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Neuronal plasticity of hippocampal and cortical circuitry modulates the formation and extinction of remote adversive memories.

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CONTENTS

1. ABSTRACT

It is generally believed that in order to enable long-term episodic memory, the information is temporarily stored in the hippocampus where it remains vulnerable to interference. Via a slow read-out process, the information is transferred into other brain structures where the memory is established and no longer vulnerable to interference. This slow read-out is termed consolidation (Mueller and Pilzecker, 1900).

The mechanisms by which memories can be acquired and consolidated in the mammalian brain are assumed to involve modifications in structural plasticity (Cajal, 1891).

The main goal of this work is to discover the morphological modification requested in memory formation and extinction.

In study I we shown that plastic changes (i.e. dendritic spine density increase) immediately develop in CA1 field of the hippocampus after a training in the contextual fear conditioning. These modifications are only transient because they disappear 36 days later, while an inverse pattern of spine density in recent and remote memory recall were found in the anterior cingulate cortex.

In study II we block the possibility to increase the number of spines in the aCC after training and we found an early temporal window in which synaptic remodelling occurring in this region is fundamental for the correct consolidation of memory.

In study III we presented a new and conflicting memory (extinction) after the consolidation of an old one, founding a disruption of the synaptic network in the aCC field. At the same time, we found an increase of connectivity in the Infra limbic cortex induced by consolidation that persist after extinction.

Our results point on a dynamic view of memory consolidation: a regulated balance of synaptic stability and synaptic plasticity is required for optimal memory retention to allow the incorporation of new memories in neuronal circuits.

Una delle funzioni principali della memoria risiede nella sua capacità di conservare le informazioni nel tempo. Il modello più accreditato si basa sulla premessa che le informazioni vengono temporaneamente immagazzinate nell'ippocampo dove rimangono vulnerabili alle interferenze provocate da contingenze esterne. Attraverso un processo molto lento, la traccia mnestica è trasferita in altre strutture del cervello dove la memoria è resa stabile e non più vulnerabile alle interferenze. Questo processo richiede tempo per essere portato a termine ed è chiamato consolidamento (Mueller and Pilzecher, 1900). Varie teorie ipotizzano che i meccanismi attraverso i quali le memorie possono essere acquisite e consolidate nel cervello dei mammiferi implichino modificazioni nella plasticità strutturale (Cajal, 1891).

Lo scopo principale di questo lavoro è quello di ricercare le modificazioni morfologiche necessarie alla formazione e all'estinzione della memoria.

Nel primo lavoro esposto mostriamo la presenza di cambiamenti plastici (sotto forma di aumento in densità di spine dendritiche) nell'ippocampo, immediatamente dopo un addestramento in un condizionamento avversivo al contesto. Questi cambiamenti sono temporanei poiché scompaiono 36 giorni dopo. Contemporaneamente in corteccia anteriore cingolata è visibile un aumento di spine dendritiche solo dopo aver testato gli animali per la loro memoria a lungo termine ma non dopo il test di memoria a breve termine, mostrando un pattern inverso rispetto a quello trovato in ippocampo.

Nel secondo lavoro esposto, blocchiamo la possibilità di aumentare il numero di spine in corteccia anteriore cingolata e troviamo una finestra temporale durante la quale i rimodellamenti sinaptici che avvengono in questa regione sono fondamentali per un corretto consolidamento delle memorie a lungo termine.

Nel terzo lavoro presentato mostriamo come l'estinzione di un comportamento indotto dal consolidamento di una memoria precedentemente acquisita modifichi nuovamente la rete sinaptica che si era lentamente formata nella corteccia anteriore cingolata. Contemporaneamente troviamo un aumento in connettività sinaptica nei neuroni della corteccia infralimbica indotto dal consolidamento che persiste dopo l'estinzione.

I nostri risultati puntano sulla versatilità e plasticità delle reti neuronali che sottostanno ai processi di memoria.

3. Memory

3.A. Multiple memory systems

Our life is the memory we maintain of events and emotions we had.

In the evolving world we live, the ability to acquire and store particular information is essential to survive.

Learning and memory has been theme of interest to philosophers long before it became a field of psychological and biological study. In psychology, memory is an organism's mental ability to store, retain, and recall information.

Even if the earliest theory treated memory as a unitary system, William James (1890) was the first introducing a dichotomy between primary (short-term, STM) and secondary (long-term, LTM) memory, the first with a rapid acquisition as a rapid degeneration, a limited capacity but the ability to acquire a large amount of specific information of the trace; the second with durable long-term storage, higher capacity, a slow rate of acquisition and the tendency to encode more general characteristics of the information previously acquired (Waughn and Norman, 1965; Baddeley, 1966).

The two types of memory are strictly connected since an information that is previously acquired from the first STM system can be consolidated into the second one.

The first empirical evidence of the existence of multiple memory systems stems from the work of Milner and colleagues with the patient HM (Scoville and Milner, 1957; Penfield and Milner, 1958). HM was treated for his epilepsy by removal of his medial temporal lobes. This caused a extensive form of anterograde amnesia: although his working memory and procedural memory were intact, he could not commit new events to long-term memory.

Since then, many studies focused their attention on patients with hippocampal lesions showing impairments in spatial memory while sparing procedural learning.

These studies suggest a selective role of medial temporal lobe (MTL) and more specifically of the hippocampus in declarative memory since many patients with lesions in the MTL exhibited anterograde amnesia sparing memories for information acquired sufficient time before the lesion.

The dissociable effects of focal lesions on declarative versus non-declarative memories support the idea that there are multiple anatomically-segregated memory systems (Scoville and Milner, 1957; Penfield and Milner, 1958; Squire and Zola-Morgan, 1991; Squire and Alvarez, 1995; Milner et al., 1998).

Subsequent studies were carried on to clarified the correlation between different types of memory and the anatomical correlated memory system. In 1982, McCormick and colleagues showed that the cerebellum is essential for delay eyeblink conditioning (McCormick, 1982). The striatum was found to be involved in habit formation (Packard and McGaugh, 1996), and the amygdala was discovered to be essential for fear conditioning and conditioned place preference (Davis, 1992; McDonald and White, 1993; White and McDonald, 1993; Fanselow and Kim, 1994).

These data contributed to classified long-term memories in different sub-components. Cohen and Squire first introduced the distinction between declarative (explicit) and non-declarative (implicit or procedural) memory (Cohen and Squire, 1980; Squire et al., 1993). Declarative memory was defined by conscious recollection: memory content such as facts and events can be recalled to consciousness. Non-declarative memory causes behavioural changes (such as the acquisition of skills, habituation or phenomenon of priming) but the memory content remains inaccessible (Squire and Zola-Morgan, 1991). Tulving (1987) suggests another division within declarative memory between episodic memory and semantic memory. Episodic memories are memories of specific learning experience with strong autobiographical aspects. Semantic memory constitute world-knowledge as meaning and relationships between objects, people, places and concepts often not directly related to personal experiences.

Squire and Zola-Morgan (1991) proposed a taxonomy based on psychological data dividing memory into a declarative and procedural, with declarative memory divided into episodic and semantic (Schachter and Tulving, 1994) and procedural memory subdivided into skills, priming, simple classical conditions and other elementary categories (Everitt and Robbins, 2005; Schultz and Dickinson, 2000) (Figure 1).

Figure 1

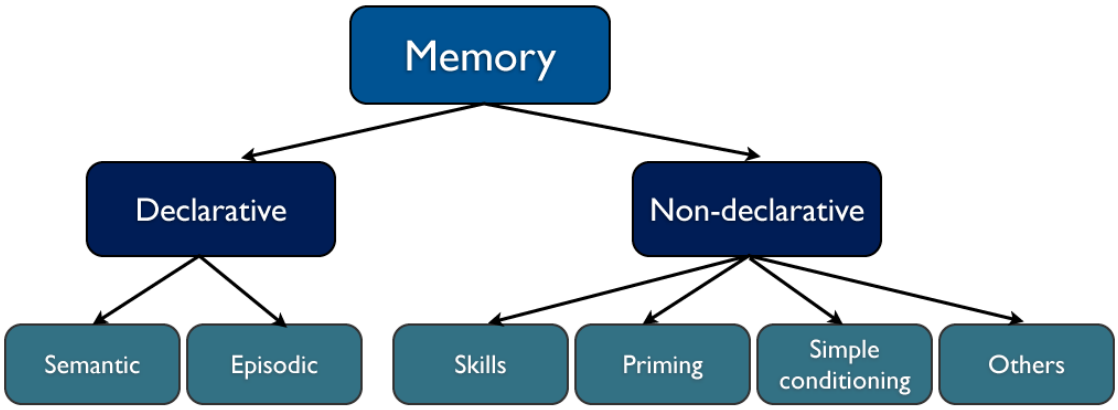

Figure 1: Memory classification and structures involved. Adapted from Squire (1992).

3.B. Memory Consolidation

The process of stabilization that progressively take place after the acquisition of a trace is called "consolidation". The term consolidation is attributed to Muller & Pilzecker, who discovered, in a series of studies carried out between 1892 and 1900, that memory takes time to fixate, or undergo "Konsolidierung" (Muller & Pilzecker 1900). Muller & Pilzecker found that correct recall of the target material improved during the first few minutes after training, and that if in this short delay new stimuli are presented, it tends to impair recall of the target material (a phenomenon they termed "retroactive inhibition"). They suggested that this reflects a posttraining time window during which associations are consolidated.

The quest for the neurobiological foundations of both slow and fast consolidation gained real momentum only in the second half of the last century. Quantitative, systematic studies of retrograde amnesia started to appear in the 1960s and 1970s (Sanders & Warrington 1971). These studies were accompanied by the development of animal models of human amnesia and attempt to identify brain substrates critical for slow consolidation (Squire et al. 2001). In parallel, neuropharmacological studies, first systemic and later targeted to selected brain areas, began to unravel molecular candidates for the cellular machinery that subserves fast consolidation (Dudai and Morris 2000; McGaugh 1966, 2000). Cellular preparations and advanced molecular biology and neurogenetics have together revolutionized the field in the past two decades (Dudai 2002, Milner et al. 1998).

Consolidation is currently used in the neuroscience literature to refer to two types of processes (Dudai 1996, Dudai & Morris 2000; Dudai, 2004). One type is believed to involve reorganization over time of the brain circuits, or the systems, that encode the memory. During the process the trace may spread to new locations in the brain while at the same time may became independent from parts of the circuits that have sub-served its acquisition. This type of process is termed "system consolidation". Sometimes, it is referred as "slow consolidation" but it is questionable because there might be cases in which system consolidation is accomplished within a time frame close to that of the other type of consolidation process: the synaptic consolidation. It starts within the first minutes to hours after the encoding has occurred or practice ended. Much attention has been devoted to processes and mechanisms of consolidation in synapses. The following two main sessions will describes this two processes more in detail.

3.B.1. System consolidation

System consolidation theory has been developed, hypothesizing that the hippocampus is required to strengthen the initially weak connections among cortical modules/areas that are encoded in parallel with the potential index sites in the hippocampus (Teyler and DiScenna 1986).

Human and animal studies starting from H.M case are been collected in support to this thesis. As previously described patients with medial temporal lobe damage shown temporal-graded amnesia, impairing recent but not remote memory (Scoville and Milner, 1957; Zola-Morgan et al., 1986). Recent functional brain imaging data in human suggested that during the recall of semantic memories, hemodynamic activity in the hippocampus is higher for recent news events related to older ones (Smith and Squire, 2009; Takashima et al., 2006).

Several studies in animals investigated extra-hippocampal area implied in storage of consolidated memories.

Different models raised in the last years with the purpose of explain the role of hippocampal and other regions in the consolidation process.

The following three sessions are committed to explain these theories and their validity.

3.B.1.1 The standard consolidation model

The standard theory is the most accepted hypothesis of system consolidation and it holds that consolidation is a process that involves a dynamic interaction between the hippocampus and cortex that gradually - over weeks or months - enables a stable associative network of traces that are later used for memory retrieval (Squire and Zola-Morgan, 1991).

According to this view the hippocampus stores a "snapshot" or "index" of experience through fast learning, while the neocortex acts as a slow learner.

At support to this theory Bontempi and colleagues tracked changes in the organization of spatial discrimination memory in mice using (14C)2-deoxyglucose uptake to map changes in brain metabolic activity at the regional level (Bontempi et al.,1999). It was also detected the expression of genes such as c-fos and Zif268 to visualize changes in neuronal activity at the cellular level after contextual fear conditioning (Frankland et al., 2004). The recall of recent spatial memories was associated with activation of the hippocampus and entorhinal cortex. By contrast, the recall of remote spatial memories was predominantly associated with activation of cortical regions such as the prefrontal, frontal, anterior cingulate, retrosplenial and temporal cortices.

These imaging studies indicate that spatial and contextual memories are represented in distributed cortical networks.

Such remodelling might be mediated by either weight plasticity (that is, rapid modification of existing connections between neurons) or wiring plasticity (that is slower structural changes leading to the addition/elimination of synapses and modulation of axonal and dendritic growth) (Chklovskii et al., 2004). Growth-associated protein 43 (GAP43), a marker of synaptogenesis (Benowitz and Routtenberg, 1997), is induced in the cortex following recall of fear memories (Maviel et al., 2004), which is consistent with the idea that cortical consolidation involves rewiring.

The model hypothesize that over time the trace previously acquired is reactivated (during sleep or inactivity as demonstrated by Wilson and McNaughton, 1994 and Buzsaki and Solt, 1995), leading the reinstatement of the activity caused by past experiences.

This reactivation strengthens the neural interconnections between parts of the representations so that eventually the neocortical memory network can be responsible

for declarative memory retrieval by itself, becoming independent from the hippocampus (Marr, 1971; Squire and Alvarez, 1995; McClelland, 1994; McClelland et al., 1995).

It is not yet known what triggers system reorganization, but the most parsimonious account is that over time, upon recurrent activation of the hippocampal trace either in explicit recall or in implicit processing (e.g., sleep), the hippocampal formation and related structures send synaptic messages to neocortical neurons, and these messages trigger synaptic consolidation locally (synaptic consolidation).

Figure 2

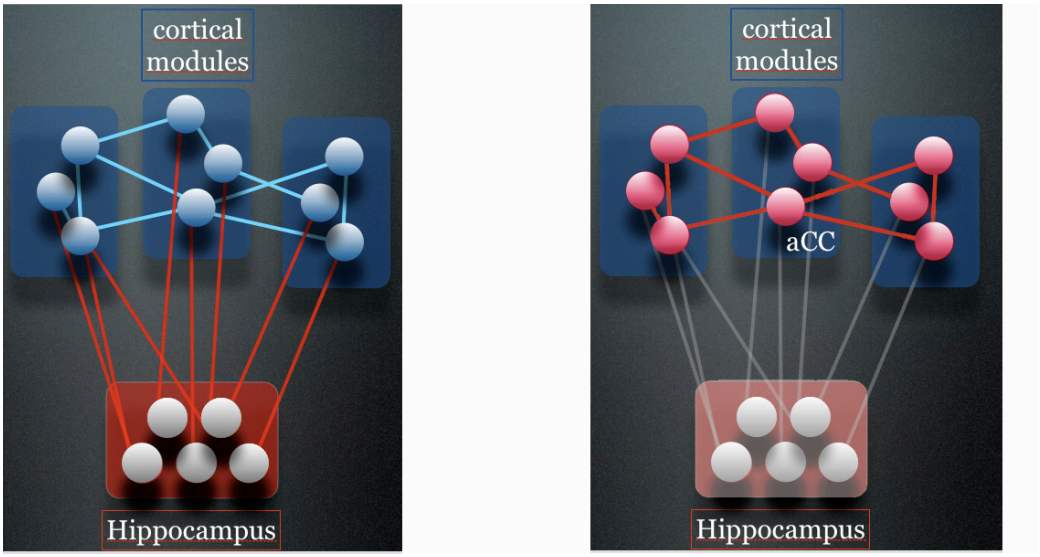

Figure 2: The standard consolidation model: Initially, memories are encoded in hippocampal-cortical networks. At this early time point, the hippocampus is crucial in integrating information from distributed cortical modules, each representing individual components of a memory (a). However, as the memory mature connections between the different cortical modules are strengthened, allowing the memory to function independently of the hippocampus (b). Adapted from Frankland and Bontempi, 2005

3.B.1.2. The multiple trace theory

Nadel & Moscovitch (1997) proposed a multiple trace theory (MTT) of memory. According to this theory, the hippocampal complex rapidly encodes all information that is attended (Moscovitch and Umiltà, 1990; Moscovitch, 1992) and connect the neocortical (and other) neurons that represent that experience into a memory trace. This information is sparsely encoded in a distributed network of hippocampal complex neurons that act as a pointer, or index, to the neurons representing the attended information (Teyler & DiScenna, 1986). A memory trace of an episode, therefore, consists of a organized network of neocortical and medial temporal lobe (MTL) neurons which represent a memory of the experience. As noted earlier, formation and

consolidation of these traces, or cohesion (Moscovitch, 1995), is relatively rapid, lasting on the order of seconds or at most days.

In this model, there is no prolonged consolidation process, as the standard model asserts, that slowly strengthens the neocortical component of the memory trace, so that with time the trace becomes independent of the hippoampus. Instead, each time an old memory is retrieved, a new hippocampally mediated trace is created so that old memories are represented by more or stronger MTL–neocortical traces than are new ones and, therefore, are less susceptible to disruption from brain damage than are more recent memories.

Although the gist of a memory may be intact after hippocampal damage, the theory asserts that the detail and vividness of memory requires the hippocampus (Nadel et al. 2000). Specifically, it suggests that the hippocampus is always required for storage and retrieval of allocentric and spatial memories (Rosenbaum et al. 2001), whereas semantic memory is mediated by neocortex alone, subject to the completion of a systems consolidation process after learning.

In this view hippocampal index traces, retained by synaptic consolidation mechanisms, guide the process by which new information is subject to systems consolidation, possibly by altering the synaptic weights of initially ‘silent’ connections to allow for rapid incorporation of new information in schema. Such intercortical connections may take time to develop (Chklovskii et al. 2004). However, once built, relevant new information can be assimilated into schema very rapidly.

3.B.1.3. Rapid system consolidation model

Alternative new perspective proposed by Wang and Morris (2010) considers the place of prior knowledge or mental schemas in determining the speed with which systems consolidation takes place. According to the standard model, it is widely thought that it takes a long time before intercortical connections become strong enough to support unaided memory retrieval.

Supporting evidence comes from studies in animals, which show that a dynamic shift in maximal immediate early gene (IEG) expression after learning—from hippocampus to cortex— takes place over weeks (Frankland and Bontempi 2005). This new model considered whether new information processed by the hippocampus can be consolidated

into the cortex more easily, or in a different way, if this new information is relevant to prior knowledge.

Like the standard model of consolidation, it is reasonable to suppose that such growth processes take time. However, once a framework or schema is created, it may then be possible to assimilate relevant new information relatively easily.

The new schema idea about consolidation has emerged from Tse et al. (2007) work where rats trained for a single trial on each of two new paired-associates 48 hr prior to being given bilateral HF lesions, having previously learned six paired-associates and developed a schema, could successfully recall the correct location at which to dig for more food when given a recall trial two weeks later.

The work point to the possibility that systems consolidation can occur in a much shorter time scale, challenging the concept of "fast" and "slow" learning systems.

The short interval that emerged from this result also points to the potential importance of sleep in consolidation, in keeping with much current theorizing in humans (Stickgold and Walker 2007) and in animals (Buzsaki 1989, Sutherland and McNaughton 2000).

In particular during sleep, replay of a neural firing pattern that was previously recorded during training in the awake period is observed in the rat hippocampus (Skaggs and McNaughton 1996) and prefrontal cortex (Euston et al. 2007). Takehara-Nishiuchi and McNaughton (2008) further showed that prefrontal cortical neurons maintain increased activity for up to six weeks after training. Evidence for hippocampus-neocortical interaction comes from a study showing that signatures of neural activity in the hippocampus coincide with neocortical activity (Battaglia et al. 2004), suggesting an orchestration of activity between hippocampus and neocortex consolidation needs future studies.

3.B.2 Synaptic consolidation

First speculations and empirical experiences attempting to relate how neural mechanisms may promote the storage of information have to be traced back to Donald Hebb. In his seminal book (The organization of Behavior, 1949) he introduced the concept of learning as a time-dependent, local and highly interactive process increasing synaptic efficacy. In particular, Hebb suggested that the repetition of coincident activation of pre- and post-synaptic cells causes changes in their reciprocal connections,

making them more likely to be activated in concert - “Cells that fire together, wire together”. In particular, Hebb proposed that repeated stimulation of specific receptors during learning leads slowly to the formation of ”cell-assemblies” which can act as a closed system after stimulation has ceased. This continuous cerebral activity serves not only as a prolonged time for structural changes to occur during learning, but also as the “simplest instance of a representative process” (Hebb, 1949).

The cell assembly theory seems to work well with the known data of synaptic plasticity, cortical modularity and the formation of integrated networks of activity across the brain. In particular a phenomena that fit well with this model is the long-term potentiation (LTP) of synaptic activity. In fact, LTP can be induced e.g. by tetanic stimulation or by theta-burst stimulation. This form of associative plasticity is of particular interest because it is an instantiation of Hebb’s postulate—essentially, that simultaneous pre- and postsynaptic activity results in the strengthening of the synaptic connection. In fact, only in presence of Glutamate and at the same time postsynaptic depolarization, the NMDA receptors permit the influx of Ca2+, which is a critical early step in the induction of LTP.

To date long-term potentiation (LTP) is considered the most popular and widely researched model of synaptic plastic changes that might occur during learning (Holscher, 1999).

LTP is a long-lasting enhancement of synaptic transmission and represents an electrophysiological correlate of processes attributed to learning and memory (Bliss and Collingridge, 1993; Bliss and Lomo, 1973; Malenka and Nicoll, 1999). LTP can be subdivided into distinct phases. Early LTP (E-LTP) is short-lasting (about 1h) and requires post-translational modification of synaptic proteins but is independent of protein synthesis. Late LTP (L-LTP) represents the long-lasting phase of LTP that is both translational and transcriptional dependent (Reymann and Frey, 2007; Voronin et al., 1995).

Very interesting LTP seems to be associated with increased spine densities (Muller et al., 2000) and with the formation of new, mature and probably functional synapses (Toni et al., 1999). Long-term depression (LTD) is thought to play an integral role in the processing and retention of information but, in contrast to LTP, LTD is long-lasting reduction in synaptic transmission. LTD is associated with declines in spine densities (Bastrikova et al., 2008; Monfils and Teskey, 2004) and with shrinkage of dendritic

spines in the hippocampus (Zhou et al., 2004). Thus, dendritic spines can undergo bidirectional morphological changes in response to neuronal activity (Nagerl et al., 2004).

Although LTP has been studied in great detail since its discovery, only recently it was observed in vivo in the hippocampus following inhibitory-avoidance learning in rats, using multi-electrodes recording (Whitlock et al., 2006). Until then, it was only observed LTP-like changes that occur during and after learning (Martin & Morris, 2002).

Many data has accumulated showing that structural plasticity (i.e. enduring changes in the structure of both synapses and cells altering the pattern of connectivity between neurons) may be effectively involved in the persistent alteration of synaptic efficacy and hence in the representation of information. In particular it seems increasingly obvious the importance of spines and of their long-lasting modifications in the process of synaptic consolidation.

The structural modifications accompanying learning and memory can be grouped into two major categories: changes in pre-exisiting synapses; and changes in the number of synapses (Xu et al., 2007; Pan and Gan, 2008; Nishiyama et al., 2007).

Experience-related manipulations produce changes in the size and vesicle complement of the active zone, in the total number of vesicles per presynaptic terminal, in the geometry of apposition between pre- and postsynaptic components (Bonhoeffer and Yuste, 2002). More interesting and enduring processes produced by experience are structural modifications in postsynaptic dendritic spines (Terry et al., 1991; Segal, 2002; Lendvai et al., 2000; Sin et al., 2002; Nimchinsky et al., 2002; Crair et al., 1998; Fischer et al., 2000)

In the following session will be largely described the characteristics of enduring changes in spines and their role in consolidating memories.

3.B.2.1. Dendritic Spines

Because dendritic spines are the key elements for information acquisition and retention, understanding how spines are formed and maintained, particularly in the intact brain, will likely provide fundamental insights into how the brain possesses the extraordinary capacity to learn and to remember.

In 1888, Cajal first described the dendritic spines, which had been discovered using a silver-impregnation protocol developed by Golgi. The Golgi impregnation is still one of the methods used to examine dendritic spines today and is the main method we used in our experiments.

It was not until the late 1950s that electron microscopy (EM) identified the synapse and the dendritic spine as associated structures (Gray, 1959). A couple of decades later, contractile actin was found to be ubiquitously present in dendritic spines (Crick, 1982; Matus et al., 1982). We now know that the vast majority of excitatory synapses are made on the heads of dendritic spines, which can be found on both excitatory and inhibitory neurons throughout the central nervous system and in an array of diverse species (Yuste and Bonhoeffer, 2004; Dailey and Smith, 1996). For this reason the size of a spine is closely related to the size of its excitatory synapse.

