 100 mm transversal sections of hippocampi were cut using a McIlwain tissue chopper. Sections analyzed in this study were within 15% and 30% along the anterior-posterior axis. At least 4 sections were analyzed per mouse, and the data are based on 300-500 mm regions along the anterior-posterior axis. All LMTs that could be resolved in 3D within any given optical field (100x) were analyzed for filopodial contents. Filopodia were defined as processes emanating from LMTs of at least 2 mm length; varicosities were defined as end-swellings of at least 1 mm in diameter.

The immuno-electron microscopy analysis was according to a published procedure[34]. For c-Fos analysis, mice were perfused for 90 min after the last memory recall. Quantitative analysis of Bassoon puncta and c-Fos-positive nuclei was performed using a computerized image analysis system (Imaris 7, Bitplane). Nuclei were detected automatically as spheres of 8 mm, and the software yielded distributions of c-Fos-positive nuclei. Intensity thresholds for CA3 were defined as follows: low (> 280, < 450), medium (> 450, < 700), high (> 700; the highest values were about 1400).

Statistical analyses were performed using Student's *t*-tests and one-way ANOVA; *post hoc* comparisons were at the $P<0.05$ level of significance. Results are presented as mean ± s.e.m.

All behavioral experiments were carried out with male mice that were 55-65 days old at the onset of the experiment, and were according to standard procedures. Data from training sessions and probe trials were collected and analyzed using Viewer2 Software (Biobserve).

Cued and contextual fear conditioning was carried out in the Mouse Test Cage (Coulbourn Instruments). Freezing behavior was scored using Ethovision software (Noldus). Mice were excluded from the dataset if they failed at the behavioral analysis; this was the case when mice failed to extinguish fear responses to TR (2 mice), exhibited weak freezing to TR in the recall experiments at 15d (3 mice), exhibited signs of behavioral extinction upon recall of TR at 15d (7 mice), or failed to learn the Morris water maze (1 mouse). All subsequent morphological and immunohistochemical analyses of behaviorally treated mice were carried out blind to behavioral conditions.

Reagents and immunocytochemistry

Antibodies were from the following sources, and were used as follows: parvalbumin, Swant, 1:5'000; VGluT1, SySy (Göttingen), 1:1'000; GAD65/67, Millipore, 1:1'000; c-Fos, Santa Cruz, 1:10'000; NeuN: Chemicon, 1:200; Bassoon, Millipore, 1:200; Alexa-labeled secondary antibodies, Molecular Probes, 1:500. For immunocytochemistry, tissues were permeabilized with 0.2% Triton X-100 in PBS with 10% BSA. Antibody incubations were overnight at 4^{o}C. Fluorescence was imaged on either an upright spinning disk microscope consisting of a Yokogawa CSU22 confocal scanning head mounted on a Zeiss Axioimager M1 using a 100x alphaPlan-Apochromat 1.45 (Zeiss) oil-immersion objective, or on an LSM510 confocal microscope (Zeiss) using a 63x (1.4) oil-immersion objective.

c-Fos analysis

For c-Fos analysis, all samples belonging to the same experimental set were processed in parallel. Occasional sections in which NeuN signals were lower than average, or where c-Fos signal intensities varied within different regions of the section were discarded as technically poor. All images were acquired with the same settings, which were defined in order to avoid saturation of the highest c-Fos signals in CA3 and DG, and to still detect background levels outside cell clusters. Cells were binned according to labeling intensities using an automatic procedure, and the same threshold settings were used for all experiments. For DG granule cells, the thresholds were as follows: low (> 280, < 450), medium (> 450, < 700), high (> 700, < 1000), very high (> 1000; the highest values were about 2200). c-Fos immunoreactive neurons

were counted using a minimum of four sections per animal, and normalized to the total number of NeuN positive nuclei within the neuronal layers in CA3 or DG. In a first series of experiments, batches of naïve and FC control mice (TR, no US) were tested for inter-animal variability, which was found to be very low.

Behavioral experiments

The behavioral experiments were in accordance with institutional guidelines, and were approved by the Veterinary Department of the Canton of Basel-Stadt. Mice were kept in temperature controlled rooms on a constant 12h light/dark cycle, and experiments were conducted at the approximate same time during the light cycle.

For the MWM test, the 140 cm pool was surrounded by black curtains, and by 4 different objects. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface, and was kept in a fixed position. Mice were trained to find the platform during 4 trials a day, during up to 8 days. During training, mice were released from pseudo-randomly assigned start locations; they were allowed to swim for up to 60 sec, when they were manually guided to the platform in the case of failures. Inter-trial intervals were 5 min. Single probe trials to test reference memory were conducted 1 day after the last training session. Mice were released at a random start position, and were allowed to swim during 60 sec in the absence of the platform.

The conditioning context (TR) was rectangular, and was cleaned with 1% acetic acid before and after each session; the neutral context (N) had a cylindrical shape and was cleaned with 70% ethanol. Freezing was defined as the absence of somatic motility, except for respiratory movements. Exploratory activity was measured as body distance travelled over time. Once placed in the conditioning chamber, the mice were allowed to freely explore for 2.5 minutes, and they received 5 presentation of CS and US (1 sec foot shock, 0.8 mA; where indicated, 1000 Hz tone for 10 s, 70 dB sound pressure level, inter-trial interval 30s). The last 1 sec of each tone was paired with the US. Contextual fear conditioning involved the same protocol, but without the tone component. To test for contextual fear memory, mice were returned to TR (or N) during a test period of 2.5 min. To test for cued fear conditioning, mice explored for 2 min, followed by 5 tone presentations. The test was performed either in the

conditioning context (context- and tone-dependent freezing), or in a novel context (tone-dependent freezing). To test for context discrimination after FC, a within subjects design was used. On the test day, freezing was assessed in TR during 2.5 min, and 5 h later in N. Were indicated, mice were tested for generalization in N, followed 5 h and 24 h later by two brief recall sessions (TR or N). Subsequently, discrimination was tested in a second novel context (novel room shape; 0.25% benzaldehyde/ethanol).

Transmission electron microscopy

This procedure is described in detail elsewhere[34]. Briefly, mice were transcardially perfused with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS 0.1M pH 7.4. Right and left hippocampi were dissected, and 60 mm vibratome (Leica) sections were obtained, rinsed, cryoprotected and freeze-thawed in liquid nitrogen. Sections were incubated in first antibody (GFP, chemicon 1:1000) overnight, followed by biotinylated secondary antibody (Invitrogen 1:500). After incubation in the avidin-biotin peroxidase complex (ABC elite, Vector Laboratories), labeling was performed with DAB and hydrogen peroxide. Following the revelation of the labeling, sections were stained in osmium tetroxide and dehydrated. After impregnation with Durcupan resin (FLUKA) sections were flat-embedded between 2 silicon-coated glass slides and cured in a 60 degree oven for 48 h.

Transmission light microscopy was performed in stratum lucidum to search for large mossy fiber terminals with more than 3 filopodia. Appropriate blocks were then trimmed, and 60 nm serial sections were cut and collected on formvar coated slot-grids. Images of labeled terminal were acquired with a side-mounted digital camera (Veleta, Olympus) on a Philips CM10 transmission electron microscopy at 80kV, and a pixel size of 2.63 nm. In order to reconstruct the structure in 3D, images were aligned (Autoaligner, Bitplane, Zurich), and contours were drawn manually using Imaris 7.1.2 (Bitplane, Zurich). Surface rendering was achieved using Geometry converter (Johanna Wolf, FMI) and Blender.

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Author contributions

S.R. devised and carried out the analysis of hippocampal learning and connectivity; C.V. carried out the cerebellar experiments; E.B. devised and carried out the behavioral and rescue experiments on *b-Adducin*$^{-/-}$ mice; C.G. carried out the immuno-electron microscopy experiments; B.S. provided advice in planning and interpreting the fear conditioning experiments; P.S. provided advice on the cerebellar experiments; P.C. helped devise the experiments and wrote the manuscript. All authors discussed the results and commented the manuscript.

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2. RESULTS

2.2 GOAL-ORIENTED SEARCHING MEDIATED BY VENTRAL HIPPOCAMPUS IN TRIAL-AND-ERROR LEARNING

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Abstract

Most behavioral learning in biology is trial-and-error, but how these learning processes are influenced by the functions of individual brain systems is poorly understood. Here we show that ventral-to-dorsal hippocampal subdivisions have specific and sequential roles in trial-and-error maze navigation, with ventral hippocampus mediating early task-specific goal-oriented searching. We investigated how naive mice learn to navigate a water maze, a hippocampus-dependent task. Although performance and strategy deployment progressed continuously at the population level individual mice exhibited discrete learning phases each characterized by search habits (repeated deployment of the same strategy). Learning phase transitions reflected feedforward inhibitory connectivity (FFI) structural plasticity in ventral (vH), then intermediate (iH), and finally dorsal hippocampus (dH). FFI growth at vH occurred abruptly upon behavioral learning of goal-task relationships. Excitotoxic vH lesions, or absence of vH FFI growth in mice lacking β-Adducin, delayed early learning and disrupted performance consistency. iH lesions impaired intermediate place learning, and delayed but did not prevent spatial learning, whereas dH lesions or absence of dorsal FFI growth specifically disrupted late spatial learning. Trial-and-error navigational learning processes in naive mice thus involve a stereotype sequence of increasingly precise subtasks learned through distinct hippocampal subdivisions. Because of its unique connectivity, vH may relate goals to internal states in learning under healthy and pathological conditions.

Introduction

Trial-and-error forms of learning and memory produce sustained modifications in behavioral output[1,2], but whether and how the sequences of learning processes that lead to mastering of complex biologically relevant tasks underlie principles reflected in the organization and recruitment of distinct brain systems has remained unclear. In biology, trial and error learning has mainly been investigated in the context of striatal circuits, which support cumulative learning of skill and habits[3,4]. These studies revealed that series of increasingly effective habits support optimization of learning processes, and avoid cognitive overload during learning of complex tasks[3,5]. Such habits may be implemented by parallel loops of striatal circuits, which in turn may be recruited according to partially hierarchical principles[6,7].

Effective trial and error learning is not likely to involve random sequences of learning processes. Thus, theoretical studies have provided evidence that because trial-and-error learning unfolds without presentation of correct models, it depends critically on the effective implementation of appropriate explorative strategies[1,2]. Furthermore, in order to effectively address complex tasks, the learning process should be broken down into subtasks addressed independently by separate subsystems[8]. Finally, machine and animal learning studies have suggested that effective early strategies should focus on local associations to goals, but whether and how such goal-oriented searching occurs has remained unclear[1,8].

The hippocampus has a critical role in rapid episodic learning[9-11]. It thus provides an attractive system to investigate implementation mechanisms of trial-and-error navigation tasks that involve combining episodes in space and time. Although local circuits are comparable throughout the hippocampus, tuning, connectivity, gene expression and functions of hippocampal ventral, intermediate, and dorsal subdivisions differ substantially[12-18]. Through their connectivity within distinct brain networks[19,20], vH (anterior in humans) is thought to be more concerned with emotions and body states[14], whereas dH (posterior in humans) predominantly has visuo-spatial and cognitive roles[12,13,15]. While dH has an important role in spatial learning[12,13,15], the function of vH in learning has remained unclear. This may be due to the fact that most studies have focused on the endpoint of complex hippocampus-dependent tasks,

which is often highly spatial and cognitive, and on average population performances, instead of how individual animals learn a task.

Hippocampal learning can be associated with a robust local growth of new excitatory synapses by large mossy fiber terminals (LMTs) in CA3 onto fast-spiking GABAergic interneurons (FFI growth), which have a critical role for memory precision[21]. The new synapses fail to stabilize in mice lacking β-Adducin[22], and local virus-mediated reintroduction of β-Adducin in granule cells restores the structural plasticity and memory precision[21]. At the physiological level, the FFI growth is required to recruit comparatively small ensembles of c-Fos expressing pyramidal neurons in CA3 upon learning, a function that appears to underlie the role of FFI growth in memory precision[21]. Here we exploited these structural traces of learning to map and probe the involvement of hippocampal subdivisions during navigational trial-and-error learning. We investigated longitudinally how individual naive laboratory mice learn to navigate a Morris water maze[23], and combined these detailed behavioral analyses with anatomical and local genetic rescue studies in order to dissect the roles of hippocampal subdivisions in this complex trial-and-error learning process.

Results

Strategy analysis in individual mice reveals three phases of maze learning

We first analyzed the average learning patterns of mice in the Morris water maze. In this task a hidden platform is kept at a fixed position, and mice each time start from different positions in the circular maze. Performance is recorded as escape latency to locate the platform during training trials (**Fig. 1a**), and spatial memory is assayed as persistence to search within the platform quadrant in the absence of the platform (reference memory)[12,23]. In addition to these conventional readouts, and in order to augment the power of the analysis, we segmented the behavior of individual mice according to the incidence of distinct search strategies[24-26] as a function of training trial (**Fig. 1b**, Methods). Consistent with previous reports[24-26], this detailed behavioral analysis revealed that mice applied qualitatively different search strategies as they became increasingly proficient at this spatial task, with global (random swim) and then

local search strategies (scanning, chaining) predominant during early phases of learning, and spatial search strategies (directed search, focal search, direct swim) taking over during late phases (**Fig. 1c**). Average latencies and strategies evolved continuously as a function of trial number (**Figs. 1a, c**).

We then analyzed the learning curves of individual mice. In contrast to what could be detected at the population level, individual mice exhibited striking search habits, consisting of the repeated deployment of the same search strategy (at least 3 consecutive trials, see Methods), interrupted by 1-2 trials involving alternative, in most cases "more advanced" search strategies (**Fig. 1d**). Individual latency curves exhibited substantial oscillations during the first half of the learning process (**Fig. 1d**). To provide average measures of these individual behaviors, we then determined whether features of the search habits might be shared among cohorts of mice during learning. A detailed analysis of 28 such individual learning curves revealed that a majority of mice (21/28) ended a first block involving strategy 2 (random swim) after trial 7 (i.e. just before the end of day 2), initiated a second block involving strategy 3 (scanning) or 4 (chaining) at trial 10-12 (i.e. during day 3), which was followed by a third series of blocks involving strategies 5 (directed search) and 6 (focal search) at trial 22-26 (i.e. between days 5 and 6) (**Fig. 1e**). A majority of mice (21/28) exhibited the characteristic 2-4-5/6 (13/25) or 2-3-5/6 (7/25) strategy progression, and a smaller fraction of mice (3/28) exhibited 2-5/6 patterns (**Fig. 1d**, 4th mouse; **Suppl. Fig. 1**). When cohorts of mice were compared, individual search habits thus appeared to begin and end at comparable stages during learning. Consistent with this notion, average total block lengths per mouse and search habit were also comparable (strategy 2: 5.2$\pm$1.2 trials; strategy 3: 6.7$\pm$0.95 trials; strategy 4: 3.65$\pm$0.85 trials; strategies 5+6: 5.46$\pm$1.17 trials; **Fig. 1f**; Methods). Furthermore, even when mice explored alternative strategies during a particular search habit, corresponding individual latencies matched average values for the particular learning phase across the mouse cohort (**Fig. 1e**). Taken together, these results suggested that the learning processes of individual naive mice might be structured into distinct learning phases characterized by distinct search habits. In most mice, the learning phases corresponded to training days 1-2 (trials 1-8; first phase), days 3-5 (trials 9-20; second phase) and days 6-9 (trials 21-36; third phase).

