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Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective

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Pavlovian fear conditioning has emerged as a leading behavioral paradigm for studying the neurobiological basis of learning and memory. Although considerable progress has been made in understanding the neural substrates of fear conditioning at the systems level, until recently little has been learned about the underlying cellular and molecular mechanisms. The success of systems-level work aimed at defining the neuroanatomical pathways underlying fear conditioning, combined with the knowledge accumulated by studies of long-term potentiation (LTP), has recently given way to new insights into the cellular and molecular mechanisms that underlie acquisition and consolidation of fear memories. Collectively, these findings suggest that fear memory consolidation in the amygdala shares essential biochemical features with LTP, and hold promise for understanding the relationship between memory consolidation and synaptic plasticity in the mammalian brain.

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Memory consolidation is a process in which short-term memory (STM) is transformed, over time, into stable long-term memory (LTM)[1]. Fearful experiences are rapidly acquired and thus easily consolidated into LTM, probably because they convey vital information about danger in the environment that might be important for survival. In this article, we discuss recent advances in our understanding of fear memory consolidation. These findings provide new insights into the cellular and molecular mechanisms of short- and long-term memory storage.

The amygdala and fear conditioning

Much of what we know about the fear learning system of the brain comes from studies of Pavlovian fear conditioning. In this learning paradigm, an initially neutral conditioned stimulus (CS), such as a tone, acquires the ability to elicit defensive responses after association with a noxious unconditioned stimulus (US), such as a brief electric shock to the feet. A large body of evidence suggests that the amygdala, and in particular the lateral amygdala (LA), is a likely site of the plasticity underlying memory storage of fear conditioning[2–5]. For example, CS and US inputs converge onto individual cells in the LA (Ref. 6). Furthermore, damage to, or reversible functional inactivation of, the LA and nearby regions prevents fear acquisition and the expression of previously acquired fear[7–12]. Finally, pairing of CS and US inputs during fear conditioning leads to alterations in synaptic transmission and neuronal activity in the LA (Refs 13–16) that are long-lasting[17].

During fear expression, the LA engages the central nucleus of the amygdala (CE), which, as the principal output nucleus of the fear system, projects to areas of the hypothalamus and brainstem that control behavioral (e.g. freezing, startle), endocrine and autonomic conditioned responses (CRs) associated with fear learning[2,18–20]. Several nuclei within the amygdala might be involved in fear conditioning[2-5], but only the LA and the CE appear to be crucial[21]. We will therefore focus mainly on findings from the LA in the present review. For an alternative view about the role of the amygdala in fear conditioning, see Cahill *et al.*[22]

Cellular mechanisms of fear memory storage: why is LTP important?

How might neurons within the LA store memories of the CS–US association during fear conditioning? In 1949, Hebb[23] proposed that when two interconnected neurons fire at the same time, the synapses between

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them become stronger, and remain so for a long time afterwards. At the time Hebb proposed his influential theory, there was little evidence to support it. Later studies, however, showed that high-frequency stimulation of afferents to the hippocampus led to a long-term enhancement of synaptic transmission[24], a form of plasticity that has become known as long-term potentiation (LTP).

For nearly 30 years since its discovery, LTP has been the leading cellular model of the events underlying memory formation in the mammalian brain. The traditional reasons that support this include the associativity, cooperativity and synapse-specificity of LTP (Ref. 25), essential features of any cellular model of memory formation. However, also important is the more recent discovery that LTP, like memory consolidation[1], has temporal phases[26,27]. In brain slice experiments, these phases are readily distinguished by the type of stimulation used at the time of LTP induction. For example, a single high-frequency train of stimulation can produce an 'early' phase of LTP (E-LTP) that lasts for minutes, is independent of protein or RNA synthesis, and is thought to involve modifications of existing proteins. By contrast, multiple high-frequency trains of stimulation will produce a 'late' phase of LTP (L-LTP) that lasts minutes to hours and depends on protein and RNA synthesis[28–32]. L-LTP, unlike E-LTP, is thought to involve structural modifications of the synapse[26,27]. Thus, just as memory can be separated into short- and long-term components that differ with respect to their requirement for RNA and protein synthesis[1], LTP often seems to occur in distinct temporal phases. This, in turn, suggests that the consolidation process can be represented at the cellular level and understood through studies of LTP (Refs 26,27).

