Neurotox Res (2010) 18:377–385
DOI 10.1007/s12640-010-9155-5

Persistence of Long-Term Memory Storage: New Insights into its Molecular Signatures in the Hippocampus and Related Structures

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Received: 11 November 2009 / Revised: 19 January 2010 / Accepted: 19 January 2010 / Published online: 12 February 2010
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Abstract Although much is known about long-term memory (LTM) consolidation, what puts the "long" in LTM is the exclusive feature of persisting over time. However, until recently the molecular mechanisms underneath memory persistence had never been properly studied. In rats, the protein translation inhibitor anisomycin impaired memory persistence when injected into the dorsal hippocampus 12 h after inhibitory avoidance (IA) training without affecting memory formation. Here, we also show learning-induced changes in hippocampal c-Fos, Homer 1a, Akt, CamKIIα, and ERK2 levels around 18–24 h after IA training. Thus, memory persistence is associated with a late phase of plasticity-related protein synthesis in the hippocampus.

Keywords Memory · Hippocampus · Persistence · BDNF · Consolidation

Introduction

The history of memory research has been extensively and successfully devoted to the study of the mechanisms and brain circuits involved in long-term memory (LTM) formation. LTM is conventionally defined as the one that lasts more than several hours and there is a vast amount of information regarding the participation of many biochemical pathways and brain areas in LTM formation. In 1900, Müller and Pilzecker (1900) proposed that the formation of permanent memory takes time, and that during this time, memory remains vulnerable to disruption. The process of developing stable memory is referred to as "consolidation" (McGaugh 1966, 2000).

The term "consolidation" is currently used to describe two types of processes. One process is fast and completed within minutes to hours after training and is termed "synaptic" or "cellular consolidation." This process is thought to take place in synapses of the neuronal circuits that encode the experience-dependent internal representation. Cellular consolidation involves posttranslational modification of synaptic proteins, activation of transcription factors, modulation of gene expression at synapses and cell body, and reorganization of pre- and postsynaptic proteins which finally ends in synaptic remodeling that makes the trace stable. In other words, cellular consolidation has been defined as the transition of memory from protein synthesis and gene expression dependence to independence in specific brain regions involved in acquisition of a particular learning experience (Medina et al. 2008).

The other type of consolidation process is slow and takes several days, weeks, or even months to conclude (Squire and Alvarez 1995), but see Tse et al. (2007). It is thought to involve reorganization of the brain circuits or systems that encode the memory and it is termed "systems consolidation." Stabilization of the memory trace in the brain is achieved by gradually binding together the multiple cortical regions that store memory for a whole event. However, when consolidation of new information involves

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interactions with an already stored associative "schema" systems consolidation may well be very rapid (Tse et al. 2007). Studies involving lesions suggest that hippocampus damage affects recent memories more severely than remote ones. This is consistent with the idea that the hippocampus plays a time-limited role in the storage and/or retrieval of memory. Therefore, "systems consolidation" is normally referred as the process by which memory becomes independent of the hippocampus. However, some studies have challenged this view suggesting that some memories never become totally independent of the hippocampus (Moscovitch and Nadel 1998; Rudy and Sutherland 2008).

Although much is known about LTM consolidation, what puts the "long" in LTM is its exclusive feature: its persistence over time. Most of the acquired information is bound to disappear or may leave an undetectable trace. Therefore, the matter of memory persistence is central in understanding the neurobiology of learning and memory. Only recently some authors have started to address this issue, focusing mainly in the neocortex as the region of permanent storage of memories (Frankland et al. 2001; Tischmeyer et al. 2003; Burwell et al. 2004; Cui et al. 2004; Frankland et al. 2004; Maviel et al. 2004; Woodside et al. 2004). Since memories may last for long periods or even a lifetime, for them to achieve persistence, cellular and molecular changes must occur after acquisition. Therefore, which mechanisms are involved in memory persistence? Is the hippocampus involved in this process? So far, these questions have been poorly addressed by the scientific community. Recently, we demonstrated that late molecular events that take place in the rat hippocampus 12 h after acquisition of inhibitory avoidance (IA) memory affect memory persistence at 7–14 days without affecting memory expression at 2 days post-training (Bekinschtein et al. 2007; Bekinschtein et al. 2008). We also found that several hippocampal proteins raise their levels 18–24 h after training, which correlate with the postulated existence of a late post-training cellular mechanism for memory persistence in the hippocampus. This late consolidation phase depends on new protein synthesis, an increased expression of the neurotrophin BDNF, and is controlled by dopaminergic inputs from the VTA (Rossato et al. 2009). In this short essay, we will review the available information about molecular signatures related with memory persistence and give some new findings on the role of these signatures in the maintenance of long-lasting memory traces.

