Neuron, Vol. 36, 727–738, November 14, 2002, Copyright ©2002 by Cell Press

Fear Memory Formation Involves p190 RhoGAP and ROCK Proteins through a GRB2-Mediated Complex

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Summary

We used fear conditioning, which is known to alter synaptic efficacy in lateral amygdala (LA), to study molecular mechanisms underlying long-term memory. Following fear conditioning, the tyrosine phosphorylated protein p190 RhoGAP becomes associated with GRB2 in LA significantly more in conditioned than in control rats. RasGAP and Shc were also found to associate with GRB2 in LA significantly more in the conditioned animals. Inhibition of the p190 RhoGAP-downstream kinase ROCK in LA during fear conditioning impaired long- but not short-term memory. Thus, the p190 RhoGAP/ROCK pathway, which regulates the morphology of dendrites and axons during neural development, plays a central role, through a GRB2-mediated molecular complex, in fear memory formation in the lateral amygdala.

Introduction

Changes in synaptic efficiency are believed to underlie learning and memory (Dudai, 1989; Kandel, 2001). A challenge is to identify molecules involved in sustaining synaptic changes and to elucidate their role in memory formation. One emerging view is that coordinate regulation of neural processes occurs by molecular complexes that couple extracellular cues to multiple intracellular signal transduction pathways (Grant and Husi, 2001; Grant and O'Dell, 2001). Although the identity of these complexes and their role in neuronal signal transduction are being elucidated, few studies have addressed their role in memory (Zhao et al., 1999).

Classical fear conditioning, a procedure in which an animal associates a neutral stimulus, such as a tone, with an aversive event, typically a mild footshock (LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001), is especially useful as a tool for studying the molecular basis of long-term memory because the putative site of memory, the lateral nucleus of the amygdala (LA), has been identified (Fanselow and LeDoux, 1999; Schafe et al., 2001). For example, damage or functional inactivation of the LA and adjacent areas during acquisition prevents the learning from taking place (e.g., LeDoux et al., 1990; Nader et al., 2001; Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 1999), and neural activity changes in LA during learning (Quirk et al., 1995, 1997; Collins and Pare, 2000; Repa et al., 2001). Thus, fear conditioning provides a behavioral tool and anatomical site within which to assess molecular mechanisms that might mediate changes in synaptic efficacy during long-term memory formation.

GRB2 is an adaptor molecule that brings various proteins into close proximity, forming molecular complexes that propagate intracellular signals and induce cellular responses (Buday, 1999). These complexes have been implicated in cytoskeletal rearrangement, cellular adhesion, and vesicle docking, cellular events that contribute to changes in synaptic efficacy during development (Luo, 2000). Given the importance of GRB2 in signal transduction in neurons and the essential role of LA in the formation of long-term memory of fear conditioning, in the present study we examined whether GRB2 forms a molecular complex in LA following fear conditioning, and if so which molecular pathways might be involved.

Our finding that GRB2 forms a molecular complex with the GTPase-activating protein, p190 RhoGAP, led us to pursue the nature of the contribution of this complex. p190 RhoGAP is involved in the regulation of Rho GTPase. Specifically, Rho GTPase is inactivated by p190 RhoGAP (Ridley et al., 1993). In its active state, induced by guanine nucleotide exchange factors (RhoGEFs; Luo, 2002), Rho GTPase activates downstream kinases (Luo, 2002), including the serine/threonine Rho-associated kinase, ROCK. Given that activation of p190 RhoGAP can lead to inhibition of ROCK through inactivation of Rho GTPases, the level of p190 RhoGAP activity regulates ROCK function. We therefore examined the effects of ROCK inhibition on fear conditioning and found that memory formation was impaired. Together these findings suggest that the Rho pathway, presumably through ROCK, in the lateral amygdala is involved in the formation of long-term memory of fear conditioning. In view of evidence implicating p190 RhoGAP and ROCK in regulating dendritic and axonal growth (Threadgill et al., 1997; Luo, 2000; Bito et al., 2000; Brouns et al., 2000, 2001; Billuart et al., 2001), we suggest that these molecules play a similar role in the neuronal changes in LA during the formation of the memory of a fear-arousing experience.

Results

Fear Conditioning Induces the Association of GRB2 with Tyrosine Phosphorylated Proteins in Lateral Amygdala

Five pairings of the tone with shock in rats induced long-term memory, as assessed by the amount of defensive behavior (freezing) elicited by the tone 24 hr after training (Figure 1A; * = $p < 0.05$ between paired and naïve or unpaired groups). In contrast, animals receiving non-overlapping (unpaired) presentations of the tone and shock exhibited little freezing to the tone. Thus, although both groups were exposed to the same environmental stimuli, a behaviorally expressed associative memory of the tone-shock relation was only formed in the paired group. Naïve rats placed in the conditioning apparatus without exposure to either stimulus also exhibited minimum freezing.