Thus, spine surface area, spine volume, bouton volume and number of presynaptic vesicles correlate with the synaptic area, while the length of the spine and the neck diameter are independent of the synapse size (Fiala et al., 2002).

Neurotransmitter receptors are largely restricted to the spine head and concentrated close to the presynaptic active zone. This zone is indicated by the postsynaptic density (PSD). The PSD is a complex electron dense structure opposed to the presynaptic actve zone that is composed of a lot of components, including receptors, cytoskeletal and adaptor proteins and associated signalling pathways involved in synaptic plasticity (Nimchinsky et al., 2002).

One of the most striking characteristics of their morphological diversity. Depending on the shape, the dendritic spines can be subdivided into different categories. The most widely used nomenclature divides the dendritic spines into 3 categories based on the relative sizes of the spine head and neck (Peters and Kaiserman-Abramof, 1970):

Mushroom (large head and visible neck), Thin (small head and long neck) and Stubby (large head without neck).

Other authors have added a further category, the so-called Chubby spines to refer at spines with small head and no neck (Jedynak et al., 2007) (Figure 3).

There are ultrastructural evidences that larger spines have larger PSDs (Harris and Stevens, 1989), and larger PSDs contain more AMPA receptors (Nusser, 2000; Takumi et al., 1999; Kasai et al., 2003) (Figure 4).

Figure 3

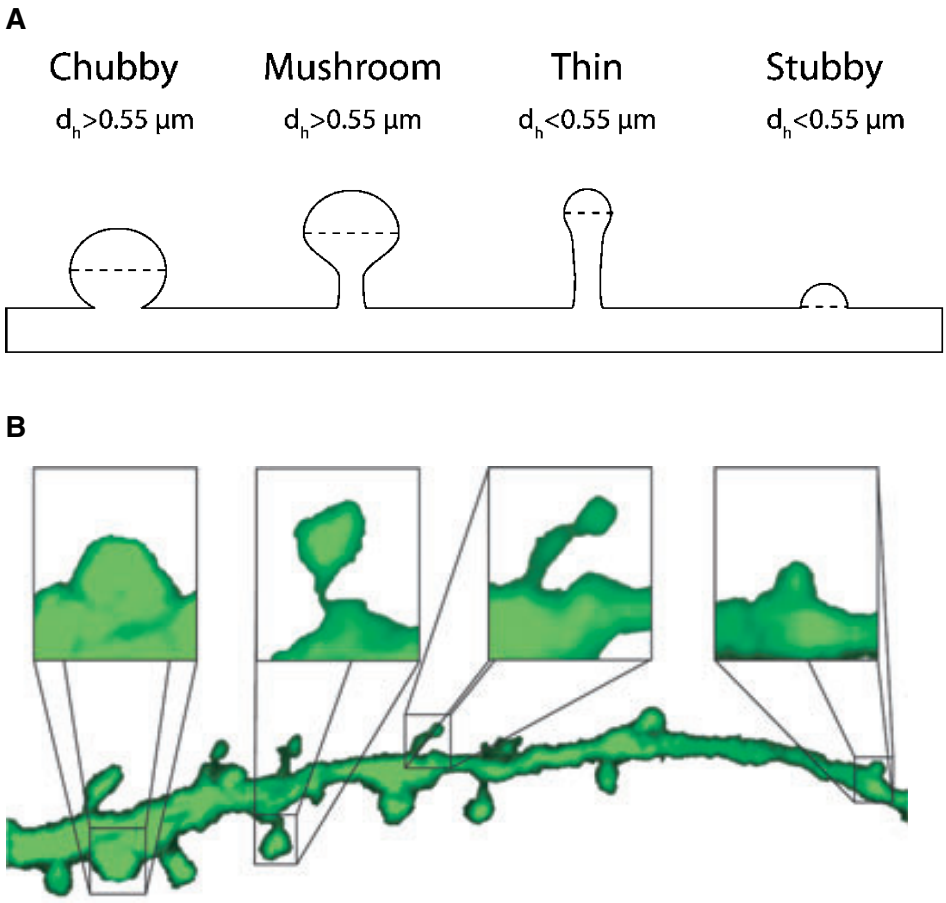

Figure 3 (a) Criteria applied to separate spines in four morphologically distinct groups. (b) Example of a dendrite that contain all four spine types. From Jedynak et al., 2007

Based on this observations, Matsuzaki et al (2004) suggest that small spines are preferential sites for LTP induction, whereas large spines might represent physical traces of long-term memory. There is no evidence to support this idea yet. In particular our results are in contrast with this drastic point of view. An hypothesis more convincing is that spines with large heads are stable and contribute to strong synaptic connections, whereas spines with small heads are motile and unstable and contribute to weak or silent synaptic connections.

Furthermore, not only the size and shape of individual spines seems to be important in the context of plasticity but also the number of spines. For example, Moser et al. (1994) demonstrated that spatial training was associated with a significant increase in spine densities of CA1 pyramidal neurons .

The density of spines is related to the amount of connectivity between the neurons with the dendritic spines and the axons from other neurons that built up synaptic contacts. Thus, one role of the dendritic spines is to establish and maintain these connections. Currently it is thought that spine density seem to reflect excitatory input density (Konur et al., 2003).

Figure 4

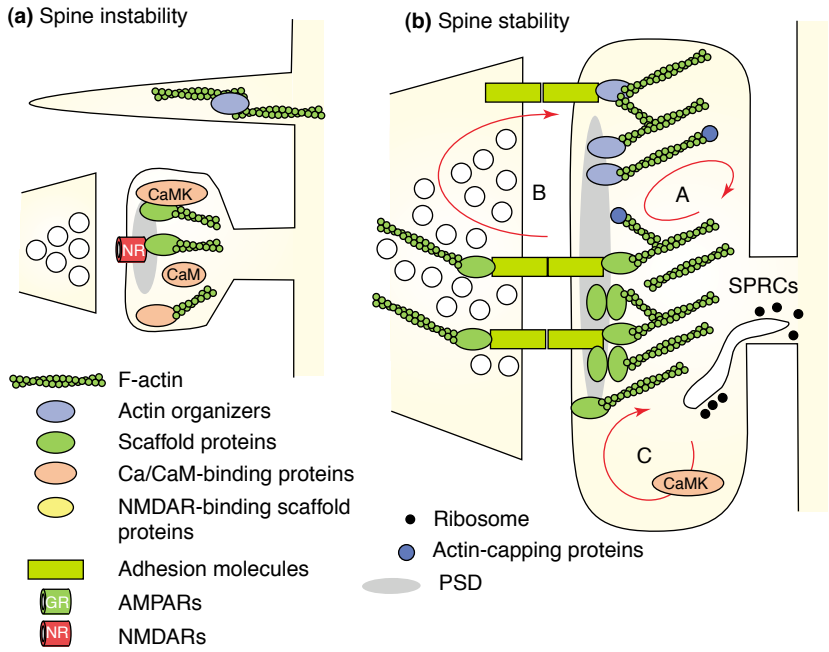

Figure 4: Spine structure -stability-function relationship. (a) Spine instability: The cytoskeleton, various actin-organizing enzymes and Ca2+/calmoduline -binding proteins contributes to spine genesis, motility, enlargement and elimination. (b) Spine stability. Hypothetical mechanisms of spine stability include the positive feedback effects via filamentous actin organization within the spine (A), via spine interaction with the presynaptic terminal (B) and via protein synthesis inside or close to a spine (C). From Kasai et al, 2003.

With the advent of new technical advances, specifically, the use of fluorescent proteins in specific neuronal types and in a sparse pattern that creates a living "Golgi stain" (Feng et al. 2000), several research groups have published studies that examine the dynamics and motility of dendritic spines in vivo (Grutzendler et al. 2002, Trachtenberg et al. 2002, Majewska & Sur 2003, Oray et al. 2004, Holtmaat et al. 2005, Zuo et al. 2005a, Zuo et al. 2005b, De Paola et al. 2006, Holtmaat et al. 2006, Knott et al. 2006, Majewska et al. 2006, Oray et al. 2006).

From these data it was clear that dendritic spines are highly dynamic structures, particularly during postnatal development, when enormous numbers of synaptic

connections are being rapidly made (Dailey and Smith, 1996; Grutzendler et al., 2002; Matsuzaki et al., 2004; Rakic et al., 1986) confirming data previously collected with Golgi-staining analysis. As animals mature into adulthood, substantial changes in spine number and morphology may still occur during the learning process (Carlisle and Kennedy, 2005; Zuo et al., 2005; Zuo et al., 2005; Restivo et al., 2006).

Spinogenesis: Dendritic spines appear during the early phases of development immediately after dendritic processes are extended from neurons and synaptic contacts already exist between spines and presynaptic axons, suggesting that the process of spine formation is intimately associated with the process of contact formation between neurons and the establishment of neural circuits.

Several lines of evidence suggest that dendritic filopodia, long and thin protrusions without bulbous heads, play a pivotal role in the initial stages of spinogenesis and synaptogenesis. First, during early development, when extensive spine formation occurs, filopodia are highly abundant and undergo rapid extension and retraction within minutes to hours (Yuste and Bonhoeffer, 2004; Dailey and Smith, 1996; Dunaevsky et al., 1999; Ziv and Smith, 1996; Portera Cailliau and Yuste, 2001; Portera Cailliau et al., 2003; Tashiro et al., 2003; Jontes and Smith, 2000). These highly dynamic dendritic filopodia initiate contacts with presynaptic axons and are occasionally transformed into spines (Dailey and Smith, 1996; Ziv and Smith, 1996). Bhatt et al. (2009) from these data extrapolate the following model: Long and thin dendritic filopodia rise from dendrites and this protrusions exhibit dynamic growth, allowing them to sample some of the nearby axons. Choosing and capturing the appropriate presynaptic axon via activity-dependent or independent signaling would result in stabilization of the contact and maturation of the filopodia into dendritic spines. The absence of proper signals (or the presence of alternative ones) would result in the regression of the filopodia back into the dendritic shaft (Yuste and Bonhoeffer, 2004; Portera Cailliau et al., 2003; Jontes and Smith, 2000; Korkotian and Segal, 2001; Lohmann and Bonhoeffer, 2008; Richards et al., 2005).

Spine Pruning: In the cerebral cortex of mammals, including that of humans, rapid synaptogenesis during early postnatal life is followed by a substantial (~50%) loss of synapses/spines that extends through adolescence (Huttenlocher, 1990; Rakic et al., 1986; Huttenlocher, 1979; Markus and Petit, 1987; Rakic et al., 1994; Lubke and Albus, 1989). In adulthood the number of spines remains relatively constant until aging-related loss of synapses occurs (Duan et al., 2003; Terry et al., 1991).

Observations of dynamicity in vivo of spines corroborate previous studies from fixed tissues showing that synaptic density in the mammalian cortex decreases substantially from infancy until puberty (Huttenlocher, 1990; Rakic et al., 1986; Markus and Petit, 1987). These *in vivo* imaging studies indicate that the major reorganization of the cortex during late postnatal life involves the elimination of existing connections between neurons (Figure 5).

Figure 5

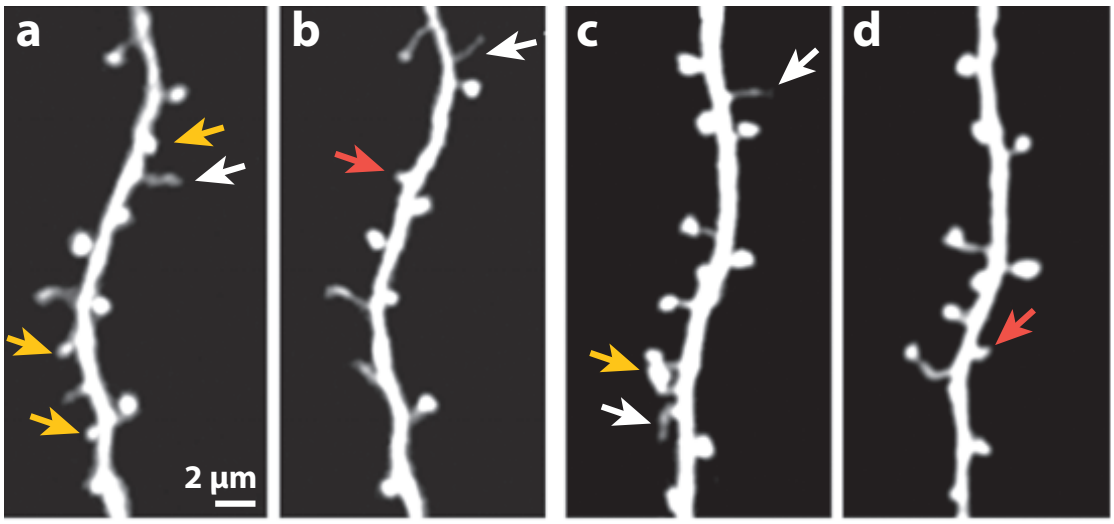

Figure 5: In vivo time- lapse imaging of dendritic spine dynamics and stability in the mouse cortex. (a-d) Repeated imaging of two dendritic branches from mice that are four to six weeks of age reveals spine elimination (Yellow arrows) and formation (red arrows) as well as filopodium turnover (white arrows) in control (a,b) and sensory-deprived (c,d) barrel cortices. From Bhatt et al, 2009.

Experience-dependent spine formation and elimination: A wide variety of experimental evidence has shown that experience/neuronal activity plays a critical role in regulating synaptogenesis (Engert and Bonhoeffer, 1999; Zuo et al., 2005; Yuste and Bonhoeffer, 2001; Buonomano and Merzenich, 1998; Bailey and Kandel, 1993; Gan and Lichtman, 1998; Katz and Shatz, 1996). For example, long-term sensory deprivation from birth

continuing to young adulthood often reduces the number of synapses, whereas enriched environments during the same period increase dendritic branching and synapse number in various brain regions (Greenough et al., 1973; Knott et al., 2002; Grossman et al., 2002; Winkelmann et al, 1977; Fiala et al., 1978; Valverde, 1967; Valverde, 1971; Lendvai et al., 2000). Zuo et al. (2005) showed that, in young adolescence, when extensive spine loss occurs, sensory deprivation over weeks preferentially reduces the rate of spine elimination rather than the rate of formation (Figure 5).

However, the degree to which experience modifies synaptic connectivity in the adult brain is still in question.

Dendritic spines possess an inherent plasticity, as is evidenced by significant and rapid changes in spine number in response to environmental challenges and under pathological conditions (Duan et al., 2003; Fiala et al., 2002; Greenough et al., 1973; Knott et al., 2002; Terry et al., 1991; Tsai et al., 2004; Zhang et al., 2005; Kirov et al., 2004; Restivo et al., 2005).

Some forms of learning have been shown to increase the number of dendritic spines (Geinisman, 2000; Leuner et al., 2003; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2001; Engert and Bonhoeffer, 1999; Leuner and Shors, 2004; Knafo et al., 2004; Muller et al., 2000).

Spine density dynamics: One important extrapolation from studies on experience-dependent spine dynamics is that a small degree of spine turnover does occur and can be modified by experience in the mature brain (Bhatt et al., 2009).

Revealing the dualism plasticity/stability of spines in adulthood will provide important insights into how long-term information is stored (and lost) in neural circuits.

If most adult spines remained throughout life, memory and basic cortical functions could be stably maintained through synaptic connections that were established during development. In contrast, findings that adult spines were highly dynamic and showed a high degree of turnover over the lifetime of an animal would suggest that long-term information might instead be stored in a dynamic fashion in constantly and rapidly rewiring synaptic networks.

An important observation is that the addition of a new stable spine and synapses is a rare event in adult mice.

Bailey & Chen (1991) in Aplysia's synapses found that not all structural changes persist as long as the memory.

The finding that some aspects of synaptic structure are transient while others endure suggests these changes are not all synchronously regulated. At the structural level, the cell appears to have several mechanisms of plasticity available to it.

These findings indicate that some component, perhaps the growth of new synapses, once in place can carry the facilitation without requiring the continued persistent activity of proteins and suggest that the structural changes may represent the final and perhaps most stable phase of long-term memory storage. The stability of long-term memory storage may be achieved, in part, because of the relative stability of synaptic connections.

Despite that, the idea that plastic changes of synapses are completed and maintained permanently imply a kind of "synaptic phrenology" (Abraham and Robins, 2005) assuming that trace is stored in a fixed configuration of synaptic weights in specific neural circuits. Such organization would allow a limited range of configurations and available connections and would saturate very quickly (Bramham et al., 2008). Instead, continued updating of synaptic weights within a dynamic network would greatly increase its capacity to process and store information. Beyond possible advantages in capacity, studies using artificial neural networks have shown that ongoing alteration in connection weights within network ensembles are necessary to maximize maintenance of previously stored information (Abraham and Robins, 2005). Activity at the time of initial encoding must be repeated to maintain changes in connections. The same study also suggested that such iterative processing of synaptic weights could take place during quiet periods or sleep. Evidence of such offline updating processes are derived from studies of neural reactivation during quiet awake periods and sleep (Foster and Wilson, 2006; Louie and Wilson, 2001; Wilson and McNaughton, 1994). These evidences shows that long-lasting memories are continuously rearranged and may repeatedly enter labile state when reactivated.

According to this view, the duration of synaptic change does not necessarily define the persistence of a memory; rather, it is likely that a regulated balance of synaptic stability and synaptic plasticity is required for optimal memory retention in real neuronal circuits (Abraham and Robins, 2005).

Wiring plasticity through spine growth may take place in the architecture of the memory formation because of the dynamic nature of spines and synapses as we will try to demonstrate in our works.

Moreover, although the vast majority of spines may persist throughout adulthood, spine morphology undergoes change in the living cortex (Grutzendler et al., 2002; Zuo et al., 2005; Majewska et al., 2006; Holtmaat et al., 2005). Because spine size correlates with synaptic strength, changes in spine morphology indicate that synaptic strength can be modified without synapse turnover (Harris and Stevens, 1989; Matsuzaki et al., 2004).

In a study that combined in vivo time-lapse imaging over one month with retrospective reconstruction of imaged dendrites by serial electron microscopy (EM), Knott and collaborators showed that new protrusions that have not formed synapses have smaller volumes and larger surface-to-volume ratios than do persistent stable spines (Knott et al. 2006). In addition, newly formed spines grow in volume as they become stable. Conversely, most spines show a reduction in volume before disappearing (Holtmaat et al. 2006). Given the correlation between spine head size and PSD area (Harris & Stevens 1989, Holtmaat et al. 2006), the observation that the volume of persistent stable spines is larger than those of transient spines suggests that persistent spines have larger PSDs with higher numbers of AMPA-type glutamate receptors and are associated with stronger synapses.

However, in adult mice, the standard deviation of the mean diameter change increases over time (Zuo et al. 2005), possibly indicating that, in adult mice, spines that are shrinking or enlarging at one time point continue to shrink or enlarge over time, respectively.

3.B.2.2. Molecular regulation of synaptic and spines plasticity

One major focus of research in spine plasticity is to understand how and to what degree spine formation and elimination are regulated by intrinsic genetic programs. By removing or over-expressing individual genes, studies in the past two decades have made important progresses in identifying genes that are important for regulating the number and size of dendritic spines (Calabrese et al., 2006; Carlisle and Kennedy, 2005; Tada and Sheng, 2006; Arikkath and Reichardt, 2008). These genes encode a wide variety of proteins such as neurotransmitter receptors (Alvarez et al., 2007), adhesion

molecules (Arikkath and Reichardt, 2008), postsynaptic density proteins (Marrs et al., 2001; Penzes et al., 2003), protein kinases and phosphatases (Pak and Sheng, 2003), and actin cytoskeleton and its regulatory elements (Matus, 2000; Ackermann and Matus, 2003; Fischer et al, 1998).

One emerging scenario regarding the genetic control of spine plasticity is that various gene products converge to regulate actin polymerization and depolymerization in dendritic spines, leading to spine formation or elimination (Calabrese et al., 2006; Matus, 2000; Tada and Sheng, 2006; Fischer et al., 1998; Oertner and Matus, 2005; Fischer et al., 2000; Feng et al., 2000). Actin filaments form the main cytoskeleton of dendritic spines and underlie rapid spine motility. The Rho family of small GTPases, including RhoA, RhoB, and Rac, regulates the dynamics of the actin cytoskeleton and is an important contributor to spine formation and elimination. Over-expression or suppression of these molecules as well as their interacting proteins results in changes in the density of dendritic filopodia and spines in vivo and in vitro (Penzes et al., 2003; Tolias et al., 2007; Newey et al., 2005; Ma et al., 2008). In addition, neuronal activity can induce extensive remodeling of the actin cytoskeleton via calcium influx through glutamate receptors of the N-methyl-d-aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subtypes, which are highly concentrated at the postsynaptic spines (Dunaevsky et al., 1999; Zuo et al., 2005; Ackermann and Matus, 2003; Fischer et al., 2000). Much evidence has shown that NMDA and AMPA receptor activation is essential for synapse formation and/or maintenance and that the calcium/calmodulin-dependent protein kinase II pathway at least partly mediates the effect of these receptors on actin dynamics (Segal, 2001; Wu and Cline, 1998).

In this thesis we are interested in particular into two new proteins that has been discovered and studied for their link with the modulation of spine density: GAP-43 and MEF2

GAP-43: Several lines of investigation over the past several years have helped clarify the role of GAP-43 (F1,B-50 or neuromodulin) in regulating normal learning and memory.

GAP-43 is a presynaptic protein that controls axon growth and sprouting and that is used as a marker of newly formed synapses (Benowitz and Routtenberg, 1997; Routtenberg et al., 2000). Studies by Routtenberg and colleagues identified GAP-43 as a presynaptic protein kinase C (PKC) substrare that is phosphorylated during LTP in the dentate gyrus. They showed that Gap-43 is required for normal learning and memory as assessed in GAP-43 heterozygous knockdown mice (Rekart et al., 2005).

Indeed, transgenic overexpression of GAP-43, but not the PKC phosphorylation site mutant form of GAP-43, has been shown to increase both dentate gyrus LTP and learning in an 8-arm radial maze (Routtenberg et al., 2000).

Several possible mechanisms for GAP-43's involvement in learning and memory have been proposed, including direct modulation of presynaptic neurotransmitter release (Routtenberg et al., 2000). GAP-43 interacts directly with components of the synaptic release machinery including the SNARE complex proteins (SNAP-25, syntaxin, and syna- ptobrevin) and synaptotagmin (Haruta et al., 1997).

GAP-43 phosphorylation may lead to increased neurotransmitter release (Dekker et al., 1990; Heemskerk et al., 1990), while decreasing GAP-43 may decrease evoked neurotransmitter release (Hens et al., 1995; Ivins et al., 1993).

In the brains of humans and other primates, high levels of GAP-43 persist in neocortical association areas and in the limbic system throughout life, where the protein might play an important role in mediating experience-dependent plasticity.

A very interesting work by Maviel et al (2004) reports that in a spatial memory task animals from the 30-day memory retention group exhibited increased GAP-43 labeling in the anterior cingulate cortex as compared with the 1-day retention group. This results is in line with the believe that consolidation of memories required a reorganization of anterior cingulate cortex synaptic connectivity (Figure 6).

Figure 6

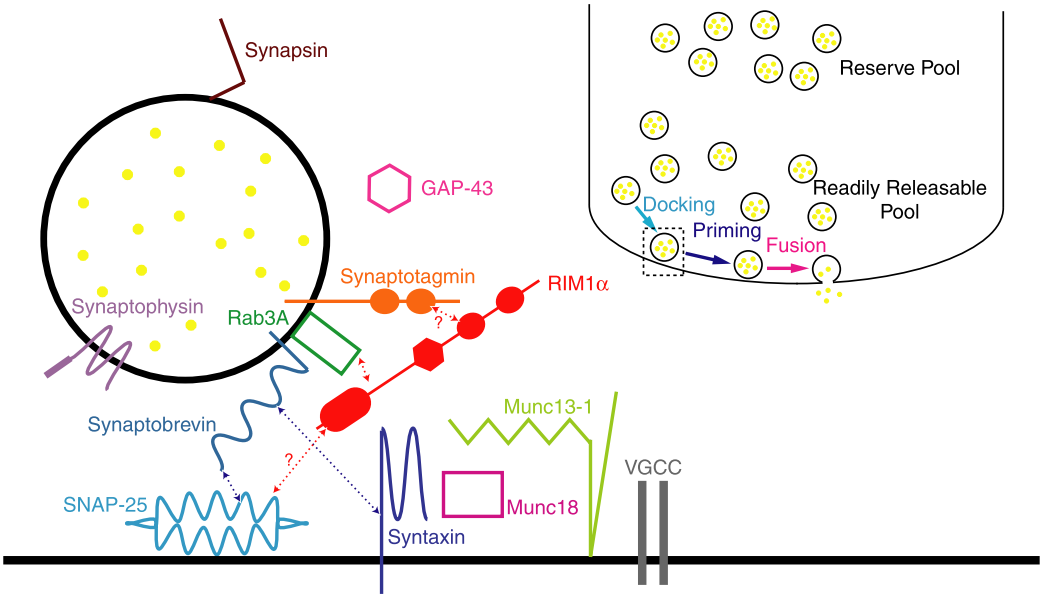

Figure 6: Schematic model of SNARE proteins, including Gap-43 and their location near the membrane.