Protein Synthesis in CA1 Region of Rat Hippocampus is Required for Persistence of IA Memory Trace

It is widely accepted that cellular consolidation for several learning tasks is dependent upon hippocampal de novo protein synthesis (Grecksch and Matthies 1980; Grecksch et al. 1980; Abel et al. 1997; Bourtchouladze et al. 1998; Quevedo et al. 1999). In this sense, LTM for different tasks in several species including a one-trial IA learning in rodents requires de novo protein synthesis in at least two time periods after training, one around the moment of acquisition and the other one 3 to 6 h after it (Quevedo et al. 1999; Igaz et al. 2002). The existence of additional waves of protein synthesis, though postulated many years ago, has only been recently described (Bekinschtein et al. 2007).

It is thought that cellular consolidation of LTM implies the growth of new connections and the rearrangements of existing ones (Bailey et al. 2004). IA training induces changes in the level of several proteins 24 h after acquisition (Igaz et al. 2004). These results led to the question of whether there were more waves of protein synthesis involved in consolidation of this task. In other words: had cellular consolidation ended 6–8 h after training or did it continue? In order to answer this question, we did some experiments by injecting the protein synthesis inhibitor anisomycin (ANI) (Bekinschtein et al. 2007), which has been broadly used to block memory formation in a wide variety of behavioral paradigms (Montarolo et al. 1986; Tiunova et al. 1998; Schafe and LeDoux 2000; Berman and Dudai 2001; Luft et al. 2004; Santini et al. 2004). We found that de novo protein synthesis in the CA1 region of the dorsal hippocampus 12 h after training was crucial for memory persistence, but not for memory formation. Here, we confirmed and extended these data (Fig. 1) showing that infusion of ANI (80 μg/side) in CA1 region of the rat hippocampus 12, 18, or 24 h after IA training did not prevent memory formation and expression when rats were tested 48 h after acquisition (Fig. 1a, $n = 9$–13, $P = 0.80$, 0.99, and 0.87, respectively). These findings are consistent with current views in the field postulating that memory for one-trial tasks would be fully consolidated (cellular consolidation) around 12 h after training. Therefore, new protein synthesis does not seem to be required 12–24 h post-training for cellular consolidation of IA LTM. On the other hand, inhibition of protein synthesis 12 h after training, caused a severe amnesia when rats were tested a week later in an independent experiment (Fig. 1a, $n = 12$–13, $P = 0.0009$). Another group of ANI-injected animals tested 4 days after training did not reach significance but showed a trend to lower step-down latencies than control animals (Fig. 1b, $n = 10$, $P = 0.057$). In contrast, no effect was seen at 7 days on retention scores when ANI was infused at 18 or 24 h after training (Fig. 1a, $n = 9$–13 per group, $P = 0.49$ and 0.71). These results indicate that protein synthesis in CA1 region of rat hippocampus is required in a restricted time window around 12 h after training for persistence of IA memory expression.