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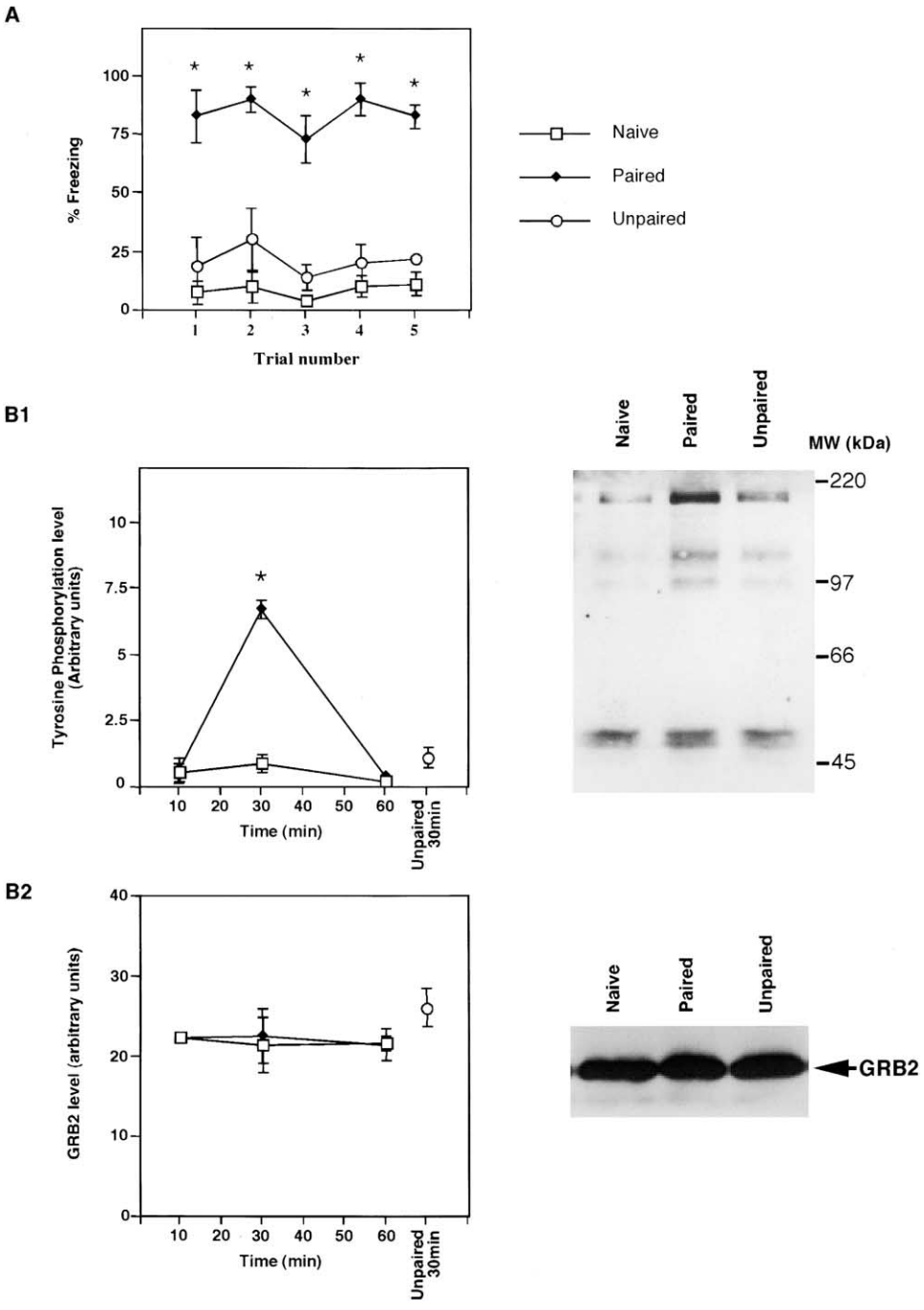

Figure 1. Fear Conditioning Induces the Association of Tyrosine Phosphorylated Proteins with GRB2

(A) Long-term memory of fear conditioning tested 24 hr after training. Rats in the paired group exhibited significantly more conditioned fear behavior (freezing) than rats in the unpaired and naïve groups (values are mean ± SEM, * = $p < 0.05$ Scheffe test; n = 4 for each group). (B1) Representative immunoblot of homogenates from lateral amygdala that were immunoprecipitated with anti-GRB2 antibodies and blotted with anti-phosphotyrosine antibody. The level of tyrosine phosphorylated proteins that coimmunoprecipitated with GRB2 was monitored 10, 30, or 60 min following naïve or paired training or 30 min following unpairing (values are mean ± SEM for the p190 kDa phosphoprotein; * = $p < 0.001$ Scheffe test between paired and naïve or unpaired groups; n = 5 for naïve, n = 6 for paired, and n = 4 for unpaired groups for the 30 min time point and n = 3 for the 10 and 60 min time points). (B2) Same as for (B1) but blotted with anti-GRB2 antibody.