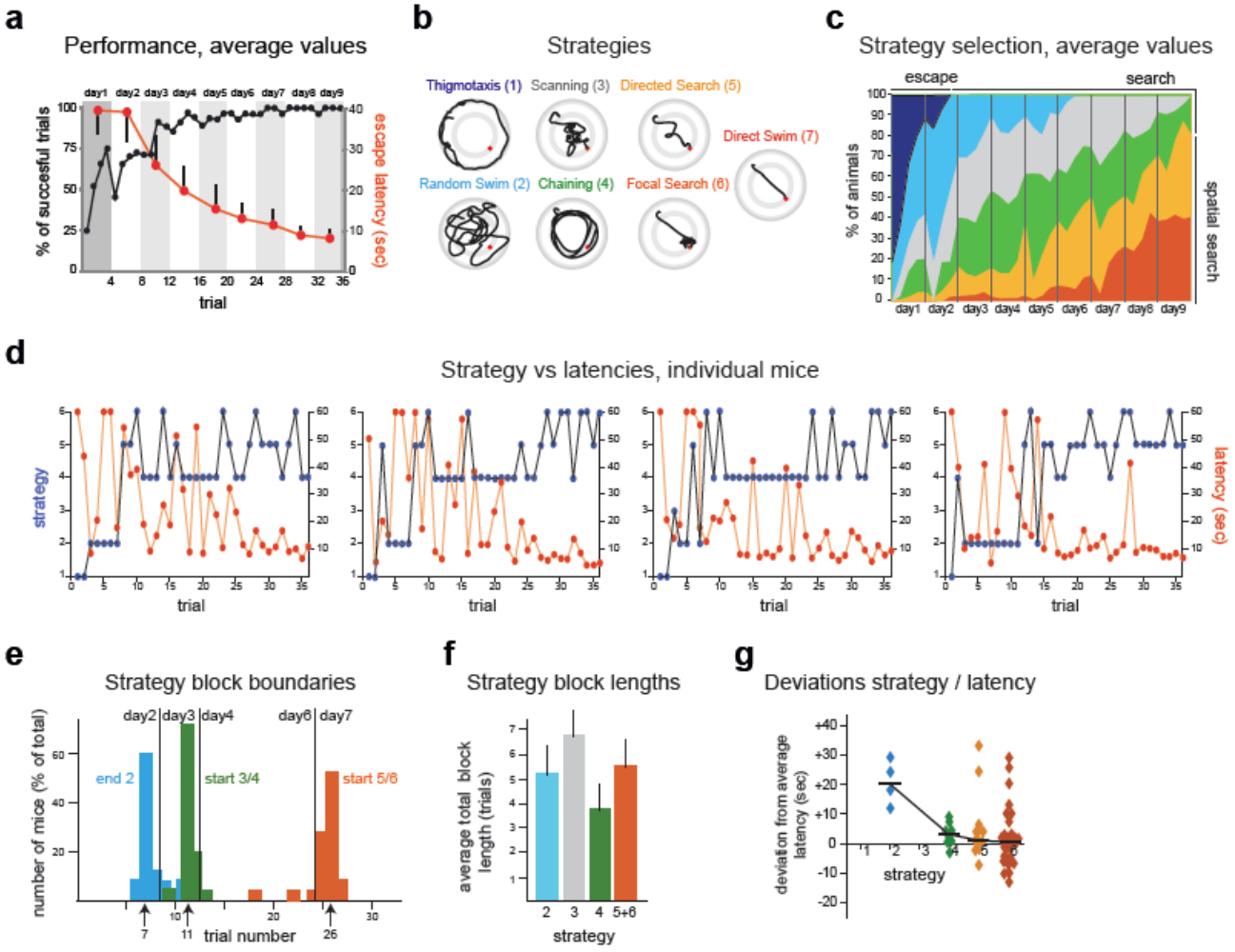
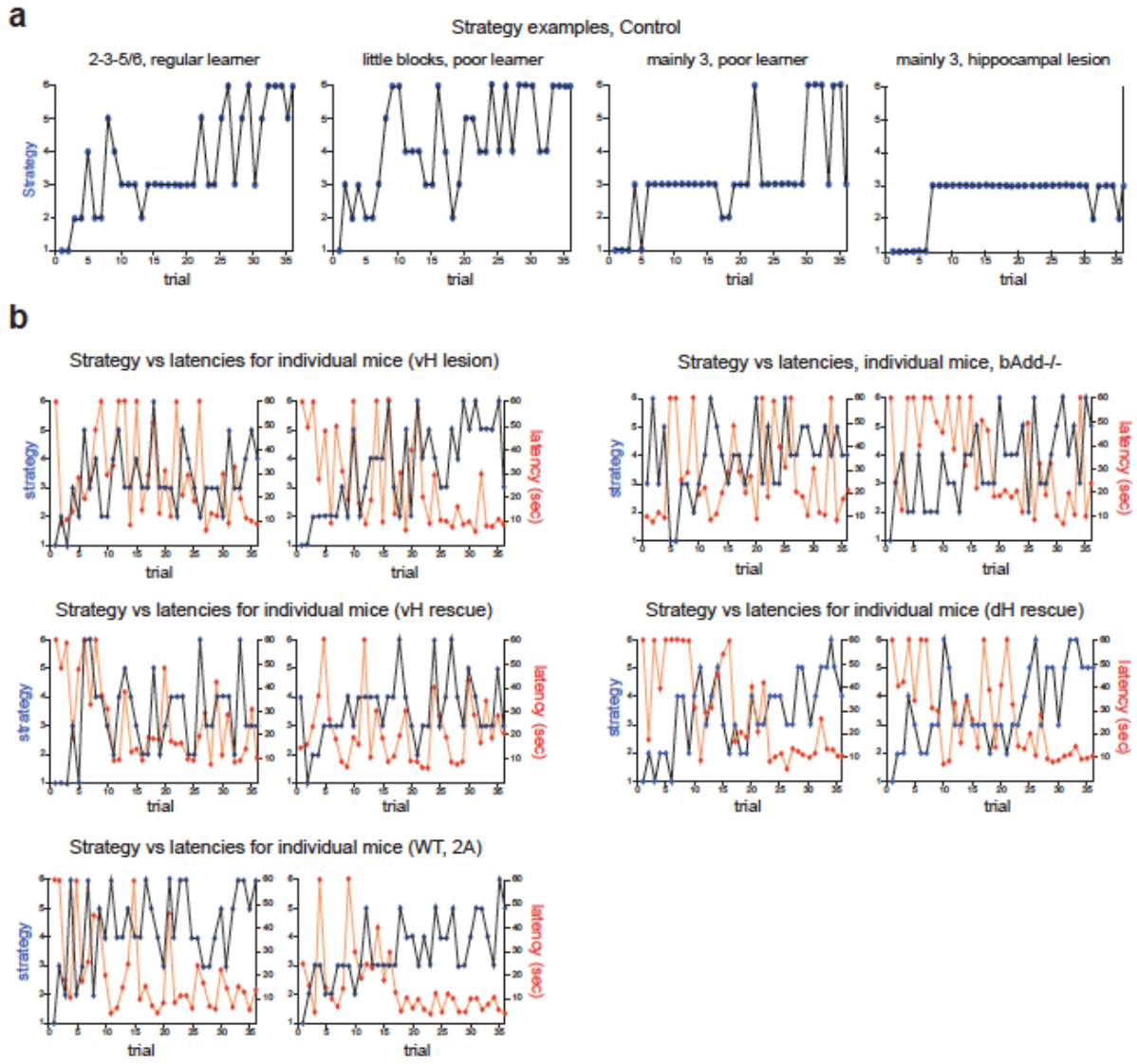


Figure 1. Sequential deployment of strategy habits during water maze learning. a-c, Behavioral analysis of water maze learning at population level. Mean values for 30 mice. Performance (**a**): average latencies to reach the platform, and fraction of successful trials at each trial and training day. Strategy selection (**b, c**): schematic representation and color code for each strategy (**b**); average prevalence of individual strategies for each trial (**c**). **d-g**, Behavioral analysis of water maze learning at level of individual mice. **d**, Strategy/latency versus trial plots for four representative mice. **e**, Distribution of strategy block boundaries for 25 individual mice. The percentages of mice for which a strategy 2 block (blue) ends, blocks 3 or 4 begin (green), and blocks 5 or 6 begin (orange) are represented as a function of trial number. **f**, Total block length values for individual mice, averaged over 25 mice. **g**, Extent to which the latencies of individual trials involving the exploration of alternative strategies differed from the mean latencies for that phase of the training process. The individual values in the plot represent the latency deviations for individual trials, ordered according to the strategy of those exploratory trials (25 mice; see also Methods).

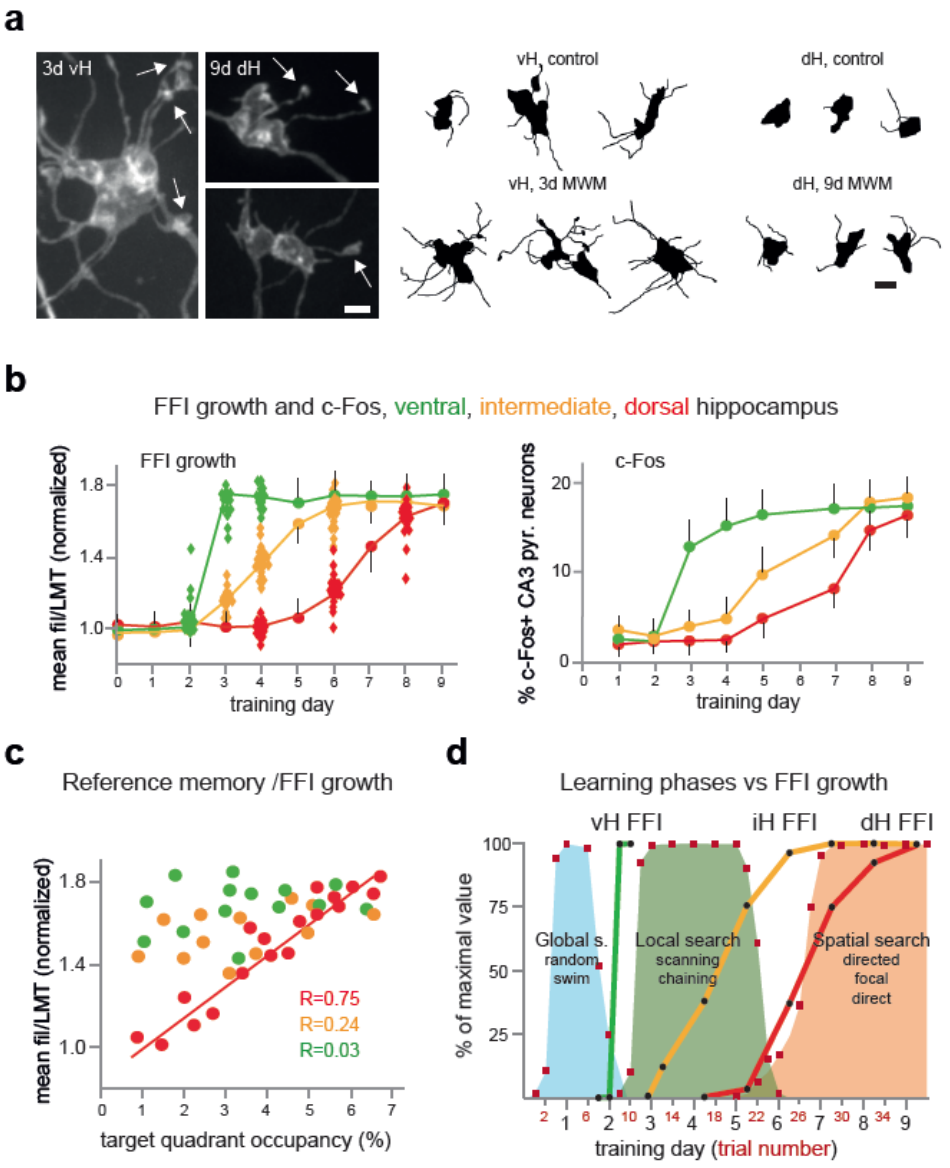


Supplementary Figure 1. Sequence of subtasks involving ventral-to-dorsal hippocampal subdivisions in complex trial-and-error learning. The schematic summarizes how an unsupervised trial-and-error learning process such as maze navigation is structured stereotypically into subtasks, which are implemented by individual hippocampal subdivisions (e.g. dorsal hippocampus (dH) for global search). The subtasks build upon previously learned relationships (arrows), and lead to learning about increasingly defining features of the particular task. FFI growth upon learning focuses subsequent searches, supporting the deployment of strategy habits. The results suggest that the connectivities and functions of hippocampal subdivisions preconfigure how individual members of a species address declarative trial-and-error tasks involving the hippocampus.

FFI growth at hippocampal subdivisions during maze learning

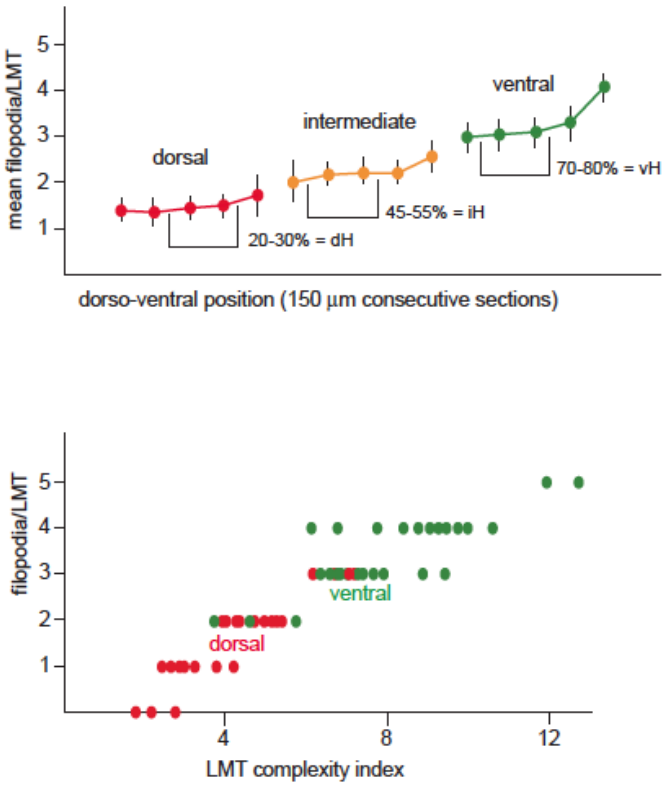
In a search for a neural basis for the distinct phases during maze learning, we investigated hippocampal patterns of FFI growth at LMTs in CA3b[21]. Cohorts of mice underwent repeated daily training of different total durations (4 trials per day, as described above), and filopodia/LMT values were determined on the day that followed the last training day. We detected distinct baselines and learning-related patterns of filopodia/LMT values in vH, iH and dH (mean baseline number of filopodia/LMT: 1.74±0.1 dH, 2.32±0.1 iH, 3.11±0.15 vH; **Figs. 2a, b, Suppl. Fig. 2**; Methods). In vH, filopodial values increased abruptly after day 2 (12/12 analyzed mice), to reach plateau levels 1.8-fold higher than ventral baseline values by day 3 (**Fig. 2b**). No filopodial increases were detected hours after the last trial on day 2, suggesting that this FFI growth reflected an overnight memory consolidation process (not shown). The dramatic switch in synapse numbers was accompanied by a corresponding increase of c-Fos recruitment upon training in CA3b pyramidal neurons from day 3 in vH (% c-Fos+ vH neurons: day1: 2.34±1.2, day2: 3.2±1.6 n.s., day3: 13.4±2.4 $p<0.001$; **Fig. 2b**). In iH, filopodial growth was first detectable on day 3, and increased gradually to reach plateau values (again ca. 1.8-fold higher than intermediate baseline levels) by day 5-6 (**Fig. 2b**). FFI growth in iH was specifically correlated to increasing c-Fos recruitment upon training in the same subdivision (% c-Fos+ iH neurons: day1: 2.68±1.3, day6: 16.95±1.7 $p<0.01$; **Fig. 2b**). Finally, in dH, filopodia/LMT values exhibited no significant increase up to day 5 of the training protocol, and then increased gradually to reach plateau values (again ca. 1.8-fold higher than dorsal baseline values) at day 8-9 (% c-Fos+ dH neurons: day1: 1.8±1.2, day9: 15.4±2.2, $p<0.01$; **Fig. 2b**). As for vH and iH, FFI growth in dH was specifically correlated to increased c-Fos recruitment in that hippocampal subdivision (% c-Fos+ dH neurons: day1: 1.8±1.2, day9: 15.4±2.2, $p<0.01$; **Fig. 2b**). Furthermore, FFI growth in dH was correlated with the quality of reference memory in individual mice, whereas FFI growth in vH or iH was not (Pearson correlation: dH $R=0.75$ $p<0.001$, iH $R=0.24$ n.s., vH=0.03 n.s.; **Fig. 2c**).

Figure 2



Sequential recruitment of hippocampal subdivisions during maze learning.

a, Representative examples of GFP-positive LMTs (left: photographs; right: camera lucidas) in CA3b of vH or dH on day 3, respectively day 9 of the maze training procedure. Arrows: varicosities (putative presynaptic terminals[21]) at the tips of filopodia. Bars: 5 □m. **b**, FFI growth and CA3b pyramidal neuron c-Fos during maze training in vH, iH and dH. N= 6-10 mice. Individual dots at days 2, 3, 4, 6 and 8: filopodia/LMT values for individual mice shown for transition time points. **c**, FFI growth at dH, but not iH or vH is correlated to spatial learning (reference memory). Dots represent values from individual mice, collected between day 5 and day 9 of the training procedure. **d**, Schematic illustrating the relationship between the prevalence of search habits of defined strategy (blue, green and orange areas) and FFI growth at vH, iH and dH as a function of trial number (red, search habits) and training day (black, FFI growth) during maze learning. Individual values are averages from the data shown in **Figs. 1e** and **2b**.



Supplementary Figure 2. Strategy/latency analysis of individual mice during maze learning. a, Additional examples of strategy deployment by individual mice under control conditions. The second (nearly no strategy blocks) and third (extensive strategy 3 blocks) mouse were among the 20% slowest learners, whereas the fourth mouse did not reach a latency of 25sec at trial 36, and exhibited an obvious reduction in hippocampal volume. **b**, Examples of strategy/latency plots for mice with vH lesions, vH or dH β-Adducin rescue, and for mice learning a maze with a 2A platform.

Maze learning phases correlated to FFI growth at vH, then iH, and finally dH

A comparison of how average search strategy habit distributions (per trial number) and FFI growth at hippocampal subdivisions (per day) evolved during maze learning is shown in **Fig. 2d**. In the majority of mice, vH FFI growth (at day 3, but not yet at day 2) anticipated the onset of local search habits such as scanning and chaining (at trial 10, > 80% of mice). Gradual FFI growth at iH (from day 3 to day 6) coincided with the deployment of local search habits, and reached plateau values when most mice switched to spatial search habits. Finally, gradual FFI growth in dH coincided with the consistent deployment of spatial search strategies. Together, these findings suggested that, in mice, trial-and-error learning to navigate a water maze involves

distinct learning phases. The results were consistent with a specific role for dH in fine scale spatial map learning late in maze navigation, and suggested that first vH, and then iH may have distinct roles during earlier phases of maze learning.

vH FFI growth reflects behavioral learning of specific task-goal associations

What aspects of trial-and-error tasks may be learned through vH? Since this hippocampal subdivision is connected to goal-related networks, and recent studies have provided evidence that it contains single units tuned to goal[14,17,18], we explored the possibility that vH may support the learning and implementation of task-specific associations to reward-related goal[27]. We further considered the possibility that vH may learn task-specific associations to rewards regardless of whether these may be positive or negative.

First, to determine whether any form of hippocampus-dependent learning may produce FFI growth at vH LMTs, we investigated mice that underwent a novel object recognition (NOR) task in the absence or presence of a positive food reward. In this mossy fiber-dependent task[22] mice explore an environment that contains two identical objects on day one. On day two they re-explore the same environment in which one of the objects has been replaced with a novel one, recognize the old object, and spend more time exploring the novel object. We repeatedly exposed mice to a novel object each day for up to 15 days, in the presence or absence of a food reward associated with the familiar object. In the absence of reward (incidental learning), mice exhibited no alterations in novel object discrimination upon subsequent training sessions, and no alterations in filopodia/LMT values in neither vH nor dH (**Fig. 3a**; NOR). However, when a food reward was repeatedly associated with the familiar object, relative exploration time values for the familiar object increased gradually from day 3-4 on (**Fig. 3b**; reinforced FOP). From day 11-12 on, but not yet on day 10, mice exhibited a strong preference for the familiar object even when tested in the absence of food reward (reference memory: day10: NOR 0.38$\pm$0.2, FOP, 0.21$\pm$0.15, n.s.; day11: NOR 0.34$\pm$0.1, FOP -0.17$\pm$0.34, n.s.; day12: NOR 0.35$\pm$0.05, FOP -0.42$\pm$0.18 $p<0.05$; day15 NOR 0.38$\pm$0.02, FOP -0.42$\pm$0.09, $p<0.01$), indicating that the familiar object-reward association had been learned, and had become a behavioral bias (**Fig. 3b**). In close correlation with this reward-controlled learning, mean filopodia/LMT values

increased selectively between days 10 and 12 in vH but not dH (filopodia/LMT: dH NOR12d 1.77±0.1, dH FOP12d 1.72±0.1, n.s.; vH NOR12d 2.89±0.1, vH FOP12d 4.97±0.3 $p<0.01$; **Fig. 3b**). When familiar object preference was compared to mean filopodia/LMT values in individual mice, FFI growth was correlated with successful reference memory (i.e. learning), and not with training day (**Fig. 3b**). In control experiments, 15 days protocols that included the same reward positioned randomly in the test box and no objects to explore, did not affect filopodia/LMT values (not shown). These results suggest that converting an incidental goal-free task into a reward-based one is sufficient to induce FFI growth at vH upon behavioral learning (tested as reference memory). The substantial delay between preferred exploration of the familiar object associated with reward, and the development of a behavioral bias, may reflect an evaluation that takes into account the relative values of the novel object and the familiar object with reward, and the confidence that the familiar object will invariantly involve a reward. Because acquisition of a behavioral bias and FFI growth were closely correlated at the level of individual mice, the results further suggest that in these experiments FFI growth occurred in vH within one day of bias learning.