MEF-2: The myocyte enhancer factor 2 (MEF2) family of transcription factors is implicated in the regulation of different cellular programs, such as muscle differentiation, neuronal survival and T-cell apoptosis. In mammals, four MEF2 isoforms are known: MEF2A, MEF2B, MEF2C and MEF2D (McKinsey et al., 2002). Shalizi et al. (2006) found that MEF2A is required for an early step in dendritic morphogenesis, the development of dendritic claws, which are the branched tips of granule cell dendrites at which glutamatergic inputs terminate. Ca2+ influx triggered by neuronal activity causes dephosphorylation of Ser408 of MEF2 by calcineurin and results in reduced sumoylation and increased acetylation of a nearby residue, Lys403. The phosphorylated and sumoylated form of MEF2A is transcriptionally active and suppresses transcription of NUR77 genes. NUR77, a transcription factor, has a negative effect on dendritic differentiation (Scheschonka et al., 2007). Thus, sumoylation of MEF2A results in the suppression of the negative regulator NUR77 and thereby enhances synapse formation (Figure 7). Because sumoylation depends on the phosphorylation of Ser408, which, in turn, is regulated by calcineurin, this study presents in vivo evidence of how sumoylation is involved in activity-dependent synaptic differentiation (Shalizi et al., 2006; Scheschonka et al., 2007).

Flavell et al. found that dephosphorylated MEF2 activates two other target genes in cultured hippocampal neurons: activity-regulated cytoskeletal associated protein (ARC)

and synaptic RAS GTPase-activating protein (synGAP) (Flavell et al., 2006). These genes have crucial roles in synaptic disassembly by promoting internalization of glutamate receptors and inhibiting postsynaptic Ras-mitogen-activated protein kinase signaling, respectively; as a result, the number of synapses between hippocampal neurons decreases. In hippocampal cells, the effect of sumoylation has not yet been shown but led Beg and Scheiffele (2006) to speculate that this pathway represents the counterpart of activity-dependent synapse formation. Neuronal activity is well documented to trigger both strengthening and weakening of synapses (Malenka and Bear, 2004). It seems that balanced phosphorylation, sumoylation and acetylation of MEF2 enable precisely controlled transcription of important target genes during developmental synaptogenesis and pruning. Conceivably, similar mechanisms might also contribute to the regulation of other neuronal transcription factors, and thereby of distinct neuronal functions.

Figure 7

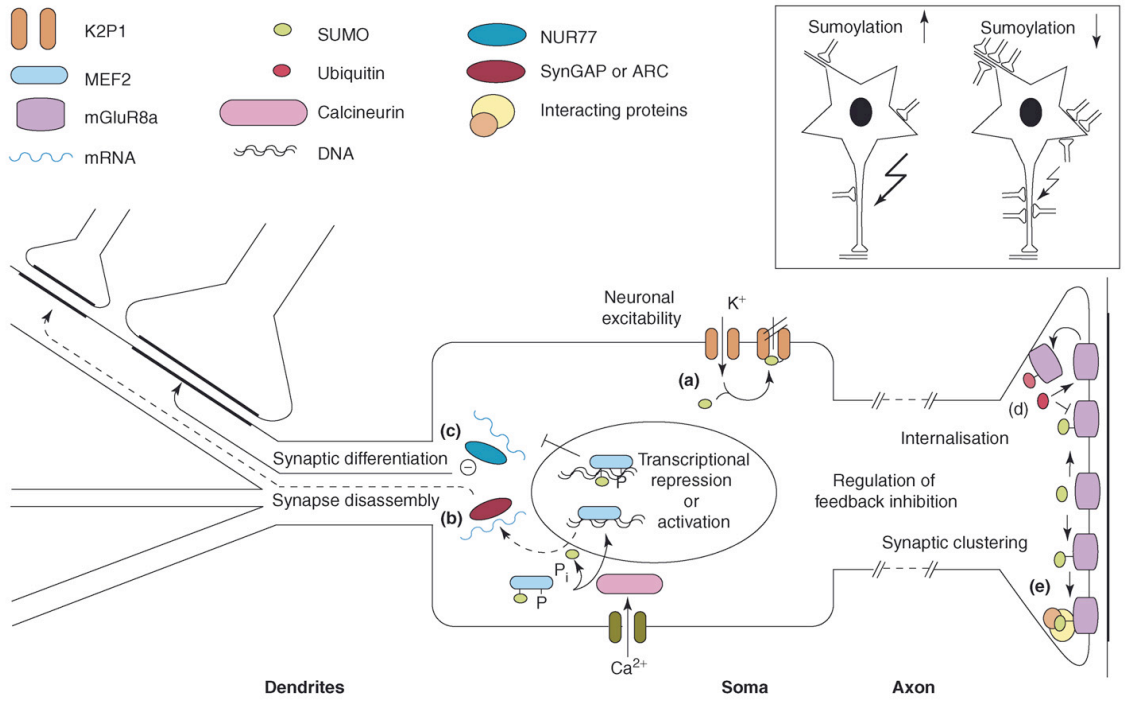

Figure 7: Model of MEF2 activation in neurons. Activity and calcineurin- dependent dephosphorilation and desumoylation of MEF2 promotes the transcription of the genes that encode synGAP and ARC which leads to synapse disassembly. From Scheschonka et al, 2007.

3.C. Memory Stability and Plasticity

A remaining question about consolidation theory is whether memory traces are permanently stabilized once they are consolidated.

For example, the loss of an apparently consolidated fear memory was demonstrated when an electroconvulsive shock was applied immediately after memory recall (Misanin et al. 1968, Schneider & Sherman 1968). This cue-induced amnesia suggested that a once-consolidated memory could still be plastic, leading to the concept of a reconsolidation process. During reconsolidaton, the act of retrieving previously consolidated memories can, in certain situations, put those memory traces back into a labile state such that they are again sensitive to the inhibition of protein synthesis and that they might be strengthened, overridden, or incorporated with new information. Lewis (1979) proposed the idea of active and inactive states of memory to describe the lability of the memory. Two decades later, interest in this concept has been reawakened (Nader et al. 2000, Sara 2000). This is what Dudai (2004) and others (Alberini 2005, Sara 2000) have referred to as memory updating.

Reconsolidation occurs when there is new information at the time of memory retrieval — information that might potentially require an established long-term memory trace to be altered. Hence, the function of re-engaging lability is to change or strengthen the ostensibly consolidated trace.

Paradoxically, although a nonreinforced trial at reactivation may be necessary for observing reconsolidation, multiple nonreinforced trials may give rise to extinction (Pavlov 1927)—and with it, new learning. Thus, fourth, when reactivation is a prolonged non-reinforced session that triggers extinction, the extinction process can dominate, and reconsolidation will fail to occur (Suzuki et al. 2004).

Memory maintenance is widely believed to involve long-term retention of the synaptic weights that are set within relevant neural circuits during learning. This issue is explained in the “system consolidation” session of this thesis.

3.D. Memory Extinction

Fear extinction is the decrease in conditioned fear responses that normally occurs when a conditioned stimulus (CS) is repeatedly presented in the absence of the aversive unconditioned stimulus (US). Extinction does not erase the initial CS-US association, but is thought to form a new memory. After extinction training, extinction memory competes with conditioning memory for control of fear expression (Milad et al., 2006).

Neuroscientific research on extinction has advanced rapidly over the past decade, uncovering the neural mechanisms that regulate this form of learning.

Extinction is not a simple process of erasing the previously acquired memory, but rather is a form of new learning that inhibits the expression of a still present long-term memory (Bouton, 2004). Evidence supporting this comes from the observation that once a memory is extinguished, it can reappear if the animal is provided with appropriate cues (Miller and Kraus, 1977).

The processes of acquisition, consolidation and retrieval of extinction require the interplay of several key structures, including the basolateral amygdala, infralimbic prefrontal cortex, and hippocampus (Quirk and Mueller, 2008).

The retrieval of extinction involves the expression of an inhibitory memory, and is highly context-specific. Accordingly, retrieval of extinction would be expected to activate inhibitory networks, as well as the hippocampus. These retrieval circuits are beginning to be understood for extinction of conditioned fear.

The important role in the extinction process of infralimbic region (IL) of the vmPFC is been largely described (Quirk et al., 2000; Lebron et al., 2004; Morgan et al., 2003; Weible et al., 2000).

A possible mechanism of action for extinction process requires the cortical control of amygdala inhibition. Amygdala ITC cells receive a strong projection from the IL mPFC in both rodents (McDonald et al., 1996) and primates (Chiba et al., 2001; Ghashghaei and Barbas, 2002). During extinction retrieval, IL activity is potentiated and is correlated with the extent of extinction retrieval (Milad and Quirk, 2002; Barrett et al., 2003; Herry and Garcia, 2002). Potentiated IL output could inhibit amygdala output via activation of ITC cells (Maren and Quirk, 2004; Pare et al., 2004).

3.E. The Anatomy of memories

3.E.1 MTL anatomy, connectivity and general functions

The mammalian hippocampal formation can be divided into four anatomically distinct parts: the subicular complex (subdivided in subiculum and parasubiculum and the presubiculum), the entorhinal cortex, the hippocampus proper (subdivided into CA1, CA2 and CA3) and the dentate gyrus (Amaral and Witter, 1989). Although there are several reciprocal connections, the circuitry inside the hippocampus is essentially unidirectional, forming the so called trisynaptic circuit. The entorhinal cortex projects via the perforant pathway mainly to the dentate gyrus. The granule cells of the dentate gyrus project through their mossy fiber to CA3, and CA3 pyramidal cells project to CA1 through the Schaffer collateral pathway. CA1 cells project to the subicular complex, which then project back to the entorhinal cortex, closing the circuit (Amaral and Witter, 1989). In addition to these major projections, the entorhinal cortex also connects directly with the CA1 and CA3 fields and the subiculum. The CA3 has associational connections terminating within the CA3. The hippocampus formation has also reciprocal connections with several cortical regions (Amaral and Witter, 1989).

The hippocampus and related systems appear to have strong ties to navigation in both rodents and humans (Maguire et al., 1998; O'Keefe and Nadel, 1978). In rodents place cells fire when the rodent is in a particular location in the environment (O'Keefe and Dostrovsky, 1971; Wilson and McNaughton, 1993). It appears likely that this system does represent general cognitive maps of stimulus features.

Morris (1996) argues that having a learning system with fast plasticity (such as the hippocampus) it can be able to represent in every moment of the life a single event or learning as it appears, while a slower learning can be committed to a system able to represent more invariant or consistent features of the world.

The role of the hippocampus in consolidation of extinction has been extensively studied in two rodent paradigms in which the hippocampus is also required for conditioning: inhibitory avoidance, contextual fear conditioning and eye-blink conditioning.

Numerous lesion studies, specifically those using neurotoxic techniques (Jarrard 1989) aimed at complete removal of the hippocampus have resulted in learning deficits (Morris et al., 1990). But as is clear from recordings of single units, the hippocampus is not a uniform structure and cells differ with respect to their physiological properties in that place cells of the dorsal hippocampus have much more confined place fields as

compared to those in the ventral hippocampus, where place fields are more disperse. As a result, lesioning the ventral hippocampus had no measurable consequence on acquisition learning or retention of a spatial paradigm in the Morris water maze (Moser et al., 1993). By contrast, dorsal hippocampal lesions completely abolished learning and it was argued that the dorsal but not ventral hippocampus was involved in the encoding of new spatial information.

Damage to the hippocampus results in impairment in the duration and/or capacity of STM primarily in animals for spatial and temporal information and in humans for spatial, temporal and linguistic information. Rarely are impairments found in animals and humans for nonspatial information (Bohbot et al., 1998; Kesner et al., 1998). This suggests that the hippocampus may be particularly important in mediating STM representations of spatial, temporal and linguistic information.

3.E.2 mPFC anatomy, connectivity and general functions

The ventromedial prefrontal cortex is a part of the prefrontal cortex in the mammalian brain and is located in the frontal lobe.

The prefrontal cortex is subdivided in different regions: the first is the medial part divided in a dorsal region including the precentral cortex (PrC) and the anterior cingulate cortex (ACg) and a ventral region including the PreLimbic cortex (PrL), the InfraLimbic cortex (Il) and the Medial Orbital cortex (MO); the second is the lateral region including the insulgraunal dorsal and ventral cortices (AID, AIV) and the lateral orbital cortex (LO) (Dalley et al., 2004).

The primate dlPFC and its rat equivalent of mPFC receive extensive innervations from the mediodorsal nucleus of thalamus and send prominent projections to dorsal striatum, nucleus accumbens, and ventral tegmental area (VTA) (Groenewegen and Uylings, 2000; Ongur and Price, 2000; Brown and Bowman, 2002).

This pattern of connections is consistent with a key role at the top of the executive hierarchy and as the ultimate regulator of goal-directed behavior (Fuster, 2001). Accordingly, these regions are critical for key executive functions such as set-shifting and inhibitory control over behavior (Ragozzino et al, 1998; Brown and Bowman, 2002; Stefani et al, 2003). In addition, dlPFC and mPFC have been strongly implicated in the processing of working memory (Mishkin and Pribram, 1955; Sakurai and Sugimoto, 1985). Moreover, damage to the prefrontal cortex in nonhuman primates and rodents

generally results in severe behavioral deficits in a variety of delayed-response tasks, such as delayed alteration, and delayed matching and nonmatching to sample tasks, associated with an increased distractibility by sensor cues, and behavioral inflexibility characterized by an impaired capacity to switch strategies.

The medial prefrontal cortex, however, is not homogenous structure, and the specific contribution of identified subdivisions is still a matter of debate. It consists of several highly interconnected regions, including the anterior cingulate, prelimbic, and infralimbic cortices. These regions are reciprocally connected to sensory, motor, and limbic cortices, and they are therefore ideally situated to integrate and synthesize information from a large number of different sources. This potential for integration has led to the hypothesis that the ability of the mPFC to process remote memories might mirror that of the hippocampus to process recent memories (Frankland and Bontempi, 2005)

Here, we will focus our attention in 2 particular mPFC regions and in their role in modulating memory: the anterior cingulate cortex (aCC) and the Infra Limbic cortex (IL).

The anterior cingulate has been primarily implicated in attentional processes and in conflict monitoring (Dalley et al, 2004). Functional imaging studies in human subjects shown that, besides processing pain stimuli, the ACC is involved in a large number of different cognitive processes (e.g., attention, error monitoring, target detection and effortful recall) (Bush et al., 2000).

Recent works shown a specific role of aCC in remote fear memories demonstrating the involvement in the storage and maintenance of information across a time delay (Seamans et al., 1995). While other rodent studies have also provided evidence that the ACC processes Pavolvian fear memories, these have emphasized the role of the ACC in memory recall rather than memory formation (Frankland et al., 2004; Takehara et al., 2003). Furthermore, these studies provide evidence that the ACC is preferentially involved in the recall of remote (rather than recent) fear memories. Though decisive evidence is still lacking at present, Jung et al (2008) proposed to assign a term 'control memory' (i.e., memory for top-down control processes) as a new type of memory function for the PFC.

The role for the IL cortex in consolidation was suggested by the observation that rats with lesions of IL could acquire extinction within a session, but had difficulty retrieving

extinction the following day (Quirk et al., 2000). IL infusions of the Na+ channel blocker TTX (Sierra-Mercado et al., 2006), NMDAr antagonist CPP (Burgos-Robles et al., 2007), PKA inhibitor (Mueller et al., 2008), or protein synthesis blocker anisomycin (Santini et al., 2004) do not impair acquisition of extinction, but lead to impaired retrieval of extinction the following day. It is been shown that the IL cortex is involved in reactivation of remote memories with analysis of i.e.g. as *c-fos* and *zif268* (Frankland et al., 2004).

3.E.3 Structural plasticity in Hippocampal and Cortical networks

The most extensively examined region in which plastic events are thought to occur is the hippocampal formation. There are many instances of changes in hippocampal synaptic neurotransmission in response to learning (McNaughton and Morris, 1987; Power et al., 1997) and many examples of learning-induced changes in structural plasticity that involve either the production of new synapses or a reorganization of existing synapses (Bailey and Kandel, 1993; Moser, 1999) as like as LTP and LTD inductions (Whitlock et al., 2006).

Evidences in our and other laboratories report that training mice in hippocampal-dependent tasks (Contextual fear conditioning (Restivo et al., 2009), MWM (O'Malley et al., 2000), simultaneous olfactory (Restivo et al., 2006, Knafo et al., 2004) trace eyeblink conditioning (Leuner et al., 2003)) induces a rapid, and robust, increase of dendritic spine density in the CA1 field of the hippocampus. Moreover, by blocking excitatory transmission in the hippocampus, Leuner et al (2003) prevented memory acquisition and abolished spines formation thus confirming that the induction of memory is dependent on the augmented excitatory transmission.

The idea that plasticity, or experience-induced lasting changes in synaptic strength or structural features, is a component of PFC function has historically received little attention because a lasting effect is somewhat inconsistent with the dynamic and flexible function of the PFC.

Nevertheless, studies performed in primate somato-sensory and auditory cortex have long shown plasticity or 'reorganization' in cortical columns in response to obliteration of sensory input to these regions (Harrington and Merzenich, 1970). Somato-sensory cortical systems also show experience-induced plasticity by increasing neuronal representation to a sensory event after the animals learn to associate that event with a

reinforced outcome (Blake et al, 2005). More recent electrophysiological studies in primates and rodents have shown that this form of plasticity generalizes to OFC as well as medial and lateral PFC by demonstrating that neurons in these regions encode novel visuo-motor, audio-motor, and olfacto-motor associations (Rolls et al, 1996; Asaad et al, 1998; Passingham et al, 2000; Schoenbaum et al, 2003; Boettiger and D'Esposito, 2005). Collectively, these findings indicate that neurons in different PFC subregions are plastic and play a role in associative learning. Thus, the PFC in addition to its classic executive functions that include assignment of attentional reserves, shifting between competing behavioral sets, conflict monitoring, and sensorimotor integration- also takes part in the acquisition of simple associations. This dual function suggests that the same PFC neuronal networks that represent the stimulus-outcome associations may subserve the executive functions such as attentional tuning and decision making that are related to these associations.

Recent studies in rodents have shown that repeated periods of traumatic experiences during the first weeks of life induce alterations of synaptic connectivity in the limbic anterior cingulate cortex and hippocampus (Helmeke et al., 2001a,b; Ovtscharoff and Braun, 2001; Poeggel et al., 2003).

Phases of pronounced synaptic reorganization, i.e. proliferation and elimination of synaptic connections, have also been described for sensory and prefrontal cortical areas of human and non-human primates (Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997)

The existence of a direct monosynaptic pathway from the ventral CA1 region of the hippocampus and subiculum to specific areas of the prefrontal cortex provides a useful model for conceptualizing the functional operations of hippocampal-prefrontal cortex communication in learning and memory.

Several anatomical studies using retrograde and anterograde tracers have shown that the hippocampus projects to restricted regions of the prefrontal cortex, as the medial and orbitofrontal cortices in rats, cats and monkeys (Rosene and Van Hoesen, 1977; Cavada et al., 1983; Goldman-Rakic et al.,1984; Barbas and Blatt, 1995; Sesack et al.,1989).

In vivo electophysiological studies have also shown that stimulation of the CA1/ subicular region evokes a characteristic monosynaptic negative-going field potential recorded extracellularly in the prelimbic cortex (Laroche et al.,1990). In the awake,

freely moving rat, LTP at the hippocampal to prelimbic cortex synapse results in an enduring increase in synaptic strength which persists for several days (Jay et al., 1996).

In one study based on monitoring variations in synaptic efficacy during learning, a delayed increase in synaptic transmission was found in the hippocampo-prefrontal cortex pathway during training in an associative learning task (Doyère et al.,1993).

Importantly, while the hippocampal inputs synaptic potentiation occurred very early during learning, changes at the hippocampal to prefrontal cortex synapses were delayed, developing only when maximal learning was reached. These results provided the first electrophysiological evidence indicating that synaptic potentiation in this pathway may serve a process of late consolidation by which the hippocampus can help the stabilization of a cortical representation of learned events (Laroche et al., 1990).

Another important finding suggesting a role of synaptic plasticity at hippocampal to prefrontal cortex synapses during consolidation derives from studies investigating learning-induced changes in the expression of genes associated with specific aspects of plasticity.

In line with the many theories which postulate a critical role of the hippocampus in directing and organizing cortical representations (Wickelgren, 1979; Teyler and DiScenna, 1986; Rolls et al., 1989), this mechanism may play an active role in configuring distributed circuits in hippocampo-cortical networks.

3.F. Study outline

Many studies have suggested that the hippocampus is involved in first phases of consolidation and that information become consolidated and stored in a more permanent form at neocortical connections (Squire and Alvarez, 1995; Bontempi et al., 1999). After an interval of time during which memories are integrated in the cortico-cortical networks, the hippocampus can be no longer necessary (Teng and Squire, 1999).
Experimental evidence supporting this model display modifications of mPFC neural activity upon reactivation of the remote memory trace suggesting that connectivity changes might occur within cortical networks during the consolidation process.

Our main goal in these studies is to bring to light wiring rearrangement (i.e. changes in spine density and morphology) that are induced by training in a fear memory and persist during recent and remote memory expression. Also, there is a critical window necessary for the consolidation of memories in the cortical networks?
Moreover, we asked whether modifications in spine density that we found in cortical areas are stable and how they can be erased. Are these modifications persistent and resistant to external contingencies?
In study I we performed a contextual fear conditioning paradigm and tested experimental animals for their recent and remote memory. Then we analysed morphological changes (i.e.: changes in spine density) induced by consolidation via Golgi-Cox staining in various areas that seems to be involved/ or not involved in retrieving recent and remote information: the hippocampus, the anterior Cingulate and the Visual Cortex.
In study II we blocked the formation of new spines injecting specifically in the anterior cingulate cortex an herpes simplex virus overespressing the protein Mef2, a regulator of structural synapse plasticity in critical phases of consolidation process.
In study III we extinguished remote memories with the intention to introduce new and conflicting information in the consolidated neocortical network. We then analysed morphological changes in aCC, Infra Limbic cortex and hippocampus as well as the expression of the Gap-43 protein during extinction process to determinate the dynamic nature of spines spreading in IL cortex network.

4.A. EXPERIMENT I: The Formation of Recent and Remote Memory Is Associated with Time-Dependent Formation of Dendritic Spines in the Hippocampus and Anterior Cingulate Cortex

4.B. EXPERIMENT II: Myocyte enhancer factor 2 (MEF2) reduces dendritic spine density in the anterior cingulate cortex and disrupts long-term memory consolidation

4.C. EXPERIMENT III: Extinction of remote memory traces promotes structural remodeling of neurons in anterior cingulate and infralimbic cortices

Behavioral/Systems/Cognitive

The Formation of Recent and Remote Memory Is Associated with Time-Dependent Formation of Dendritic Spines in the Hippocampus and Anterior Cingulate Cortex

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Although hippocampal– cortical interactions are crucial for the formation of enduring declarative memories, synaptic events that govern long-term memory storage remain mostly unclear. We present evidence that neuronal structural changes, i.e., dendritic spine growth, develop sequentially in the hippocampus and anterior cingulate cortex (aCC) during the formation of recent and remote contextual fear memory. We found that mice placed in a conditioning chamber for one 7 min conditioning session and exposed to five footshocks (duration, 2 s; intensity, 0.7 mA; interstimulus interval, 60 s) delivered through the grid floor exhibited robust fear response when returned to the experimental context 24 h or 36 d after the conditioning. We then observed that their fear response at the recent, but not the remote, time point was associated with an increase in spine density on hippocampal neurons, whereas an inverse temporal pattern of spine density changes occurred on aCC neurons. At each time point, hippocampal or aCC structural alterations were achieved even in the absence of recent or remote memory tests, thus suggesting that they were not driven by retrieval processes. Furthermore, ibotenic lesions of the hippocampus impaired remote memory and prevented dendritic spine growth on aCC neurons when they were performed immediately after the conditioning, whereas they were ineffective when performed 24 d later. These findings reveal that gradual structural changes modifying connectivity in hippocampal– cortical networks underlie the formation and expression of remote memory, and that the hippocampus plays a crucial but time-limited role in driving structural plasticity in the cortex.

Introduction

One influential theory of memory consolidation posits that the storage and the retrieval of recent memory depends on the hippocampus and that, at later stages, the hippocampus interacts with neocortical sites where corticocortical connections progressively develop and, ultimately, self-govern the storage and the retrieval of remote memory (Squire and Alvarez, 1995; Squire et al., 2004; Frankland and Bontempi, 2005; Squire and Bayley, 2007). To date, experimental evidence supporting this model comes from studies revealing sequential activation of hippocampal and medial prefrontal cortex (mPFC) regions by means of glucose uptake imaging (Bontempi et al., 1999), immediate early genes induction (Frankland et al., 2004), NMDA activity (Takehara-Nishiuchi et al., 2006), or expression of proteins involved in axonal growth and sprouting (Routtenberg et al., 2000; Frankland et al., 2004; Maviel et al., 2004; Frankland and Bontempi, 2005). These findings support, therefore, the view that consolidation of remote memory requires extra-hippocampal structures (Teng and Squire, 1999; Rosenbaum et al., 2000) and that transformation of initially vulnerable traces into resistant ones involves the coordinated activation of hippocampal and distributed cortical areas including prefrontal, anterior cingulate, and retrosplenial cortices.