The level of tyrosine phosphorylated proteins associated with GRB2 markedly increased in the immunoprecipitants derived from LA of paired rats as compared to naïve or unpaired animals 30 min following training (Figure 1B1, * = $p < 0.001$ between paired and naïve or unpaired groups for the prominent 190 kDa phosphoprotein). The level of tyrosine phosphorylation of the 190 kDa protein in paired groups at 10 or 60 min after training

was at basal level (Figure 1B1). The level of GRB2 protein in the immunoprecipitants was similar in all groups, indicating that the increase of the phosphoproteins is due to a difference in their recruitment to GRB2 rather than to an increase in the level of GRB2 protein itself (Figure 1B2). Thus, a GRB2-mediated complex is formed specifically following associative pairing of the tone and shock. The fact that the unpaired group was exposed to the same stimuli indicates that the results are not due to non-associative factors such as shock-induced stress responses. Furthermore, immunoprecipitation with nonimmune rabbit IgG revealed a very low background of tyrosine phosphorylated proteins (lower than the level detected in naïve group immunoprecipitated with anti-GRB2) that was not different between paired and naïve groups at 30 min ($n = 3$ each). This observation indicates that the tyrosine phosphorylated proteins, immunoprecipitated with anti-GRB2 antibodies, are pulled down specifically through their interaction with GRB2.

The timecourse of GRB2-mediated complex formation in Figure 1B1 is consistent with previous studies showing that learning induces a significant increase in phosphorylation of proteins tens of minutes following training that rapidly subsides to basal level (e.g., Berman et al., 1998).

The 190 kDa Tyrosine Phosphorylated Protein that Becomes Associated with GRB2 following Fear Conditioning Is the p190 RhoGAP

The most prominent tyrosine phosphorylated protein that interacts with GRB2 following fear conditioning has a molecular weight of 190 kDa (Figure 1B1). To understand how the formation of the GRB2 complex might subserve synaptic plasticity induced by fear conditioning in LA, we sought the identity of this protein. Modulation of cell contact during early stages of neuronal development triggers the interaction of the 190 kDa tyrosine phosphorylated protein p190 RhoGAP with RasGAP, p66Shc, and GRB2 (Dupont and Blancq, 1999). p190 RhoGAP was also shown to be involved in the regulation of dendritic and axonal morphology (Threadgill et al., 1997; Brouns et al., 2000, 2001; Billuart et al., 2001) and to become phosphorylated on tyrosine in response to extracellular stimuli (Nakahara et al., 1998). We therefore studied whether p190 RhoGAP is the phosphoprotein that forms the complex with GRB2 in LA following fear conditioning training.

The interaction of GRB2 with p190 RhoGAP increased in LA 30 min, but not 10 or 60 min, following pairing, as compared to naïve and unpaired groups (Figure 2A). Thus, immunoprecipitation of GRB2 from the LA of paired animals pulled down significantly more p190 RhoGAP than immunoprecipitation of GRB2 from the LA of naïve or unpaired groups (Figure 2A; $p < 0.004$ between paired and naïve groups and $p < 0.01$ between paired and unpaired groups). The level of GRB2 in the immunoprecipitants was similar in all groups, indicating that the interaction of p190 RhoGAP is not due to changed levels of GRB2. Furthermore, the level of p190 RhoGAP in total homogenates before immunoprecipitation was not changed by training (Figure 2B). Other molecules of similar molecular weight, such as the NMDA subunit NR2B, a prominent tyrosine phosphorylated protein in the postsynaptic densities (PSDs), or the insulin receptor substrate (IRS) that is known to be phosphorylated on tyrosine and to bind GRB2, did not differ between, or was not detected in, the various GRB2 immunoprecipitants (data not shown).