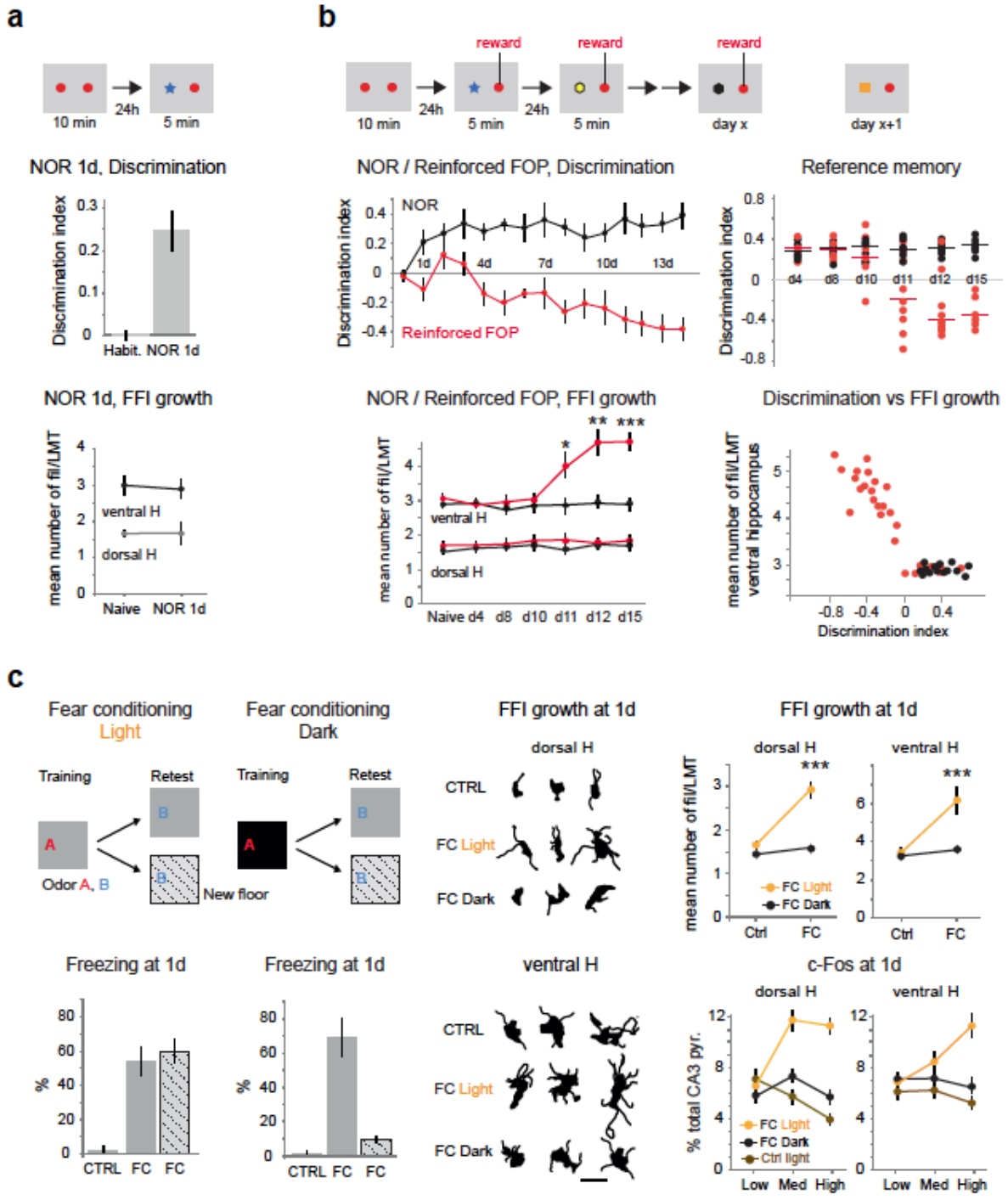


Figure 3. vH FFI growth reflects behavioral learning of specific task-goal associations.

a, Absence of FFI growth at vH and dH upon novel object recognition (NOR). Top: schematic of experimental protocol, involving habituation (10 min) on day 0, and discrimination test (5 min) on day 1. Familiar object in red. Mice explore identical objects equally (Habit.), but preferentially explore the novel object (NOR) on day 1. N=20. **b**, Converting an incidental NOR task into a reward-based one is sufficient to induce FFI growth specifically at vH upon learning. Top graphs: schematic of experimental protocols. Left, middle graph: training protocols with (red traces; Familiar Object Preference, FOP) and without (black traces; NOR) reward. Negative discrimination index values reflect longer exploration of familiar object. N=30. Left, lower graph: corresponding FFI growth values. N=3-7. Right: reference

memory test (in the absence of reward) 1 day after last training day, as indicated; each dot represents an individual mouse, with (red) and without (black) reward. **c**, FFI growth in vH and dH upon contextual, but not upon explicit fear conditioning (FC). Left: schematic of FC protocols under light (contextual) and dark (explicit) conditions (top row), and corresponding freezing behavior 1d after learning (lower row). Center: representative camera lucidas of LMTs in CA3b of vH and dH. Bar: 10 □m. Right: contextual, but not explicit FC induces FFI growth (upper graphs) and enhanced CA3b pyramidal neuron c-Fos recruitment (lower graphs) in vH and dH. FC values 1 day after learning. N=20.

To investigate whether learning to persistently associate context to reinforcers and exhibit learning-related FFI growth in vH involves specific types of experiences, we carried out fear conditioning experiments (negative reward) in which we varied the contextual conditions. Protocols in which mice received a sequence of mild footshocks (negative reinforcer) upon exploring a new environment that included a defined odor produced rapid (within hours) undistinguishable 1.80-fold increases in filopodia/LMT values at vH and dH (1.83-fold changes, $p<0.001$ in dH and vH; **Fig. 3c**). We then removed visuo-spatial components from the protocol by pairing an odor to footshock in complete darkness (explicit, but non-contextual learning). Mice that had been conditioned with this olfactory learning protocol in the dark froze when re-exposed to the same odor or the same cage floor under daylight conditions in an otherwise novel context, indicating that they learned to associate these explicit features to the aversive stimulus (**Fig. 3c**). However, the mice did not freeze when both odor and cage floor had been changed in the original context, and they exhibited no alterations in mean filopodia/LMT values, and no alterations in c-Fos recruitment in vH or dH (filopodia/LMT dark FC: dH 1.67$\pm$0.1, vH 3.22$\pm$0.1, n.s.; **Fig. 3c)**. These results are reminiscent of the perceptual requirements for hippocampal encoding[28], and suggest that FFI growth upon behavioral learning is only induced in vH if the behavioral protocol involves conditions producing (possibly spatial) hippocampal associations between context and reward. The results further suggest that FFI growth at hippocampal subdivisions is preceded by learning-related plasticity at the same hippocampal subdivisions.

Specific task-goal association reflected by vH FFI growth during maze learning

We then determined whether vH FFI growth in maze navigation might involve learning about any goal, or rather about a task-specific association to reward-related goal[27]. As expected, when mice were subjected to the maze protocol in the absence of a platform, and hence in the absence of a reward-related goal, no increase in filopodia/LMT values was detected at any time in vH (filopodia/LMT vH: naive 3.25±0.2, free swim 2d 3.29±0.3, n.s.; **Fig. 4a**). In further support of an association with reward-related goal, vH FFI growth was induced to undistinguishable extents whether the platform was hidden or visible (**Fig. 4a**). Notably, however, when the position of a visible platform was changed from a peripheral position on day 1 to a position closer to the center of the pool on day 2, no FFI growth was detected in vH on day 3 (**Fig. 4b**). By contrast, changing the position of the visible platform within a comparable position from the wall did not suppress vH FFI growth on day 3 (**Fig. 4b**). We further found that: (a) omitting the platform on the first or second day of training suppressed FFI growth (**Fig. 4a**); (b) two days of training each involving 4 trials were required for FFI growth (**Fig. 4c**); (c) the second day of training did not have to immediately follow the first day in order to elicit FFI growth (**Fig. 4c**); (d) introducing a goal-free training day in the absence of a platform between the first and second day of goal-oriented learning inhibited ventral FFI growth upon the second platform training day (**Fig. 4c**). Taken together, these result suggested that in maze learning structural plasticity in vH on day 3 involves establishing a consistent association on day 1 between the goal (the platform) and a specific task-related aspect (e.g. the distance of the platform from the wall), and confirming that association on day 2 (**Fig. 4d**).

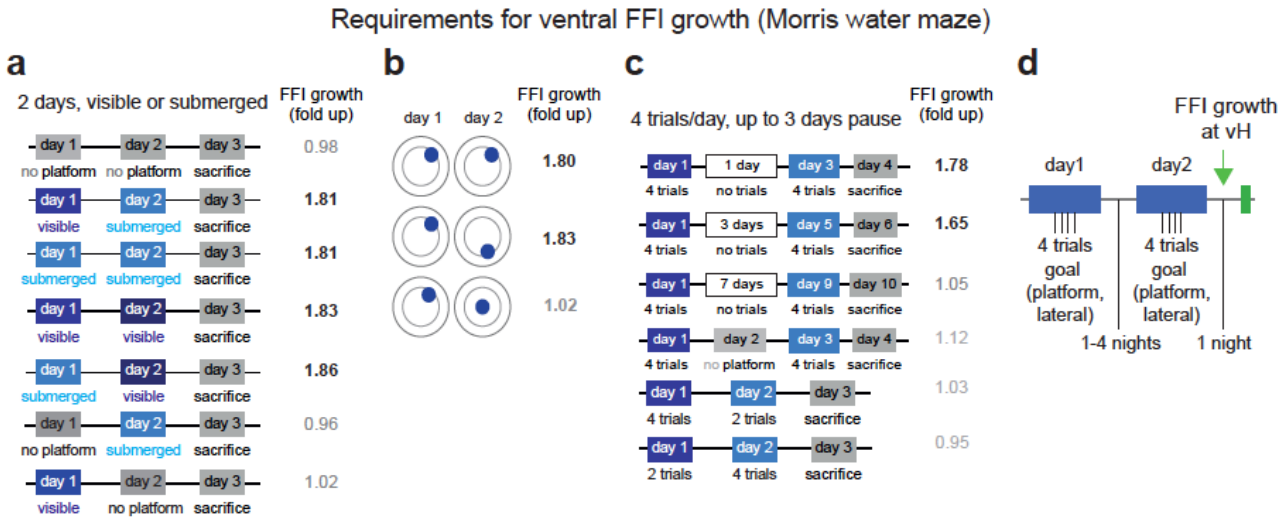
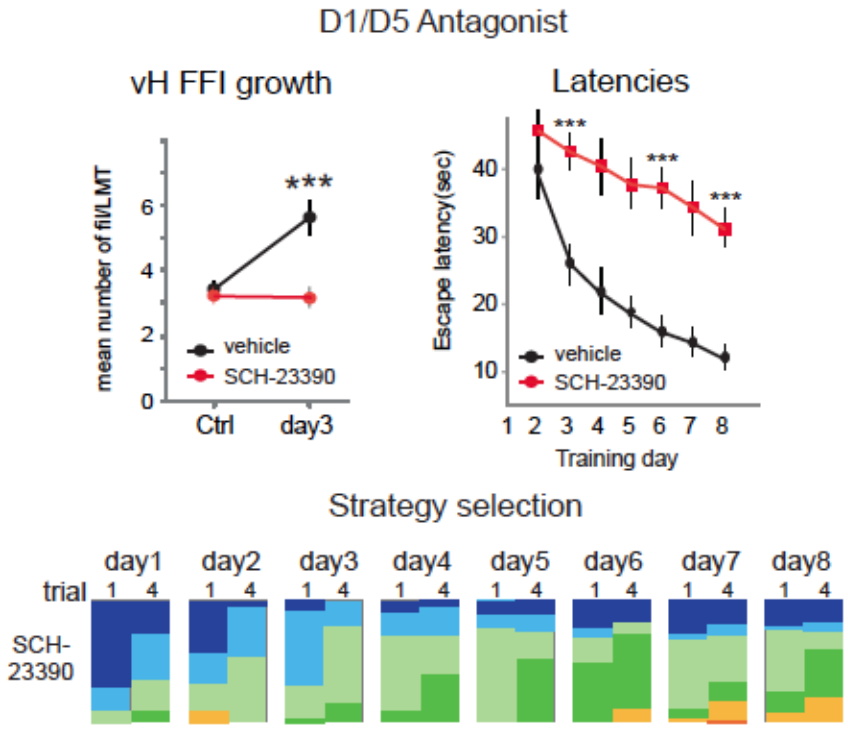


Figure 4. Specific task-goal association reflected by vH FFI growth during maze learning.

a-c, Analysis of experimental conditions that produce FFI growth at vH. No platform: four 60sec swim trials in the maze; no trials: mice kept in home cage. **b**, Role of platform position constancy on vH FFI growth. Moving a visible platform to a new position, but at a comparable distance from the wall on day 2 does not compromise FFI growth on day 3 (middle row), whereas moving the platform towards the center of the maze on day 2 suppresses FFI growth on day 3 (lower row). Mean values; N= 5-8 mice. **d**, Schematic of requirements for FFI growth at vH.

To provide additional evidence that FFI growth at ventral hippocampus does involve a classical reward mechanism, we interfered with signaling by the reward neuromodulator dopamine[29] and its D1/D5 receptor during water maze learning[30]. To this end, the D1/D5 antagonist SCH23390 was applied systemically 20min before each training day, and control mice were treated with vehicle lacking the drug. The antagonist interfered with learning and strategy selection throughout the training procedure, and completely blocked FFI growth in ventral hippocampus (**Suppl. Fig. 3**).



Supplementary Figure 3. Distinct filopodia/LMT baseline values correlated to LMT complexities in dorsal, intermediate and ventral hippocampus. Upper graph: Mean filopodia/LMT values along the dorso-ventral axis in the hippocampus of mice housed under standard cage conditions. Isolated hippocampi were sectioned transversally, and the dorsal and ventral ends (ca. 250□m and 460□m respectively) were excluded from the analysis. The connected bars indicate the regions along the dorso-ventral axis of the hippocampus that were defined as dorsal (20-30% of total length, dH), intermediate (45-55%, iH) and ventral hippocampus (70-80%, vH) in this study[16]. Note how mean filopodia/LMT values increase substantially along the dorso-ventral axis, but are comparable within hippocampal subdivisions. The ventral end of ventral hippocampus exhibited mean filopodia/LMT values that were substantially higher than those in the rest of ventral hippocampus. Lower graph: Positive correlation between filopodia/LMT values and LMT complexities for individual LMTs (Methods). This finding suggests that at the level of individual LMTs filopodial numbers (which are proportional to FFI synapses onto parvalbumin interneurons[21]) are correlated to the numbers of feedforward excitatory synapses onto pyramidal neurons in CA3.

Role of vH in water maze learning

Having defined features of the task that lead to FFI growth at vH early during maze training, we next addressed the role of this hippocampal subdivision during water maze learning. We reasoned that the hippocampal subdivisions might be involved in the learning process throughout training; alternatively, each subdivision may make its specific contribution at distinct phases of the learning process.

We first analyzed mice with excitotoxic bilateral lesions of vH. All mice included in the analysis exhibited nearly complete lesions of vH CA3 (and CA1), and less than 15% losses at iH CA3 (**Suppl. Fig. 4**). Ventrally lesioned mice exhibited strongly compromised latency values at days 3-5 of training (escape latency day3: vehicle 23.2±5.5s vH lesion 36.5±4.1s, $p<0.01$), and improved later to reach values comparable to controls at days 10-11 (day10 vehicle 10.3±2.2s, vH lesion 12.2±3.1s n.s.; **Fig. 5a**). The progression of local search strategy deployment was disrupted during days 2-4 (strategy4: day4 Ctrl 28±3.3%, vH lesion 15±6.5, $p<0.01$); the onset of spatial search strategies was delayed, but their deployment during late phases of maze learning was comparable to control mice (strategy5+6: day9 Ctrl 33±3.8%, vH lesion 29±5.1%, n.s.; **Fig. 5b**). Strategy habits were less prominent; the lesions particularly affected random swim, whereas scanning habits were comparatively preserved (**Suppl. Fig. 1**; **Fig. 5c**). Furthermore, vH lesions produced a loss of correlation between single trial strategies, single trial latencies, and mean latencies (**Suppl. Fig. 5**). Likely reflecting this inconsistency of individual trial latencies, individual lesioned mice exhibited strikingly unpredictable trajectories of daily variations in latency values through most of the training procedure (**Fig. 5d**). In spite of these disruptions in performance consistency, ventrally lesioned mice exhibited delayed but ultimately normal reference memory[15] and extents of FFI growth in dorsal hippocampus (**Fig. 5e**). These results suggest that vH is important to support learning during days 2-5, when global and local search strategies predominate, and to support search and performance consistency throughout maze learning.

To further define the role of vH in maze learning, we devised experiments in which learning-related FFI growth was confined to vH. In □-*Adducin*$^{-/-}$ mice deficient in learning-related FFI growth and synaptogenesis[22,31] improvements in latency values

were reduced throughout the maze learning procedure (latency day3: wildtype 24.5±1s, mutant 38.3±3.4s p<0.01; day5: wildtype 14.8±3.2, mutant 32.1±2.6s p<0.001; day7: wildtype 12.3±2s, mutant 23.8±2.3s, p<0.001; **Fig. 5f**). Furthermore, β-*Adducin*$^{-/-}$ mice exhibited very little learning-related habits throughout training (**Fig. 5h**). In mutant mice in which □-Adducin had been reintroduced locally in vH, but not iH or dH granule cells using a lentivirus expressing GFP-β-Adducin[22], learning-related FFI growth was specifically rescued in vH (filopodia/LMT: day3 mutant 2.78±0.3, vH rescue 5.96±0.3, p<0.0001; **Suppl. Fig. 6**). Supporting the notion that behavioral learning involving vH has a critical role specifically during early phases of maze learning, ventral rescue restored average latency and strategy curves to wildtype values from day 3 and up to day 5 of training (latency day5: wildtype 14.8±3.2s, vH rescue 20.1±4s, n.s.), whereas further improvements beyond that early phase were still inhibited (latency day11: wildtype 10.2±1.1s, mutant 19.4±1.9s, vH rescue 16.1±1.7, p<0.05; **Figs. 5f, g**). The analysis of individual mice revealed that the second learning phase (local search habits) was specifically rescued (average block length strategy 4: wildtype 3.65±0.85, mutant 1.1±0.41, p<0.01, vH rescue 2.45±1.1 n.s.), whereas the first phase (average block length strategy 2: wildtype 5.2±1.2, mutant 0.2±0.1 p<0.001, vH rescue 0.51±0.4 p<0.01) and the third phase (average block length strategy 5+6: wildtype 5.46±1.2, mutant 0.84±0.3 p<0.001, vH rescue 0.76±0.4 p<0.001)) were not (**Suppl. Fig. 1**, **Fig. 5h**).