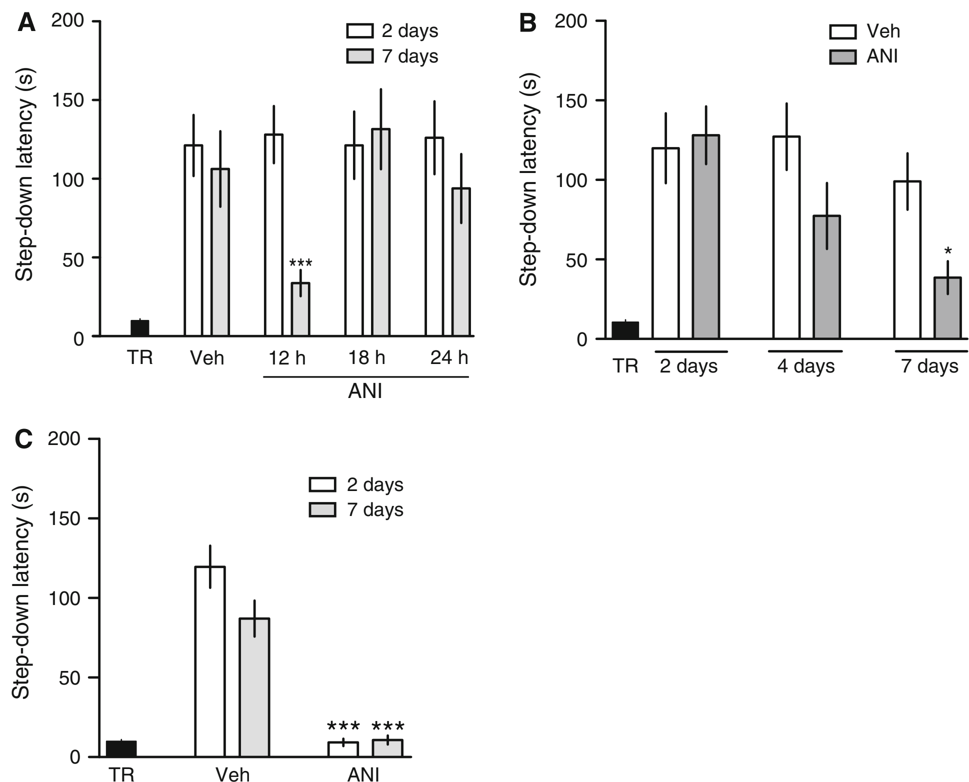

Fig. 1 A late protein synthesis phase is required for memory persistence but not formation. Male Wistar rats (2.5 months old, 220–250 g) were trained in IA and injected intra-CA1 of the dorsal hippocampus with Anisomycin (ANI, 80 µg/side, Sigma) or Vehicle (Veh) at different time points. Bars represent step-down latency mean ± SEM in seconds at the training session (TR, *black bar*) or test session (*white and gray bars*). (**a**) Animals were injected at 12, 18, or 24 h post-training and tested at 2 (*white bars*) or 7 days (*gray bars*) after training. *** $P < 0.001$; $n = 9$–13 per group. (**b**) Animals were injected at 12 h post-training and tested at 2, 4, or 7 days after training. * $P < 0.05$; $n = 10$ per group. (**c**) Animals were injected 15 min pre-training and tested 2 (*white bars*) or 7 days (*gray bars*). *** $P < 0.001$; $n = 8$–10 per group

Consolidation of IA memory trace expressed 7 days after training could be independent from consolidation of the one expressed at 24 or 48 h. If this were the case, ANI injection 12 h after acquisition would be blocking consolidation of a memory that is expressed at 7 days but not at 24 h. If this was true, there should be a treatment that prevents consolidation of memory expressed at 24 h after training, but keeps memory intact 7 days later. Pre-training intra-hippocampal infusion of ANI that caused amnesia 24 h after training also affected memory when rats were tested 7 days later (Fig. 1c), indicating that the consolidation process that takes place during the first few hours is needed for later expression of IA memory.

Several Hippocampal Proteins Increased Their Level 24 h After IA Training

Since blocking protein synthesis 12 h post-training affects memory expression at 7 days (Fig. 1), there should be changes in protein expression beyond the first few hours after acquisition. Previous results from our lab indicated that several proteins showed increased levels 24 h but not 3 h after IA training (Igaz et al. 2004). We decided to perform a time course analysis of these changes to establish more precisely when they became evident. Total hippocampal homogenates of naïve, shocked or trained rats were subjected to immunoblot analysis. Shocked rats received a 3-s footshock in the training apparatus in the absence of the platform; this behavioral procedure did not increase step-down latencies and did not lead to contextual fear learning (Igaz et al. 2004). Since no true unpaired control can be designed for this task, the shocked group is a suitable control for biochemical analysis. Trained or shocked animals were killed 0, 1, 3, 9, 12, 18, 24, 30, or 72 h after the behavioral manipulation. Immunoreactivity to c-Fos, a well-known immediate early gene (IEG), reached a significant increase of 62% respect to naïve group 24 h after training (Fig. 2a, $P < 0.05$, $n = 8$) and an increase was also evident at 18 h. Another IEG, Homer 1a, also showed increased protein levels of 34% 24 h post-training ($P < 0.05$, $n = 8$), but no changes were evident at other time points (Fig. 2b).