The anatomical localization of GRB2 and p190 RhoGAP within LA was examined (Figure 3). By low power (4×; Figures 3A and 3B), labeled cells were seen throughout the amygdala. Dense patches of peroxidase labeling were associated with the intercalated nuclei. High-power Nomarski optics (100×; Figures 3A and 3B, insets) revealed thick dendritic processes emanating from labeled cells and a dense matrix of labeled dendrites and puncta. Dual-label confocal analysis showed that these proteins colocalized in LA cell bodies and dendritic processes (Figure 3C). Furthermore, electron microscopic analysis revealed that both GRB2 and p190 RhoGAP immunolabel was localized to somata, dendrites, and dendritic spines (Figures 3D and 3E). Immunolabel in glia and axon terminals was rarely observed. Though labeled somata contained abundant cytoplasm, they exhibited the morphological characteristics of projection cells (e.g., their nuclei were not invaginated and only symmetric synapses were observed along the perikaryal plasma membrane). Staining in dendrites was robust and immunolabel rimmed the microtubules and was seen throughout the dendritoplasm. Occasionally, only a patch of label corresponding to the spinous or synaptic portion of a dendrite was seen (Figures 3D and 3E). Dendritic spines were heavily labeled but the PSD was always apparent and frequently more intensely stained than the spinoplasm. The presence of these proteins in dendrites and spines is of interest since previous studies have shown that these structures are intimately involved in synaptic plasticity (Nimchinsky et al., 2002).

GRB2 Becomes Associated with RasGAP and Shc, in Lateral Amygdala, following Fear Conditioning

GRB2 becomes associated with Shc and RasGAP in various biological systems (e.g., gastrulation; Dupont and Blancq, 1999). RasGAP was also shown to be associated with p190 RhoGAP (Settleman et al., 1992). We were therefore interested to study whether GRB2 forms a complex with RasGAP or Shc following fear conditioning. Figure 4 (A1 and B1) shows that in the LA, GRB2 becomes associated with RasGAP and Shc proteins significantly more in paired rats as compared to unpaired or naïve animals 30 min following training ($p < 0.03$ between paired and naïve and $p < 0.003$ between paired and unpaired for RasGAP; $p < 0.0006$ for $p46^{Shc}$; $p < 0.05$ for $p52^{Shc}$; $p < 0.03$ for $p66^{Shc}$ between paired and naïve groups; and $p < 0.0005$ for $p46^{Shc}$; $p < 0.002$ for $p52^{Shc}$; and $p < 0.05$ for $p66^{Shc}$ between paired and unpaired groups). We could not detect complex formation between Shc, RasGAP, and GRB2 10 or 60 min following training. The levels of RasGAP or Shc proteins in the total homogenate were similar in all groups (Figures 4A2 and 4B2).

Inhibition of the Protein Kinase ROCK in Lateral Amygdala Impairs Long- but Not Short-Term Fear Conditioning Memory Formation

To further elucidate whether the regulation of the Rho pathway, downstream to p190 RhoGAP in LA, is essen-