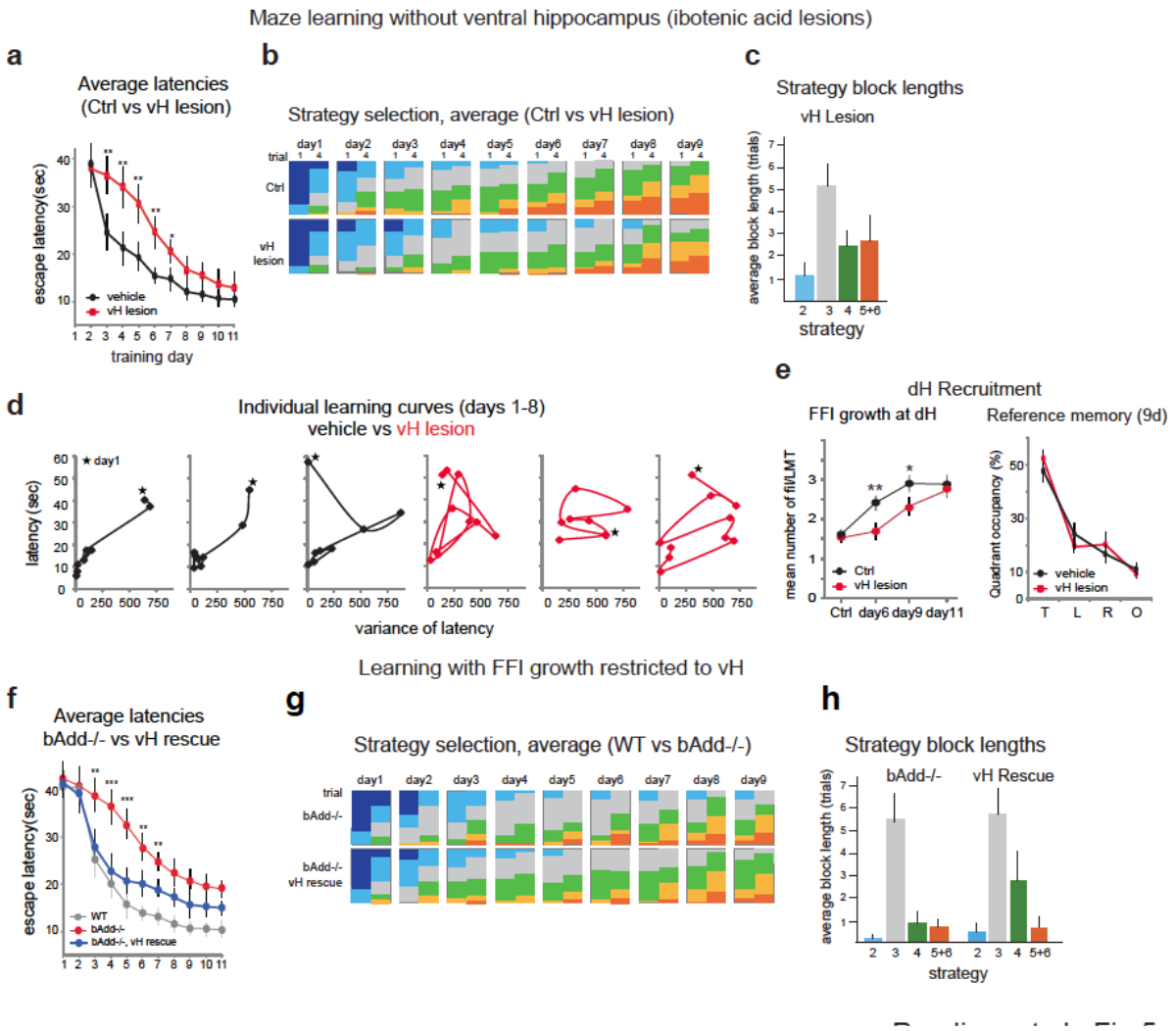


Figure 5. Role of ventral hippocampus in water maze learning.

a-e, Maze learning by mice with excitotoxic lesions of vH (vH lesion). Analysis as described in **Fig. 1**. N= 15 mice each. **a, b**: Population level analysis of latencies (**a**) and strategies (**b**). To highlight progress within and between days, the strategy plot (**b**) reflects the mean strategy recruitment values for the 1st and 4th trial of each day. **c**: Strategy block lengths, as described in **Fig. 1f**. **d**, Enhanced variability of individual trials during maze learning in vH lesioned mice. Latency versus latency variance plots for three individual mice each. Asterisk: values for first training day; lines connect values from day 1 to day 8. **e**, Delayed but undiminished dH FFI growth and reference memory in vH lesioned mice. T: target; L: left; R: right; O: opposite. N= 4 each (FFI growth) and 15 each (reference memory). **f-h**, Learning with FFI growth restricted to vH. Impaired maze learning by *β-Adducin*$^{-/-}$ mice, and specific rescue of early learning phase upon reintroduction of β-Adducin into granule cells of vH. Population and individual mouse analysis as described in (**a-c**). N= 12 mice.

Role of iH in water maze learning

To investigate the role of iH in maze learning we analyzed mice with complete bilateral excitotoxic lesions of iH (**Suppl. Fig. 4**). None of the mice exhibited neuronal losses extending for more than 10% into the anterior-posterior extension of adjacent vH or dH (data not shown). The learning curves of mice with iH lesions were specifically delayed between day 4 and day 7 of the learning process, and iH lesioned mice reached latency values comparable to non-lesioned controls by day 11 (latency day11: vehicle 9.8±2s, iH lesion 11.4±2, n.s.; **Fig. 6a**). iH lesioned mice exhibited much smaller variations in latency across individual trials than vH lesioned mice (**Fig 6c**; compare to **Fig. 5d**). A strategy analysis revealed a majorly prolonged deployment of local search habits (scanning, chaining; average block length strategy4: 6.12±0.7, $p<0.05$), and delayed deployment of spatial search strategies (**Figs. 6b, d**). Consistent with this delay in spatial search strategies, iH lesioned mice exhibited greatly impaired spatial reference memories at day 9, but control reference memory values at day 11 (target quadrant occupancy: vehicle 9d: 47.8±3%, iH lesion 9d: 34.2±4% $p<0.05$, iH lesion 11d 48.1±5%, n.s.; **Fig. 6e**). Taken together these results suggest that iH makes a specific contribution during intermediate phases of water maze learning[32], when local search habits are deployed, and before the appearance of spatial search habits. Consistent with previous reports[15], the presence of an intact iH is not an absolute requirement for spatial learning.

Role of dH in water maze learning

Mice with bilateral excitotoxic dH lesions (**Suppl. Fig 4**) were specifically impaired during the late phases (days 6-11) of maze learning (latency at day11: vehicle 9.2±2.3s, dH lesion 16.5±2.1s, $p<0.01$; **Figs. 7a, b**). dH lesioned mice exhibited no abnormally large variations in inter-trial latencies throughout maze learning (**Fig. 7d**; compare to **Fig. 5c**), and normal strategy deployments up to day 5-6 of training, but failed to consistently deploy spatial search strategies during late phases of maze learning (average block length strategy 5+6: dH lesion 0.9±0.6, $p<0.001$; **Figs. 7b, c**). Consistent with previous reports that dH is specifically required for spatial learning[13,15], mice with dH lesions failed to establish a spatial reference memory (**Fig. 7e)**.

β-Adducin rescue specifically in dH of *β-Adducin*$^{-/-}$ mice markedly improved latencies at days 7-11, but did not influence latency values at days 3-5 of training (**Fig. 7f**). Consistent with a specific role for dH in spatial learning late during maze training, reintroducing β-Adducin in dH granule cells improved the progression of global spatial search strategies (**Fig. 7g**), and rescued the deployment of spatial search strategy habits (average block length strategy5+6: mutant 0.84+0.3, dH rescue 3.95±0.7, p<0.001; **Fig. 7h**), but did not improve local strategy deployment (average block length strategy4: dH rescue 0.54±0.1 n.s.; **Figs. 7g, h**), nor the correlation between single trial and mean latencies (**Suppl. Fig. 5**). These results suggest that dH is required specifically to establish a spatial map of the task during late phases of water maze learning, and suggest that learning processes involving vH or dH may be recruited independent from each other during a hippocampal spatial task.

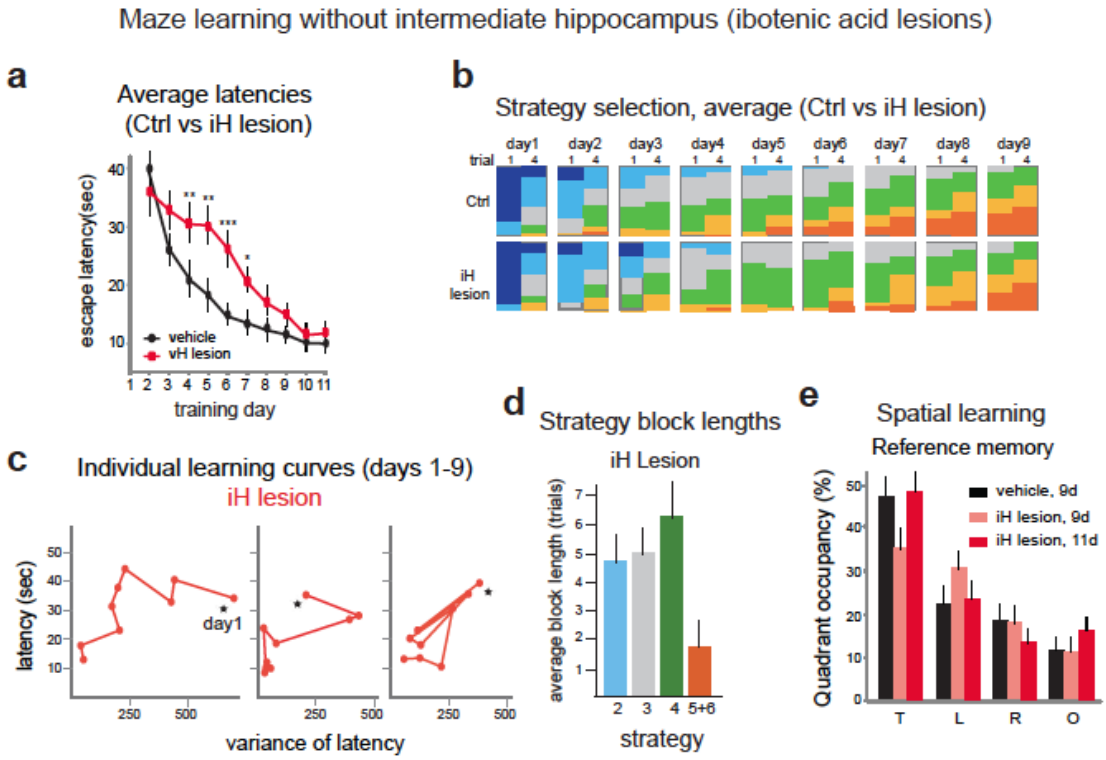


Figure 6. Role of intermediate hippocampus in water maze learning.

a-e, Maze learning by mice with excitotoxic lesions of iH. Analysis as described in **Figs. 1** and **5**. N= 8 mice each. **a, b**: Population level analysis of latencies (**a**) and strategies (**b**). **c**, Variability of individual trials during maze learning in iH lesioned mice. Latency versus latency variance plots for three individual mice (analysis as in **fig. 5d**). **d**: Strategy block lengths, as described in **Fig. 1f**. **e**, Delayed but undiminished reference memory in iH lesioned mice. N= 8 mice.

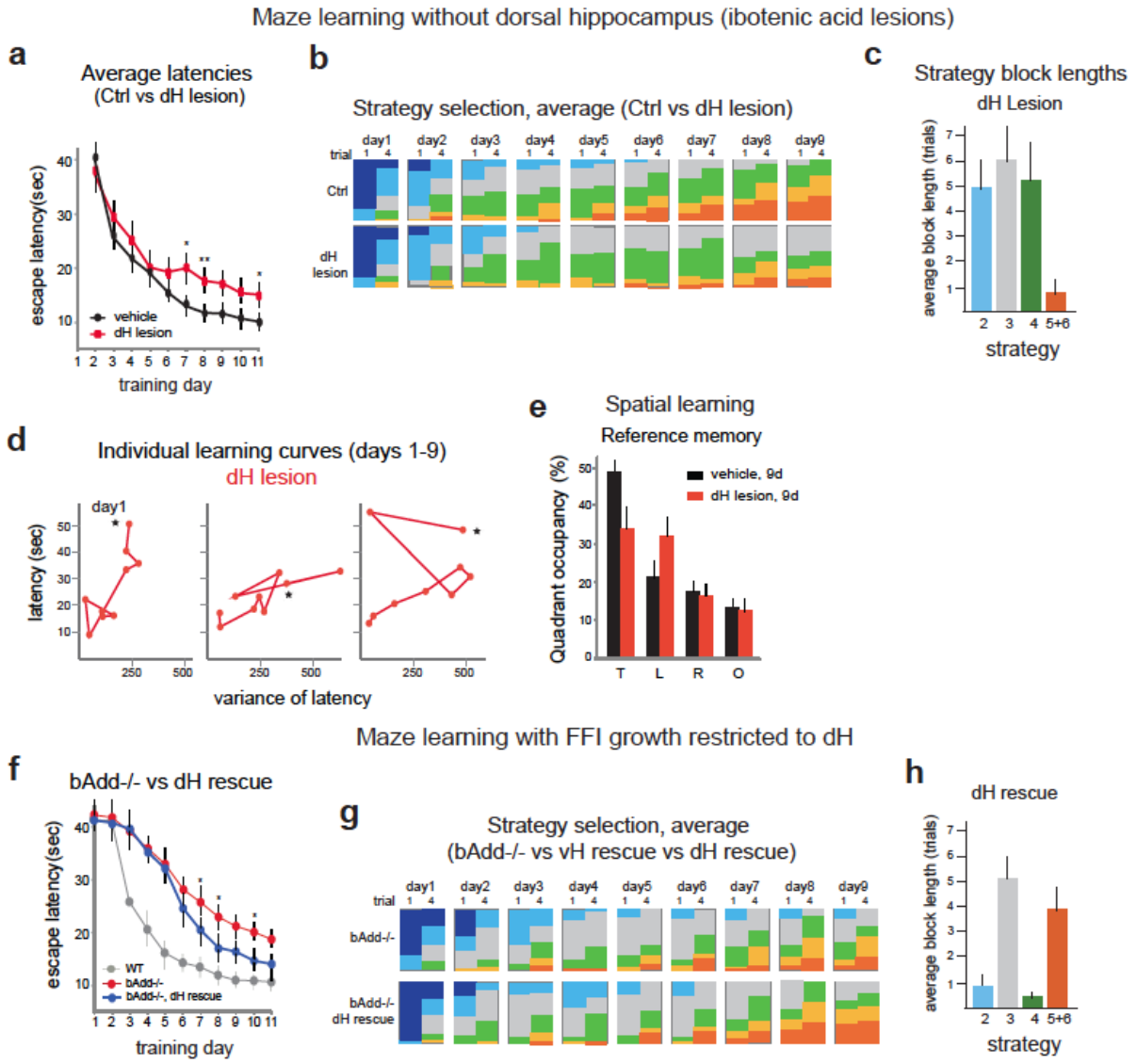
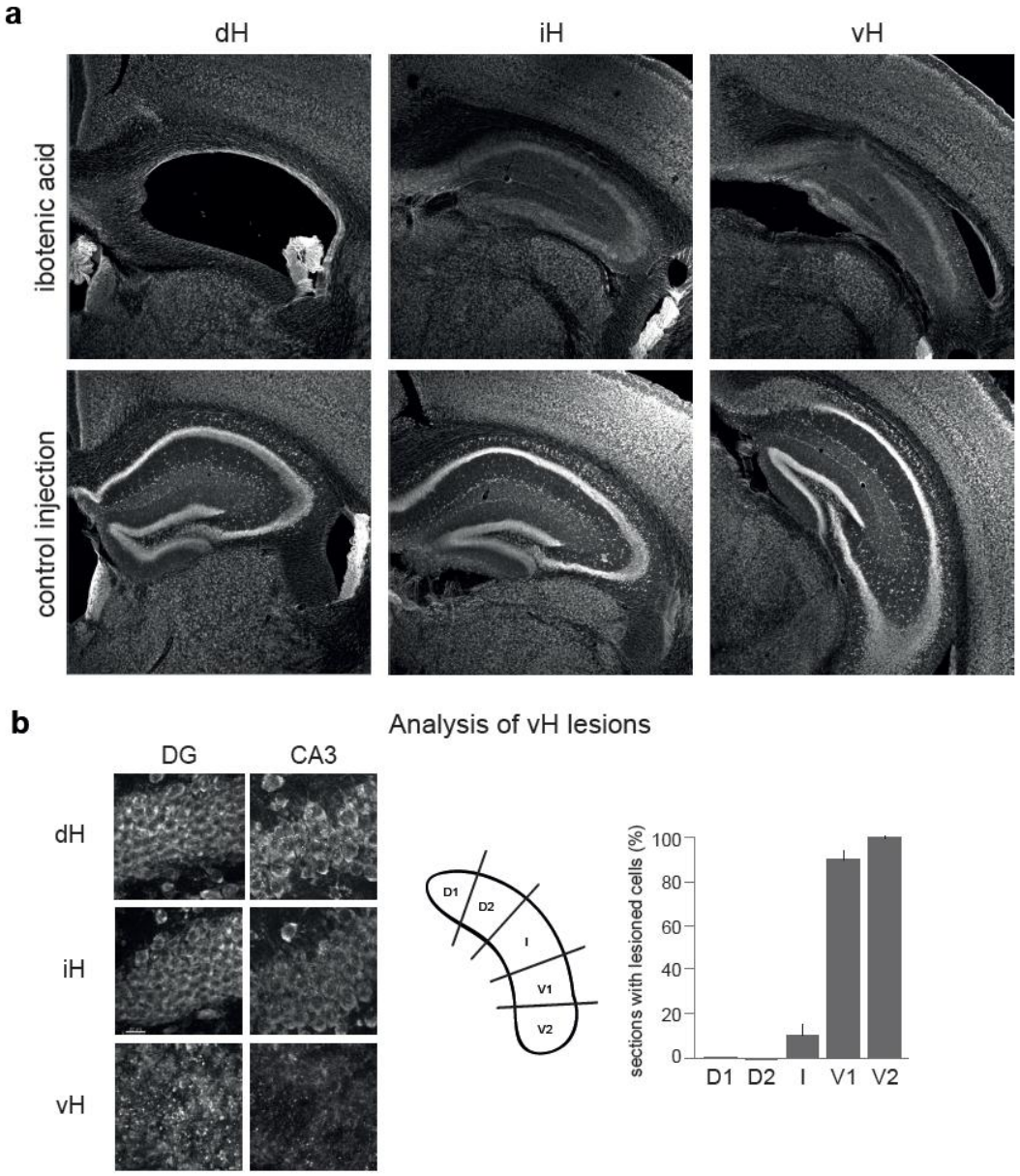


Figure 7. Role of dorsal hippocampus in water maze learning.

a-e, Maze learning by mice with excitotoxic lesions of dH. Analysis as described in **Figs. 1** and **5**. N= 8 mice each. **a, b**: Population level analysis of latencies (**a**) and strategies (**b**). **c**: Strategy block lengths, as described in **Fig. 1f**. **d**, Variability of individual trials during maze learning in dH lesioned mice. Latency versus latency variance plots for three individual mice. **e**, Absence of fine scale spatial learning (reference memory) in dH lesioned mice. **f-h**, Learning with FFI growth restricted to dH. Specific rescue of late learning phase upon reintroduction of β-Adducin into granule cells of dH. Population and individual mouse analysis as described in (**a-c**). N= 12 mice.