Total levels of three protein kinases, two of which are known to be activated shortly after IA training (Cammarota et al. 1998; Alonso et al. 2002; Quevedo et al. 2004), were augmented 24 h after IA acquisition (Fig. 3a–c). α Subunit of calcium calmodulin-dependent protein kinase II (CamKIIα) showed greater immunoreactivity at both 18 and 24 h after training (35 and 55%, respectively) when compared to naïve animals (Fig. 3a; $P < 0.05$, $n = 8$). ERK-2 member of the MAPK family significantly raised its level at 18–24 h by 32–39% respect to naïve (Fig. 3b; $P < 0.05$, $n = 8$). A 28% increase in total level of Akt/PKB kinase was detected

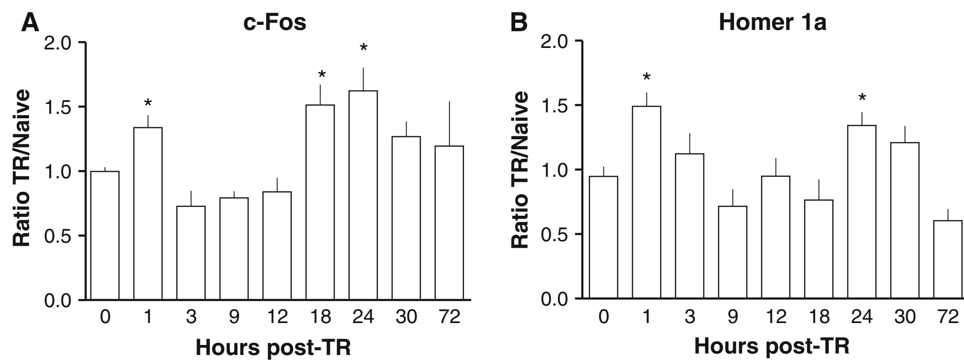

Fig. 2 Biphasic increases in the IEGs c-Fos and Homer 1a levels after IA training. Bars represent the mean ± SEM hippocampal c-Fos (**a**) or Homer 1a (**b**) levels of animals trained in IA and killed 0, 1, 3, 9, 12, 18, 24, 30, and 72 h after training respect to naïve rats which were withdrawn from their home cages at the same time points that the trained animals and killed immediately thereafter (group not shown in the figure). Dorsal hippocampi were dissected out and rapidly homogenized in iced-chilled buffer, subjected to SDS–page, electrotransfered to PVDF membranes, and then incubated with anti c-Fos (1:2000; Santa Cruz Biotechnology Inc) or anti Homer 1a (1:1000; Santa Cruz Biotechnology Inc). * $P < 0.05$ versus naïve; $n = 5$ per group