Importantly, LTP exists in each of the major sensory input pathways to the amygdala that are essential for fear conditioning[28,33–38]. Furthermore, fear conditioning enhances neuronal activity at sensory inputs to the LA in a manner similar to artificial LTP induction[13,17], and LTP in the LA is sensitive to the same stimulus contingencies as fear conditioning[39]. Collectively, these findings suggest that an LTP-like process in the LA could underlie fear conditioning, which in turn suggests that fear acquisition and consolidation might be understood at the cellular level through studies of LTP (Refs 3,40–42).

Biochemical mechanisms of short- and long-term fear memory

The biochemical and molecular events that underlie LTP have begun to be elucidated in detail, especially in the hippocampus[26,27,43], but also more recently in the LA (Refs 28,35,44,45). In both structures, LTP is thought to involve activation of a variety of protein kinase signaling pathways, either directly or indirectly, by increases in intracellular Ca^{2+} in the postsynaptic cell at the time of LTP induction. Depending on the pathway and type of stimulation, either the N-methyl-D-aspartate (NMDA) receptor[46,47] or the L-type voltage-gated calcium channel (VGCC)[33,48,49], or both[50], have been implicated in activity-dependent increases in intracellular Ca^{2+}. In addition, several protein kinases have been implicated in LTP induction and in E-LTP, whereas others have been implicated primarily in L-LTP. Here, we review what is known about the biochemical processes that underlie each of these phases of LTP, and how these processes might contribute to acquisition and consolidation of fear memories in the LA.

E-LTP and short-term fear memory

E-LTP is a short-lasting, RNA- and protein-synthesis-independent form of LTP that does not persist unless it becomes consolidated into a more permanent form, namely L-LTP (Refs 28–31). E-LTP can be induced by a single train of tetanic stimulation in either the hippocampus[29,30] or the LA (Refs 28,35). In both structures, E-LTP requires Ca^{2+} entry into the postsynaptic cell through the NMDA receptor at the time of induction[30,35]. NMDA receptor-mediated elevations in Ca^{2+} are thought to induce E-LTP postsynaptically by activating several protein kinases, including α-calcium/calmodulin-dependent kinase II (αCaMKII) and calcium/phospholipid-dependent protein kinase (PKC)[51,52]. Once activated, each of these kinases has the capacity to become 'autophosphorylated', or persistently active in the absence of Ca^{2+} for a period of time following LTP induction[53,54]. While activated, αCaMKII and PKC can, in turn, phosphorylate a variety of target proteins. Autophosphorylation of αCaMKII on Thr^{286}, for example, leads to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor phosphorylation, thereby increasing excitatory current influx into the postsynaptic cell[53]. Thus, activation of αCaMKII by NMDA receptor-mediated Ca^{2+} entry could be a biochemical mechanism for short-term synaptic plasticity, which in turn might underlie some forms of STM (Refs 53,55).

Although the roles of αCaMKII and PKC have been extensively studied in other memory systems[56–60], few studies have systematically examined the role of these kinases in either amygdala E-LTP or STM of fear conditioning. However, there is indirect evidence that NMDA receptor-mediated activation of either αCaMKII or PKC in the amygdala might be involved. It has long been established, for example, that NMDA receptor blockade in the LA disrupts fear conditioning[61–64]. Furthermore, recent studies have shown that intra-amygdala infusion of an NMDA receptor antagonist[65] or of a selective antagonist of the NR2B subunit of the NMDA receptor[66] impairs both STM and LTM of fear conditioning. This finding is consistent with a recent study in which mice that overexpressed NR2B had

facilitated STM and LTM of fear conditioning[67]. Because autophosphorylated αCaMKII has been linked to Ca^{2+} entry through the NMDA receptor[53] and can in turn target the NR2B subunit[68,69], this suggests that αCaMKII in the LA could play an essential role in fear conditioning, particularly in STM formation. Although direct evidence for this hypothesis is currently lacking, it is of interest that regulated expression of a αCaMKII transgene targeted to the LA and striatum results in impaired fear conditioning[70]. Furthermore, mice deficient in either αCaMKII or the β isoform of PKC have impaired fear conditioning[71,72]. Based on evidence from the hippocampus, it is reasonable to assume that Ca^{2+} entry through NMDA receptors in the LA might support STM by activating αCaMKII and PKC to induce LTP. However, additional experiments are needed to evaluate these possibilities.