Memories, however, are not to be seen as being literally transferred from the hippocampus to the neocortex as they consolidate over time. Several models have proposed that the hippocampus plays a privileged role in organizing remote memory storage that would consist in actively modifying connectivity in distributed cortical networks (McClelland et al., 1995; Squire and Alvarez, 1995). This role intuitively suggests that changes in the morphology of hippocampal or cortical neurons should take place during the formation of recent or remote memories. Insofar, there is evidence that synaptic rearrangements rapidly achieved through an increase in spine density on hippocampal cell dendrites occur at early stages of memory formation (Leuner et al., 2003; Knafo et al., 2004; Restivo et al., 2006). However, whether such rearrangements occur in cortical regions during remote memory formation is unknown.

To address this issue, we trained mice for contextual fear conditioning and processed their brain for Golgi–Cox impregnation at a recent (24 h) or a remote (36 d) memory time point. Here, we show that structural plasticity, i.e., dendritic spine growth, se-

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quentially develops in the hippocampus and the anterior cingulate cortex (aCC) during the formation of recent and remote contextual fear memories. The increase in spines was achieved even in the absence of the memory tests, suggesting that off-line neural activity triggered by initial learning was sufficient to promote time- and region-specific structural changes. Ultimately, hippocampal lesions performed shortly, but not late, after the conditioning prevented spine density changes in aCC neurons and impaired remote memory, thus pointing to a crucial but time-limited role for the hippocampus in driving structural plasticity in the cortex.

Materials and Methods

Animals. A total of 118 male C57BL/6J@Ico mice purchased from Charles River Italy (Calco) were used. At the beginning of the experiments, mice were 9 weeks old, and their weight ranged from 24 to 26 g. They were housed five per cage and maintained in a temperature-controlled facility (22 ± 1°C) on a 12 h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were conducted in accordance with the official European guidelines for the care and use of laboratory animals (86/609/EEC).

Contextual fear-conditioning protocol. Mice were first handled for 3 d in the vivarium and then for 2 additional days in the experimental room. Contextual fear conditioning consisting of one single session of 7 min began on the next day. Each mouse was placed in a transparent Plexiglas cage (28 × 28 × 10 cm) with a removable grid floor made of stainless steel rods. After 120 s of free exploration, the mouse was exposed to a series of five non-signaled footshocks (duration, 2 s; intensity, 0.7 mA; interstimulus interval, 60 s) delivered through the grid floor. Control mice were treated identically, except that they were not shocked. Contextual fear memory tests were run by placing the mice back in the conditioning chamber for 4 min, either 24 h (recent memory) or 36 d (remote memory) after the conditioning session. Behavior during conditioning or testing was recorded using an automated procedure described previously (Anagnostaras et al., 2000). Briefly, activity was recorded by means of a video camera mounted 60 cm above the ceiling of the cage and connected to a computer equipped with the Ethovision software (Noldus). Activity suppression ratios and percentage of time spent freezing (absence of all but respiratory movements) were used to score fear memory. The following formula was used to calculate activity suppression ratios: activity testing/(activity conditioning plus activity testing) (the lower the ratio, the better the memory).

Golgi–Cox staining and tissue preparation. Golgi–Cox staining was used to assess postconditioning changes in neuronal morphology at the recent and remote memory time points. Mice were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with a solution of 0.9% saline. Brains were dissected and impregnated using a Golgi–Cox solution, according to the method described by Glaser and Van der Loos (1981). Briefly, they were first immersed in the Golgi–Cox solution at room temperature for 6 d, transferred to a 30% sucrose solution for 2 d, and then sectioned using a vibratome. Coronal sections (100 μm thick) were mounted on gelatinized slides, stained according to the method described by Gibb and Kolb (1998), and coverslipped with Permount.

Quantification of neuronal morphology and imaging. Spine density was measured on pyramidal neurons located in the CA1 region of the dorsal hippocampus and in layers II/III of the anterior cingulate and of primary visual cortices that has been shown not to be involved in contextual fear memory. These structures were defined according to the Franklin and Paxinos (2001) mouse atlas (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Neurons, identified with a light microscope (Leica; DMLB) under low magnification [×20; numerical aperture (NA), 0.5], were chosen by first locating, among all the stained sections, the regions of interest in their respective coronal sections. Three neurons showing at least fourth-order branches for both apical and basal dendrites in each region and within each hemisphere were selected. Since no significant interhemispheric difference was observed, measurements were pooled so that six neurons per region were studied in each animal. Only neurons which satisfied the following criteria were chosen for analysis in each of the experimental groups: (1) presence of untruncated dendrites, (2) consistent and dark impregnation along the entire extent of all of the dendrites, and (3) relative isolation from neighboring impregnated neurons to avoid interference and ensure accuracy of dendritic spine counting. Subsequently, dendritic spines were analyzed under a higher magnification (×63; NA, 0.75). Series of sequential photomicrographs of dendritic segments were generated on a computer screen by means of a video camera connected to the microscope. These photomicrographs were acquired using at least five serial focal planes (2–3 μm apart) by focusing in and out with the fine adjustment of the microscope to create a stack of sequential images. This ensured the accurate reconstruction of entire dendritic segments and enabled counting of these segments with all their visible spines on two-dimensional images. To further minimize any missing of spines, both apical and basal dendrites of the selected segments had to appear as much as possible in the series of focal planes selected for counting. Spines were counted on secondary and tertiary branches of apical dendrites in the stratum radiatum and on secondary and tertiary branches of basal dendrites in the stratum oriens of the CA1 hippocampal field. The same categories of dendrites were analyzed on aCC and visual cortex (VC) pyramidal neurons lying in the II/III layer (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material). On each neuron and for each dendrite category, five 20 μm dendritic segments were randomly selected using a two-dimensional sampling grid made of 20 μm squares to generate values of spine density (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In some cases, segments were from the same branch (Leuner et al., 2003). Segments were sampled 50 μm away from soma to exclude the spine-depleted zone which arises from the cell body. Only protuberances with a clear connection of the head of the spine to the shaft of the dendrite were counted as spines. Since this method has proven to provide reliable results (Horner and Arbuthnott, 1991), no attempt was made to introduce a correction factor for hidden spines. As no difference in spine counts was observed between secondary and tertiary branch segments for each group, data were pooled for each dendrite category (basal and apical) to generate the final spine density results. All measurements were performed by an experimenter blind to the experimental conditions.

Spine density measurements after recent and remote memory tests. Mice ($N = 40$) were subjected to contextual fear conditioning ($N = 20$) or pseudoconditioning ($N = 20$). In each subgroup, half of the mice were tested for recent or remote memory, respectively, 24 h or 36 d after the conditioning episode. Twenty-four hours after the completion of the retention tests, their brains were processed for Golgi staining. Dendritic spines were counted on pyramidal neurons in the CA1 hippocampal subfield, the anterior cingulate cortex, and the primary visual cortex.

Spine density measurements in mice that were not subjected to memory tests. Mice ($N = 20$) were conditioned ($N = 10$) or pseudoconditioned ($N = 10$) without undergoing recent or remote memory tests. In each subgroup, half of the mice were killed 48 h after the conditioning and the other half 37 d later and their brains processed for Golgi staining. Dendritic spines were counted on CA1 (recent time point) and anterior cingulate cortex (remote time point) pyramidal neurons.

Effect of early or late postconditioning hippocampal lesions on remote memory and cortical spine density. Mice ($N = 58$) were conditioned or pseudoconditioned and then randomly allocated to early ($N = 32$) or late ($N = 26$) lesion groups. Mice in these four groups were further subdivided in mice receiving lesions (early, $N = 20$; late, $N = 16$) or sham lesions (early, $N = 12$; late, $N = 10$). Early lesions were performed immediately after the conditioning and late lesions 24 d later. Hippocampal lesions were produced by inserting stainless steel cannulae bilaterally in the dorsal hippocampus using the following stereotaxic coordinates: anteroposterior relative to bregma, −2.1 mm; lateral to midline, ±2.1 mm; ventral from the skull surface, −1.8 mm. The excitotoxic agent ibotenic acid (0.2 μl per side over 2 min) was slowly infused bilaterally using a perfusion pump connected to the cannulae. Sham-lesioned mice underwent the same procedure, except that sterile saline (0.9% NaCl) was infused. After completion of early or late surgery, mice were returned to their home cage and tested for remote memory 36 d after the condition-

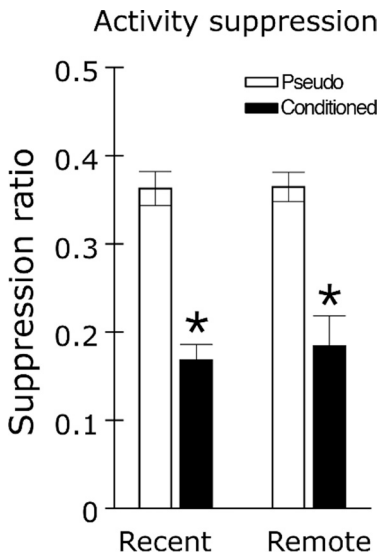

Figure 1. Contextual fear conditioning elicits a robust fear response 24 h or 36 d after the conditioning. Conditioned mice (black bars) showed lower activity suppression ratios during the recent and remote memory tests compared with pseudoconditioned mice (white bars) that were not shocked. No forgetting occurred across retention intervals. $*p < 0.05$; $N = 10$ mice per group.

ing. Twenty-four hours after the remote memory test, their brains were processed for morphological analyses. After the perfusion, the anterior part of the brain was Golgi stained to count dendritic spines on aCC neurons, whereas the posterior part of the brain was stained according to the Nissl method to estimate hippocampal neuronal loss attributable to ibotenic acid lesions. Only mice with cannula tips correctly located, and whose extent of lesion was circumscribed to the dorsal hippocampus, were included in this study. Accordingly, six early lesioned mice out of 20 (three conditioned and three pseudoconditioned) and five late-lesioned mice out of 16 (two conditioned and three pseudoconditioned) were excluded.

Statistical analyses. Results were expressed as means ± SEM. Retention performance (activity suppression and freezing) was compared across groups by means of a two-way ANOVA with training condition (conditioned, pseudoconditioned) and retention interval (recent, remote) as main factors. Differences in spine density were assessed by means of a three-way ANOVA with training condition (conditioned, pseudoconditioned, naive), retention interval (recent, remote), and brain region (CA1, aCC, VC) as main factors. *Post hoc* analyses were performed using the Fisher's protected least significant differences test. Cumulative frequencies of spine density in conditioned, pseudoconditioned, and naive mice were compared across groups using, first, the normal distribution Kolmogorov–Smirnov (K–S) fitting test and then K–S two-sample tests for subsequent paired comparisons. Differences in spine density between conditioned and pseudoconditioned mice that were not subjected to the retention tests were estimated in the hippocampus at the recent time point and in the aCC at the remote time point by means of Student's *t* tests. The effect of hippocampal lesions on remote memory and spine density in the aCC was evaluated by means of a two-way ANOVA with training condition (conditioned, pseudoconditioned) and treatment (lesioned, sham lesioned) as main factors. Separate analyses were performed for mice receiving early or for late lesions. Values of $p < 0.05$ were considered as significant.

Results

Conditioned mice show robust contextual fear responses during the recent and the remote memory test

Mice exposed to the footshocks showed greater activity suppression ratios (training condition effect, $F_{(1,36)} = 63.80$; $p < 0.0001$) (Fig. 1) and freezing responses (training condition effect, $F_{(1,36)} = 1595.86$; $p < 0.0001$) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) relative to non-shocked mice at each retention interval. No forgetting occurred across retention intervals (retention interval effect, $p > 0.5$ for each variable).

Contextual fear conditioning promotes a time-dependent increase in dendritic spine density in the CA1 hippocampal region and the anterior cingulate cortex

Spine density was measured on apical and basal dendrites of CA1, aCC, and VC neurons at each retention interval in conditioned, pseudoconditioned, and naive mice. Results are shown in Figure 2. Structural rearrangements that were specific to the conditioned mice developed sequentially in the hippocampus and the aCC during the formation of recent and remote memory (training condition × retention interval × brain region interaction, $F_{(2,48)} = 4.36$; $p < 0.05$ for apical dendrites and $F_{(2,48)} = 5.22$; $p < 0.01$ for basal dendrites). *Post hoc* comparisons of spine density in the hippocampus revealed that conditioned mice exhibited a significant increase in spines on apical (spines/20 μm, 16.6 ± 0.21; $p < 0.01$) and basal (spines/20 μm, 16.9 ± 0.42; $p < 0.01$) dendrites of CA1 neurons compared with pseudoconditioned mice (apical, spines/20 μm: 14.9 ± 0.81; basal, spines/20 μm: 14.7 ± 0.84) after the recent memory test (Fig. 2*a*). This increase, however, was only transient, since hippocampal spine values counted after the remote memory test were similar in conditioned (apical, spines/20 μm: 11.7 ± 0.61; basal, spines/20 μm: 11.6 ± 0.36; raw data) and pseudoconditioned mice (apical, spines/20 μm: 10.9 ± 0.27; basal, spines/20 μm: 11.4 ± 0.32) (Fig. 2*b*). Interestingly, a significant increase ($p < 0.05$) in spine density was found in the hippocampus of pseudoconditioned mice (apical, spines/20 μm: 14.9 ± 0.81; basal, spines/20 μm: 14.7 ± 0.84) compared with naive mice (apical, spines/20 μm: 12.8 ± 0.57; basal, spines/20 μm: 11.5 ± 0.84) at the short retention interval, possibly reflecting the encoding of a nonaversive contextual representation. *Post hoc* comparisons of spine density in the aCC indicated an inverted temporal pattern of structural changes. At the recent time point, spine density values were in the same range in conditioned (apical, spines/20 μm: 14.9 ± 0.23; basal, spines/20 μm: 13.4 ± 0.13) and pseudoconditioned mice (apical, spines/20 μm: 15.3 ± 0.09; basal, spines/20 μm: 13.8 ± 0.13) (Fig. 2*c*). These values, however, were significantly higher ($p < 0.01$) in the conditioned (apical, spines/20 μm: 16.7 ± 0.33; basal, spines/20 μm: 14.2 ± 0.28) than in the pseudoconditioned group (apical, spines/20 μm: 14.4 ± 0.48; basal, spines/20 μm: 13.1 ± 0.48) at the remote time point (Fig. 2*d*). *Post hoc* comparisons of spine density in layer II/III of primary VC neurons showed that spine density values were similar in all groups at any interval (Fig. 2*e*,*f*).

Spine density changes in hippocampal and cortical networks are extensive

Cumulative frequency distribution curves of spines measured on hippocampal, aCC, and VC neuron dendrites and photomicrographs of Golgi-impregnated dendritic segments in each condition are shown in Figure 3. For the hippocampus, a stronger shift of the curves to the right was observed in conditioned mice compared with pseudoconditioned and naive mice at the recent memory time point ($p < 0.01$ for both dendrite categories), a pattern revealing that spines were increased on the majority of sampled neurons. At the same time point, the curves of pseudoconditioned mice also showed a significant shift on the right compared with those of naive mice ($p < 0.05$ for both dendrite categories), further indicating that encoding of a nona-

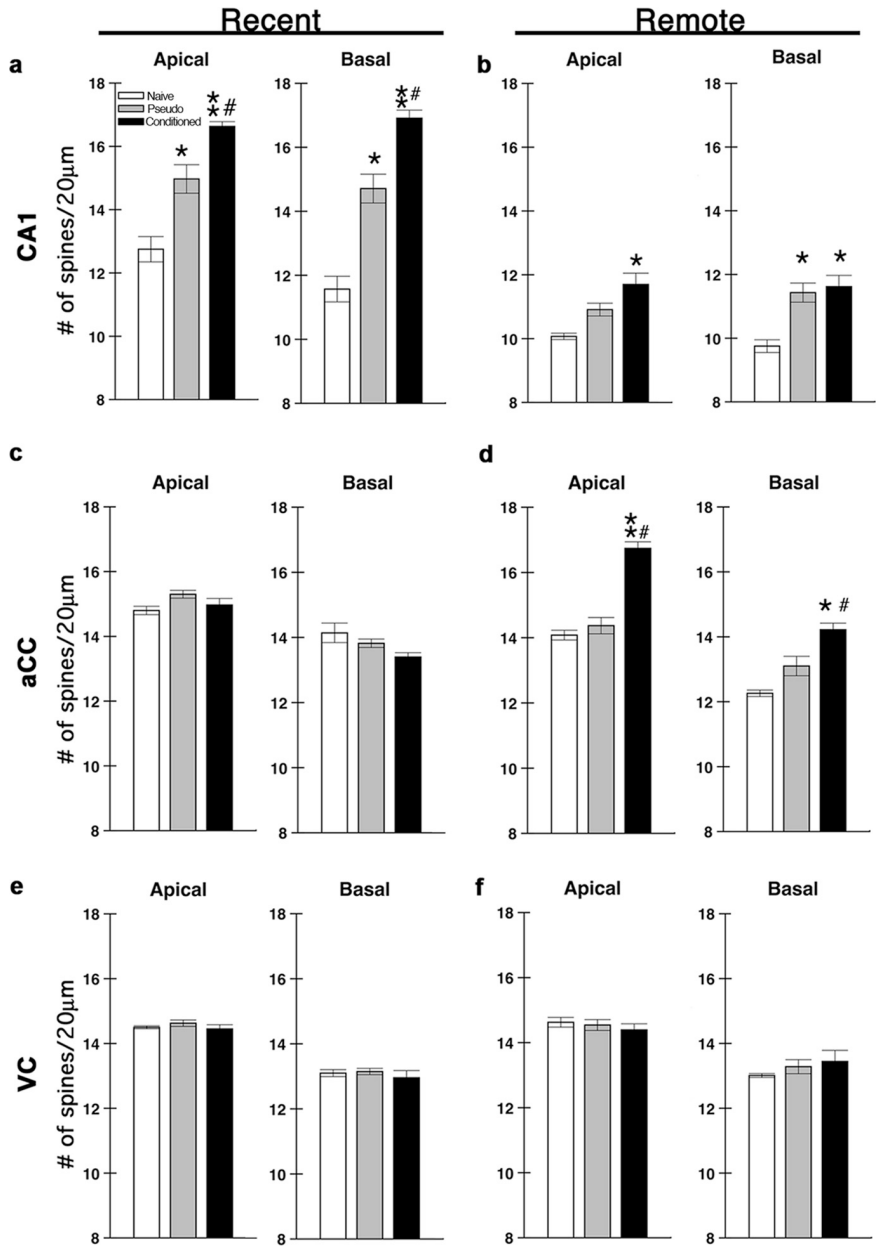

Figure 2. Recent and remote memory formation triggers region-specific and time-dependent morphological changes in hippocampal and cortical networks. ***a–d***, Retrieval of recent (***a***), but not remote (***b***), memories was associated with an increase in spine density on apical and basal dendrites of pyramidal CA1 neurons, whereas an inverse temporal pattern of structural changes occurred on aCC pyramidal neurons (***c***, ***d***). Spine density of VC pyramidal neurons did not vary between groups at any retention intervals (***e***, ***f***). $^{\#}p < 0.01$ versus respective pseudoconditioned controls; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus respective naive controls; $N = 5$ mice per group.

versive contextual representation elicited structural changes in the hippocampus. For the aCC, a significant shift to the right was observed for the curves of conditioned mice at the remote memory time point ($p < 0.01$ for both dendrite categories). The finding that, at this time point, the curves of pseudoconditioned and naive mice overlapped suggests that cortical remodeling was a net remote memory effect. In the VC, the curves fully overlapped at the recent and remote memory time points.

Structural remodeling of hippocampal–cortical networks is independent from memory retrieval

Conditioned mice that were not subjected to the recent memory test showed an increase in spine density (apical, spines/20 μm: 17.1 $\pm$ 0.60; basal, spines/20 μm: 17.3 $\pm$ 0.90) on hippocampal dendrites 48 h after they were exposed to the footshocks relative to their pseudoconditioned counterpart (apical, spines/20 μm: 16.4 $\pm$ 0.80; basal, spines/20 μm: 14.8 $\pm$ 0.14) (basal, $t_{(8)} = 2.4$; $p < 0.05$) (Fig. 4*a*,*b*). Similarly, conditioned mice that were not subjected to the remote memory test showed an increase in spine density on aCC dendrites 37 d after they were exposed to the footshocks (conditioned: apical, spines/20 μm: 16.2 $\pm$ 0.15; basal, spines/20 μm: 14.8 $\pm$ 0.24; pseudoconditioned: apical, spines/20 μm: 14.6 $\pm$ 0.15; basal, spines/20 μm: 14.0 $\pm$ 0.15; apical: $t_{(8)} = 7.91$; $p < 0.001$); basal: $t_{(8)} = 2.74$; $p < 0.05$) (Fig. 4*c*,*d*).

The hippocampus drives structural changes in the cortex, but its role is time limited

The data are shown on Figure 5. Histological controls of ibotenic hippocampal lesions performed immediately (early) or 24 d (late) after the conditioning revealed similar robust cell loss in CA1, CA3, and dentate gyrus, whereas the subiculum was spared (Fig. 5*a*,*b*; supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Statistical analysis performed on activity suppression ratios of mice receiving early lesions revealed a significant effect of the training condition ($F_{(1,22)} = 35.23$; $p < 0.001$) and of the training condition $\times$ treatment interaction ($F_{(1,22)} = 4.53$; $p < 0.05$). *Post hoc* tests then indicated that conditioned mice with early postconditioning hippocampal lesions showed a higher activity suppression ratio than conditioned mice with sham lesions ($p < 0.05$). No effect of the lesion on this variable was observed in pseudoconditioned mice (Fig. 5*c*). In the conditioned mice, the amount of freezing was also significantly lower in the lesioned than in the sham-lesioned group ($t_{(11)} = 3.57$; $p < 0.01$) (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Remarkably, early hippocampal lesions completely abolished late development of dendritic spine growth in aCC neurons in the conditioned mice. Specifically, a significant effect of the training condition $\times$ treatment interaction was found for each dendrite category ($F_{(1,19)} = 28.74$; $p < 0.001$ for apical dendrites; $F_{(1,19)} = 4.89$; $p < 0.05$ for basal dendrites) with *post hoc* tests showing that conditioned mice receiving sham lesions exhibited more spines on aCC neuron dendrites than the other groups (conditioned plus sham, spines/20 μm: 16.1 $\pm$ 0.14; conditioned plus lesion, spines/20 μm: 14.6 $\pm$ 0.19; $p < 0.01$ for apical dendrites; conditioned plus sham, spines/20 μm: 13.5 $\pm$ 0.37; conditioned plus lesion, spines/20 μm: 12.7 $\pm$ 0.21; $p < 0.05$ for basal dendrites) (Fig. 5*d*). Also, the shift to the right of cumulative frequency distribution curves found in sham-

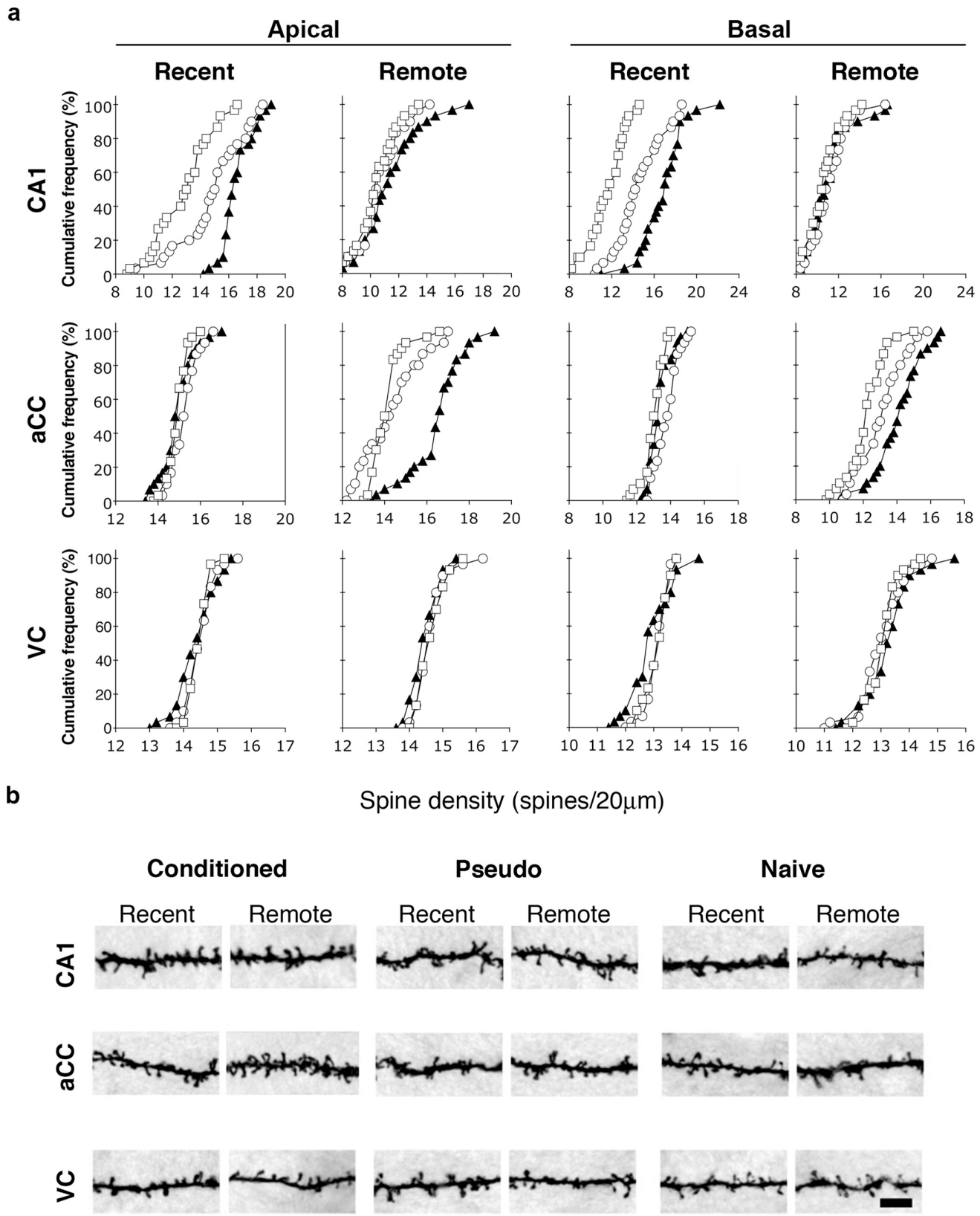

Figure 3. Morphological changes in hippocampal– cortical networks after recent and remote memory tests are extensive. ***a***, Cumulative frequency of spine density values along apical and basal dendrites of CA1, aCC, and VC pyramidal neurons are shown for conditioned (filled triangles), pseudoconditioned (open circles), and naive mice (open squares). Each point represents the average spine density (number of spines counted along 20 μm dendritic segments) of a single neuron. A shift of the curve to the right indicates that the majority of the sampled neurons showed an increase in spines. Such an increase was found on CA1 neurons at the recent, but not the remote, time point in conditioned mice compared with pseudoconditioned and naive mice. A milder increase was also present in pseudoconditioned mice compared with naive mice. An inverted pattern of structural changes was observed in pyramidal neurons of the aCC where a shift to the right of the cumulative frequency curve of conditioned mice was found at the remote, but not the recent, time point. Cumulative frequency curves in the VC fully overlapped at each time point. ***b***, Photomicrographs of Golgi-impregnated basal dendritic segments showing spine density in the CA1 region of the hippocampus, the aCC, and VC after the recent and remote memory tests. Scale bar, 5 μm.