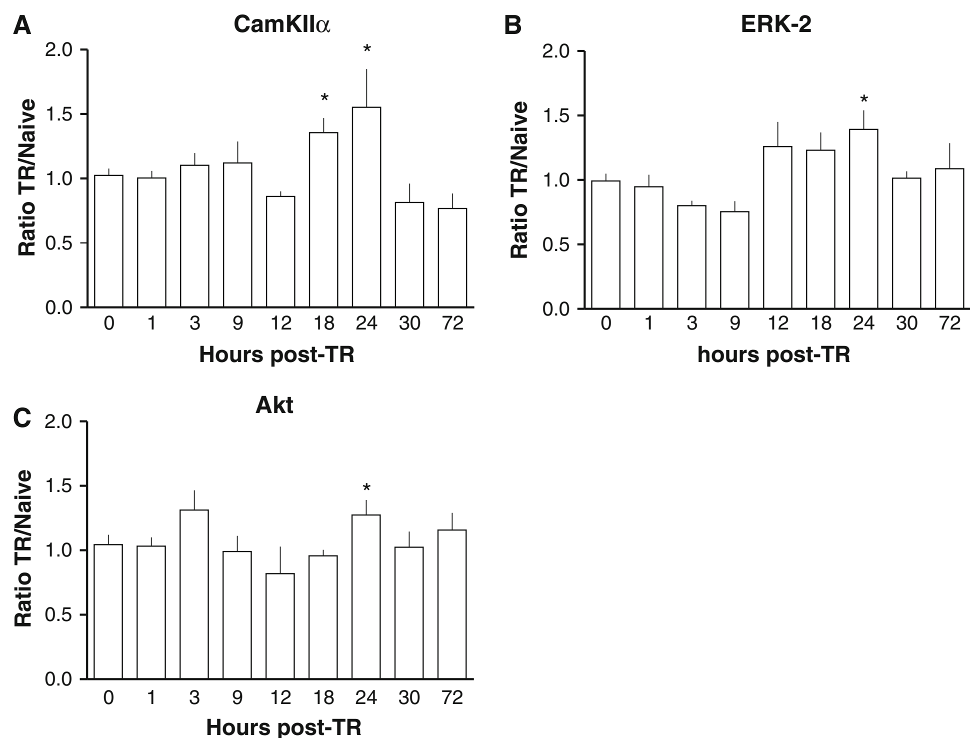

Fig. 3 Delayed increase in CamKIIα, ERK-2, and Akt levels after IA training. Bars represent the mean ± SEM hippocampal CamKIIα (**a**), ERK-2 (**b**), and Akt (**c**) levels of animals trained in IA and killed 0, 1, 3, 9, 12, 18, 24, 30, and 72 h after training respect to naïve group (not shown in the figure). PVDF membranes were incubated with anti CamKIIα (1:1000, Santa Cruz Biotechnology Inc), anti ERK-2 (1:3000, Santa Cruz Biotechnology Inc), or anti Akt (1:3000, Santa Cruz Biotechnology Inc). * $P < 0.05$ versus naïve; $n = 5$ per group

24 h after IA (Fig. 3c; $P < 0.05$, $n = 8$). An apparent non-significant increase in Akt/PKB immunoreactivity was seen at 3 h. In all the experiments, the shocked group did not differ significantly from the naïve one.

Protein kinases' increase should imply an increase in activity. Although we did not measure their phosphorylated forms, it is expected that the increase in total protein levels implies an increase in both the phosphorylated and non-phosphorylated forms, and therefore these increases in protein levels may probably represent increased activity.

These results indicate that IA learning is associated with late changes in the level of several proteins in rat hippocampus that reach a peak value around 24 h after training and then they return to basal levels. The following question arises: if consolidation is thought to be concluded a few hours ago, why would there be protein expression 24 h after training and what does it have to do with memory? Results from Fig. 1 might give hints to clarify this issue: are these increases in protein levels related to a hippocampal mechanism involved in memory persistence described earlier?

Intrahippocampal Infusion of Anisomycin 12 h After Training Abolishes c-Fos and Homer 1a Changes at 24 h

According to our hypothesis, there is a wave of protein synthesis that starts around 12 h after training involved in a hippocampus-dependent mechanism of memory persistence. If protein changes seen at 18–24 h are a part of this process, then injection of ANI at 12 h should prevent them. In order to test this prediction, animals received infusion of ANI (80 μg/side) (Ani group) or vehicle (Veh group) in the CA1 region of the dorsal hippocampus 12 h after IA training and were killed 12 h later for immunoblot analysis of total hippocampal homogenates. This dose of the inhibitor is capable of producing the amnesic effect seen at 7 days. In addition to the naïve (N) and shocked (Shock) groups, one more control was carried out: to rule out that any decrease in protein levels caused by ANI was due to a change in protein turnover, another group of naïve rats was injected with the inhibitor and killed 12 h later (N Ani).

c-Fos increase in immunoreactivity 24 h after IA training was detected in the Veh group (Fig. 4a; $P < 0.01$ respect to N, $n = 4$) and was completely abolished by ANI injection (Fig. 4a; $P < 0.01$ Ani vs. Veh, $n = 4$). N Ani group did not show any differences with the N group in c-Fos levels (Fig. 4a; $P > 0.05$ N Ani vs. N, $n = 5$). Therefore, decreased c-Fos levels detected in the Ani group were not caused by a diminished basal level of protein expression. There was no significant increase in c-Fos levels in the Shock group (Fig. 4a; $P > 0.05$ Shock vs. N, $n = 5$).

The same pattern was observed for hippocampal Homer 1a protein levels. Injection of ANI 12 h after training completely blocked the increase detected at 24 h in the Veh group (Fig. 4b; $P < 0.05$ Ani vs. Veh, $n = 5$). All control animals showed significantly lower amount of Homer 1a than Veh animals (Fig. 4b; $P < 0.05$ Veh vs. N, $n = 5$) and did not differ from the N Ani group (Fig. 4b; $P > 0.05$ N Ani vs. N, $n = 5$). Although increase in Akt levels in Veh animals did not reach significance when compared to N (Fig. 5a; $P > 0.05$, $n = 4$), infusion of ANI produced a significant decrease 24 h post-training with respect to Veh group (Fig. 5a; $P < 0.01$, $n = 4$).