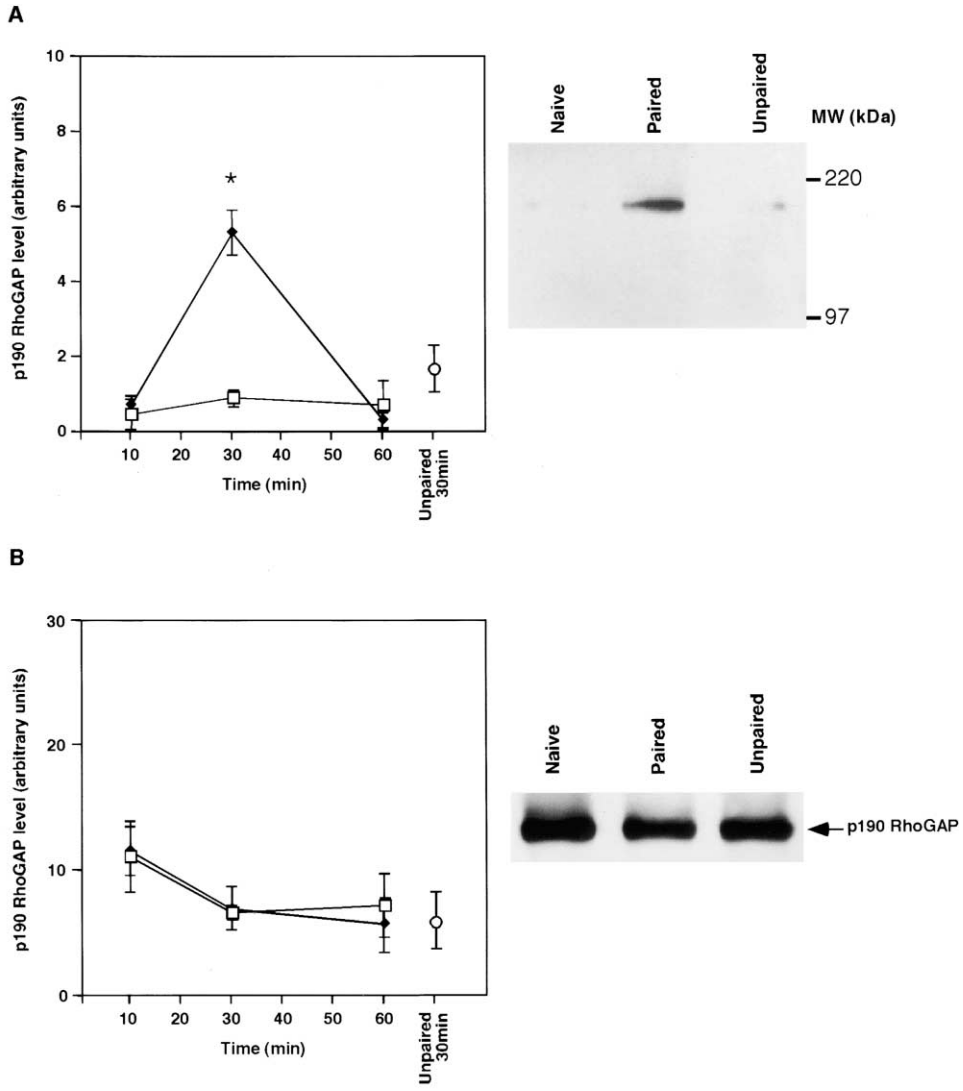

Figure 2. p190 RhoGAP Becomes Associated with GRB2 Protein following Fear Conditioning Training

(A) Representative immonoblot of protein samples extracted from lateral amygdala 30 min following training and immunoprecipitated with anti-GRB2 antibodies followed by immunoblotting with anti-p190 antibodies. The level of p190 RhoGAP that coimmunoprecipitated with GRB2 was monitored 10, 30, and 60 min following naïve or paired training and 30 min following unpairing (values are mean ± SEM; * = $p < 0.004$ between paired and naïve and * = $p < 0.01$ between paired and unpaired; Scheffe test; n = 5 for naïve, n = 6 for paired, and n = 4 for unpaired groups for the 30 min time point and n = 3 for the 10 and 60 time points). (B) The same samples as (A) before immunoprecipitation (total homogenate). Blots were incubated with anti-p190 antibodies. Square—naïve group; diamond—paired group; circle—unpaired group.

tial for the formation of fear conditioning memory, we inhibited the protein kinase ROCK which is one of the best-characterized Rho effector molecules (Amano et al., 2000). We utilized the ROCK inhibitor Y-27632, which has been shown to be cell permeable, nontoxic, and highly specific for ROCK (Ki = 0.14 μM; Uehata et al., 1997; Narumiya et al., 2000). The inhibitory effect of Y-27632 is rapid (within 30 min of application) and reversible (within hrs; Uehata et al., 1997).

Microinjection of Y-27632 into the LA 30 min before fear conditioning training significantly impaired long-term memory (LTM) for the tone (tested 24 hr after training) as compared to microinjection of the vehicle (artificial cerebral spinal fluid, ACSF) (Figure 5A; $p < 0.008$). The Y-27632 microinjected animals were not significantly impaired in tone memory when tested 1 hr after training (short-term memory; STM) when compared to the ACSF microinjected animals (Figure 5A). This result shows that the animals can acquire fear conditioning but cannot consolidate the STM into LTM. These animals, when retrained drug free, showed intact LTM (Figure 5B). This observation indicates that Y-27632 caused no functional damage to amygdala.

Microinjection of Y-27632 into the LA 1.5 hr before the LTM test (similar to the time before the 1 hr STM test) did not affect the retrieval of tone memory as compared to the ACSF animals (Figure 5C). This finding shows that Y-27632 has no effects on brain faculties