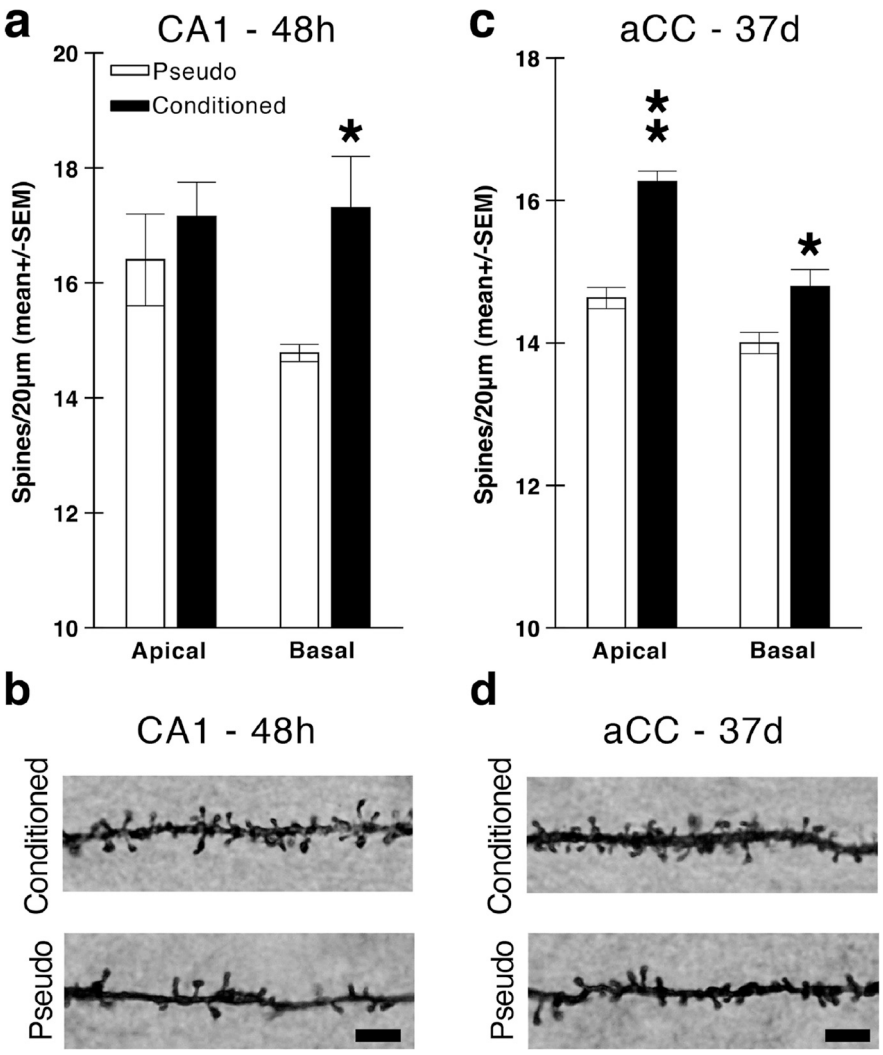

Figure 4. Consolidation-induced synaptic remodeling in hippocampal–cortical networks is independent of retrieval processes. ***a***, Conditioned mice that were not subjected to the recent memory test show an increase in spine density on CA1 neuron dendrites 48 h after the conditioning. ***b***, Photomicrographs of Golgi-impregnated apical dendritic segments showing changes in the number of spines counted in the hippocampal CA1 of mouse brains at the same postconditioning interval. Scale bar, 5 μm. ***c***, Conditioned mice that were not subjected to the remote memory test show an increase in spine density on aCC neuron dendrites 37 d after the conditioning. ***d***, Same as ***b*** but for the aCC examined 37 d after the conditioning. Morphological changes observed in CA1 and aCC neurons were similar in magnitude to those observed in mice subjected to retrieval (Figs. 2, 3). *$p < 0.05$, **$p < 0.001$; $N = 5$ mice per group.

lesioned conditioned mice relative to pseudoconditioned mice ($p < 0.01$ for apical dendrites; $p < 0.05$ for basal dendrites) was completely abolished by the hippocampal lesions (Fig. 6*a*,*b*).

Conversely, mice receiving late postconditioning hippocampal lesions did not show any reduction in activity suppression ratio (Fig. 5*e*) or freezing ($t_{(9)} = 0.91$; $p > 0.38$) (supplemental Fig. 5, available at www.jneurosci.org as supplemental material) compared with sham-lesioned conditioned mice during the remote memory test, since the analysis revealed only a significant effect of the training condition ($F_{(1,17)} = 126.00$; $p < 0.001$). Late hippocampal lesions did not prevent conditioned mice to show an increase in spine density on aCC neurons (apical dendrites, conditioned plus sham, spines/20 μm: 16.7 $\pm$ 0.44; conditioned plus lesion, spines/20 μm: 16.2 $\pm$ 0.44; pseudoconditioned plus sham, spines/20 μm: 14.1 $\pm$ 0.63; pseudoconditioned plus lesion, spines/20 μm: 13.7 $\pm$ 0.45; basal dendrites, conditioned plus sham, spines/20 μm: 14.7 $\pm$ 0.45; conditioned plus lesion, spines/20 μm: 13.5 $\pm$ 0.67; pseudoconditioned plus sham, spines/20 μm: 12.6 $\pm$ 0.43; pseudoconditioned plus lesion, spines/20 μm: 11.6 $\pm$ 0.61; lesion effect: $F_{(1,17)} > 0.83$; $p > 0.5$ for both dendrite categories) (Fig. 5*f*) or to display a normal pattern of cumulative frequency distribution of spine density (Fig. 6*c*,*d*) at the remote time point.

Discussion

Memory traces are not definitively fixed at the time of encoding but undergo a gradual process of stabilization and consolidation (Dudai, 2004; Squire et al., 2004). Recent memories are thought to be initially dependent on protein synthesis-related modifications of hippocampal synaptic connections (Bailey et al., 2004). Their storage in this brain region is, however, time limited, since damage to the medial temporal lobe, which includes the hippocampus, disrupts recent memory retrieval, whereas remote memories are left unaffected (Zola-Morgan and Squire, 1990; Kim and Fanselow, 1992; Martin and Clark, 2007; Squire and Bayley, 2007). Consistent with this model, we provide direct evidence that time-dependent changes in spine density occur in hippocampal and cortical networks during the formation of recent and remote memory. Specifically, our findings point to a highly dynamic process of memory consolidation triggering complex time- and region-dependent structural rearrangements that involve both the formation and elimination of spines. First, we observed that the formation of a recent aversive memory was associated with an increase in dendritic spines on CA1 pyramidal neurons that was specific to the conditioned mice. Importantly, this increase was found to be transient, since the number of spines counted in the conditioned and pseudoconditioned groups 36 d after the conditioning was no longer different. Second, a robust fear response to the context was still present at the remote time point and was now associated with an increase in spines on aCC pyramidal cell dendrites. Indeed, whether the aCC actively contributes to memory storage per se or, as hypothesized (Simons and Spiers, 2003; Frankland and Bontempi, 2005), exerts an executive role in integrating and coordinating memories from distributed cortical modules processing selective aspects of the memory trace (Ross and Eichenbaum, 2006) remains to be determined.

A central tenet of the systems memory consolidation theory is that the recruitment of cortical networks in remote memory storage requires early activation of hippocampal–mPFC circuits. According to this view, hippocampal lesions performed shortly after the conditioning would prevent the increase in spines on aCC neurons and disrupt remote memory. We directly tested this prediction by lesioning the hippocampus after completion of the conditioning episode and then examining conditioning performance and cortical morphology 36 d later. Results showed that

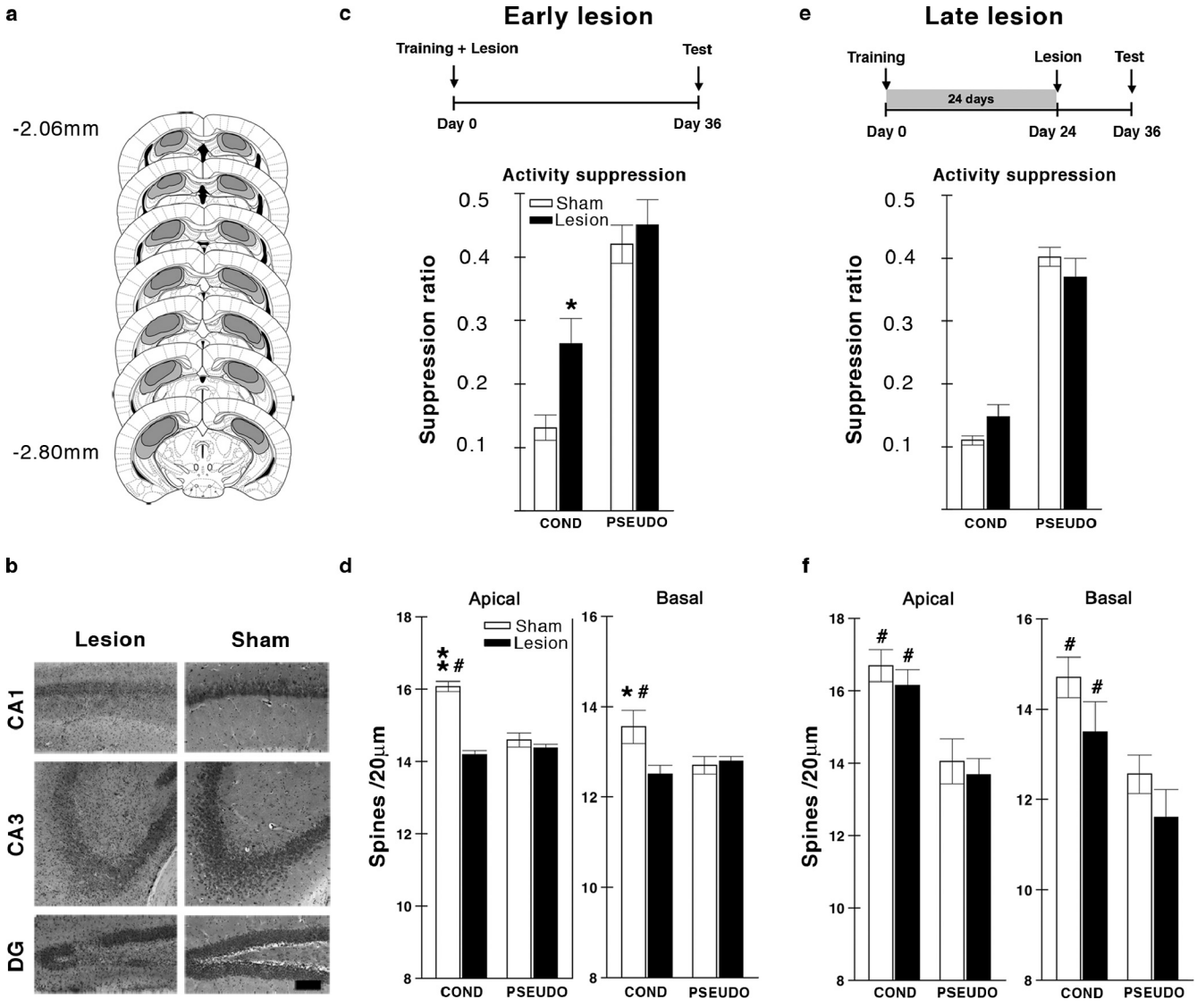

Figure 5. Early, but not late, hippocampal lesions impair remote memory and prevent spine density changes on aCC neurons. ***a***, Coronal diagrams of mouse brain sections [adapted from Franklin and Paxinos (2001)] showing the extent of hippocampal lesions (maximal extent is shown in light gray, whereas minimal extent appears darker). Lesions were specific and mainly extended through the CA1, CA3, and dentate gyrus (DG) fields of the dorsal hippocampus, whereas the subiculum was spared. ***b***, Representative photomicrographs of hippocampal tissue in lesioned and sham-lesioned mice. Cresyl violet staining revealed consistent and similar cell shrinkage and microglial activation in the early and late lesion groups (see also supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Scale bar, 100 μm. ***c***, Mice with early hippocampal lesions exhibited lower activity suppression ratios than sham-lesioned mice when tested for remote memory. ***d***, Early hippocampal lesions (black bar) prevented the increase in spine density on aCC neuron dendrites after the remote memory test, whereas this increase was still present in sham-lesioned mice (white bars). ***e***, Mice with late hippocampal lesions showed intact remote memory performance. ***f***, Late hippocampal lesions did not prevent the increase in spine density on aCC neuron dendrites after the remote memory test. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus lesion group; $^{\#}p < 0.05$ versus respective pseudoconditioned sham or lesion controls; $N = 5$–7 mice per group.

not only remote memory was impaired, as it could be expected from studies showing retrograde amnesia gradients after hippocampal damage (Squire et al., 2004; Squire and Bayley, 2007) but, more importantly, that hippocampal lesions prevented consolidation-induced spine growth on aCC neurons at the remote time point. Furthermore, to ascertain that remote memory storage requires only a time-limited activation of hippocampal–mPFC circuits, we performed hippocampal lesions long after (24 d) the conditioning with the prediction that spine density changes would occur on aCC neurons and remote memory would be spared. The results of our experiment support this prediction, since mice undergoing late lesions showed both robust conditioning and an increase in spines on cortical neurons. Our findings, therefore, show that formation and expression of long-lasting memories entail structural plasticity in aCC and that consolidation-induced cortical spine rearrangements require an intact hippocampus only at early stages of memory formation. Indeed, how the hippocampus manages to activate the experience-relevant set of cortical neurons is still a matter of debate, although potential mechanisms, including synaptic tagging, have been proposed (Frey and Morris, 1998; Martin and Kosik, 2002).

To ensure that remodeling of hippocampal and cortical neurons was actually a correlate of contextual fear memory, our mice were previously subjected to recent and remote memory tests raising the possibility that the observed morphological rearrangements were triggered by retrieval processes. To determine whether consolidation per se would yield comparable results, we tested additional groups of mice that did not undergo postconditioning tests. We found that similar time-dependent structural

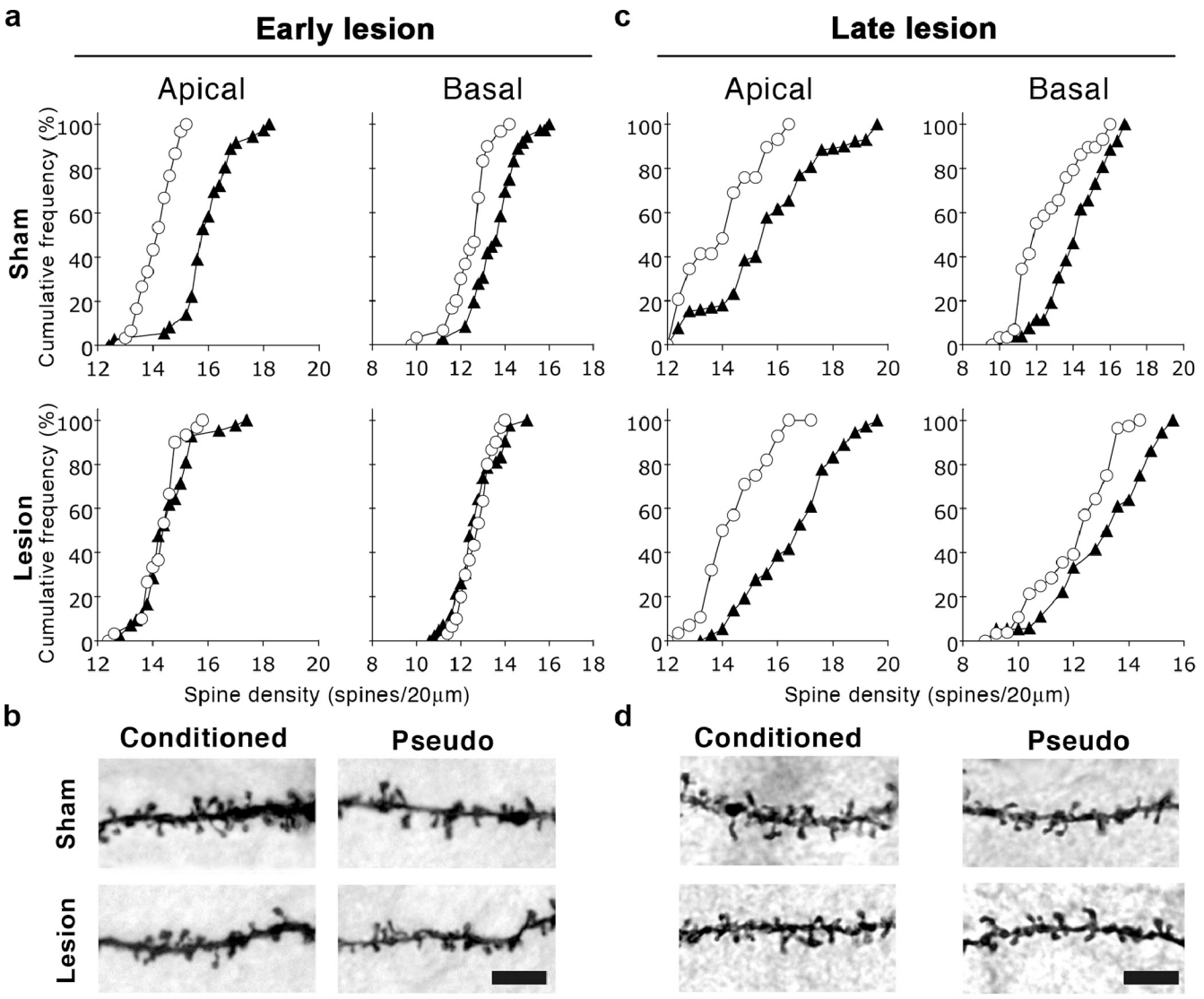

Figure 6. Early hippocampal lesions produce widespread disruption of spine growth in aCC neurons. ***a***, A shift to the right of the curves depicting the cumulative frequency of spine density values on apical and basal dendrites of aCC neurons was observed in sham-lesioned conditioned mice (filled triangles) compared with pseudoconditioned mice (open circles), indicative of extensive structural changes in the conditioned group (top). Conversely, spine growth was disrupted on the majority of sampled neurons in early lesioned conditioned mice, as shown the overlapping of their cumulative frequency distribution curves with the one of pseudoconditioned mice ($N = 30–42$ neurons per group; bottom). ***b***, Photomicrographs of Golgi-impregnated apical dendritic segments of aCC neurons. Scale bar, 5 μm. ***c***, Late hippocampal lesions did not prevent widespread spine growth in the aCC, since the cumulative frequency distribution curves of conditioned mice were shifted to the right compared with the curves of pseudoconditioned mice; either they were lesioned or sham lesioned ($N = 30–36$ neurons per group). ***d***, Same as ***b*** but for mice that received late hippocampal lesions.

rearrangements developed in conditioned groups. Thus, off-line neural activity triggered by initial learning alone is sufficient to elicit structural plasticity in the hippocampus and the aCC during recent and remote memory consolidation. These observations fit well with a large body of evidence now showing that replay of encoding-related activity during off-line phases of sleep, periods of quiet wakefulness, or conscious recollection are a core mechanism for driving plasticity in hippocampal–cortical networks (Squire and Alvarez, 1995; Frankland and Bontempi, 2005; Walker and Stickgold, 2006; Euston et al., 2007). For instance, repeated activation of hippocampal circuits during slow-wave sleep has been shown to reinstate, in a coordinated and synchronized manner, neuronal activity in a wide set of cortical regions (Ji and Wilson, 2007). Assuming that this phenomenon stabilizes hippocampal–cortical connections and enables a refinement of cortical memory traces, it might explain why information relevant to an existing cortical wiring diagram (or mental schema) requires less time to be consolidated. Direct support for this proposal comes from recent data showing that the rate at which systems consolidation occurs in the neocortex is dependent on previously acquired knowledge (Tse et al., 2007).

Collectively, our data show that sequential remodeling within hippocampal–cortical circuits occurs during consolidation of fear memory. Nevertheless, several aspects of this structural reorganization need clarification. For example, the mechanisms controlling the disengagement of the hippocampus remain elusive, although a top-down inhibitory control, presumably arising from cortical regions which are actively engaged in remote memory storage and retrieval, has been hypothesized (Wiltgen et al., 2004; Frankland and Bontempi, 2005). Also, once established in the cortex, do consolidated memories remain in their original form and retain their precision? Recent data suggest that they do

not, since rats tested for late retrieval of contextual fear show comparable levels of freezing when exposed to the original or a different context (Winocur et al., 2007). This observation implies that an initially hippocampus-dependent and context-specific memory is gradually transformed into a more gist-like and schematic memory, as embedding into cortical networks occurs over time. This notion is fully consistent with the multiple trace theory, which holds that information is initially processed by the hippocampus and then by neocortical regions where multiple traces of the initial experience are formed (Moscovitch and Umiltà, 1990; Moscovitch, 2005). This theory, however, also assumes that a new hippocampally mediated trace is created each time an old memory is retrieved. Although we did not observe that hippocampal spines were increased at the remote memory time point, new traces based on hippocampal synaptic changes other than spine formation cannot be excluded. In fact, contrasting with the traditional view that changes in synapses may be permanent once consolidation of enduring memories has been achieved (Anagnostaras et al., 2000), several lines of evidence now point to a continual updating of post-transcriptional modifications of proteins to ensure long-term memory storage (Routtenberg and Rekart, 2005). These modifications seem to include phosphorylation of proteins already present at the synapse, as highlighted in a recent study, which shows that persistence of long-term associative memory requires ongoing protein kinase M zeta enzymatic activity in the cortex (Shema et al., 2007). Accordingly, future studies will tackle the question of cortical networks stability over time and their resistance to perturbations (interference) or extinction. One stake of these studies is that unveiling the dynamics of neuronal circuits mediating remote memory persistence might help in dampening abnormally long-lasting fear memories, especially those generating post-traumatic stress disorder.