Supplementary Figure 4. Ibotenic acid lesions of ventral hippocampus. a, Representative example of ventrally lesioned hippocampus. Nissl staining. Note near to complete absence of intact cells in dentate gyrus and CA3 of ventral hippocampus, and absence of detectable damage in intermediate and dorsal hippocampus. **b**, Analysis of excitotoxic damage spread along the hippocampus of ventrally lesioned mice. Left: schematic of hippocampal regions along dorso-ventral axis. Right: fraction of sections with at least 10% damaged cells in CA3. N=20 mice (five 50□m sections each per hippocampal region).

Maze learning depending on vH, independently of dH

To investigate whether maze learning involving vH can occur in the absence of a requirement for dH, we developed modifications of the water maze task aimed at removing a requirement for fine-scale spatial learning. We reasoned that augmenting the area of the hidden platform might produce conditions under which mice would no longer need to systematically apply spatial search strategies to effectively locate the hidden platform. Indeed, when the circular platform area was doubled from a standard size of A (78.5cm^2, corresponding to 10cm diameter) to 2A (157cm^2, 14cm diameter, compared to a pool diameter of 140cm), mice learned the task more rapidly (latency at 2 days: 38.9$\pm$4.6s (A), 23.8$\pm$2.1s (2A), $p<0.001$; **Fig. 8a**), exhibited directed search and direct swim on each 4th trial throughout the training process (i.e. they took “shortcuts”), but no consistent increase in the application of spatial search strategies during the second half of the training procedure (**Figs. 8b, c**). Likely reflecting less challenging learning conditions and the successful application of "shortcuts", the analysis of individual mice revealed a reduced deployment of habits (**Suppl. Fig. 1**; **Fig. 8c**). Notably, while vH FFI growth was undistinguishable under A or 2A platform conditions (filopodia/LMT at 3 days: 5.94$\pm$0.7 (A), 6.1$\pm$0.8 (2A) n.s.), mice developed no reference memory of the platform quadrant (average target quadrant occupancy at 9d: 49.8+4% (A), 28.3+2% (2A) $p<0.01$), did not learn to directly head for the platform between day 7 and day 9 of training (fraction of heading angle error <10^o at 9d: 41.5$\pm$3.4% (A), 13.2$\pm$1.3%, $p<0.001$), and exhibited no increased filopodia/LMT contents in dH under 2A platform conditions (filopodia/LMT dH: 1.7$\pm$0.1 Ctrl, 2.88$\pm$0.2 (A, 9d), 1.74$\pm$0.1 (2A, 9d) n.s.; **Fig. 8d**). iH filopodia/LMT contents were also not increased upon 2A platform conditions (day 9; not shown). Consistent with the notion that dH is not required to learn to navigate a maze with a 2A platform, mice with dH lesions exhibited learning curves and strategy progressions undistinguishable from control mice under 2A conditions (latency at 2d: Ctrl 22.5$\pm$2.1, dH lesion 20.8$\pm$1.8, n.s.; **Figs. 8a, b**).

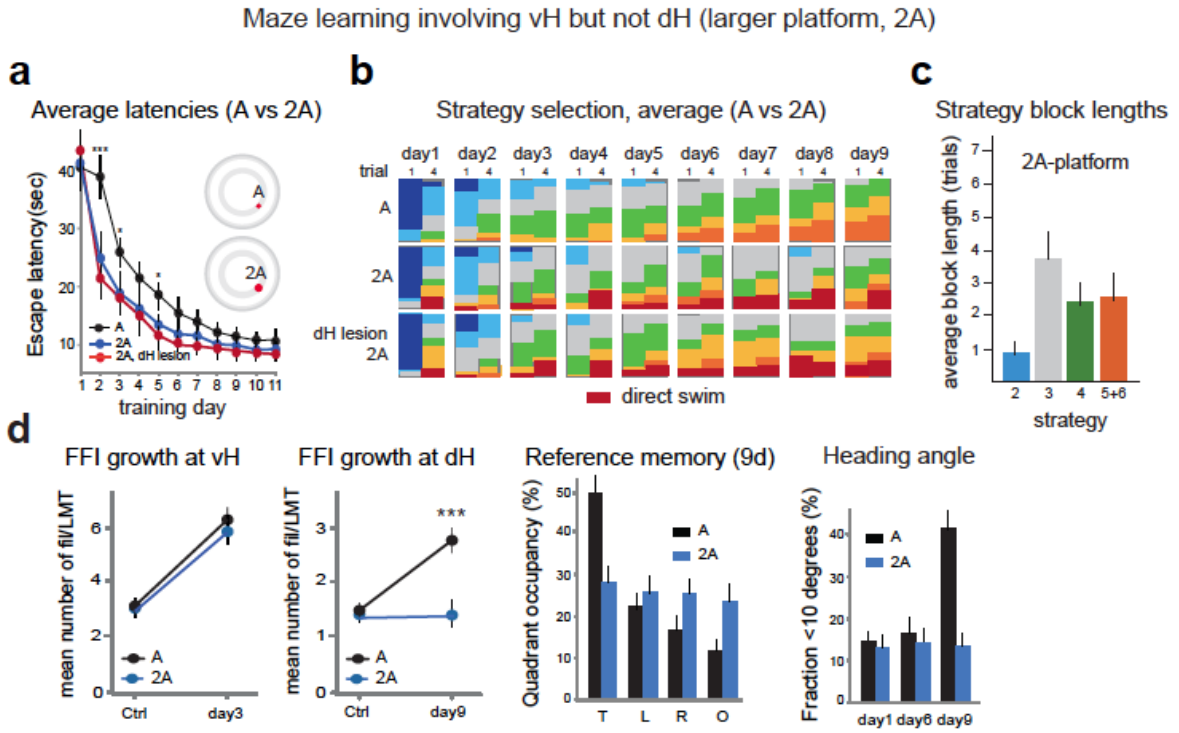
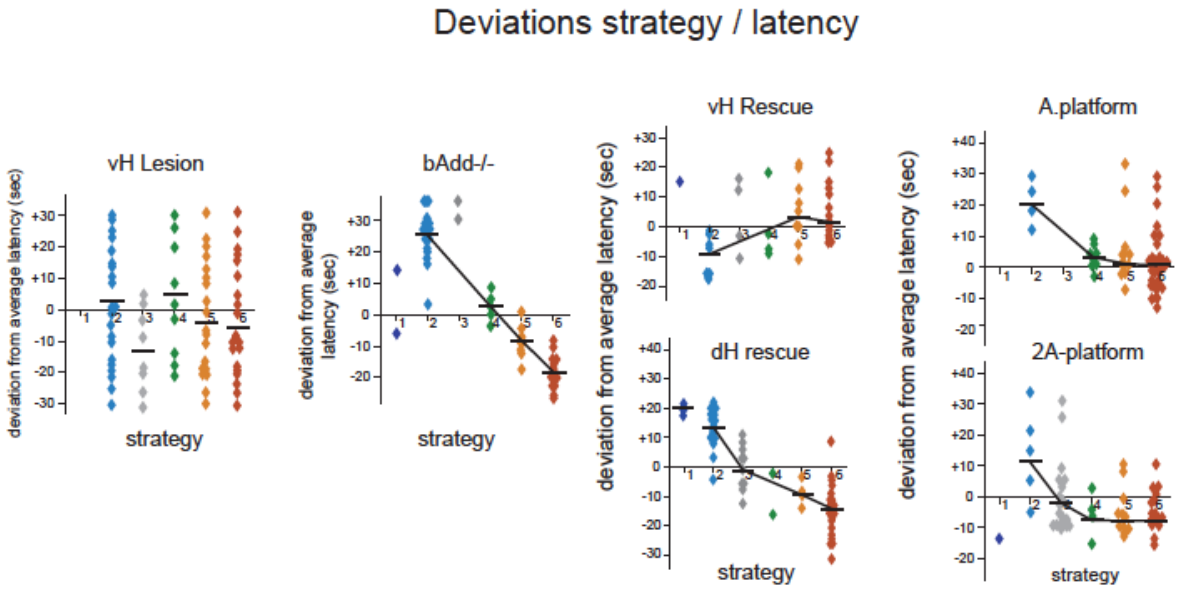
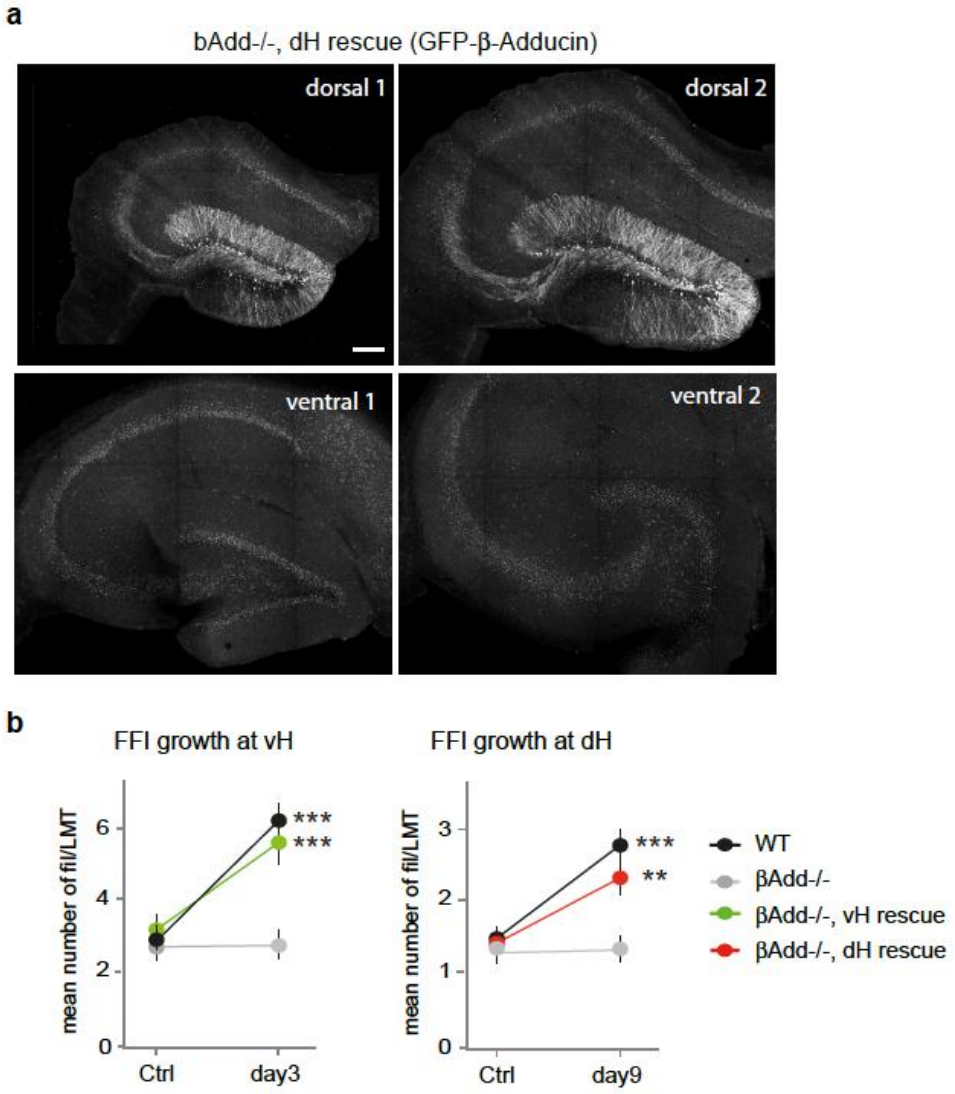


Figure 8. Maze learning depending on vH, independently of dH.

a-d, Maze learning involving ventral but not dorsal hippocampus. The analysis of maze learning with platform areas of A and 2A is as described in **Figs. 1** and **5**. The schematic (**a**) is in scale. N= 15 (2A, A, control) and 8 (2A, dH lesion) mice. **d**, Absence of FFI growth in dH, but not vH, and corresponding absence of spatial learning (reference memory, heading angle) in mice navigating a maze with a 2A platform. N=15 mice.



Supplementary Figure 5. Relationship of strategy deviations and escape latency during maze learning. Plot represents single trial latency deviations from the means for explorative strategies. Extent to which the latencies of individual trials involving the exploration of alternative strategies differed from the mean latencies for that phase of the training process. The individual values in the plot represent the latency deviations for individual trials, ordered according to the strategy of those exploratory trials (25 mice; see also Methods).



Supplementary Figure 6. Local rescue of β-Adducin expression and FFI growth in ventral or dorsal hippocampus granule cells of *β-Adducin*$^{-/-}$ mice. a, Lentivirus-mediated expression of GFP-□-Adducin specifically in dorsal hippocampus granule cells of *β-Adducin*$^{-/-}$ mouse. The panels show representative examples of GFP-β-Adducin signal in granule cells (note labeled dendrites in the dentate gyrus, and labeled mossy fibers in the hilus and along CA3) in dorsal, but not ventral hippocampus. Lentivirus-mediated rescue led to GFP-□-Adducin expression in 18-25% of the granule cells in dorsal hippocampus. Bar: 100µm. **b**, Rescue of learning-related FFI growth upon GFP-□-Adducin expression in ventral or dorsal hippocampus. Data from water maze learning experiments. As previously reported[21], lentivirus-mediated GFP-□-Adducin transduction rescued FFI growth specifically in those (GFP+) granule cells that exhibited virus-mediated expression. N=3 (rescue, control), 10 (rescue, trained) and 30 (non-rescued or wildtype) mice each.

Discussion

Our detailed behavioral analysis of how individual naïve mice learn to navigate a water maze, a biologically relevant task, combined with the analysis and local manipulation of its specific anatomical counterparts in the hippocampus have provided insights into mechanisms of complex trial-and-error learning. We provide evidence that maze navigation involves a stereotype sequence of subtasks that are learned sequentially through distinct hippocampal subdivisions. The connectivities and functions of hippocampal subdivisions may thus influence how the individuals of a species address declarative trial-and-error tasks involving the hippocampus. Our results further reveal that vH has a critical early role in goal-oriented learning and searching. In doing so, we assign a key function to vH (anterior hippocampus in humans) in complex behavioral learning.

Our longitudinal analysis of water maze learning has revealed the presence of a structured learning process throughout the training procedure, which was reflected in the sequential roles of vH, iH, and then dH during maze learning, and in the sequential deployment of increasingly sophisticated spatial search habits. Our results suggest that mice may: 1) learn on day 1 that there is a goal (platform), leading to a global search strategy; 2) learn on days 1-2 (vH) that the platform is consistently at a certain distance from the wall, supporting the deployment of local search strategies and local search habits; 3) learn on days 3-5/6 (iH) that the platform is consistently at the same position within the pool, supporting the deployment of spatial search strategies and spatial search habits; 4) learn on days 6-9 (dH) a fine scale spatial map of the task, leading to direct swim from any position in the maze (**Suppl. Fig. 7**). The mechanisms linking successful subtask learning to FFI growth at hippocampal subdivisions remain to be determined and may differ among individual networks. For example, vH switches appear to occur abruptly, whereas incremental FFI growth at iH and dH during maze learning may be coupled to gradual error function mechanisms[3,29]. In a possibly related issue, the mechanisms underlying strategy selection and the establishment of search habits[3,5] also remain to be determined. Because of the prominent roles of basal ganglia systems in adjusting learning and habits to performance through dopamine-mediated reward systems[3,5,29], it is tempting to speculate that the strategy selection processes discovered in this study may involve costs/rewards computations

at striatal circuits. This possibility seems particularly plausible for learning involving vH, which exhibits extensive connectivity with striatal circuitry[14,20]. Follow-up studies focusing on the possible roles of hippocampal-striatal loops may thus provide novel insights into how animals rapidly acquire and exploit biologically relevant knowledge in reinforced learning tasks that include complex contexts and no extensive repetitions.

Our study assigns a key role to vH in complex trial-and-error learning. We provide evidence that FFI growth at vH reflects previous learning of consistent task-specific goal-context relationships in behavioral learning, that it supports deployment of local search habits during further learning, and that an intact vH is critically important to support performance consistency throughout maze learning. These results tie in well with previous reports that “simplified learning”, consisting of pre-training rats with a visible platform, accelerates subsequent maze learning, and reduces its requirement for NMDA-mediated plasticity[33,34]. Surprisingly, no functions had been assigned unequivocally to vH in behavioral learning by previous studies. That may have been due to a predominant focus on the endpoint of complex hippocampus-dependent learning, which usually involves cognitive and highly spatial aspects depending on dH, and on the fact that most studies have not focused on how learning is achieved longitudinally by individual animals[35]. While providing evidence for a role of vH in maze learning up to day 6, our results suggest a partially overlapping and later role for iH. The function of this hippocampal subdivision is poorly understood, but it can mediate rapid place learning[32], and has been suggested to integrate ventral and dorsal functions in hippocampus-dependent behavioral learning[14,32]. One possibility consistent with our findings is that FFI growth at vH on day 3 is important to support place learning between days 3 and 6 by iH (**Suppl. Fig. 7**).

The assignment of a critical role to vH in goal-oriented learning has implications for future research. Thus, efficient goal-oriented learning and searching, and rapid mastering of intermediate goals are likely to be key determinants of success in realistic biological settings. Furthermore, linking emotional processes to declarative learning through vH may affect behavioral learning within emotionally complex settings, including social interactions. Accordingly, it will be of interest to determine how vH influences learning of complex trial-and-error tasks as a function of internal states under healthy and pathologic conditions.

Material and Methods

Reagents and anatomical procedures

β-Adducin$^{-/-}$ mice[31] were from Jackson laboratories, Bar Harbor, Maine; the reporter line *Thy1-mGFP(Lsi1)* was as described[36]. The GFP-β-Adducin construct was cloned into a lentivirus vector, and dentate gyrus infections were as described[9]. We analyzed structural traces of learning at GFP-positive Large Mossy fiber Terminals (LMTs) of ventral and dorsal hippocampus (CA3b) using the "sparse" transgenic reporter line *Thy1-mGFP(Lsi1)*[21]. In parallel, the main findings were confirmed in mice in which mossy fibers were labeled randomly using a lentivirus expressing mGFP[21]. Based on boundaries identified by a previous gene expression study[16], subdivisions along the dorso-ventral axis of the hippocampus were defined as follows: dorsal, within 20% and 30% of total length; intermediate, within 45-55%; ventral, within 70-80%.