On the other hand, ANI did not block IA training induced increases in CamKIIα or ERK-2 protein levels measured at 24 h (Fig. 5a, b).

In addition to these late IA training-induced changes in several synaptic proteins in the hippocampus, a growing body of evidence strongly suggests that delayed and/or protracted changes in transcription factors, IEGs, protein kinases, protein phosphatases, and neurotransmitters receptors are associated with persistent memories (see Table 1).

Long-Lasting Perseverance to Unravel Memory Persistence

The first study that demonstrated a lasting process in the hippocampus required for LTM consolidation is that of Riedel et al. (1999). They found that ongoing activity in the hippocampus is necessary for consolidation/storage of spatial information during 5 days after training rats in a water maze. In this context, there is mounting evidence demonstrating that different types of glutamate receptors undergo long-lasting changes and/or are needed at very late post-training time points in the hippocampus (Cammarota et al. 1996; Riedel and Micheau 1999). Using classical conditioning in rabbits, Tocco et al. (1991) found that in the first couple of hours after subjecting rats to different

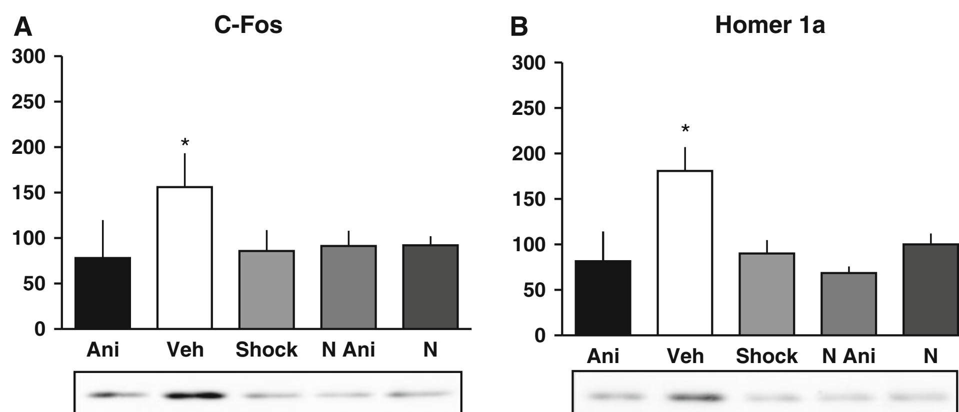

Fig. 4 Late increases in c-Fos and Homer 1a are blocked by anisomycin injections 12 h after training. Bars represent the mean ± SEM hippocampal c-Fos (**a**) or Homer 1a (**b**) levels 24 h after training respect to naïve group (N). Animals were either trained in inhibitory avoidance and injected 12 h after training with Anisomycin (80 μg/side, Ani) or Vehicle (Veh) and killed at 24 h or placed over the grill without the platform (shock group, S). In the N Ani group, naïve animals were injected with anisomycin 80 μg/side. * $P < 0.05$ versus naïve; $n = 5$ per group