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4.B. EXPERIMENT II: Myocyte enhancer factor 2 (MEF2) reduces dendritic spine density in the anterior cingulate cortex and disrupts long-term memory consolidation

Introduction

While the initial encoding of contextual fear memories depends critically on the hippocampus, over time memories may become permanently consolidated in the cortices, including the anterior cingulate cortex (aCC) (Squire and Alvarez, 1995; Frankland and Bontempi, 2005).

This consolidation process is thought to involve the gradual and incremental remodeling of synapses in cortical networks in the days and weeks following training.

Restivo et al. (2009) observed that gradual structural changes modifying connectivity in hippocampal–cortical networks underlie the formation and expression of remote memory. In particular they observed that in the aCC the number of spines in neurons of animals tested 1 day after training were similar to control mice neurons but in animals tested 36 days later they observed an increase in spine density related to control group.

Moreover, they found that ibotenic lesions of the hippocampus impaired remote memory and prevented dendritic spine growth on aCC neurons when they were performed immediately after the conditioning suggesting the existence of a dialogue between the hippocampus and the anterior cingulate cortex that permit the structural reorganization in the aCC and the proper formation of consolidation process.

However, to date evidence that blocking directly structural plasticity in aCC cause the impairment of memory consolidation is missing as unknown is the time window in which this structural changes occurs.

Takashima et al. (2006) with a functional imaging study in humans found that, within the space of 1 month, the circuits supporting memories undergo major reorganization, with activity shifting from the hippocampus to the mPFC. (McClelland et al., 1995; Eichenbaum, 2001; Eichenbaum, 2004).

Their study suggests that the rate of memory reorganization (at least for some types of information) may be much faster than originally thought.

This observation elicits that early after the training the hippocampus starts a dialogue with the cortex that induces an increase of spine density necessary for the consolidation

and retrieval of memories. To verify this thesis we may blockade the possibility to form new spines in the neocortex after training.

To this aim we injected specifically in the aCC bilaterally a virus coespressing EGFP with a constitutively active form of MEF2 (MEF2-VP16), a protein that in an activity-dependent way modulate synaptic plasticity. Indeed, new data shown the role of Mef2 in blocking neuronal remodeling in hippocampal neuronal culture negatively regulating excitatory synapse number (Flavell, Science 2006) as well as in vivo decreasing spine density in NAc neurons (Pulipparacharuvil et al., 2008).

We blocked the possibility to increase spine density 1 day after training or 6 weeks later and analyzed the impact in spine density levels and the performances 1 week after surgery. We expected to block remodeling only if we inject the virus 1 day after training because at a remote time point no new remodeling seems to be necessary for consolidation. We also predict an impairment in performances in mice with failed remodeling.

Material and Methods

Animals. Male offspring from a cross between C57Bl/6NTacfBr [C57B6] and 129Svev [129] mice (Taconic, Germantown, NY) were used in these experiments. Mice were bred in our colony and were maintained on a 12 hr light/dark cycle with free access to food and water. Mice were at least 8 weeks of age at the start of experiments, and behavioral procedures were conducted during the light phase of the cycle. Experiments were conducted blind to the treatment condition of the mouse, and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

HSV amplicon vectors. Plasmids encoding GFP-MEF2-VP16, MEF2-VP16, and MEF2-VP16Δ were obtained from Dr. Michael Greenberg (Harvard Medical School, Boston, MA) (Flavell et al., 2006). GFP-MEF2-VP16 cDNA was subcloned into pHSVprPUC (Geller et al., 1993); MEF2-VP16 was subcloned into p1005(+) (Russo et al., 2009). All cloning was done using standard techniques. HSV amplicons were produced using 2-2 cells and the helper virus 5dl1.2 as described (Lim and Neve, 2001). Control vectors expressing GFP-lacZ (Han et al., 2008) or GFP alone have been described (Russo et al., 2009).

Cell culture and reporter assays. Gli36 cells were provided by Dr. Alberto Epstein (Université Claude Bernard Lyon 1, Villeurbanne, France). NIH-3T3, Gli36, and 2-2 cells were maintained in high glucose Delbucco's Modified Eagle Medium supplement with 10% fetal bovine serum, penicillin/streptomycin, and GlutaMAX (all from Invitrogen, Carlsbad, CA) at 37oC with 5% CO2.

The MEF2 reporter plasmid was provided by Eric Olson (University of Texas Southwestern, Dallas, TX) (Wu et al., 2001). Gli36 cells (5x104) were seeded in 24-well plates, and 24 h later, were transfected using Lipofectamine 2000 and OptiMEM (both from Invitrogen), according to the manufacturer's instructions. Each well was transfected with 100 ng of reporter plasmid and 400 ng of p1005-derived plasmids expressing GFP alone or MEF2-VP16. Twenty-four hours post-transfection, GFP expression was visualized to confirm that cell in each well were transfected with similar efficiencies (i.e., 60-80%), medium was removed, and cells lysed in 1x Reporter Lysis Buffer (Promega, Madison, WI). Lysate was cleared by centrifugation (5 min at 20,000g) and luciferase expression was determined using the Firefly Luciferase Assay System (Promega), according to the manufacturer's instructions. All transfections were performed in duplicate within each experiment.

Surgical procedures. Mice were treated with atropine (5 mg/kg, i.p.) and anesthetized with chloral hydrate (400 mg/kg, i.p.). Mice were placed in a stereotaxic frame (ASI...), the skin was retracted and holes drilled in the skull above the aCC [AP = +0.8, ML = ±0.3, V = -1.75]. HSV-MEF2 vector [volume 1.5 µl] was infused at a constant rate [0.1 µl /min] via a 32-guage injection needle connected to a Hamilton microsyringe (Hamilton, Reno, NV). The injection needle was left in place for 5 minutes following the completion of the infusion to ensure diffusion. For sham surgeries, mice were treated identically except HSV-GFP vector was infused. Mice were treated post-operatively with the analgesic ketoprofen (5 mg/kg, i.p., Sigma, St. Louis, MO).

Contextual fear conditioning. Context fear conditioning experiments were performed in a windowless room equipped with 4 conditioning chambers (31 cm °— 24 cm °— 21 cm; Med Associates, St. Albans, VT) containing stainless steel shock-grid floors. Shock grid bars (diameter 3.2 mm) were spaced 7.9 mm apart. The grid floor was positioned

over a stainless-steel drop-pan, which was lightly cleaned with 70% ethyl alcohol to provide a background odor. The front, top, and back of the chamber were made of clear acrylic and the two sides made of modular aluminum. Mouse freezing behavior was monitored via four overhead cameras.

Mice were handled for three days in the fear conditioning room to minimize non specific emotional reactivity to the experimental room. Fear conditioning, consisting of one single 3-minute session, began the next day. After 120 sec of free exploration, the mouse was exposed to a non signaled foot shock (duration: 2s; intensity: 0.75 mA) delivered through the gridfloor. Mice remained in the context for an additional minute before being returned to their home cage. Mice underwent surgery 1 day or 42 days after training. Contextual fear memory tests were run 7 days after surgery by placing the mice back in the conditioning chamber for 5 minutes . Freezing was assessed using an automated scoring system (Actimetrics, Wilmette, IL), which digitized the video signal at 4 Hz and compared movement frame by frame to determine the amount of freezing.

Tissue processing - Exclusion critera. At the completion of behavioral testing, mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA, transferred to 30% sucrose solution and stored at 4 ºC until processed. Seventy um thick slices were cut using a Vibratome, counterstained with Hoechst, mounted on gelatinized slides and coverslipped.

Only brains showing bilateral infection on layer 2/3 of the aCC were included in the analysis. More subjects were excluded from both behavioral and morphological analyses using the following criteria: (i) number of infected cells - animals showing less than 100 GFP+ cells in the layer 2/3 were excluded from the analysis (ii) number of damaged slices - mice showing more than 5 damaged slices were excluded from the analysis (damage exceeding 5% of aCC area on the section)

Immunofluorescence. Mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA and then transferred to 30% sucrose solution and stored at 4 ºC until processed. Coronal and sagittal sections [40µm] covering the entire anterior-posterior extent of the aCC were cut and immunohistochemically processed using primary

antibodies against neuronal-specific nuclear protein (NeuN) (mouse anti-NeuN Alexa Fluor 488 conjugated; 1:500; Chemicon, Temecula, CA) and Myocyte enhancer factor 2 (MEF2) (1:200, rabbit anti-MEF2 sc313) diluted in blocking solution containing 2% goat serum, 1% bovine serum albumin, and 0.2% Triton X-100 dissolved in PBS . Sections were incubated for 2 h at room temperature with primary antibodies and 48h at -4C with secondary antibodies (Jackson abs, 1:500). Nuclear staining was performed using DAPI counterstain. Sections were mounted on slides (VWR, West Chester, PA) with Permafluor anti-fade medium (Lipshaw Immunon, Pittsburgh, PA). The aCC field was anatomically defined according to a mouse brain atlas.

Golgi-Cox staining. Golgi-Cox staining was used to assess experience-dependent changes in neuronal morphology. Ninety minutes after the completion of the test session, brains from each experimental group were dissected and impregnated using a Golgi-Cox solution according to the method described by Glaser & Van der Loos (1981). Briefly, they were first immersed in the Golgi-Cox solution at room temperature for 6 days, transferred to a 30% sucrose solution for 2 days, and then sectioned using a vibratome. Coronal sections (100-µm thick) were mounted on gelatinized slides, stained according to the method described by Gibb and Kolb (1998), and coverslipped with Permount.

Spine density analysis. Spine density measurements were performed on layer II/III pyramidal cells of the aCC. Neurons, identified with a light microscope (Leica DMLB) under low magnification (x20, NA 0.5), were chosen by first locating, among all the stained sections, the region of interest in the respective coronal sections. Spines were counted under a high magnification (100X / 1.25 NA). Measurements were performed on secondary and tertiary branches of apical dendrites and spine densities were quantified by counting the number of spines along 20–100 µm segments of dendrites (two to five dendrite segments/neuron, six neurons /animal). Segments were sampled 50 mm away from soma. Only protuberances with a clear connection of the head of the spine to the shaft of the dendrite were counted as spines. As no difference in spine counts was observed between secondary and tertiary branch segments for each group, data were pooled for each dendrite category to generate the final spine density results.

All measurements were performed by an experimenter blind to the experimental conditions.

Statistical analyses. Results were expressed as means ± SEM. Retention performance (freezing) was compared across groups by means of a two-way ANOVA with training condition (trained, pseudotrained) and retention interval (recent, remote) as main factors. Differences in spine density were assessed by means of a two-way ANOVA with training condition (trained and pseudotrained), and retention interval (1 day group, 42 days group) as main factors. Differences in spine density between trained and pseudotrained mice that were not subjected to the retention tests were estimated by means of Student's t tests. Values of $p < 0.05$ were considered as significant.

Results

Memory consolidation is associated with spine density increase

Spine density levels were measured on apical dendrites impregnated with Golgi-Cox staining of aCC in trained and pseudo-trained mice to detect morphological changes induced by consolidation. Results are shown in Fig. 4.B.1.

Mice trained for the contextual fear conditioning and tested 1 day after training shown similar level of spine density of mice pseudo trained ($t(115)= 0.487$, $p=0.627$ N.S.).

However, we shown higher level of spine density in mice trained in a contextual fear conditioning and tested 42 days later related to that of mice pseudo-trained ($t(104)=5.565$, $p<0.001$).

No difference between pseudo trained mice tested 1 day or 42 days after training were found ($t(99)=0.028$, $p<0.978$ N.S.) showing no age effect.

Our data replicate the results found in Restivo et al (2009) and it is consistent with the idea that remodeling of aCC circuits plays a role in gradual consolidation of contextual fear information.

Figure 4.B.1.

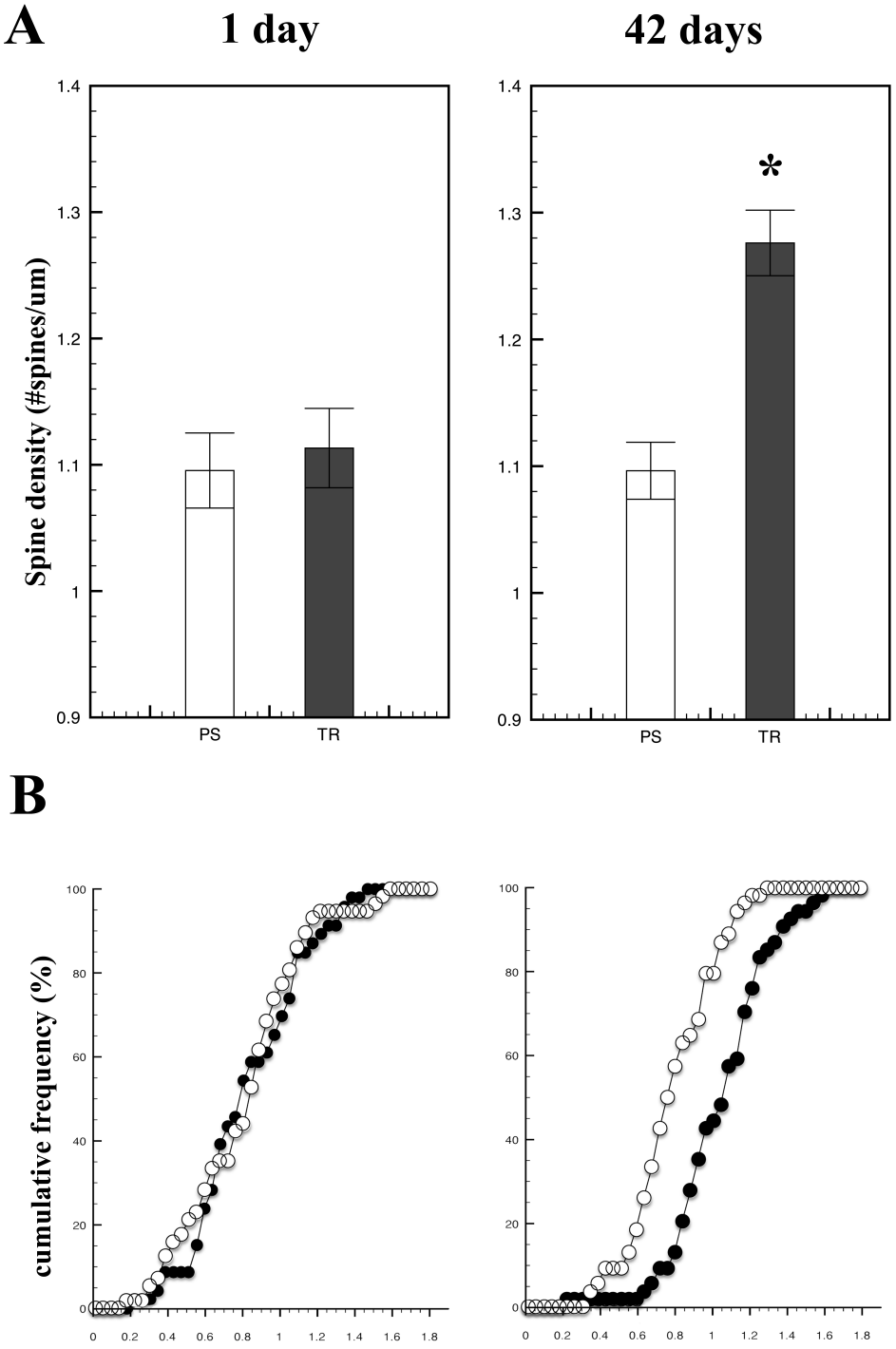

Figure 4.B.1. Memory consolidation is associated with spine density increase. (a) Spine density measured in mice trained (black bars) and tested 1 day after training where similar at spine density of mice pseudo trained (white bars). Conversely, Spine density analyzed 42 days after training in trained mice (black bars) were higher related to that of mice pseudo-trained (white bars). (b) Cumulative frequency of spine density values of apical dendrites of aCC pyramidal neurons are shown for trained (black circles) andpseudotrained (open circles) mice. Each point represents the average spine density (number of spines/ um) of a single neuron. A shift of the curve to the right indicates that the majority of the sampled neurons showed an increase in spines. Such an increase was found only in mice tested 42 days after the training session. *p<0.001

Mef2 is highly expressed in the aCC

To test the role of Mef2 in regulating dendritic spine density in the aCC, we first analyzed the expression of Mef2 proteins in adult aCC (Figure 4.B.2.). We observed strong, nuclear Mef2 immunostaining in this region. We clearly shown a selective expression of this protein in neuronal cells that colocalize with NeuN staining, without label in glial cells (no colocalizatoin with GFAP immunofluorescent staining).

Figure 4.B.2

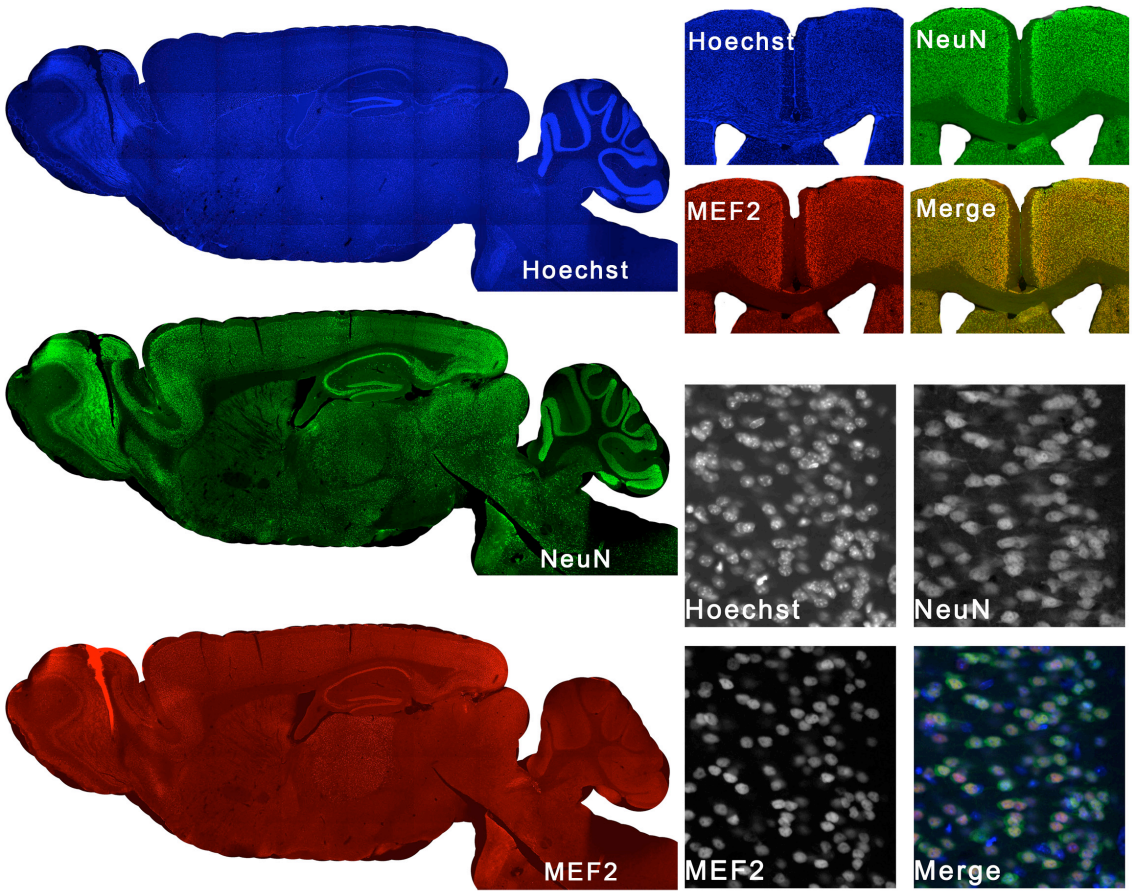

Figure 4.B.2 Mef2 is highly expressed in the aCC of control cage mice. Moreover, MEF2 colocalize with NeuN and Hoeschst showing its nuclear localization in the neuronal cells. MEF2 is no present in glial cells.

Dendritic spines are necessary for memory consolidation

Mice were trained in a contextual fear conditioning and they were injected bilaterally with the MEF2-VP16 or the GFP-P1005 in the aCC 1 day or 42 days after training.

We processed the brains after test session and analyzed the presence of fluorescence in the brain tissue. Mice with a number of slices presenting fluorescence < of 5 as well as mice with damaged tissue where excluded from this analysis (Figure 4.B.3.).

Mice were tested 7 days after surgery and their performances were evaluated by means of freezing responses to the context (Figure 4.B.4). Mice exposed to a footshock showed impaired freezing responses during the testing session 7 days after training if injected with MEF2-VP16 virus in the aCC field 1 day after training related to mice injected with control GFP-P1005 ($t(18)=2.26$; $p<0.05$). Mice injected with the GFP-P1005 or with the MEF2-VP16 virus 42 days after training do not shown any differences ($t(27) = 0.00168$; $p= 0.998$ N.S.). No significant differences where found between GFP injected mice at 1 day or 42 days ($t(11) = 0.733$; $p = 0.471$ N.S.).

Figure 4.B.3

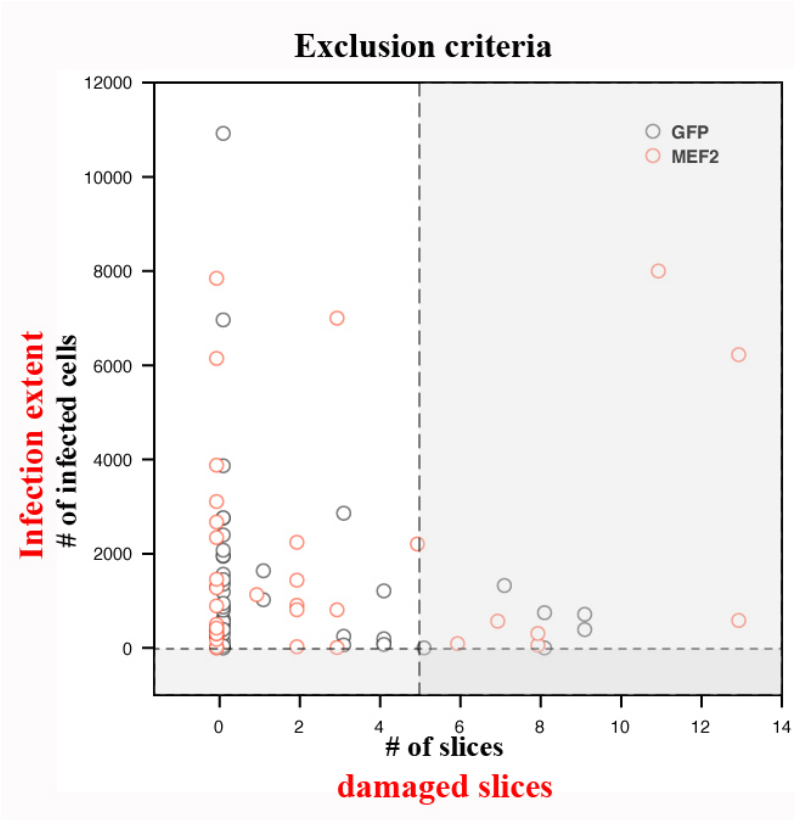

Figure 4.B.3. Exclusion criteria. Mice with a number of slices presenting fluorescence < of 5 as well as mice with damaged tissue where excluded from this analysis. Animals in the clearest grey rectangle were included in the analysis.

Figure 4.B.4.

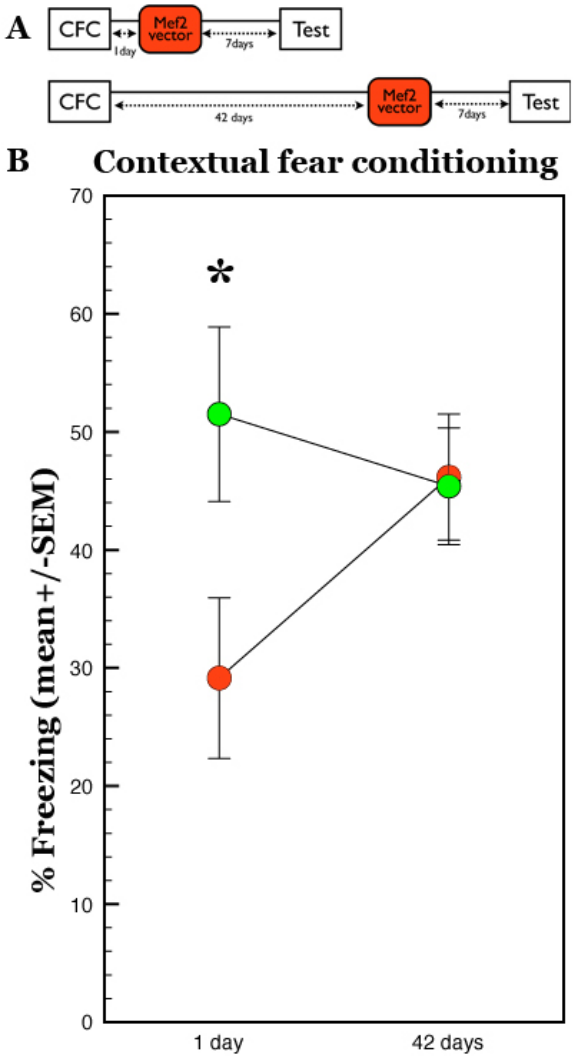

Figure 4.B.4. Dendritic spines are necessary for memory consolidation. (a) behavioural and surgical protocol: mice were trained in a contextual fear conditioning and they were injected bilaterally with the MEF2-VP16 or the GFP-P1005 in the aCC 1 day or 42 days after training. (b) Mice showed greater freezing responses during the testing session 7 days after training if injected with control GFP-P1005 (green circles) in the aCC field 1 day after training related to mice injected with MEF2-VP16 virus (red circles). While, Mice injected with the GFP-P1005 or with the MEF2-VP16 virus 42 days after training do not shown any differences. *p<0.05

Mef2 prevents consolidation-induced spine growth

Spine density was measured on apical dendrites of aCC in mice injected with MEF2-VP16 or GFP-P1005 1 day or 42 days after training. Results are shown in Figure 4.B.5. Statistical analysis performed on spine density values of neurons injected revealed a significant effect of the group-injection condition ($F(84) = 4.9926$, $p < 0.05$) and of the delayed-injection condition ($F(84) = 21.21$, $p< 0.001$) was found.