Behavioral procedures and their analysis

All behavioral experiments were carried out with male mice that were 55-65 days old at the onset of the experiment, and were according to standard procedures[21,22]. Data from training sessions and probe trials were collected and analyzed using Viewer2 Software (Biobserve, Bonn, Germany). The software Viewer III (Biobserve) was used to sample animal positions during Morris water maze trials. Search strategies were as defined in previous studies[24,26]. For quantitative identification of search strategies, we developed an algorithm in collaboration with Biobserve. In addition, all assignments were verified through direct observation in order to make adjustments for short latency trials (<15sec), in which the algorithm tended to overestimate the impact of small deviations in search strategy. Search strategies were defined as follows[26]: Thigmotaxis: >35% of swim time (60sec) within closer wall zone (5cm from pool wall), >65% of time within wider wall zone (10cm from pool wall); Random Search: >70% surface coverage; Scanning: <70% surface coverage, and > 15% surface coverage <0.7[StdU] distance to the pool center; Chaining: >65% of time within annulus zone; Directed Search: >80% of time within goal corridor (rectangular goal corridor of 20cm width, centered along direct connection between start and platform positions); Focal search: <0.35 body angle STE, <0.25 [StdU] mean distance to present goal; Direct Swim: 100% within goal corridor. Using these definitions and the algorithm, in

combination with adjustments for short latency trials, only <2% of the trials could not be assigned univocally to one strategy. Strategy blocks were defined as a sequence of at least three trials with the same strategy (two trials for strategy 5 and 6 blocks). For block lengths, one-trial interruptions were tolerated, but not counted (e.g. a 2225223422 sequence was scored as a block length of 5 for strategy 2). Total block lengths were the sum of all blocks for one strategy and one mouse. For strategy/latency deviation values, mean latencies within days 1-2, days 3-6 and days 7-9 for a particular condition or genotype were subtracted from corresponding single trial latencies when the strategies of those trials deviated from those of a sequence of at least two same strategy trials by at least two strategy levels (e.g. latency of the 4th trial in the sequence 222422). Statistical analyses were performed using Student's *t*-tests and one-way ANOVA; *post hoc* comparisons were at the $P<0.05$ level of significance. Results are presented as mean $\pm$ s.e.m.

Behavioral protocols

Mice were kept in temperature-controlled rooms on a constant 12h light/dark cycle, and all experiments were conducted at the approximate same time of the light cycle. Prior to the behavioral experiment, mice were housed individually during 3-4 days and provided with food and water ad libitum, unless otherwise stated. All animal procedures were approved and performed in accordance with the Veterinary Department of the Canton Basel-Stadt.

The training procedures for Morris water maze and contextual fear conditioning were as described[21]. For fear conditioning experiments, the conditioning chamber was cleaned with 2% acetic acid before and after each session. Once placed inside the training chamber, mice were allowed to explore the apparatus for 2.5 min and then received a series of five foot shocks (1s and 0.8 mA each, intertrial interval of 30s). Control mice were subjected to the same procedure without receiving foot shocks. The contextual fear memory was assessed by returning mice to the training chamber 24h after fear conditioning during a test period of 2.5 min. Freezing was defined as the complete absence of somatic mobility, except for respiratory movements. Fear conditioning in the dark involved the same procedure, but with all lights switched off. Recall 24h after conditioning was in the training context in the presence of odor A (2% acetic acid) or B (0.25% benzaldehyde), shock floor or plastic floor.

The Morris water maze consisted of a circular (diameter: 140 cm) pool filled with milky water at 24^{o}C. The circular escape platform (10 cm diameter) was positioned at a fixed position 0.5 cm above (visible platform) or 0.5cm below (hidden platform) the water. Training involved four 60sec trials separated by a 5min interval every day. For each trial, mice were placed in the pool facing the pool wall from random starting locations. At the end of each trial mice were allowed to sit on the platform for 15 seconds; when trials had been unsuccessful, mice were manually guided to the platform. Latencies and reference memories were determined as described. Heading angles were defined by the start position of the mice, a line connecting that position to the platform, and a second line connecting the start position to the position of the mouse after 18cm of swimming. This distance was selected in order to exclude variations due to initial rotations, away from the pool wall.

For novel object recognition experiments, mice were first habituated to the testing arena for 10 min. On the next day, each animal was allowed to explore two identical objects placed in the arena for 10 min. 24h later, mice explored the same arena for 5min, but one of the familiar objects was replaced with a novel object. The recognition memory was expressed by the discrimination index (*D*), which was defined as D= (T(novel) – T(familiar)) / (T(novel) + T(familiar).

For the reinforced learning version of the object recognition task and its controls, all mice were only fed sufficiently after each training session in order to maintain them at 85% of their initial body weight. Wheat flakes (3-5 flakes; 100-120 mg in total) were placed as reward on top of the familiar object (a cube of ca. 4 cm height, with an inserted cylinder of additional 3 cm of height; reward flakes were placed on top of the cube and of the cylinder). To habituate mice to the reward, 2-3 wheat flakes were placed in the home cages after each habituation and training session. For all object recognition task protocols, the testing arena was cleaned with 70% ethanol after each mouse, on each day.

Drug delivery and stereotactic surgery *in vivo*

SCH23390 (Tocris Bioscence) was dissolved in saline 0.9% and injected i.p. at a dosis of 0.05mg/kg 20 min prior to water maze training at day 1 (habituation), and

throughout the training. Coordinates for lentiviral injections into mouse dentate gyrus were (in mm from Bregma): 1.70 posterior, 1.10 lateral, 1.70 down (dorsal hippocampus); 3.16 posterior, - 2.5 lateral, 2.10 down (ventral hippocampus). The lentivirus for β-Adducin rescue was as described[9]. Adducin knockout mice were trained 5 weeks after injection of eGFP-β-Adducin-Lentivirus. For hippocampal subdivision lesions, Ibotenic acid (Ascent scientific) was dissolved into PBS to a final concentration of 10mg/ml, and injections of 50nl were made at 2-3 sites. Injections coordinates were (in mm from Bregma): 3.08 posterior; 2.7 lateral; 3.2, 3.4 and 3.6 down (vH lesion); 2.3 posterior; 2.3 lateral: 1.3, 1.5 and 1.7 down (iH lesion); 1.58 posterior; 1.25 lateral; 1.3 and 1.6 down (dH lesion). Upon injection, mice were given 7 days recovery before training.

Immunocytochemistry, histology

Antibodies were as follows: rabbit anti-GFP (Molecular probes, Eugene, OR, USA), 1:1000; rabbit anti-c-Fos (Santa Cruz), 1:10'000; mouse anti-NeuN (Chemicon), 1:200. Secondary antibodies were AlexaFluor 568 or 488 (Molecular probes): 1:500.

Morris water maze, c-Fos expression: mice performed a single probe trial 24h after the last training session, and were returned to their home cage for 90min prior to perfusion (transcardially with 4% paraformaldehyde in PBS pH 7.4). Brains were kept in fixation solution overnight at 4°C. Hippocampi were dissected, embedded in 3% agarose gel and sliced transversally on a tissue chopper (McIlwain) to obtain lamellar hippocampal sections of 100µm thickness. c-Fos immunocytochemistry was performed and analyzed as described[21].

Imaging and image analysis

For high-resolution imaging of LMTs in fixed tissue, lamellar sections were imaged on an upright spinning disk microscope using an alpha Plan-Apochromat 100x/1.45 oil-immersion objective (Zeiss) and Metamorph 7.7.2 acquisition software (Molecular Devices, Sunnyvale, CA, USA). Voxel size was 0.106µm x 0.106µm x 0.2µm. For c-Fos analysis, all samples belonging to the same experimental set were processed in parallel and acquired with the same settings on a LSM700 confocal microscope

(Zeiss) using a EC Plan-Neofluar 40x/1.3 oil-immersion objective (Zeiss). LMT analysis**:** Transverse hippocampal sections at different dorsoventral levels (dorsal, intermediate, ventral hippocampus) were used for the analysis of LMT morphology and filopodial contents in CA3b. 80-100 LMTs per animal and region were analyzed; this involved 3-4 confocal stacks per section along CA3b, and an average of 3-4 sections. All objects that were completely included in the 3D stack where analyzed blind to experimental conditions. High-resolution 3D confocal stacks were analyzed using Imaris 7.0.0 (Bitplane AG) software.

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Author contributions

S.R. devised and carried out the analysis of hippocampal behavior, connectivity, vH lesions and β-Adducin rescue; D.S. carried out the analysis of FFI growth and c-Fos immunoreactivity; F.D. devised and carried out behavioral and lesion studies relating vH, iH and dH FFI growth to subdivision function in learning. P.C. helped devise the experiments and wrote the manuscript. All authors discussed the results and commented the manuscript.

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RESULTS

2.3 STRUCTURAL TRACES OF INNATE FEAR ALONG THE HIPPOCAMAL MOSSY FIBER PATHWAY (UNPUBLISHED RESULTS)

Introduction

The hippocampus is a brain structure critical for learning and memory that exhibits several forms of experience-dependent plasticity. Experiences of different nature such as chronic stress and learning differentially affect the structure and function of the hippocampus. The hippocampus is particularly vulnerable to sustained stress. In humans, the hippocampus undergoes atrophy in several neural disorders such as depression (Sheline, Wang et al. 1996), and this is accompanied by specific deficits in hippocampus-dependent memory (Sloviter 1993). In rodents, chronic stress suppresses adult neurogenesis (Gould, McEwen et al. 1997; Malberg and Duman 2003; Mirescu, Peters et al. 2004; Mirescu and Gould 2006) and triggers atrophy of dendrites in the CA3 region of the hippocampus (Sandi, Davies et al. 2003; Stewart, Davies et al. 2005). One popular view is that stress functions as a suppressor of synaptic plasticity whereas learning represents a promoter of synaptic plasticity (McEwen 1999; Kim and Diamond 2002; Sandi, Davies et al. 2003). However, under natural conditions stress is not only experienced chronically, but often occurs in acute or discrete episodes such as the attack by a predator. It is not well understood how such an acute stressful event impacts on hippocampal function and plasticity. Furthermore, it is not well established, whether acute stress and chronic stress affect hippocampal functions in comparable manners.

In order to study the interplay between acute stress and hippocampal function we used an innate fear paradigm to elicit acute fear in mice. Rodents exhibit an innate and unconditioned fear response to the exposure of predator odors such as trimethylthiazoline (TMT) (Morrow, Redmond et al. 2000; Wallace and Rosen 2000; Fendt and Endres 2008). TMT was originally isolated from fox feces and triggers characteristic autonomic and behavioral changes as unconditioned freezing and defensive burying (Wallace and Rosen 2000). Studies on the neural circuitry underlying these innate fear responses indicate that the amygdala is not necessary for TMT-induced freezing (Wallace and Rosen 2000; Fendt, Endres et al. 2003), whereas

the bed nucleus of stria terminalis (BNST) has a critical function (Fendt, Endres et al. 2003). In line with this finding, it has been suggested that BNST mediates unlearned fear, whereas the amygdala may selectively mediate conditioned fear (Davis, Walker et al. 1997).

In this study we determined the effect of innate fear on the structural connectivity at the hippocampal mossy fiber to CA3 synapse. The synaptic connectivity along the mossy fiber pathway was examined by light microscopy of mossy fiber terminals in thy1-mGFP expressing mice. In addition we also investigate the ultrastructural level of synaptic connectivity by three-dimensional electron microscopy. Furthermore we studied the behavioral function of synaptic connectivity alterations along the hippocampal mossy fiber projection and analyzed the effect of acute fear on behavioral pattern separation.

Results

TMT elicits rapid structural rearrangements along the mossy fiber pathway

In this study we determined the effect of innate fear on the structural connectivity at the hippocampal mossy fiber to CA3 synapse. We exposed mice for 5 min to the predator odor TMT and analyzed the synaptic arrangement of LMTs in the hippocampus at different time points upon acute stress using light microscopy. Structural rearrangements of LMTs were rapidly induced upon TMT exposure whereas a control odor (2% Acetic acid) did not affect the morphology of LMTs. The structural organization of LMTs was changed 6h after TMT exposure. The LMTs exhibited characteristic rearrangements such as surface texture remodeling and alterations in the LMT morphology (Fig. 1a). The fraction of LMTs with these structural features was increasing 6h after TMT exposure and reached maximum around 12h to 24h after acute stress (Percentage of LMTs with surface texture: Ctrl 2.9%±0.8%, TMT12h 29.6%±6.8%, TMT 24h 28.9%±5.4%, $p<0.01$; surface to volume ratio: Ctrl 2.71±0.1, TMT12h 3.06±0.07, $p<0.01$; Fig 1b). In contrast to fear learning, TMT exposure did not increase the FFI connectivity (average number of filopodia per LMT: Naive 1.6±0.1, TMT12h 1.52±0.18, Ctrl 24h 1.49±0.13, n.s.; Fig 1b), which is consistent with the notion that learning selectively involves growth of FFI connectivity at hippocampal LMTs. The structural plasticity upon TMT exposure was transient and 7d days after TMT exposure the LMT complexity was comparable to baseline conditions (surface to volume ratio: TMT7d 2.48±0.08, TMT25d 2.58±0.06; n.s; Fig 1b). A qualitative analysis of the postsynaptic arrangement on CA3 pyramidal cells indicated that TMT did also elicit structural plasticity on postsynaptic structures. For example, CA3 pyramidal exhibited high densities of thorny excrescences compared to control dendrites (Fig 1c). In addition, the fine structure of thorny excrescences was strongly increased (Fig 1c). However, it was not possible to characterize these fine postsynaptic rearrangements in detail based on light microscopy.

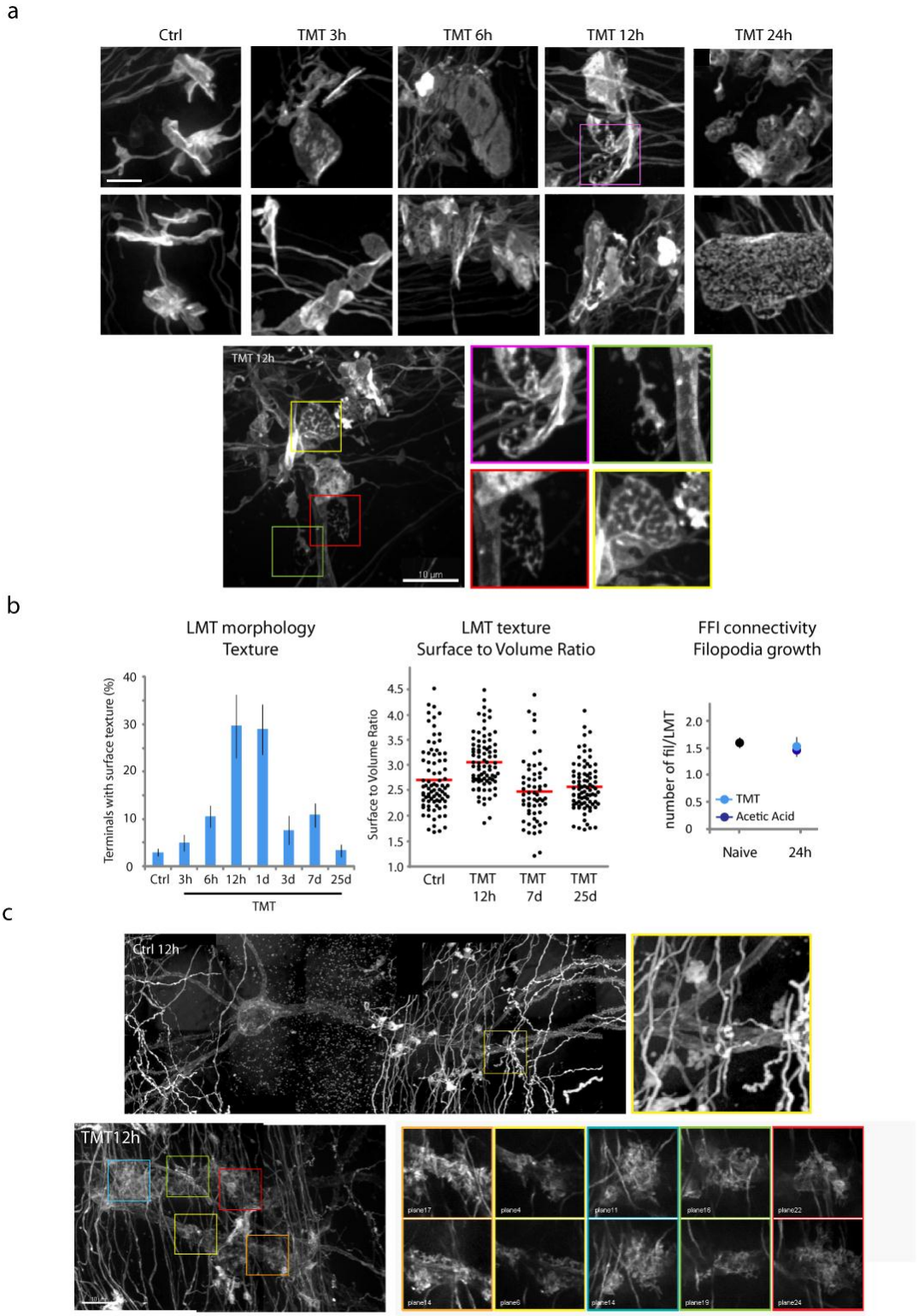

Figure 1. TMT elicits structural plasticity along the mossy fiber pathway

(**a**) Thy1-mGFP positive LMTs in CA3b of stratum lucidum. Maximum Intensity projection of confocal images Scale bar 10 µm **(b)** Left: Distribution of LMTs with surface rearrangement after TMT exposure N=4 per group. Middle: Surface to volume ratio of LMTs. N=3, 60-80 terminals per groups, Right: FFI connectivity, average number of filopodia per LMT. **(c)** GFP-positive pyramidal cell in CA3b. Inset: Thorny excrescence structure. Scale bar 10µm.

To determine the synaptic organization of CA3 pyramidal cells in more detail we performed an electron microscopic study using serial block-face scanning methodology. We reconstructed the three-dimensional organization of CA3 pyramidal dendrites in stratum lucidum and found a characteristic arrangement of thorny excrescences along the dendrite under baseline condition (Fig. 2a). Thorny excrescences were typically arranged in two main bands along the CA3 pyramidal dendrite and followed a spiral-like pattern along the dendrite. We then assay the postsynaptic organization upon acute stress. CA3 pyramidal dendrites exhibited marked structural rearrangements 12h upon TMT exposure (Fig 2b). The average density of thorny excrescences on individual CA3 pyramidal dendrites was increased (mean density of thorny excrescences: Ctrl 0.61±0.05, TMT12h 0.77±0.04, $p<0.05$; Fig 2b), indicating the rapid formation of new thorny excrescences.

To measure the complexity of individual thorny excrescences we next performed a second three dimensional analysis and reconstructed individuals thorns of CA3 pyramidal cells. This analysis revealed an increase in the complexity of thorny excrescences 12h after TMT exposure (Surface to volume ratio: Ctrl, TMT 12h; Fig. 3a), whereas the overall number of spines heads per thorny excrescence and the average volume of spines was not affected on average. These results are suggesting that acute stress causes structural plasticity of preexisting and/or newly formed spines on CA3 pyramidal cell within 12h after stress. In order to relate the synaptic complexity of thorny excrescences and synaptic strength, we also analyzed the density of active zones per spine perimeter using transmission electron microscopy. The density of active zones per spine perimeter was reduced upon TMT exposure compared to control ($p<0.05$, Fig 3b). Since the three dimensional analysis of thorny excrescences revealed no difference in the volume of individual thorns or spines this data suggests a general reduction in the number of synaptic contact site per postsynaptic element. Together, this structural data provides strong evidence for large scale alterations in the synaptic connectivity along the hippocampal mossy fiber pathway upon acute stress. However, it is not known whether and how these changes in synaptic connectivity may influence hippocampal-dependent behavior.