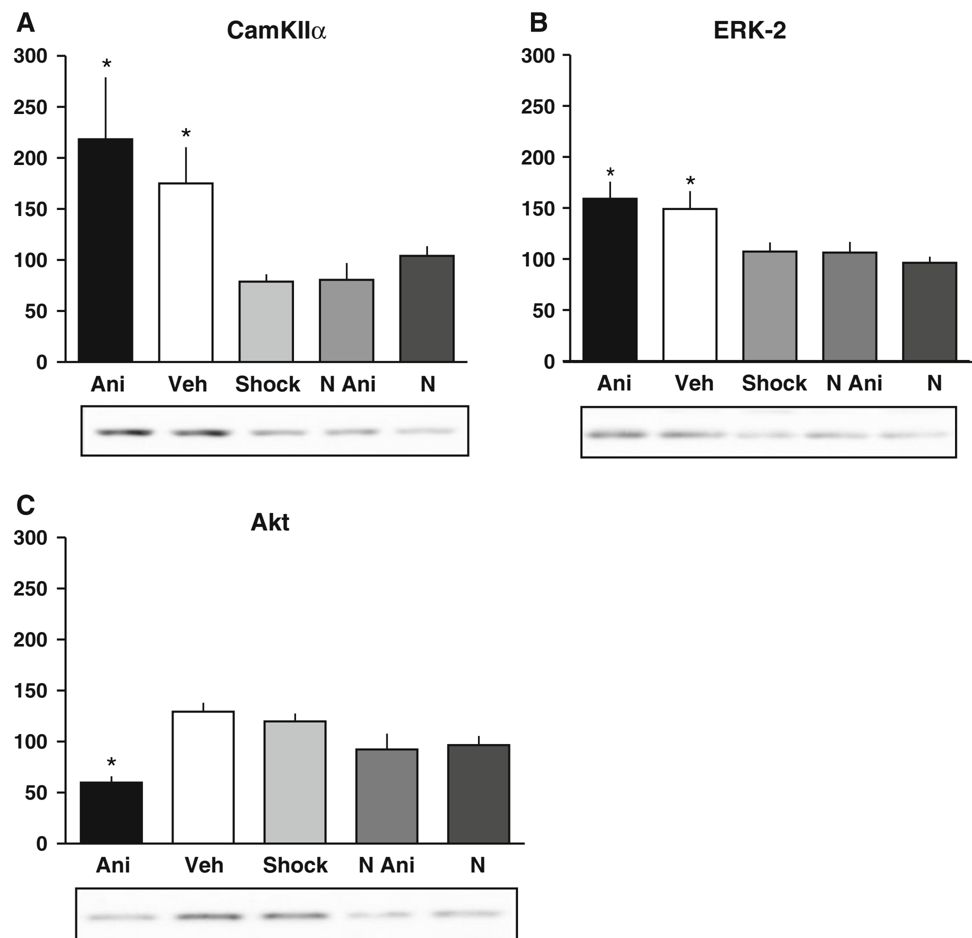

Fig. 5 Late increases in CamKIIα and ERK-2 are independent of protein synthesis 12 h after training. Bars represent the mean ± SEM hippocampal CamKIIα (**a**), ERK-2 (**b**), and Akt (**c**) levels 24 h after training respect to naïve group (N). Animals were either trained in inhibitory avoidance and injected 12 h after training with Anisomycin (80 μg/side, Ani) or Vehicle (Veh) and killed at 24 h or placed over the grill without the platform (shock group, S). In the N Ani group, naïve animals were injected with anisomycin 80 μg/side. * $P < 0.05$ versus naïve; $n = 5$ per group

learning tasks, there was a selective and region-specific increase in AMPA receptors in the hippocampus. We and others (Cammarota et al. 1995; Whitlock et al. 2006) confirmed and extended this findings using IA training in rats. More importantly, we also found that these changes lasted at least 2 days in CA3 region and dentate gyrus. No changes were seen 7 days after training. It is feasible that these long-lasting changes may well facilitate reactivations of the hippocampal circuit and may entrain extrahippocampal structures in which the memory trace is eventually stored. Metabotropic glutamate receptors (mGluRs) also undergo long-lasting modifications after training. By using specific antibodies, Riedel et al. (2000) showed an early and transient increase in the expression of mGluR 5 in CA3 region of the hippocampus. This was paralleled by an increase in mGluR 5 in CA1 and dentate gyrus 10 days post-training, suggesting that these long-lasting changes in the expression of hippocampal AMPA and mGluRs could participate in the maintenance phase of memory consolidation.

Soon after, Shimizu et al. (2000) showed that reactivation of NMDA receptors in the CA1 region of the hippocampus is required during the first several days after spatial learning or after contextual fear conditioning. These findings have opened a new avenue of research on the mechanisms of memory storage. Using an inducible, reversible, and region-specific protein knockout technique, Wang and Kelly (2001) demonstrated that reactivation of CaMKIIα activity in the forebrain during the first, but not the second or third weeks after training mice in a contextual fear conditioning is crucial for remote memory consolidation.

In 2002, Drier et al. (2002) found that overexpression of PKMζ, an atypical isoform of PKC, during a narrow time window after training enhanced the persistence of memory in odor avoidance conditioning in *Drosophila*. More recently, Sacktor and co-workers demonstrated that inhibiting PKMζ into the hippocampus (Pastalkova et al. 2006) or the neocortex (Shema et al. 2007) hampered both recent and remote memories, suggesting that persistent PKMζ may play a role in maintaining remote memories.

These findings suggest that persistent activity of NMDAr, CAMKIIα, and PKMζ for the first several days after training is important in establishing remote memories.