More in detail, MEF2-VP16 blocks consolidation-induced spine growth in the aCC in a time-dependent fashion. Spine growth in neurons injected with the virus is affected when MEF2 is injected 1 day ($t(47) = 3.145$, $p < 0.005$ but not 42 days after the training ($t(37) = 0.463$; $p < 0.646$ N.S.) related to GFP injected neurons.

Figure 4.B.5.

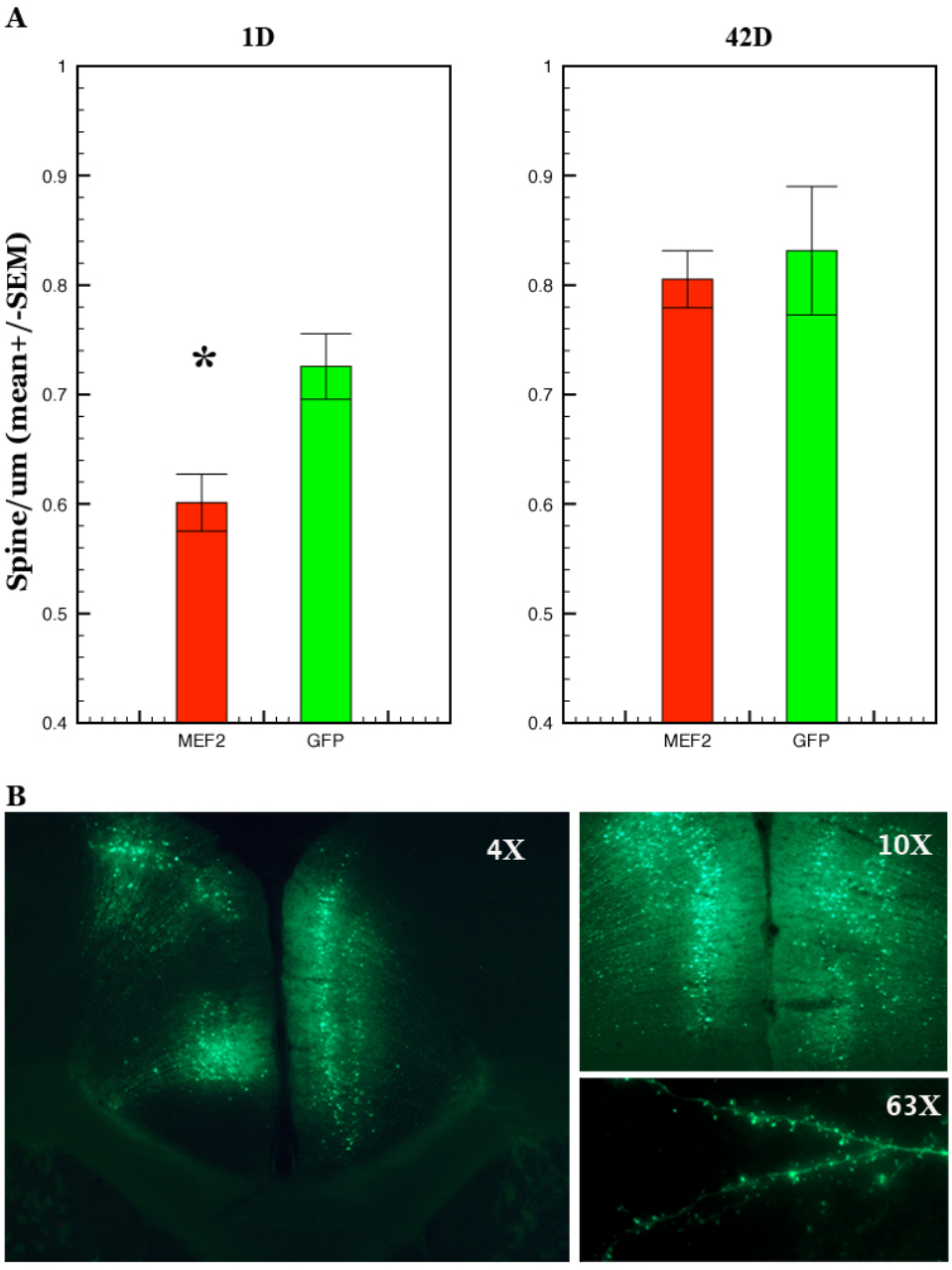

Figure 4.b.5. Mef2 prevents consolidation-induced spine growth. (a) Spine density measured in mice injected 1 day after training and tested 7 days later shown minor spine density level related to control group. Spines measured in neurons of mice injected 42 days after training and tested 7 days later do not shown differences between groups. (b) Representative pictures of injected aCC field at different magnification.*p<0.05

Conclusions

Our data shown that in vivo blockade of spinogenesis disrupted fear memory consolidation only when performed 1 day, but not 42 days after training suggesting that synapse formation and spinogenesis in the first days following training is necessary for the consolidation of contextual fear memory. These data suggest the presence of a time window opened soon after the completion of training - during which consolidation of contextual fear memory is supported by the active rewiring of aCC neurons.

In particular our experiment aims to detect the impact of morphological rearrangement in the first and last phases of consolidation in aCC. Specifically we explored the impact of post-training expression of the constitutively active form of myocyte enhancer factor, MEF2 - a negative regulator of excitatory synapse number - in the aCC on contextual fear memory consolidation through the injection of a virus vector. The virus contained MEF2-VP16, a fusion between the MEF2 DNA binding and dimerization domains and the basal transcription activation domain of the viral transcription factor VP16 (Black et al., 1996). Recently many studies described function of MEF2 in restricting the number of excitatory synapses that growth from the dendritic arborization of the neurons. In these experiments many of the MEF2 targets detected are known to contribute to the weakening/loss of excitatory synapses by regulating different aspects of signal transduction on the postsynaptic side of the synapse. In the subset of MEF2 genes targets there are Homer1a, Arc, c-jun, protocadherins, kcna1 and kcna4, c-fos, fosB, early growth response 1, nur77, histone deacetylase 5 (Flavell et al., 2006; Shalizi et al., 2006). The identification of these genes as MEF2 targets suggests that MEF2 induces the expression of a set of regulatory factors that work together with MEF2 to control synaptic function.

In particular it is been observed that MEF2A in hippocampal and cerebellar neurons in vivo regulates the formation and maintenance of spines and associated excitatory synapses by modulating the expression of some of these genes (Flavell et al., 2006; Shalizi et al., 2006).

We shown that MEF2 is highly expressed in neocortex of control cage mice and we observed a decrease of spine density levels when we block specifically the formation of new, but not the existing spines in neurons of aCC. Our data shown that the injection of MEF2-VP16 virus in the aCC 1 day after training causes a decrease in spine density and an impairment on performances during the test performed 7 days later. This finding

leads to the conclusion that morphological changes start early after training and this rewiring is necessary for the correct performances in the first week after training.

Our data are consistent with the standard theory of memory consolidation posits neocortical structures are the final repository in consolidating remote memories through changes in synaptic efficacy. Many studies founded an increase in i.e.g. expression (Frankland et al, 2004), glucose uptake imaging (Bontempi et al., 1999) and expression of proteins involved in axonal growth and sprouting (Routtenberg et al., 2000; Frankland et al., 2004; Maviel et al., 2004; Frankland and Bontempi, 2005) in the aCC following the recall of remote memories. Moreover, blocking of the mPFC NMDA receptors during the first 1 or 2 weeks after learning impairs memory retention 6 weeks after learning (Takehara-Nishiuchi et al., 2006).

All together these data reveal a general increase of activation of the aCC that subserve the recall of remote but not recent memories. In particular, studies of expression of proteins involved in synaptic remodelling are consistent with the opinion that consolidation involves a gradual strengthening of neocortical connections necessary for a correct recall (Wiltgen et al., 2004; Frankland and Bontempi, 2005; McClelland et al., 1995).

Restivo et al. (2009), report evidences for the strengthening of aCC connections in terms of increase in spine density at a remote time point. Their data represent the first evidence of specific structural remodelling in aCC following a delayed interval from the training and not dependent from the retrieval. It is not yet identified the initial time in which these morphological modification start in the aCC.

Previous works supporting the standard consolidation theory hypothesize that memories are consolidated in the aCC through a slow and progressive rearrangement in neocortical circuitry can start very early after training (Takashima et al., 2006; Frankland and Bontempi, 2006). In support to this view our data shown that the remodelling is starting in the first week after training and, more interesting, that this remodelling is necessary for a correct recall of the memory trace. At the same time our data fit well with the alternative “Multiple memory trace” theory, assuming that formation and consolidation of the traces is relatively rapid, lasting on the order of seconds or at most days (Moscovitch, 1995).

Are such dramatic changes in network organization possible on this time scale?

Sleep-dependent processes may play a key role in promoting memory reorganization. This is largely consistent with the view that memory reactivation during sleep enhance the gradual reorganization of cortical memory networks (Sutherland and McNaughton, 2000).

Another relevant topic to take in consideration is that all the virus injections affected selectively the 2/3 layers of the aCC field and all our analysis are specifically mired to reveal changes in spine density of apical aCC neurons of these layers. It is believed that apical dendrites of layers 2nd and 3rd are required for the interactions with other neocortical areas while more deeper layer are involved in connections with subcortical areas. It is in line with the theory of consolidation that hypothesize a selective increase in cortico-cortical interactions to allow the recall of consolidated memories.

Interestingly we found that injection of the virus 42 days after training do not affect memory retrieval neither the number of spine density.

The two main observations rising from this results are that: (i) at this remote time point there is no significant reorganization in spine density distribution modifiable by the interfering with the injection of MEF2; (ii) the normal increase in spines in the aCC lead to a correct performances behavior.

Although we found evidence for rapid consolidation effects, these results are fully consistent with the idea that the process of memory consolidation has not been completed at the end of the first week. In fact, we can detect an increase in spine density in neurons injected with GFP control virus and in neurons stained in Golgi-Cox solution between the group of mice tested 42 days after training related to the group tested in the first week.

4.C. EXPERIMENT III: Extinction of remote memory traces promotes structural remodeling of neurons in anterior cingulate and infralimbic cortices

Introduction

Increasing evidence indicates that the formation of remote memories is achieved through a progressive enhancement of synaptic activity in hippocampal and medial prefrontal cortex (mPFC) regions. To date, experimental evidence supporting this model comes from studies revealing sequential activation of the hippocampus and the mPFC by means of glucose uptake imaging (Bontempi et al, 1999), immediate early genes induction (Frankland et al, 2004), NMDA activity (Takehara-Nishiuchi et al, 2006), expression of proteins involved in axonal growth and sprouting (Routtenberg et al, 2000; Frankland et al, 2004; Maviel et al, 2004; Frankland and Bontempi, 2005) and structural remodeling of synapes (Restivo et al., 2009), with the latter observations revealing that the remote formation of spines in the mPFC was coupled with a pruning of hippocampal spines. These data support therefore the view that (i) memory formation proceeds in stages and (ii) newly formed memories become stable upon modifications of cortical architecture. However, despite the intuitive appeal of the stability hypothesis, which well explains the robustness of traces generated by strong emotional events, it is conceivable that the information stored in memory is frequently updated under environmental pressure, and that this process engages remodeling of mPFC networks.

To approach the synaptic changes underlying flexibility of remote memories, we trained mice in a contextual fear conditioning task and mapped the increase in spine density in different mPFC subregions 36 days posttraining. In parallel, we subjected an additional group of mice to contextual fear extinction starting from posttraining day 31, and then tracked the pruning of spines together with the expression of GAP-43, a marker of synaptogenesis in cortical regions at various steps of the extinction process. In addition, we verified the possibility that the formation of a recent novel memory (extinction) elicited spine growth in the hippocampus.

Here we show that contextual fear conditioning produced extensive remodeling in the anterior cingulate (aCC) and the infralimmbic (IL) regions of mPFC 36 days pottraining. Contextual fear extinction abolished the freezing reaction, promoted a gradual reduction in spines in the aCC region, but did not in the IL region which is noteworthy involved in mediating response flexibility (Dalley et al., 2007).

Measurements of GAP-43 in the IL then revealed the presence of newly formed spines indicating that intense wiring of IL neurons was no longer coding the remote memory trace but reflecting the IL recruitment in the extinction process. Interestingly, mice undergoing extinction showed an increase in hipppocampal spines at the time aCC spines were decreased. Our results point to a dynamic reorganization of hippocampal and cortical structural networks ensuring flexibility of the remote memory traces.

Material and Methods

Ethics statement. All experimental procedures were conducted in accordance with the official European Guidelines for the care and use of laboratory animals (86/609/EEC).

Animals. male C57BL/6J@Ico mice were purchased from Charles River Italia (Calco, Como). At the beginning of the experiments animals were 9 weeks old and their weight ranged from 24 to 26 g. They were housed 5 per cage and maintained in a temperature-controlled facility (22 ± 1°C) on a 12:12 hr light-dark cycle with free access to food and water.

Contextual fear conditioning protocol. Mice were first handled for three days in the fear conditioning room to minimize non specific emotional reactivity to the experimental room. Fear conditioning, consisting of one single session of 7 minutes, began the next day. Each mouse was placed in a transparent Plexiglas cage (28 x 28 x 10 cm) with a removable grid floor made of stainless steel rods. After 120 sec of free exploration, the mouse was exposed to a series of 5 non signalled foot shocks (duration: 2s; intensity: 0.7 mA; 60 sec apart) delivered through the grid-floor. Control mice were treated identically except that they were not shocked. In the conditioned subgroup, half of the mice were tested for extinction or not extinction memory 31 days following the conditioning episode. The group of mice subjected to the extinction protocol were placed back to the conditioning chamber for 4 days (7 minutes each day) 31 days after the training session. Memory retrieval was assessed in all the groups by placing the mice back in the conditioning chamber for 4 minutes 35 days after the training session. Computer scored-freezing during conditioning, extinction or retrieval testing was recorded using an automated procedure described elsewhere (Anagnostaras et al., 2000). Briefly, activity was recorded by means of a video camera mounted 60 cm above the ceiling of the cage and connected to a computer equipped with the Ethovision software

(Noldus, Wageningen, The Netherlands). Percentage of time spent freezing (absence of all but respiratory movements) were recorded.
Nineteen minutes after the completion of the retention tests, the brains were processed for morphological and immunofluorescence analyses.

Golgi-Cox staining and tissue preparation. Golgi-Cox staining was used to assess experience-dependent changes in neuronal morphology. Mice were deeply anesthetized with chloral hydrate (400mg/kg i.p.) and transcardially perfused with a solution of 0.9% saline. Brains were dissected and impregnated using a Golgi-Cox solution according to the method described by Glaser & Van der Loos (1981). Briefly, they were first immersed in the Golgi-Cox solution at room temperature for 6 days, transferred to a 30% sucrose solution for 2 days, and then sectioned using a vibratome. Coronal sections (100-µm thick) were mounted on gelatinized slides, stained according to the method described by Gibb and Kolb (1998), and coverslipped with Permount.

Quantification of neuronal morphology and imaging. Spine density was analysed on pyramidal neurons located in the CA1 region of the dorsal hippocampus and in layers II/ III of the anterior cingulate and infralimbic (IL) cortices. These structures were defined according to the Franklin and Paxinos (2001) mouse atlas. Neurons, identified with a light microscope (Leica DMLB) under low magnification (20X / NA 0.5), were chosen by first locating, among all the stained sections, the regions of interest in their respective coronal sections. Three neurons showing at least fourth order branches for apical dendrites in each region and within each hemisphere were selected. Since no significant interhemispheric difference was observed, measurements were pooled so that six neurons per region were studied in each animal. Only neurons which satisfied the following criteria were chosen for analysis in each of the experimental groups: (1) presence of untruncated dendrites, (2) consistent and dark impregnation along the entire extent of all of the dendrites and (3) relative isolation from neighbouring impregnated neurons to avoid interference and ensure accuracy of dendritic spine counting.
Subsequently, dendritic spines were analysed under a higher magnification (100X / 1.25 NA). Spine counts were performed on secondary and tertiary branches of apical dendrites in the stratum radiatum and on secondary and tertiary branches of basal dendrites in the stratum oriens of the CA1 hippocampal field. The same categories of

dendrites were analysed on anterior cingulate cortex and infralimbic cortex pyramidal neurons lying in the II/III layer.

Spine density counting: Spine density count were performed using an optical microscope (Leica DMLB) with a video camera (Qimaging Qicam Fast1394) connected to the microscope. Neurons were draw and analysed using the Neurolucida software. Primary dendrites were excluded from the analysis.

On each neuron and for each dendrite category, five 30–100 μm dendritic segments were randomly selected. In some cases, segments were from the same branch (Leuner et al., 2003). Segments were sampled 50 μm away from soma in order to exclude the spine-depleted zone which arises from the cell body. Only protuberances with a clear connection of the head of the spine to the shaft of the dendrite were counted as spines. Since this method has proven to provide reliable results (Horner and Arbuthnott, 1991), no attempt was made to introduce a correction factor for hidden spines. As no difference in spine counts was observed between secondary and tertiary branch segments for each group, data were pooled for each dendrite category (basal and apical) to generate the final spine density results.

Morphology of spines analysis: Apical dendrites were randomly selected for imaging. Primary dendrites were excluded from the analysis. Selected dendrites were imaged using an optical microscope (Leica DMLB) with a video camera (Qimaging Qicam Fast1394) connected to the microscope. Neurons were acquired using the Neurolucida software. Images were acquired as a series of optical z-sections at 0.4 μm increments using an oil immersion 100X objective (NA 1.25). They were exported to NIH ImageJ software for analysis. Only spines appearing continuous with their parent dendrite shaft in maximum-intensity z projection were used for quantitative

analysis. Spines were classified according to prevously described criteria (Jedynak et al., 2007): the “thin” type has a long neck and a small head (< 0.55 μm); the “stubby” type has a large head (> 0.55 μm) but does not have a neck; the “mushroom” type has a large head (> 0.55 μm) with a neck; the “chubby” type has no neck and small head (< 0.55 μm).

Analysis was performed blind with respect to the experimental conditions.

Spine densities were expressed as spines per μm.

Immunofluorescence. Ninety minutes after the end of the test, mice were deeply anaesthetized with chloral hydrate and perfused trans-cardially with PFA 4%. The brains were sectioned coronally with a cryostate and the sections incubated with Gap-43 (1:500; Abcam) and NeuN antibodies (1:500; Chemicon) coupled with Dapi staining to detect neurons bodies.

Qualitative analyses. To assess Gap-43 expression within the Il cortex, densitometric analyses of fluorescence images were performed. After background subtraction, cell associated signals were quantified by manually outlining individual neurons labeled with NeuN staining and measuring cell associated fluorescence intensity with the ImageJ software (http://rsb.info.nih.gov/ij/). The ratio, F/A, defines mean fluorescence of individual cells (F) normalized to total cellular surface (A). Quantification was done on 120 cells per group (n=5). All qualitative analyses were conducted blind to the animal's experimental group assignment.

Statistical analyses. Results were expressed as means ± SEM. Freezing values were compared by means of a one-way ANOVA with training condition (trained, pseudotrained and extinction group) as main factor. Differences in spine density were assessed by means of a one-way ANOVA with training condition (trained, pseudotrained and extinction group) as main factor. Post hoc analyses were performed using the Fisher's protected least significant differences test. Differences in GAP-43 expression were estimated in the IL cortex by means of Student's t tests. Values of $p < 0.05$ were considered as significant.

Results

Remote memory can be rapidly extinguished after 4 days of non reinforced exposure to the context.

Mice were trained or pseudotrained in the contextual fear conditioning and tested 36 days. A different group of animals were trained and 31 days later were extinguished for 4 consecutive days reexposing the group in the context without delivering the footshocks. Statistica analysis of freezing responses during the test revealed a training

condition effect F(28) = 49,282; p < 0.001. Post hoc comparisons of freezing revealed that trained mice exhibited higher levels of freezing related to pseudotrained group (p<0.001) and to extinguished group (p<0.001), while the last group do not perform differently from the control pseudo-trained group (p=0.73 N.S.).

Mice exposed to the context without footshock for 4 consecutive days, during the memory test shown a complete abolishment of the freezing behaviour (delay effect F(30) = 86,64 p<0,001) (Figure 4.C.1).

Figure 4.C.1

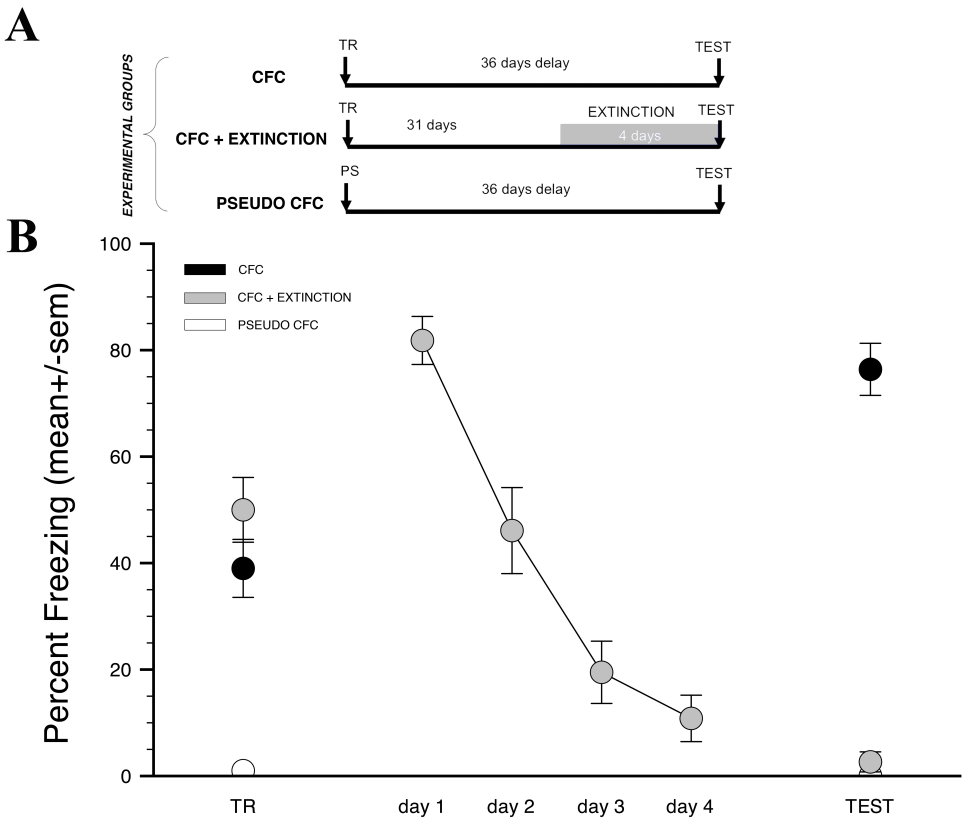

Figure 4.C.1. Remote memory is rapidly extinguished after 4 days of non reinforced exposure to the context. (a) behavioral protocol. Mice were trained or pseudotrained (PSEUDO CFC, white circles) and extinguished (CFC + EXTINTION, grey circles) or tested (CFC, black circles) 36 days after training. (b) Mice extinguished during test exhibited same levels of freezing of animals pseudotrained while trained mice without extinction showed higher levels of freezing response.

Remote memory formation increases spine density in both aCC and IL neurons.

Spine density was measured on apical dendrites of aCC, IL and CA1 neurons in trained and pseudo-trained mice. Results are shown in Figure 4.C.2.

One-way ANOVA analysis revealed significant differences in spine density in aCC ($F(99)=3,8519$ $p<0.05$) and IL neurons ($F(92)=7,2988$ $p<0.005$). Post-hoc analysis revealed structural rearrangements specific to the trained animals in the aCC and IL cortices after the remote memory test ($p<0.001$ for aCC neurons; $p<0.01$ for IL neurons) while in CA1 neurons after test no increase was observed ($F(99)=2,639$ $p=0.076$ *N.S.*).