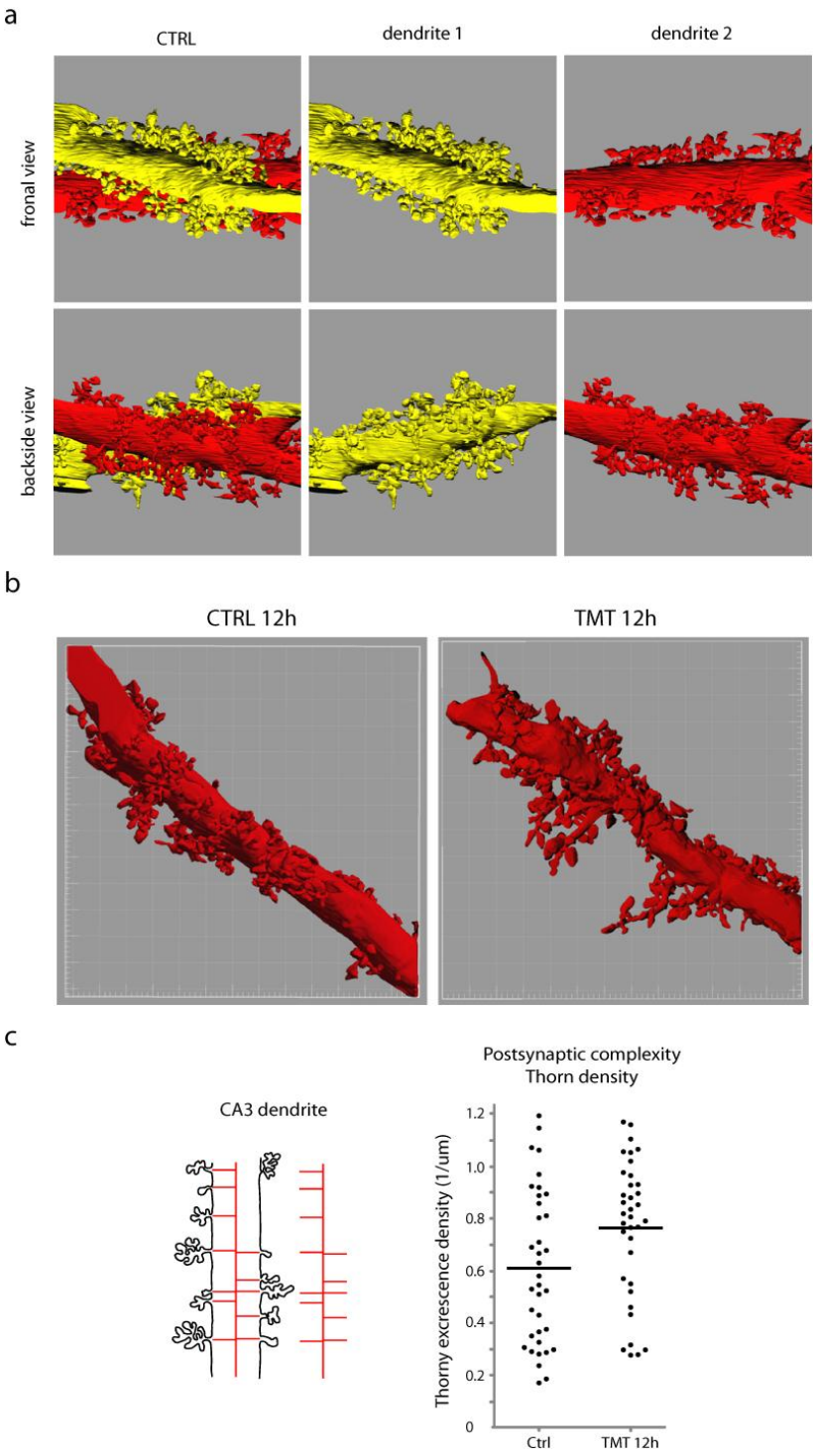

Figure 2. Three-dimensional EM reconstruction of CA3 pyramidal dendrite

(**a**) CA3 pyramidal dendrite of stratum lucidum CA3b. **(b)** Left: Control dendrite organization, Right: dendritic structure 12h after TMT treatment (c) Density of Thorny excrescences along CA3 pyramidal dendrties in stratum lucidum. N=3, 35 dendrites per group.

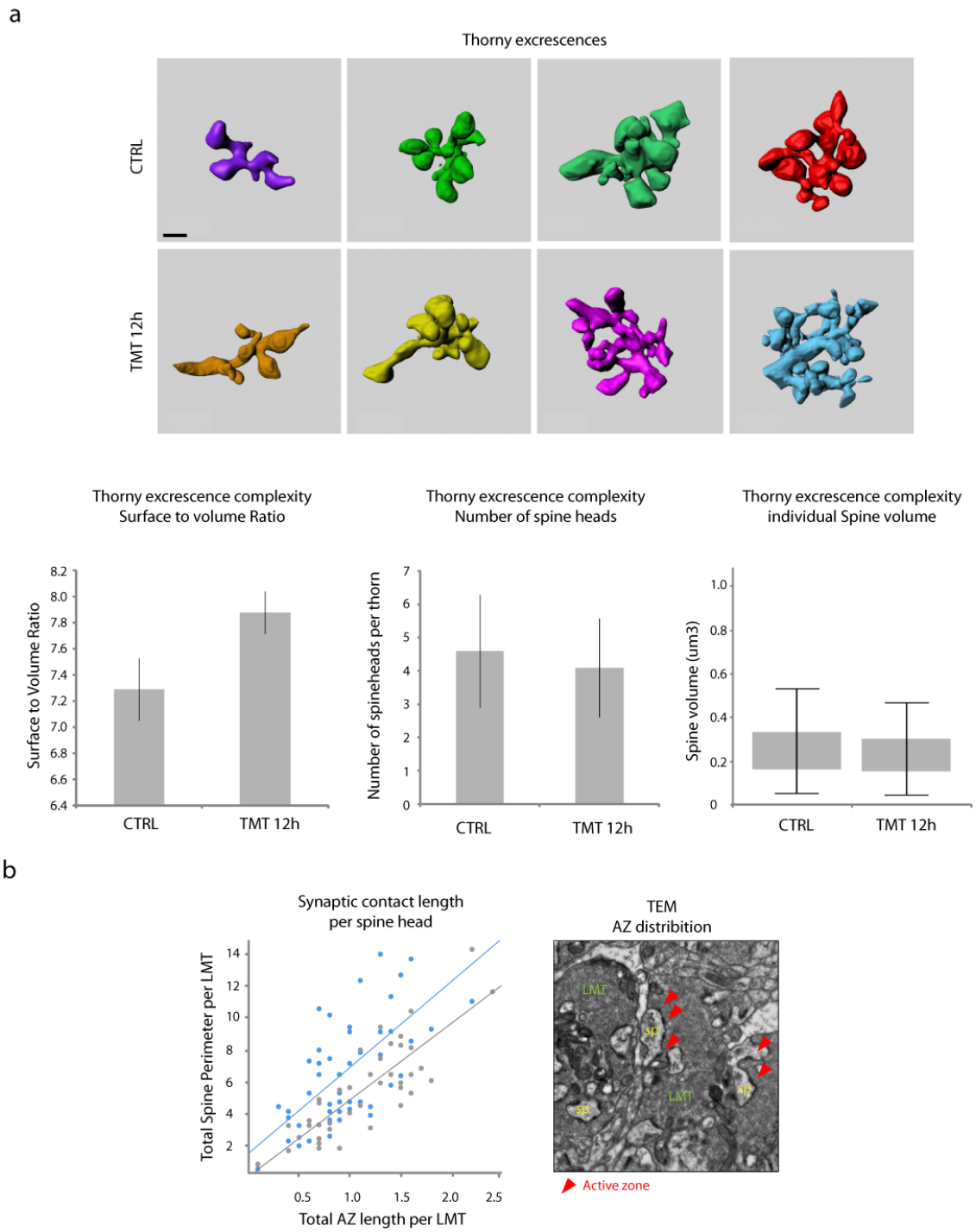

Figure 3. Three-dimensional EM reconstruction of thorny excrescences

(**a**) Top: Representative thorny excrescences under baseline and TMT conditions. Bottom Left: Surface to Volume Ratio of Thorny excrescences. Middle: Number of spines heads per thorn, Right: Volume of individual spine heads. N=3, 60 thorny excrescences per group. **(b)** Active zone density per spine perimeter. N=3, 45 spines per group.

We next assayed the behavioral effects of structural plasticity after acute predator stress. In particular, we used context discrimination upon fear conditioning as a behavioral test for pattern separation. The hippocampal circuits of dentate gyrus and CA3 play an important role in pattern separation and context discrimination (Leutgeb, Leutgeb et al. 2007; McHugh, Jones et al. 2007) and therefore provide an ideal behavioral paradigm to test the role of structural plasticity along the hippocampal mossy fiber pathway. Mice were first subjected to contextual fear conditioning, 9 days after fear conditioning mice were acutely stressed using TMT and 12h later context discrimination was assessed. Four different contexts were used to measure the context-dependent memory strength and fear specificity (Fig 4a). Mice were tested for the context memory in the training context (TR). In addition, the specificity of the memory was tested in a novel context (N) which had a different shape and contained a different odor. To test behavioral pattern separation we used two additional contexts which were related to the training context TR. The first altered TR context TR(A) contained a distinct floor and four altered walls and a different ceiling than original TR. The second TR-related context TR(B) contained a distinct floor and two altered walls (Fig 4a). Fear conditioned mice exhibited a strong contextual fear memory for the training context 12h after predator stress, which was comparable to non-stressed conditioned mice (Freezing response in TR: Ctrl 37.6%±7.5%, TMT 12h 41.2%±9.6%, n.s.; Fig. 4b). In addition, TMT exposure did not elicit generalized fear since the freezing rates in the novel context were minimal (Freezing N: Ctrl 3.4%±0.14, TMT 12h 2.3%±0.32%, n.s.; Fig 4b).

To assay the behavioral expression of pattern separation we calculated context discrimination ratios for the distinction of TR (A) versus TR (B) and TR versus TR (B). This analysis revealed a strong behavioral deficit in the discrimination of TR(A) and TR (B) (Discrimination index: Ctrl 0.69±0.05, TMT 12h 0.56±0.45, $p<0.05$; Fig 4c). In contrast, the discrimination of original TR and TR (B) was not affected 12h after exposure to predator odor (Fig 4c). To further examine the relation of LMT structure and behavioral pattern separation we performed a time course analysis of context discrimination after TMT exposure (missing graph). We found a selective impairments in context discrimination of TR(A) versus TR(B) at 12h upon predator stress whereas no deficits was measured 3h, 3d and 7d upon acute stress. This is consistent which

the time course terminals exhibit strong surface texture which also exhibited a maximal 12h after stress.

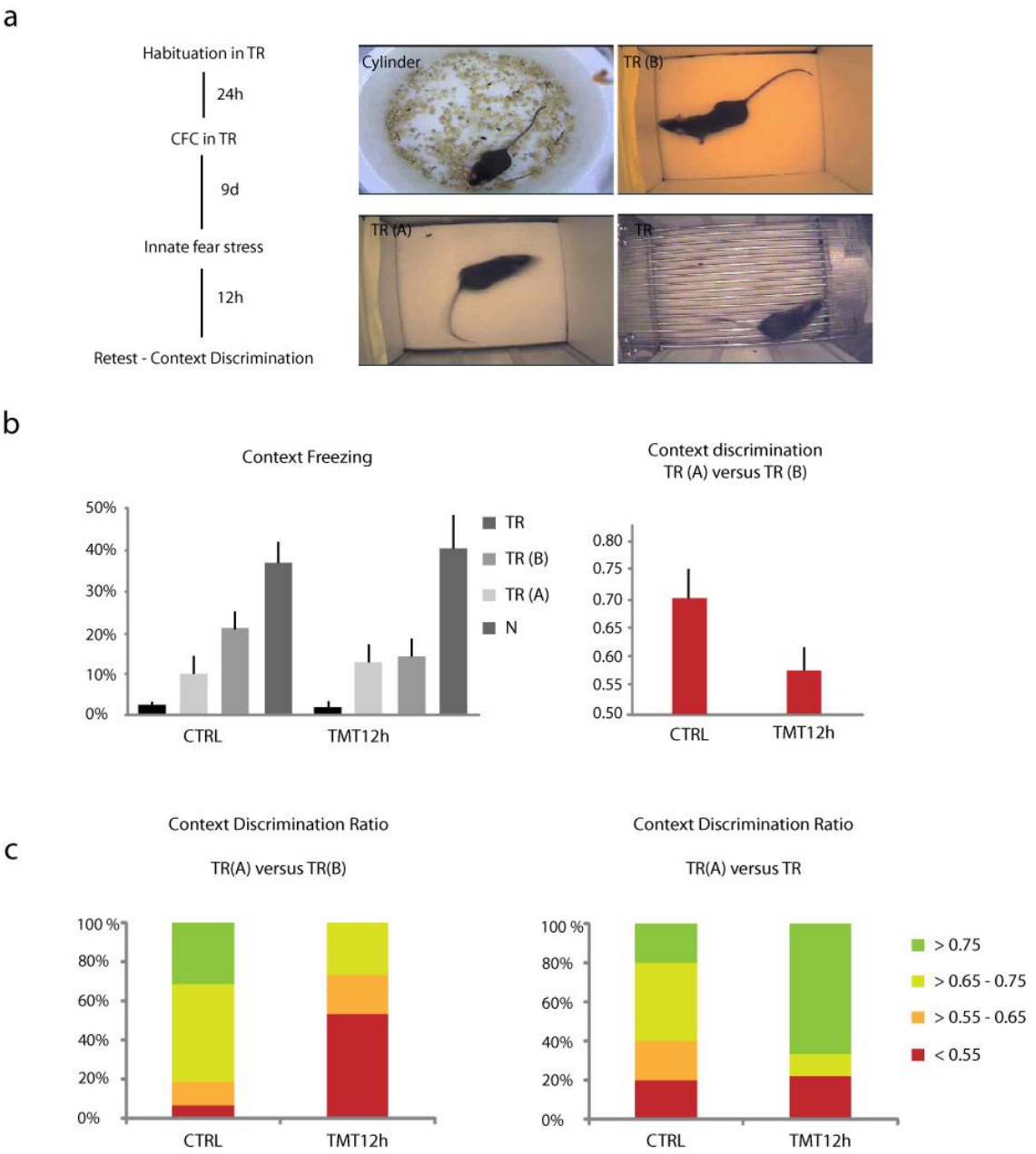

Figure 4. Three-dimensional EM reconstruction of thorny excrescences

(**a**) Schematic diagram of contextual fear conditioning upon habituation. Retest contexts: Cylinder N, All wall changes TR(B), 3 Walls changed TR(A), Training context TR. **(b)** Left: Freezing rates in retest context. N=10 per group. Right: Context discrimination between TR(A) and TR(B). **(c)** Left: Context discrimination for TR(B) and TR(A), Percentage of mice in a defined discrimination category. Right: Context discrimination for TR and TR(B). Red: poor discrimination. Green: strong discrimination. N=10 per group.

We further performed a correlation analysis of LMT complexity and context discrimination. Non-stressed conditioned mice exhibited a strong correlation of pattern separation of TR (A) versus TR (B) and the average filopodial context of LMTs 10d after fear conditioning. In stark contrast, this correlation was lost upon TMT treatment (Fig. 5a). Moreover we found that this performance deficit was related to the percentage of textured terminals in mice stressed mice. Mice exhibiting large fraction of textured terminals exhibited the strongest reduction in context discrimination, whereas mice with low fractions of textured terminals could still partially discriminate TR(A) from TR (B) (Fig. 5b).

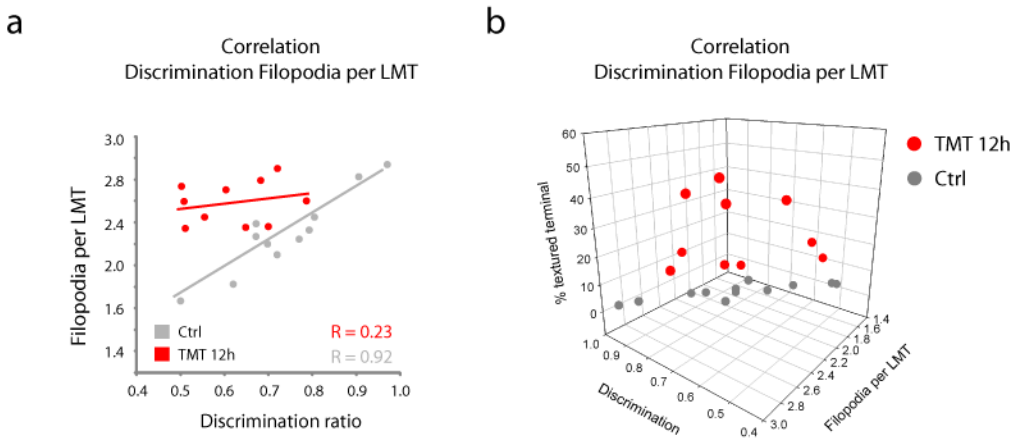

Figure 5. Correlation of LMT connectivity and pattern separation

(**a**) Correlation of FFI growth and Context discrimination of TR(A) vesus TR (B). Grey: Ctrl, Red: TMT12h. Each represents an individual mouse. (b) Relation between FFI growth and Context discrimination upon surface remodeling of LMTs. x: average number of Filopodia/LMT, y: Context Discrimination TR(A) versus TR(B), z: Percentage of texture terminals. Each dot represents an individual.

Together these data indicate a behavioral deficit in pattern separation 12h upon predator stress. However, it is not addressed whether this behavioral deficit is selectively linked to the structural rearrangements along the mossy fiber pathway or whether it is due to other stress-related mechanism upon TMT exposure. It has been previously shown that stress increase glucocorticoid levels which cause behavioral impairments in hippocampal-dependent memory retrieval (Luine, Villegas et al. 1994; Roozendaal, Griffith et al. 2003; Roozendaal, Hahn et al. 2004)

To determine whether the behavioral deficit in context discrimination upon predator stress was related to increased levels of glucocorticoid we measured the plasma

levels of corticosterone upon TMT exposure. Acute exposure to TMT rapidly increased the plasma corticosterone levels. The maximal level of plasma corticosterone levels we measured 10 min after predator stress (Fig 6a). However, 12h after TMT exposure the plasma corticosterone levels were comparable to non-stressed control mice (Fig. 6a), indicating that the behavioral deficit was not related to increased levels of corticosterone. We next tested whether the rapid increase in corticosterone levels may trigger the structural rearrangements upon TMT exposure. Systemic injections of corticosterone did only slightly increase the percentage of textured terminals (Fig 6a). In addition, blockade of corticosterone production by metyrapone did not affect the percentage of textured terminals 12h after TMT exposure. Together these results indicate that neither the behavioral deficit in pattern separation not structural plasticity along the mossy fiber pathway was caused by increased levels of corticosterone.