L-LTP and long-term fear memory

In contrast to E-LTP and STM, much has been learned about the biochemical mechanisms underlying L-LTP and LTM in the fear system. In both the hippocampus and the LA, L-LTP is a long-lasting and RNA- and protein-synthesis-dependent phase of plasticity[28–31] that requires the cAMP-dependent protein kinase (PKA) and the extracellular-regulated kinase/mitogen-activated protein kinase (ERK/MAPK). In the hippocampus, for example, both PKA or ERK/MAPK have been shown to be activated following stimulation that induces L-LTP (Refs 73,74). In addition, *in vitro* application of PKA or ERK/MAPK inhibitors has been shown to prevent the induction of L-LTP in both the hippocampus and in the LA (Refs 28–30, 44,75,76).

How might PKA and ERK/MAPK promote long-term plastic change? Following activation, possibly by the events set in motion by either αCaMKII or PKC (Ref. 55), both PKA and ERK/MAPK are thought to translocate to the cell nucleus where they can engage activators of transcription[77–79]. These nuclear transcription factors include the cAMP response-element binding protein (CREB), which, when activated by phosphorylation, can bind to the DNA machinery and induce the transcription of cAMP response element (CRE)-mediated genes and ultimately proteins that lead to the structural changes thought to underlie L-LTP (Refs 26,27,43,103). In support of this hypothesis, stimulation that leads to L-LTP in the hippocampus induces the transcription of CRE-mediated genes, an effect that is blocked, along with LTP, by inhibitors of PKA and ERK/MAP kinase[80,81]. Furthermore, LTP-inducing stimulation of the hippocampus or the LA, by either the cAMP agonist forskolin or by artificial high-frequency stimulation, leads to increases in the phosphorylation of CREB (Refs 28,82), suggesting an essential role for CRE-mediated transcription in the both structures. Among the CRE-mediated genes that have been implicated in hippocampal L-LTP are early growth response 1 (*EGR1*; Refs 83,84), brain-derived neurotrophic factor (*BDNF*)[85,86], and the CCAAT-enhancer binding protein (*CEBPB*)[87].

Several recent studies have asked whether the biochemical mechanisms known to underlie L-LTP are also necessary for fear memory consolidation in the amygdala. These studies have shown, for example, that intra-amygdala infusion of an RNA synthesis inhibitor impairs LTM of auditory and contextual fear[88]. Similarly, infusion of an inhibitor of either protein synthesis or PKA into the amygdala impairs auditory fear memory consolidation; that is, rats have intact STM, but impaired LTM (Refs. 89). Fear conditioning is also accompanied by transient activation of ERK/MAPK in the LA, and blockade of this activation by an inhibitor of ERK/MAPK activation impairs fear memory consolidation[44]. Consistent with the role for both PKA and ERK/MAPK in CRE-dependent transcription[80,81], overexpression of CREB in the amygdala using viral transfection methods facilitates LTM, but not STM, of fear-potentiated startle[90], and *EGR1* mRNA is upregulated in the LA following fear conditioning[91,92]. These findings agree with previous studies that have evaluated the role of PKA, ERK/MAPK and CREB in fear memory consolidation processes using either molecular genetic[45,93,94] or systemic or intraventricular drug infusions[76,95,96,101].

Is amygdala LTP a cellular mechanism of fear memory consolidation?

Collectively, the findings of recent behavioral and electrophysiological experiments are clearly consistent with the hypotheses that the amygdala is a likely site of fear memory consolidation and storage, and that this process shares essential biochemical features with an LTP-like mechanism (Fig. 1). However, because many, if not all, of these LTP studies have employed *in vitro* methods, it remains difficult to draw conclusions about the causal role of amygdala LTP in fear memory formation. This is especially true given that the same molecular manipulation produces impairments in LTP and in behavior with different time courses. For example, in recent behavioral studies, STM (i.e. protein synthesis-independent memory) has been found intact for several hours following conditioning and drug administration. In our own studies, STM was intact for 4 hr following protein synthesis and PKA inhibition[89] and for at least 3 hr following ERK/MAPK inhibition[44]. By contrast, LTP in the LA appears to decay with a much faster time course under the influence of the same manipulations[28,44]. This pattern of findings is also found in the molecular genetic literature, where STM in behavioral experiments almost invariably appears to last longer than LTP (Refs 45,93,94). For example, in *Ras*-deficient mice, STM is intact at 1 hr, whereas