Figure 4.C.2

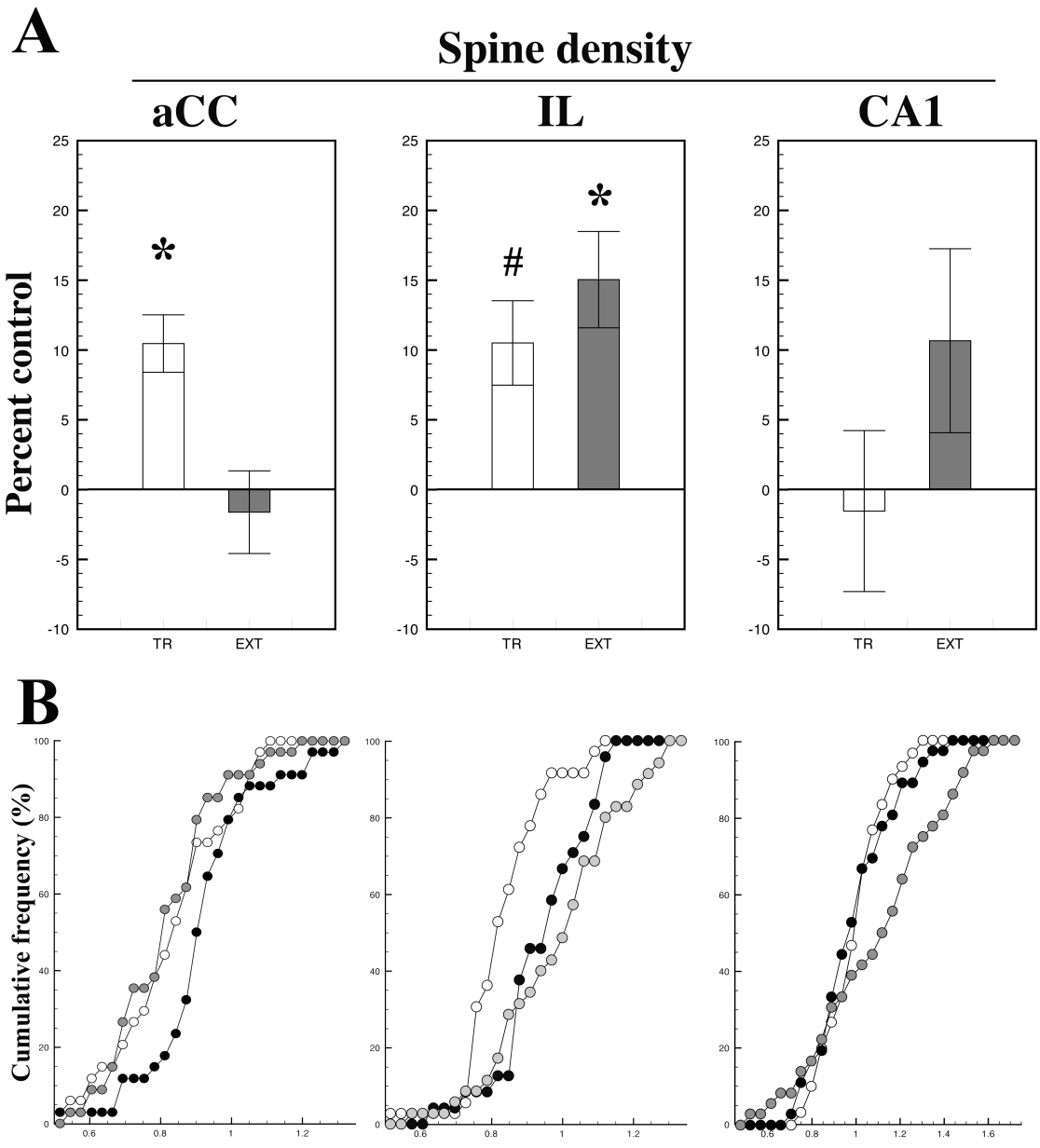

Figure 4.C.2. Remote memory formation increases spine density in both aCC and IL neurons. (a) In aCC neurons increase in spine density is visible after consolidation but not after extinction related to pseudotrained control group. In IL neurons spine density was higher in both mice trained and extinguished related to control group. In CA1 neurons not significative increase was found but a visible trend of increase was measureStructural rearrangements that were specific to the trained animals are found in the aCC and IL cortices after the remote memory test while in CA1 neurons after test an increase

in neurons of mice extinguished was observed. (b) Cumulative frequency of spine density values of apical dendrites of aCC pyramidal neurons are shown for trained (black circles), pseudotrained (open circles) and extinguished mice (grey circles). Each point represents the average spine density (number of spines/ um) of a single neuron. A shift of the curve to the right indicates that the majority of the sampled neurons showed an increase in spines. Such an increase was found in aCC neurons in mice after consolidation, in IL neurons in mice after consolidation and extinction and CA1 neurons in mice after extinction *p<0.001, #p<0.01.

Consolidation-induced spines in aCC, but not in IL, are reduced to baseline levels after the extinction of remote memories.

Post hoc comparisons revealed that structural remodelling formed during consolidation is completely deleted after extinction sessions in aCC neurons (p=0,728 N.S. between extinction group and pseudo trained group) (Figure 4.C.2a).

In animals extinguished for their remote memories, the IL neurons presented an increase in the number of spines related to control group (p<0.001) and no differences related to CFC group (p=0.3145 *N.S.*).

Post hoc comparisons of spine density in the hippocampus revealed that conditioned mice exhibited a significant increase in spine density in neurons of mice from the extinction group related to control group (p<0.05).

Cumulative frequency measured on aCC, IL and hippocampal neuron dendrites and are shown in Figure 4.C.2.b. For the aCC and IL, a strong shift of the curve to the right was observed in mice tested for their remote memory, while only in IL neurons were observed an overlap of neurons from consolidated mice group with neurons from animals that extinguished their remote memory. In hippocampus a partial shift can be observed in mice extinguished.

One extinction session lead to an increase of Gap-43 (a marker of synaptogenesis) in IL neurons

Immunofluorescence analysis shown an increase of the level of GAP-43 marker in IL structure in mice trained and subjected to a single session of extinction (t(675)=-5.557 p<0.001). This increase return to basal levels in mice that completely extinguished the trace (t(560)=0.742 p=0.458 *N.S.*) (Figure 4.C.3.).

Figure 4.C.3.

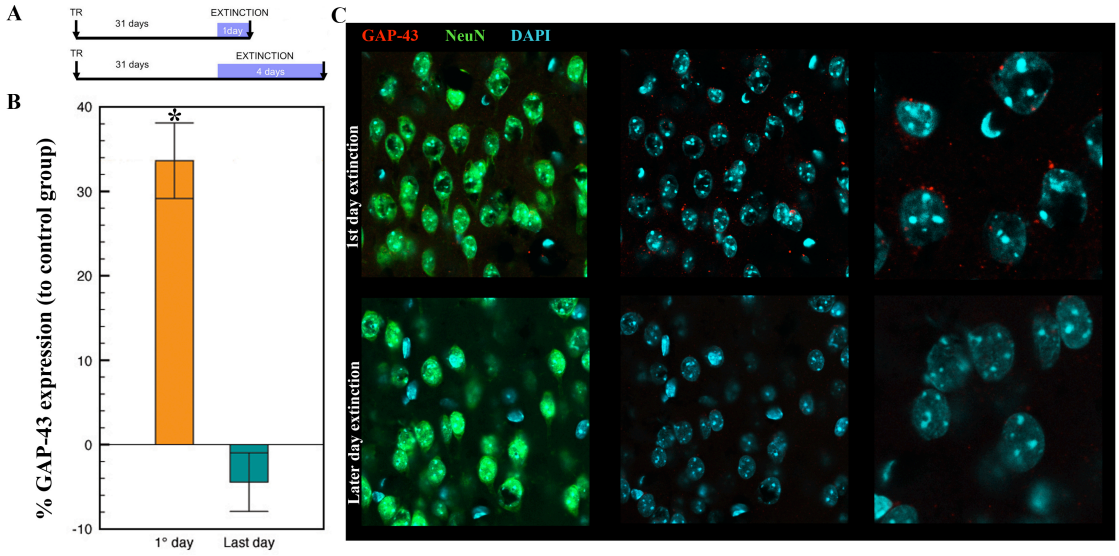

Figure 4.C.3. One extinction session lead to an increase of Gap-43 (a marker of synaptogenesis) in IL neurons (a) Behavioral protocol: mice were trained and exposed to extinction session for 1 day (orange bar) or 4 days (green bar). (b) mice exposed to one session of extinction showed higher levels of GAP-43 expressed in IL neurons related to control group or mice after total extinction. (c) representative picture of confocal acquisition from the two groups of mice analyzed showing higher expression of GAP-43 in mice from the group of 1 day extinction related to mice after complete extinction of the trace. *p<0.001

IL neurons of mice subjected to extinction present more immature spines compared to IL neurons of mice analyzed after remote memory consolidation retrieval.

The spines of IL neurons naive mice presented a distribution of shaped-like spines shown in Figure 4.C.4. The percentage of thin-shaped spines is 42%, stubby-shaped spines are the 25%, mushroom-shaped spines correspond to the 19% of and 14% of the total amount are chubby-shaped spines.

Animals subjected to the consolidation process presented a different distribution, showing an increase of the number of mushroom-shaped spines against a minor number of thin spines related to control group. Animals subjected to extinction protocol presented a percentage of spines mushroom-shaped similar to naive group while we can observe an increase of thin-shaped group related to naive mice.

Figure 4.C.4.

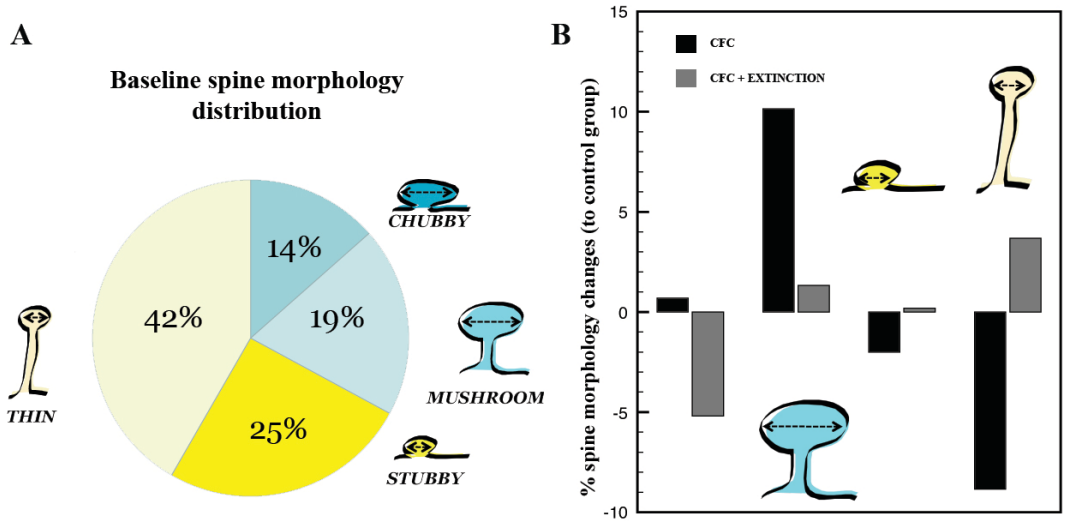

Figure 4.C.4. IL neurons of mice subjected to extinction present higher levels of immature spines. (a)Baseline spine morphology distribution shown higher levels of thin-like spines. (b) mice from the group CFC (black bars) showed higher levels of mushroom spines, while is subjected to a decrease of the number of thin spines related to control group. Mice from the group of CFC + EXTINCTION (grey bars) showed similar percentage of morphology-like spines related to control group and as even more thin-like spines related to control group.

Conclusions

The standard theory of memory consolidation posits that the hippocampus and related structures are needed for storage and recovery of the memory trace, but their contribution diminishes as consolidation proceeds, until the neocortex (and possibly other extra-hippocampal structures) alone is capable of sustaining the permanent memory trace and mediating its retrieval (Squire and Alvarez, 1995; Squire et al., 2004; Frankland and Bontempi, 2005; Squire and Bayley, 2007). However, little is known about the morphological mechanisms mediating prolonged consolidation.

Much evidence since then has accumulated showing that structural plasticity (i.e. changes in the structure of synapses and cells altering the pattern of connectivity between neurons) may be effectively involved in the persistent alteration of synaptic efficacy and, hence, in the representation of information (Maviel et al., 2004). In agreement with this, we reported (Restivo et al., 2009) that gradual structural changes modifying connectivity in hippocampal–cortical networks underlie the acquisition and retention of remote memories. Specifically, we observed that hippocampal neurons of mice trained in a contextual fear conditioning showed an increase in spine density 24 hours posttraining. This increase was only transient since it returned to basal levels 36

days later. An inverted pattern was found in the aCC at these time pointing to a correlation between sequential activation of these regions and modification of their pattern of neuronal connectivity.

The theory of memory consolidation states that the storage of remote memory requires extended cellular activity in key regions of the brain in order they become enduring. These memories, however, are known to be continuously rearranged since they re-enter in labile state upon reactivation (Foster and Wilson, 2006; Wilson and McNaughton, 1994). These data point, therefore to a more dynamical view of memory storage with traces alternating from an inactive to an active state to ensure not only retrieval but updating and interleaving of newly formed memories. According to this view, different synaptic weight configurations should be computed to incorportate novel information into the previously endoced patterns schemas.

To approach this mechanism, we trained mice in a contextual fear conditioning task and mapped the increase in spine density in different mPFC subregions 36 days posttraining. In parallel, we subjected an additional group of mice to contextual fear extinction starting from posttraining day 31, and then tracked the pruning of spines together with the expression of GAP-43, a marker of synaptogenesis in cortical regions at various steps of the extinction process. In addition, we verified the possibility that the formation of a recent novel memory (extinction) elicited spine growth in the hippocampus.

We found that spine density was increased in aCC after consolidation was achieved but that these spines returned to the basal level after extinction. This finding has several implications. At a synaptic level, we can assume that spines induced by remote memory consolidation are extremely labile considering that they can be rapidly eliminated when mice are subjected to a task leading to a novel representation conflicting with the previously stored one. At a system level we cannot argue that the synaptic changes induced in this region are the physical trace storing the memory. One possibility is that aCC is only necessary for the reactivation of the trace (Frankland et al., 2004). Another implication is that the aCC is not the only region involved in the storage of the trace. In fact, there is evidence that the IL region of mPFC is activated during retrieval of consolidated memory (Frankland et al., 2004) but is also critical for the extinction of conditioned fear (Quirk et al., 2000; Santini et al., 2004; Morgan et al. 1993; Sotres-Bayon et al. 2006, Morgan and LeDoux 1995). In agreement with these data, we found an increase in spine density in the IL region following remote memory consolidation,

but no decrease in spines following remote memory extinction. These observation suggest the following interpretations. First, the spines induced by remote memory consolidation are maintained in IL after extinction, contrary to what happens in aCC neuron. Alternatively, the high amount of spines in IL is the result of the fact that the old spines induced by consolidation were eliminated while the new spines elect the IL recruitment in response flexibility. This second possibility is consistent with the idea that a new memory can be inserted into an old one inducing synaptic and systemic rearrangement in consolidated networks. To investigate whether the abundant spines in IL are newly formed spines, we analysed the expression of GAP-43, a marker of synaptogenesis to has evidences of the formation of new spines.

Several lines of investigation have helped clarify the role of GAP-43 (F1,B-50 or neuromodulin) in regulating the growth state of axon terminals. In transgenic mice, overexpression of GAP-43 leads to the spontaneous formation of new synapses and enhanced sprouting after injury. GAP-43 appears to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organization in the nerve ending. In the brains of humans and other primates, high levels of GAP-43 persist in neocortical association areas and in the limbic system might play an important role in mediating experience-dependent plasticity.

We observed that an increase in the levels of GAP-43 is visible only after the first day of extinction but disappears at the end of the extinction process. This is a first evidence of a remodelling of the cortical networks following extinction. We then analysed the morphology of spines after consolidation or extinction of remote memory and found differences in the size of spines. Specifically, the analysis of the size of spines in naive mice pointed out a high percentage of thin-shaped spines corresponding to a 42% of the total amount, 25% of stubby-shaped spines, 19%of mushroom-shaped spines and 14% of chubby-shaped spine. This is in agreement with recent studies using *in vivo* imaging of spine morphology (two-photon laser-scanning imaging) which demonstrated remarkable stability of a subset of spines in neocortex, with large mushroom-shaped spines beeing the most stable. These studies also demonstrated a significant capacity for ongoing synaptic change. In fact, about 17% of spines had lifetimes inferior to 1 day, and 23% had a mean lifetime of about 2-3 days thus revealing intensive turnover of spines in thr control cage mice. Within this context, we consolidation the percentage of mushroom-shaped spines is higher (about 10% more) in IL cortex related to naive

group, while the percentage of thin-shaped spines was lower than in naive mice (circa 10% less).

However, by the end of extinction process, the percentage of mushroom-shaped spines are similar to the percentage in naive mice, while the thin-shaped spines are even more than in control mice. These results suggest that during consolidation there is the formation in IL cortex of new spines that after time became more stable (mushroom-like) forming strength connections between neurons that can be necessary for the retrieval of the trace. It could be therefore, that during extinction, the structural support of the trace undergoes a strong rearrangment warranting incorporation of novel information within the old network, suggesting in turn that the revised. In agreement with this view, there is growing evidence (Kasai et al, 2003) from empirical, theoretical and modelling approaches suggesting that memory retention does not require the maintenance of a specific configuration of synaptic weights for the lifetime of the memory. Moreover, we found no difference in spine density in the hippocampal neurons after consolidation. Intriguingly, we observed an increase of the number of spines after extinction that however is not significant. This can be a signal that new aspects of the previously consolidated trace must has to be acquired and this process need the interference of the hippocampus. From our data we drew the conclusion that cortical wiring plasticity in the mPFC (aCC and IL) is a prerequisite for the formation and expression of long-lasting memories, pointing to the hypothesis that the aCC might play a role in the retrieval of the remote trace, while the IL might be devoted to the switching of behavioral output according to external contingencies and previous knowledge, during consolidation activating the behaviour of freezing and after extinction disactivating the same behaviour. It is possible that extinction potentiates infralimbic circuitry/connections, which inhibits fear during subsequent exposure to fear stimuli. The hippocampus can be partially recruited to mediate only the novel components of trace. Our findings are therefore in line with the idea that, despite their apparent stability, remote memory are highly flexible and do not rigidly correlate with stable synaptic networks in the cortical regions. In particular, they suggest that new informations can be incorporated into formerly stored memory representations, and that the formation of a new trace conflicting with a previously consolidated one produces massive rearrangements of the former circuits based upon a strong synaptic a remodelling.

While the involvement of hippocampus and neocortex in storing long term memories is been largely studied in many works, the morphological changes sub-serving the recruitment of these regions even many days after the training session are still not well understood. To define the complex neural substrates enabling memory retention is at least one of the most challenging goals of modern neuroscience. The series of works that I have described in this thesis represent the first evidence supporting the idea of long-lasting changes in dendritic spines as result of a consolidated learning.

Our works are based on much evidences accumulated in the last years showing that structural plasticity may be effectively involved in the persistent alteration of synaptic efficacy and in the representation of information.

To this aim, we looked at the plasticity/stability nature of the system, analyzing morphological changes induced by consolidation (i.e. dendritic spine density and spine morphology).

We focused our attention in the dendritic spines modifications for many important reasons. In fact, dendritic spines represent the main surface of contact of neurons (Ramon j Cajal, 1888), spines host primary excitatory synapses (Yuste R, Bonhoeffer T. 2004; Dailey and Smith, 1996) and memory induction by training rodents in behavioural tasks promotes a rapid increase of dendritic spine density in specific brain structures (Leuner et al., 2003; Knafo et al., 2004; Restivo et al., 2006).

Our main goal is to detect long-lasting changes in networks that can correlate with the standard model of consolidation that posits a shift of the trace memory from the hippocampal to neocortical circuitry. In fact, as assumed by many studies, the last point of morphological changes that can occur during consolidation is the organization of connections in schema. It is supposed that these modified networks are enduring and our intention is to verify this hypothesis. To this aim, we extinguish a remote memory and reveal the stable/labile state of morphological changes induced by previous consolidation.

We are also interest in revealing the time course of modifications occurring during the consolidation process and how the blockade of synaptic plasticity can influence the retrieval of the trace.

Plastic changes during consolidation process

We shown that training mice in an hippocampal-dependent task (contextual fear conditioning) induces a rapid increase of dendritic spine density in the CA1 field of the hippocampus. This increase returns to basal levels in animals tested for their remote memory 36 days later. This finding is in line with the consolidation hypothesis posits that hippocampus plays only an initial role in acquisition and consolidation of memories while the neocortex is engaged for the maintenance of long-term memories. As aspected, we found an increase in spine density in aCC neurons only in mice tested at a remote time point.

Moreover we found that lesions of the hippocampus immediately after learning but not 24 days after lead to a disruption of correct behavioral performances during remote memory test and a subsequent failure to increase spine density in the aCC field. This result is in line with the idea that a dialogue between hippocampus and neocortex is necessary for the shift of the information from one to the other region and that the hippocampus coordinates memory consolidation in target cortical regions (Frankland and Bontempi, 2006).

This is the first evidence that shown an increase in connectivity in a specific area of the brain (aCC) that is delayed from the event (training in the contextual fear conditioning) causing this increase. This is confirmed by the observation that these changes are not dependent on the remote memory test because we shown also an increase in connectivity in mice that are not tested. While other evidences suggesting the role of aCC in remote memory are been shown following retrieval, we found that spine density modifications are independent from the retrieval. This important result lead to the hypothesis that plastic modifications are induced slowly over time by a single training event.

Morphological changes happens in a particular time window

We then examined the effects induced by a blockade of morphological changes during the retrieval of remote memories.

In particular we blocked the formation of new spines in the aCC by injecting 1 day or 42 days after training a virus that overexpress the protein MEF2. This protein has a key role in modulating synaptic and spines formation. In particular it is been demonstrated that MEF2 blocks remodelling in vivo and in vitro in NAc and hippocampal neurons.

We shown that the injection of this protein blocks the increase of spine density induced in the aCC 8 days after training. However we were not able to detect any difference in spine density number in mice injected with the virus overexpressing MEF2 42 days after training related to mice injected with the virus that do not contain the MEF2 construct. This confirm the role of this protein in modulating the ongoing remodelling. In fact, overexpression of Mef2 has no effect in neurons in which the remodelling process is been concluded.

Moreover we found that the induction of morphological changes in the aCC is strictly necessary for the correct behavior during recall of acquired memories.

Very interesting we found that these changes occurs closed to the initial training event. Our results represent an important step toward the comprehension of the time course dynamics in the complex scenario of the system consolidation process (Nadel et al. 2000; Frankland and Bontempi, 2006, Wang and Morris, 2010).

The labile state of connections

The next question we attempt to answer concerns the fate of a memory once it is acquired and consolidated: Is the stability of long-term memory achieved because of the stability of synaptic structures? If so, is loss of memory with time reflected in loss of synaptic connections in the aCC?

We can argue that changes rising from the complex process of consolidation will remain stable to permit the retrieval of memories. However it is increasingly obvious that long-lasting memories are far from being stable (Alvarez and Sabatini, 2007; Bhatt et al., 2009). Recent evidence shows that enduring memories are continuously rearranged. According to this view, our results shown that new spines formed in aCC neurons induced by consolidation can disappear after extinction.

We also found that other regions are involved in these rearrangements. In particular the Infra- Limbic cortex, as the aCC, increase spine density degree after consolidation and we still observe higher levels of spine density after extinction. This finding reveals a complete rearrangement of neocortical networks, showing a disengagement of aCC region and parallel involvement of the Infra-Limbic cortex.

We next analyzed the morphology of spines in IL neurons after consolidation and extinction of remote memories. In fact, many researches pointed to the change in the morphology of spines as a synaptic consolidation in adult neurons.

In fact it is believed that spine size correlates with synaptic strength, thus changes in spine morphology indicate that synaptic strength can be modified without synapse turnover.

We found an increase of mushroom-like spines after consolidation in IL cortex related to spines in control cage mice, while mushroom-like spines are similar to control group's spines after extinction. Mushroom-like spines has large head and it is believed that this type of spines are the more functional and mature one. The decrease in number of these spines is an index of instability of the network. Another index of plasticity is the increase in synaptogenesis we found after the first day of extinction when higher expression of GAP-43 (a marker of synaptogenesis) was found in IL cortex.

All together these data pointed to a dynamic and flexible storage of the trace in neocortical networks.

Moreover, it seems that more than one structure is involved in consolidating and extinguishing memories through changes occurring at a synaptic level as predicted by both Standard theory and Multiple trace theory of memory consolidation

From our data we can argue that memories are achieved initially in the hippocampus by increasing connections between neurons in the CA1 field. Immediately after a dialogue between the hippocampus and neocortical areas take place and caused a rearrangement in cortico-cortical circuits. This process starts very early after the training session and it is strictly necessary for the correct recall of memories.

However these morphological changes undergo immediate rearrangement if new and contrasting information interfere with the old ones and must have to be integrate in the schema previously formed.

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