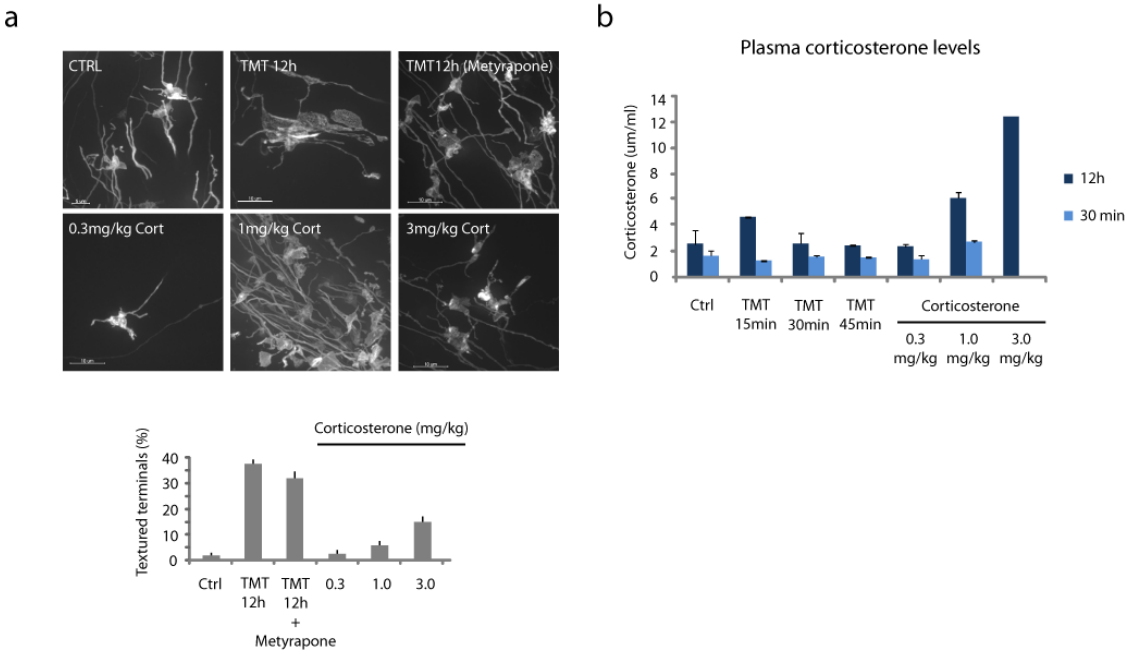

Figure 6. Corticosterone upon TMT predator stress

(**a**) Top: Maximum intensity projections of confocal images of mGFP positive LMTs upon TMT predator stress versus Corticosterone injection Scale bar 10 µm. Bottom: Percentage of textured terminals upon treatment, N=3 per group. (b) Plasma corticosterone levels upon TMT and Corticosterone injections.

At the current stage our study reveals non-learning related structural plasticity along the hippocampal mossy fiber projection which causes a behavioral deficit in pattern separation. While chronic stress has been shown to cause dendritic atrophy on CA3 pyramidal cell (Magarinos and Mcewen 1995), we found that acute exposure to a predator odor increases density and complexity of thorny excrescences on CA3 pyramidal cells. In addition, we show that acute fear leaves a transient structural trace in the adult hippocampus which suggests, that the behavioral deficit may be transient as well. Furthermore, we show that structural plasticity upon TMT is not related to classical stress signaling via corticosterone but is likely to be mediated by other specific signaling mechanisms.

Discussion

Several lines of evidence start to suggest that structural plasticity along the hippocampal mossy fiber projection exhibit characteristic features depending on the behavioral conditions. For example learning-related plasticity at mossy fiber terminals selectively involves an increase in FFI connectivity at LMTs (Ruediger et al, 2011) whereas non-learning related structural plasticity at LMTs affect the core terminal organization, which is consistent with structural plasticity of postsynaptic sites. Interestingly, we found that acute fear upon exposure to a predator odor increases density of thorny excrescences on CA3 pyramidal cells. This is in contrast to previous findings on chronic stress which resulted in dendritic atrophy of CA3 cells (Magarinos and Mcewen 1995). Other studies in rodent models of stress have been shown to impair synaptic plasticity such as LTP and LTD in the hippocampus which have been hypothesized to cause learning and memory deficits upon stress (Kim and Diamond 2002). TMT induces unconditioned freezing it does not involve a learning nor memory component. Therefore it will be interesting to test whether Rab3a mutant mice, which lack mossy fiber LTP, exhibit similar structural plasticity along the hippocampal mossy fiber projection upon TMT.

In addition, the BNST has been shown to be involved in TMT-induced freezing. In order to test whether the plasticity in the hippocampus is resulting from BNST activation during stress, target lesion experiments will provide further insight in the regulation of hippocampal plasticity upon acute stress. Moreover, our results on the

relation between structural connectivity and acute fear are on plasticity in dorsal hippocampus. We did not address the relation of TMT and plasticity in the ventral hippocampus so far. However, based on the connectivity of BNST and ventral hippocampus this may be site of plasticity in the hippocampus.

On the ultrastructural level a single mossy fiber terminal is thought to contact a single CA3 pyramidal cell. Indeed, under baseline conditions we found individual LMTs to contact a single CA3 cell by one or few thorny excrescences. However the dramatic rearrangements of LMTs upon TMT exposure suggest that LMTs may not follow the rule of one-to-one connectivity but may be contacted by two distinct dendrites. Therefore we will extend out immuno electron microscopic studies and reconstruct individual LMTs and the postsynaptic counter parts upon TMT exposure. This ultrastructural analysis of pre- and postsynaptic elements may allow a better explanation of the behavioral deficits in pattern separation upon predator stress. To complement the structural-function analysis it will be interesting to assay the expression of immediate early genes such as c-fos upon TMT exposure. The ensemble activity in the hippocampus may provide further insight in the network alterations upon acute stress and how these relate to behavior. In addition, it is not known what drive structural plasticity on the synaptic level. It is likely that the postsynaptic rearrangements of thorny excrescences are causing the morphological changes in presynaptic LMTs. In order to test this idea, it will be interesting to assay the effects of acute stress in mouse mutants which are deficient postsynaptic structural plasticity or synapse stabilization such as CamKII mutant mice or β-Adducin mutants, respectively.

Author contributions

S.R. devised and carried out the structural analysis of mossy fiber terminal connectivity upon acute fear. Martina Mitterhuber devised and carried out behavioral studies and corticosterone measurements relating structural plasticity to pattern separation.

Materials and Methods

The behavioral experiments were in accordance with institutional guidelines, and were approved by the Veterinary Department of the Canton of Basel-Stadt. Male Mice were kept in temperature controlled rooms on a constant 12h light/dark cycle, and experiments were conducted at the approximate same time during the light cycle. Mice at the age of 50 to 70 days were used for the experiments.

TMT exposure was done under a chemical hood in a rectangular plastic box of
The dimension 25cm x 15cm x 15cm. Mice were subjected to TMT (20µl) for 5 minutes. TMT was put on a tissue paper which was positioned in the center of the test box that was thoroughly cleaned with water and ethanol after every experiment. For Memory retest mice were re-exposed to an identical test box which was not used for TMT exposure. Corticosterone (0.3, 1.0 and 3.0mg/kg) and Metyparone (150mg/kg) were dissolved in Ethanol and systemically injected.

Electron microscopy

For the tissue preparation a modified protocol by Mark Ellisman was used. Animals were anesthetized and perfused with 0.15M cacodylate buffer pH 7.4 containing 2.0%, 2% formaldehyde (with 2mM calcium chloride) for 10 min. The hippocampi were removed and fixed for an additional 3 hours on ice in the same solution. Transversal hippocampus sections of 80µm were cut on a vibratome in ice-cold PBS. Sections were then washed 3 times for 5 min in cold cacodylate buffer containing 2mM calcium chloride. Right before use, a solution containing 3% potassium ferrocyanide in 0.3M cacodylate buffer with 4mM calcium chloride was combined with an equal volume of 4% aqueous osmium tetroxide (EMS). The tissue was incubated in this solution for 1 hour, on ice. During the incubation time, thiocarbohydrazide (TCH) solution was

freshly prepared. 0.1 g thiocarbohydrazide were added to 10 ml ddH2O and heated at 60° C for 1 hour. The solution was filtered through a 0.22 um Millipore filter before use. At the end of the first heavy metal incubation the tissue was washed with ddH2O at room temperature 5 x 3 minutes. The sections were then placed in the TCH solution for 20 min, at room temperature. Afterwards the sections were rinsed 5 times for 3 min in ddH2O at room temperature and thereafter placed in 2% osmium tetroxide in ddH20 for 30 min, at room temperature. Then the tissue was washed 5 x 3 min at room temperature in ddH2O was placed in 1% uranyl acetate (aqueous) and left at 4°C overnight. On the next day, *en bloc* Walton's lead aspartate staining was performed. First, aspartic acid stock solution was prepared: 0.998 g of L-aspartic acid in 250 ml of ddH2O at the pH3.8. To stain 0.066 g of lead nitrate in 10 ml of aspartic acid stock were dissolved at pH 5.5 (adjusted with 1N KOH). The lead aspartate solution was heated at 60°C oven for 30 min. The tissue was then washed 5 x 3 min in ddH2O at room temperature and then placed in the lead aspartate solution and returned to the oven for 30 min. The tissue was again washed 5 x 3 min in room temperature ddH2O and dehydrated using ice-cold solutions of freshly prepared 20%, 50%, 70%, 90%, 100%, 100% ethanol 5 min each and then placed in anhydrous ice-cold acetone and left at room temperature for 10 min. Afterwards the tissue was placed in room temperature acetone for 10 min and the Durcupan ACM resin (EMS) was prepared as previously described (Knott, Holtmaat et al. 2009)

3. GENERAL DISCUSSION AND OUTLOOK

The main focus of my PhD thesis work was on the functional role of identified neural circuit elements in learning and memory. As model circuitry we took advantage of the hippocampal mossy fiber pathway as it represents an ideal system to study the interplay of identified structural and functional circuit elements in relation to learning and memory in the adult brain. Mossy fibers exhibit specific structural features to dissect the differential contribution of excitatory and inhibitory components of synaptic connectivity by structural elements. I will briefly summarize the key results of this thesis and then discuss the general relevance of the summarized data. Finally, I will outline short-term and long-term future directions of research which may follow up from my studies.

Elucidating the relationship between structural synaptic plasticity and the processes of learning and memory in the adult brain is a major current objective in neuroscience. To address this issue, we used a series of hippocampus-dependent paradigms to characterize the nature and specificity of morphological changes in the adult brain in relation to learning and memory.

In the first study presented in this thesis, we identified a specific form of learning related plasticity, which manifested as a selective increase in structural connectivity that provides feedforward inhibition in the hippocampus and the cerebellum. In addition, we described the spatio-temporal relations between plasticity and specific aspects of behavior. For example we found that structural growth of feedforward inhibitory connectivity in the dorsal hippocampus was strongly correlated with spatial memory performance in spatial incremental learning of maze navigation. This work establishes the first evidence for a causal relationship between learning-related structural plasticity and the functional role of this kind of plasticity for behavior. We demonstrate a critical role of structural plasticity in the precision of learning and memory as revealed by a selective growth of synapses which mediate feedforward inhibition in the hippocampus and the cerebellum. In addition, this work uncovered a relationship between feedforward inhibition growth and plasticity at hippocampal mossy fibers with the precision of hippocampus-dependent memories. Together, these findings uncover a role of structural plasticity in learning and memory and add to

our understanding of the mechanistic processes underlying mnemonic functions in both health and disease.

In the second study presented in this thesis, we addressed the behavioral contributions of FFI connectivity growth along the longitudinal axis of the hippocampus upon complex trial-and-error learning during maze navigation. We provided evidence for region-specific induction of FFI connectivity growth related to distinct aspects of hippocampus-dependent learning. In particular we found structural plasticity in feedforward inhibitory connectivity in the ventral hippocampus during early stages of goal-oriented learning. In addition, we uncovered a sequential recruitment of distinct hippocampal regions along the dorso-ventral axis which progresses from the ventral to the dorsal pole of the hippocampus during learning. We identify an association between local structural plasticity of feedforward inhibitory connectivity and learning of defined subtasks during trial-and error navigation learning as revealed by a characteristic search strategy deployment.

Taken together, our results provide new entry points to the study of learning and memory. In particular, the structural readout of plasticity, which is specifically related to learning, adds a new level to the analysis of learning and memory. For example, it allows a closer investigation of learning at the level of neural circuits such as the hippocampus. In the future, combinatorial approaches whereby learning and memory are analyzed via their behavioral definition in relation to structural plasticity might enable a more precise dissection of learning deficits at the behavioral level in disease models to gain a better understanding of the circuit mechanisms underlying learning and memory.

Our findings on search strategy deployment and the modulation of task difficulty in relation to structural plasticity in the Morris water maze illustrate how useful it is to monitor several behavioral parameters during learning in order to differentiate between different aspects of learning and memory. For example, while the escape latencies in the MWM2A task suggest that the task is easily learnt, the absence of spatial reference memory indicates that this is not a classical hippocampus-dependent spatial learning task. This is in good agreement with the notion that mice with complete hippocampal lesions exhibit a performance improvement at the level of escape latencies in the water maze task, but they fail to establish a spatial reference

memory (Moser, Moser et al. 1995). In the future such a qualitative and quantitative analysis of behavior at different levels may be a useful approach to assess novel aspects of learning and memory.

Regarding the role of FFI connectivity growth at hippocampal LMTs in relation to learning an unresolved question is whether this connectivity growth is beneficial for other or new forms of hippocampal-dependent learning. For example, what happens on the structural level after prolonged training in the water maze to find a fixed hidden platform when the training rules are suddenly changed? To understand whether and how this affects FFI connectivity it would be very interesting to perform as so called reversal training, in which as new platform position has to be learnt after a prolonged training in the water maze. We currently hypothesize two potential scenarios: 1) Learning of a new platform position may recruit different granule cells and therefore the global FFI response may be enhanced after training to the second platform position. According to this idea, FFI levels from the first training would accumulate with FFI growth for the new platform position; 2) Learning of the new platform may induce a regression of previously formed filopodia and later induce a second wave of filopodial growth for the encoding of the new platform position. In addition to the potential effect on structural plasticity it will be interesting to see whether increased FFI connectivity affect the learning curves for the new platform position in comparison to the initial learning. One may also ask whether FFI growth affects other forms of hippocampal-dependent learning such as fear conditioning in order to investigate which rules apply to FFI connectivity changes upon multiple learning events. In addition, it will be interesting to assay how LMTs connectivity changes are reflected on the level of individual mossy fiber axons in comparison with the random LMT populations.

On the long term it will be interesting to define the regulatory mechanisms which orchestrate FFI plasticity along the mossy fiber projection in a spatio-temporal manner during learning. For example, it has been suggested that neuromodulation around the time of learning may affect cellular consolidation processes (Dudai 2004; Hasselmo 2006). The hippocampus receives dopaminergic projections from the ventral tegmental area and the substantia nigra (Scatton, Simon et al. 1980; Swanson 1982; Gasbarri, Packard et al. 1994), and dopamine has been shown to be a critical component of stimulus-reward learning (Flagel, Clark et al. 2011). Therefore, our

findings are in line with a potential contribution of dopamine during early goal-related learning of the ventral hippocampus in the water maze. Interestingly, pharmacological studies on acetylcholine have demonstrated that blockade of cholinergic receptors impairs the encoding of new memories whereas it does not affect memory retrieval (Atri, Sherman et al. 2004; Hasselmo and McGaughy 2004). These findings suggest that acetylcholine may underlie the encoding of novel memories. It will be interesting to dissect the spatial and temporal rules which may relate release of defined neuromodulators during learning and memory with local structural plasticity in the hippocampus.

In addition to the regulatory mechanisms which may control plasticity along the mossy fiber pathway, it would be interesting to understand whether the hippocampus depends on the interaction with other brain systems during different phases of learning. To which extent do other systems contribute to the characteristic signatures of plasticity along the longitudinal axis of hippocampus? Targeted lesions or pharmacological inactivation experiments may reveal brain candidate structures which interact with the hippocampus during specific aspect of learning and thus might contribute to specific aspects of hippocampus-dependent learning and learning-related plasticity. For example, the amygdala might interact with the ventral hippocampus in fear learning, thereby facilitating the rapid induction plasticity in the hippocampus (McGaugh 2004). In turn, the amygdala receives inputs arising from the ventral hippocampus, which may have regulatory functions on the amygdala during context dependent memory recall.

Finally, our study on the relationship between stress and plasticity in the hippocampus suggests that experiences of different nature can exhibit very distinct effects on synaptic connectivity along the hippocampal mossy fiber projection. For future experiments, it would be interesting to have a functional readout of neuronal activity in the hippocampus during behavior. Potentially one could assay neuronal properties along the mossy fiber pathway by electrophysiological means *in vivo* in order to examine the relation of synaptic organization, neuronal activity and behavior. For example, it would be interesting to investigate how structural plasticity upon specific experiences such as learning or stress may affect spatial pattern separation and pattern completion functions of individual neurons in the hippocampus.

On the long- term, future experiments will have to address changes in neuronal activity of individual cells and neuronal ensembles as a function a structural plasticity in relation to learning and memory. This will enable to gain further insight in the relationship between defined structural alterations in neuronal circuits and their function in learning and memory in both health and disease.

4. ABBREVIATIONS

BNST	Bed nucleus of the stria terminalis
CA	Cornu ammonis
CFC	Contextual fear conditioning
CS	Conditioned stimulus
DG	Dentate gyrus
DH	dorsal hippocampus
EC	Entorhinal cortex
EM	Electron microscopy
EXT	Extinction
FC	Fear conditioning
FFE	Feedforward excitation
FFI	Feedforward inhibition
FIL	Filopodia
GC	Granule cell
IH	Intermediate hippocampus
IN	Interneuron
LMT	Large mossy fiber terminal
LTD	Long-term depression
LTP	Long-term potentiation
MF	Mossy fiber
MWM	Morris water maze
N	Novel context
NMDA	N-Methyl-D-aspartic acid
NOR	Novel object recognition
OFC	Olfactory fear conditioning
PP	Perforant path
PYR	Pyramidal cell
RAB	Ras-related protein
TMT	2,5-dihydro-2, 4,5- trimethylthiazoline
TR	Training Context
US	Unconditioned stimulus
VH	Ventral hippocampus

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Ich erkläre, dass ich die Dissertation „**Structural plasticity of synaptic connectivity in the adult hippocampal mossy fiber projection ”** nur mit der darin angegebenen Hilfe verfasst und bei einer anderen Fakultät eingereicht habe.