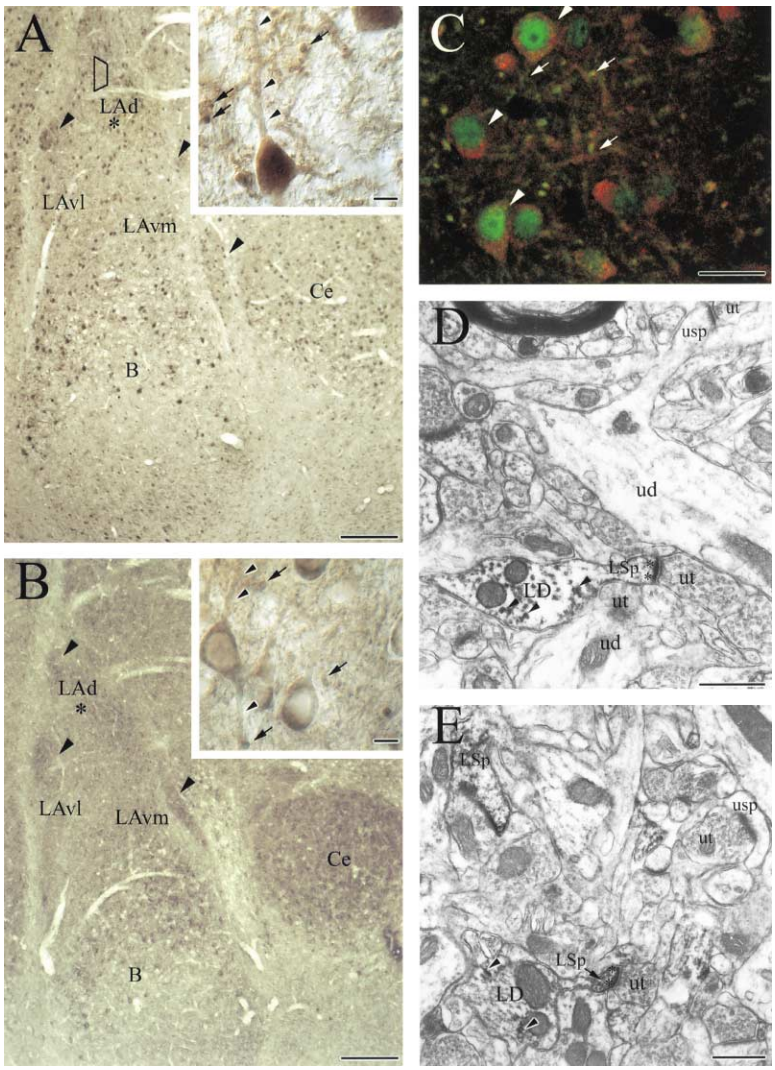

Figure 3. Photomicrographs Illustrating GRB2 and p190 RhoGAP Immunolabel in the Amygdala

(A) Low power (4×) photomicrograph shows the distribution of GRB2-ir in the amygdala. Labeled cells are distributed across the different nuclei of the amygdala. Arrowheads point to the dense patches of immunolabel associated with the intercalated nuclei. The trapezoid corresponds to region sampled for electron microscopic analysis and the asterisk to the region shown at higher magnification in inset. Inset: higher power Nomarski optics (100×) show a thick dendritic process (arrowheads) emanating from a labeled cell body. Within the dense matrix of labeled dendritic processes, stained puncta (arrows) can be seen. (B) p190 RhoGAP-labeled cells are seen throughout the various nuclei of the amygdala. Arrowheads indicate dense staining in the intercalated nuclei. Asterisk corresponds to region shown at higher magnification in inset. Inset: high power (100×) Nomarski optics reveal intensely labeled cells, their proximal dendrites (arrowheads) and labeled punctate processes (arrows). (C) Confocal micrograph shows that GRB2 (green) and p190 RhoGAP (red) are colocalized within cell bodies (arrowheads) and dendritic processes (arrows). (D) Electron micrograph shows GRB2 immunolabel within a dendrite (LD) rims the microtubules (arrowheads) and is intensely expressed within its spinous portion (LSP) and at the PSD (asterisks). Unlabeled dendrites (ud), spines (usp), and terminals (ut) are shown for comparison. (E) p190 RhoGAP immunolabel (arrowheads) is seen in discreet patches within a dendrite. The spinous portion of the dendrite is also labeled (LSp). A labeled spine (LSp), unlabeled spines (usp), and unlabeled terminals (ut) are also shown. Scale bar = 100 μm for (A) and (B), 10 μm for insets of (A) and (B), 10 μm for (C), and 500 nm for (D) and (E). B—basal nucleus of the amygdala; Ce—central nucleus of the amygdala; LAd— lateral nucleus of the amygdala, dorsal division; LAvl—lateral nucleus of the amygdala, ventral lateral division; LAvm—lateral nucleus of the amygdala, ventral medial division.

needed for sensory perception and fear memory expression.

Discussion

In the present study, we have shown that associative fear conditioning, leading to long-term tone memory, induces the formation of a protein complex in the lateral amygdala (LA) that includes GRB2 and p190 RhoGAP. Unpaired training that exposed the animals to the same sensory inputs but without inducing an association between the tone and shock did not produce such a complex. This indicates that the formation of the GRB2-mediated complex is not due to stress or other non-associative factors but is instead triggered by coincident occurrence of the tone and shock and may therefore be related to long-term memory of the tone-shock association. Indeed, inhibition of a downstream effector of the p190 RhoGAP pathway, ROCK, in LA during training impaired long-term, but not short-term, memory of the tone-shock association. These findings suggest that p190 RhoGAP and its downstream effector ROCK play an important role in fear memory formation in LA.