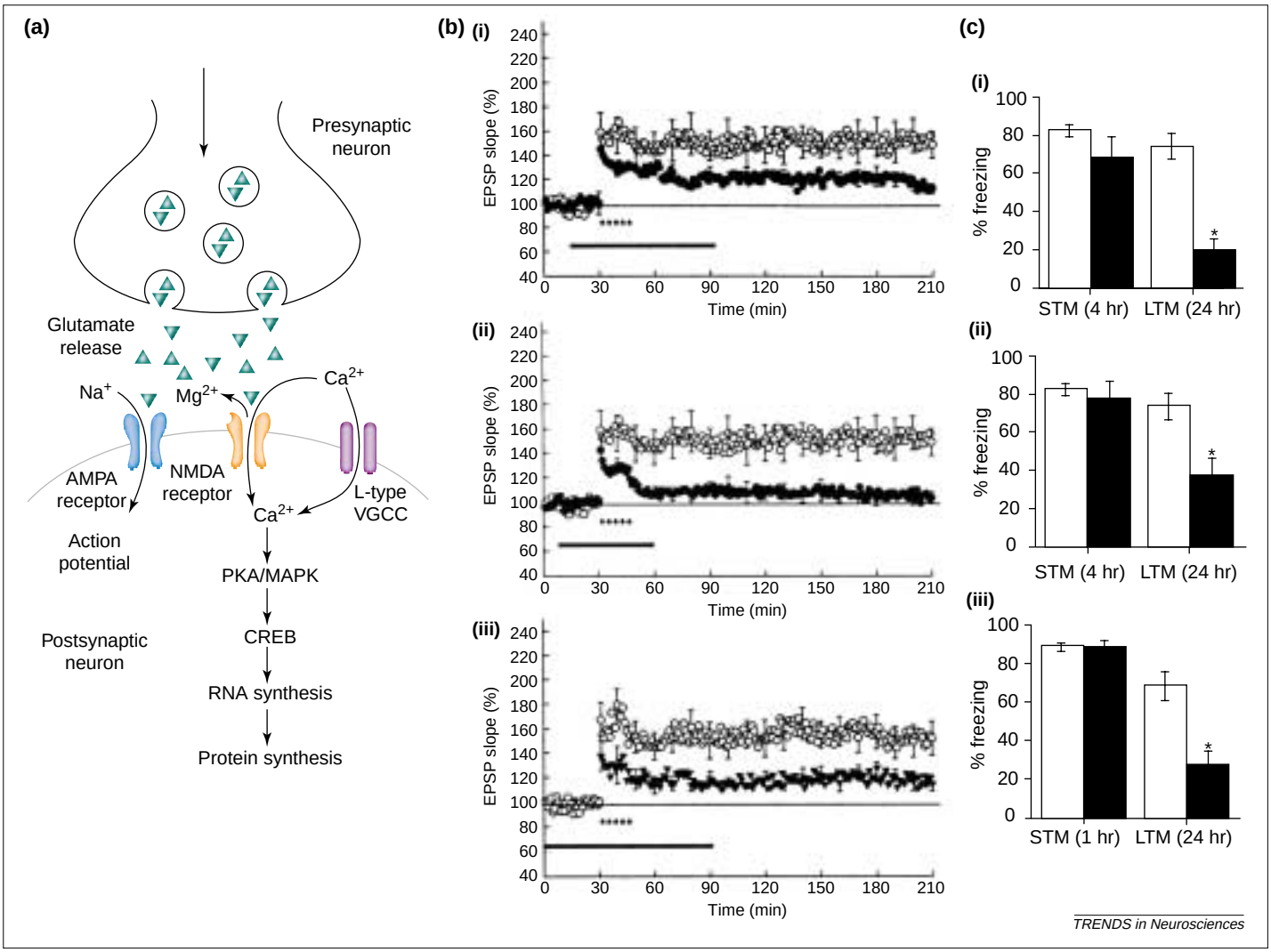

Fig. 1. Biochemical and molecular basis of amygdala L-LTP and fear memory consolidation. (a) L-LTP involves the presynaptic release of glutamate and Ca^{2+} influx into the postsynaptic cell through either NMDA receptors or L-type VGCCs. the increase in intracellular Ca^{2+} leads to the activation of protein kinases, such as PKA and ERK/MAPK. Once activated, these kinases can translocate to the cell nucleus where they activate transcription factors such as CREB. The activation of CREB by PKA and ERK/MAPK promotes CRE-mediated gene transcription and the synthesis of new proteins that are critical for the ultrastructural and/or functional changes that underlie L-LTP. (b) L-LTP in the LA, for example, has recently been shown to require protein synthesis, PKA and ERK/MAPK. In these studies, amygdala slices were treated with either (i) anisomycin (a protein synthesis inhibitor; black circles), (ii) KT5720 (a PKA inhibitor; black circles) or (iii) PD098059 (an inhibitor of MEK, which is an upstream regulator of ERK/MAPK activation; black triangles) before and during high frequency tetanus of the auditory 'thalamic' input pathway. In each experiment, field recordings were obtained from the LA and expressed across time as a percentage of baseline. In each panel, the vehicle group is represented by white circles. The black bar represents the duration of drug application, and the asterisks represent the tetanus period. Reproduced, with permission, from Ref. 28. (c) Fear memory consolidation in the amygdala has recently been shown to require the same biochemical processes. In these studies, rats received intra-amygdala infusions of (i) anisomycin, (ii) Rp-cAMPS (a PKA inhibitor) or (iii) U0126 (a MEK inhibitor) at or around the time of training (1–5 trials) and were tested for both short-term (1–4 hr later) and long-term memory (~24 hr later) of auditory fear conditioning. In each figure, vehicle-treated rats are represented by the white bars, while drug-treated animals are represented by the black bars. *P<0.05 relative to controls. Abbreviations: CRE, cAMP response-element; CREB, cAMP response-element binding protein; ERK/MAPK, extracellular-regulated kinase/mitogen-activated protein kinase; LA, lateral amygdala; L-LTP, 'late' phase of long-term potentiation; MEK, mitogen activated kinase kinase; NMDA, N-methyl-D-aspartate; PKA, cAMP-dependent protein kinase; VGCC, voltage-gated calcium channel.