More recently, several groups have shown that memories are accompanied by late modifications in the levels and/or activity of C/EBP and CREB, two well-known

Table 1 Learning induces protracted biochemical changes in the hippocampus

Protein	Levels	Structure	Time	Task	Reference
AMPAr	↑	Hipp	2–48 h	IA	Cammarota et al. (1995) and Cammarota et al. (1996)
Arc	↑	Hipp and PCx	8 and 24 h	OF	Ramirez-Amaya et al. (2005)
BDNF	↑	Hipp	12 h	IA	Bekinschtein et al. (2007)
C/EBPb	↑	Hipp	9–28 h	IA	Taubenfeld et al. (2001)
C/EBPb	↑	Hipp and ICx	18 h	CTA	Yefet et al. (2006)
CamKIIα	↑	Hipp	18–24 h	IA	Igaz et al. (2004)
CaN	↓	Amyg	3 days	CTA	Baumgartel et al. (2008)
C-Fos	↑	Hipp	18–24 h	IA	Bekinschtein et al. (2007)
ERK-2	↑	Hipp	18–24 h	IA	Igaz et al. (2004)
Homer 1a	↑	Hipp	24 h	IA	Igaz et al. (2004)
IGF-II	↑	Hipp	20 h	IA	Chen et al. 2009
mGluR5	↑	Hipp	Up to 10 days	CFC	Riedel et al. (2000)
NCAM	↑	Hipp	12 h	CFC	Sandi et al. (2003)
pCREB	↑	Hipp	9–20 h	IA	Taubenfeld et al. (2001)
pCREB	↑	Hipp	9 h	CFC	Trifilieff et al. (2006)
pCREB	↑	Amyg, Hipp and PCx	3–6 h	CFC	Stanciu et al. (2001)
pERK	↑	Hipp	9–12 h	CFC	Trifilieff et al. (2006)
pERK 1/2	↑	Amyg	9 h	UFC	Trifilieff et al. (2007)
Syntaxin 1a	↑	Hipp	18–24 h	IA	Igaz et al. (2004)
Zif-268	↑	Hipp	18–24 h	IA	Bekinschtein et al. (2007)
Zif-268	↑	Amyg	3 days	CTA	Baumgartel et al. (2008)

The table summarizes the findings regarding changes in hippocampal gene expression or activation of intracellular molecular cascades several hours after learning

IA inhibitory avoidance, *CTA* conditioned taste aversion, *CFC* contextual fear conditioning, *UFC* unpaired fear conditioning, *OF* open field, *C/EBPb* CAAT/enhancer-binding protein beta, *pCREB* phosphor-cAMP response element binding protein, *pERK* phospho-extracellular regulated kinase, *AMPAr* AMPA receptor, *mGluR5* metabotropic glutamate receptor 5, *CamKIIα* calcium/calmodulin-dependent protein kinase II, *BDNF* brain-derived neurotrophic factor, *IGF-II* insulin-like growth factor 2, *NCAM* neural cell adhesion molecule, *Arc* activity-regulated cytoskeleton-associated protein, *CaN* Calcineurin, *Hipp* Hippocampus, *PCx* prefrontal cortex, *ICx* insular cortex, *Amyg* amygdala

transcription factors (Taubenfeld et al. 2001; Trifilieff et al. 2006), Zif-268 (Bekinschtein et al. 2007; Baumgartel et al. 2008), of ERK-2 (Igaz et al. 2004; Trifilieff et al. 2006; Bekinschtein et al. 2008; Eckel-Mahan et al. 2008), Calcineurin (Baumgartel et al. 2008), BDNF (Bekinschtein et al. 2007; Rossato et al. 2009), and several other synaptic proteins (see Table 1). Interestingly, pilot experiments from our lab reveal that persistent memories are associated with delayed increases in two important plasticity-related proteins in the hippocampus (unpublished data): Arc, an IEG activated by spatial exploration and involved in AMPAr trafficking (Bramham et al. 2008) and PKMζ, mentioned above (Sacktor 2008).

Conclusions

The findings on a critical phase involved in persistence of LTM storage open a potential avenue of research on the mechanisms of late memory consolidation in the hippocampus. For instance, is this process the end of the cellular consolidation phase? Is this phase a necessary link between cellular and system consolidation? Regardless of the involvement of the hippocampus in storage or retrieval of many memories, the late protein synthesis- and BDNF-dependent phase is surely not the end of the story. In other words, it is possible that this critical window about 12 h after acquisition is only the first in a series of recurrent rounds of protein synthesis in the hippocampus and extrahippocampal areas necessary to persistently store new information. The study of some of the potential signatures outlined here may help understand the maintenance of LTM storage.

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