The involvement of the Rho-regulatory protein p190 RhoGAP in LA in fear conditioning is interesting in light of the fact that this protein has been shown to play a central role in molecular regulation of dendritic and axonal morphology and synaptic connectivity during development (Threadgill et al., 1997; Brouns et al., 2000, 2001; Billuart et al., 2001), events also believed to underlie changes in synaptic efficiency during learning (Dudai, 1989; Kandel, 2001). Mice lacking functional p190 RhoGAP exhibit defects in axon guidance and fasciculation (Brouns et al., 2001). Conversely, overexpression of this protein in neuroblastoma cells promotes extensive neurite outgrowth (Brouns et al., 2001). It has also been

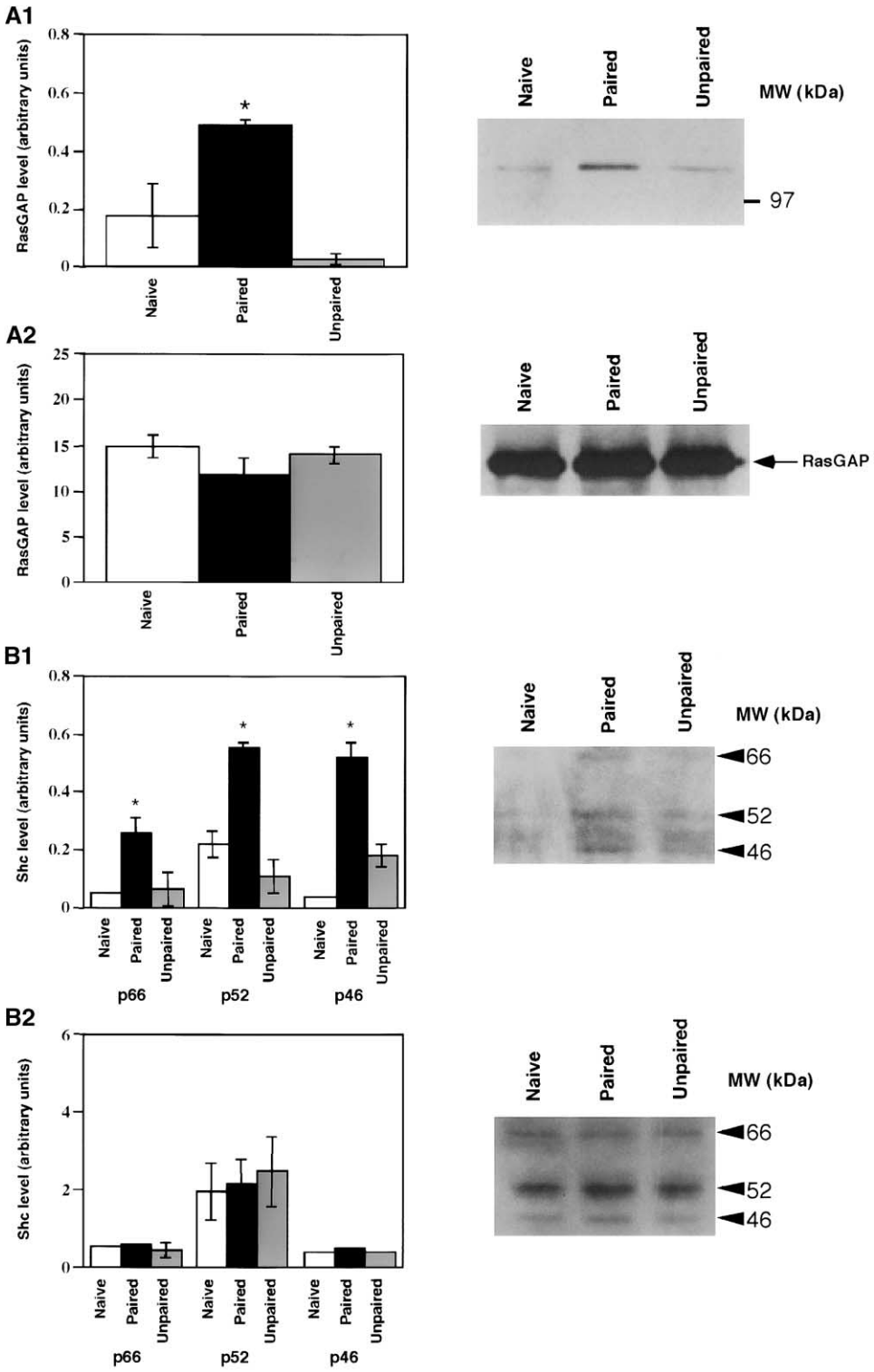

Figure 4. Fear Conditioning Induces the Interaction of RasGAP and Shc with GRB2 Protein