LTP in amygdala slices from these animals is decayed to baseline within 30 mins[45].

Thus, despite clear correlation in mechanism between LTP and fear memory, these temporal discrepancies might present a challenge to the theory that LTP provides a neural substrate for LTM in the LA, and possibly also in other learning systems. However, as just discussed, LTP induction at a synapse is known to depend upon several interacting biochemical signaling pathways, and the time course of the establishment of protein-synthesis dependent LTP and LTM might be quite sensitive to the manner in which these pathways are engaged. For example, *in vitro* studies employ artificial patterns of electrical stimulation to induce LTP, which could be very different from natural activity patterns that occur in the LA of behaving animals during CS–US pairing. Furthermore, neurons undergo significant trauma during preparation of brain slices for *in vitro* experiments[102], and they are disconnected from many of the modulatory inputs that are normally present *in vivo*. These factors could be responsible for quantitative differences in the time course of the effects of drugs on protein-synthesis dependent LTP and LTM formation, even though both phenomena involve qualitatively similar molecular signaling pathways. Future studies employing *in vivo* LTP recording techniques and using naturalistic

patterns of stimulation will be necessary to evaluate these possibilities.

A model of fear memory consolidation in the amygdala

Despite the questions that remain, at this stage we can begin to envision a model of the cellular and molecular events that underlie memory formation and consolidation of fear conditioning in the LA. In brief, the existing behavioral and electrophysiological data are consistent with a model wherein pairing of CS and US inputs onto LA principal cells during training leads to Ca^{2+} influx through the NMDA receptor[61–66]. This increase in intracellular Ca^{2+} leads to the activation of a variety of protein kinases. Some of these, possibly αCaMKII and/or PKC, might be important for STM. Others, such as PKA and ERK/MAPK[44,89], appear to be exclusively involved in the formation of LTM, possibly via translocation to the cell nucleus and activation of transcription factors such as CREB (Ref. 90). The activation of CREB by PKA and ERK/MAPK promotes CRE-mediated gene transcription[88], including *EGR1* (Ref. 91), and the synthesis of new proteins[89].

However, many important questions remain. For example, are L-type VGCCs, like NMDA receptors, necessary for fear memory, and, if so, in what way? What are the biochemical mechanisms underlying STM of fear conditioning and how are these coupled to Ca^{2+} entry through the NMDA receptor and/or L-type VGCCs? How might αCaMKII, PKC, PKA and ERK/MAPK interact in the LA during signal transduction to promote a shift from short- to long-term plasticity and memory? Finally, what are the downstream nuclear targets of CREB and EGR-1, and how might transcription of these gene products alter the structure and/or function of the LA neuron such that it now responds differently in the face of danger?

Retrieval and reconsolidation of fear memories in the amygdala

Although we have begun to piece together a model of the cellular and molecular events underlying memory formation and consolidation in the LA, it currently applies only to the initial phases of memory consolidation following training. Indeed, this model will no doubt require modification to account for the process of reconsolidation of fear conditioning, which we currently know very little about.

As discussed earlier, memory consolidation is typically thought of as a process in which labile, protein synthesis-independent short-term memories are transformed over time into stable long-term traces that are resistant to further manipulation. In the last several decades, however, several studies have been published that appeared to challenge this fundamental linear notion of memory formation. In these studies, amnesic manipulations at or around the time of memory retrieval, rather than at the time of initial learning, appeared to result in loss of the memory on subsequent recall tests[97–99]. However, because many of these studies used gross systemic manipulations, such as electroconvulsive shock, and behavioral paradigms that were poorly defined at the systems level, the concept of reconsolidation was not readily integrated with progress in understanding the biology of memory consolidation.

In recent studies, we revisited the question of memory reconsolidation using an approach that offered several distinct advantages over past studies[100]. First, we used Pavlovian fear conditioning, a behavioral paradigm for which a putative site of plasticity had been defined – namely, the LA. Second, we had implicated several intracellular processes, including protein synthesis, PKA and ERK/MAPK, in the LA in the initial phases of memory consolidation[44,89]. Thus, we already had at our disposal an established set of tools and behavioral protocols to ask questions about reconsolidation.

Following the logic of our consolidation studies, we infused the protein synthesis inhibitor anisomycin into the LA immediately after recall (i.e. exposure to the CS). Rats treated this way at the time of retrieval showed marked impairment of conditioned fear on subsequent recall tests. This effect was dependent on activation of the memory; that is, no memory deficit was observed if exposure to the CS was omitted. Furthermore, the effect was observed not only when the initial recall test and drug infusion were given shortly after training (i.e. one day), but also if given 14 days later, suggesting that the effect could not be attributable to disruption of late phases of protein synthesis necessary for the initial consolidation period. Finally, additional controls suggested that reconsolidation of fear, like initial consolidation, had phases. For example, post-recall STM (assessed 4 hr after retrieval and anisomycin infusion) was intact, whereas post-recall LTM (assessed ~24 hrs later) was impaired[100]. Thus, fear memories appear to return to a labile state after retrieval that appears very similar to STM after new learning. However, if they are to persist, these reactivated memories must be put back into long-term storage via a protein synthesis-dependent mechanism in the amygdala.

At this point, we have more questions than answers about the cellular and molecular mechanisms by which reconsolidation might be accomplished. For example, is reconsolidation simply a recapitulation of the biochemical events that are known to underlie initial consolidation? Future studies will be necessary to determine the point in the biochemical signaling cascade at which these two phenomena diverge, if at all.

Concluding remarks

Progress in elucidating the neural system underlying fear conditioning has in recent years

been paralleled by great strides in our understanding of the cellular and molecular basis of synaptic plasticity, including LTP. The application of the knowledge gained by LTP studies to the fear conditioning paradigm has revealed a great deal about the cellular and molecular mechanisms of fear conditioning in the LA, suggesting that similar mechanisms might be involved. These findings provide a foundation for the continued study of the neural basis of emotional learning and memory at the cellular level, and also for bridging the gap between studies of memory formation and synaptic plasticity in the mammalian brain. Further, recent studies provide a framework for unraveling the mechanisms underlying reconsolidation of fear and determining whether it shares essential biochemical features with initial memory consolidation.

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