Lateral amygdala protein samples extracted 30 min following training were immunoprecipitated with anti-GRB2 antibodies. Blots were incubated with anti-RasGAP antibodies (A1) or anti-Shc (B1) antibodies (values are mean ± SEM; * = $p < 0.03$ between paired and naïve and * = $p < 0.003$ between paired and unpaired for RasGAP; and * = $p < 0.0006$ for $p46^{Shc}$, $p < 0.05$ for $p52^{Shc}$, $p < 0.03$ for $p66^{Shc}$ between paired and naïve and $p < 0.0005$ for $p46^{Shc}$, $p < 0.002$ for $p52^{Shc}$, and $p < 0.05$ for $p66^{Shc}$ between paired and unpaired; Scheffe test; $n = 4$ for all groups). (A2) and (B2) are representative blots from total homogenates of same samples of (A1) and (B1) that were incubated with anti-RasGAP or anti-Shc antibodies, respectively.

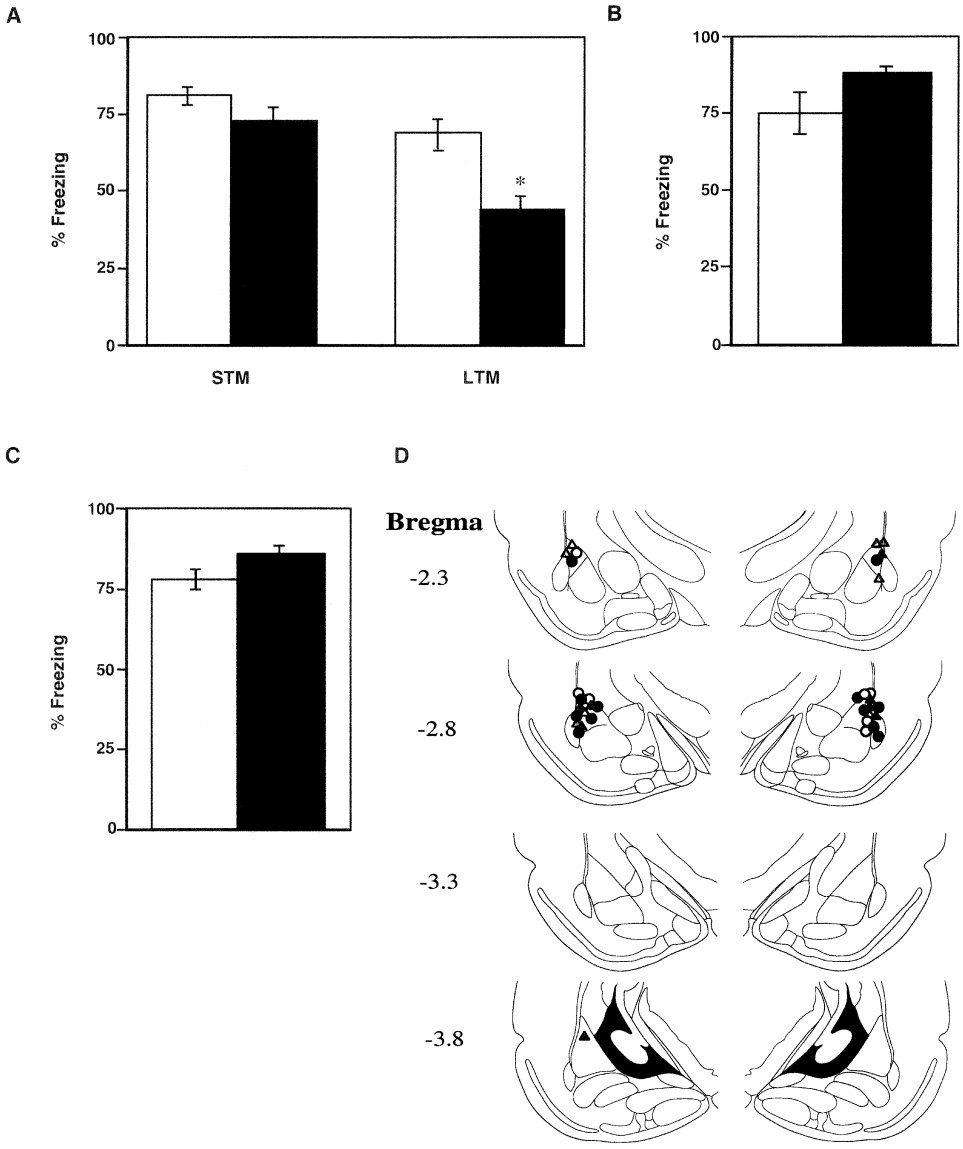

Figure 5. Inhibition of ROCK Activity in LA Impairs Long- but Not Short-Term Fear Conditioning Memory Formation

(A) Microinjection of Y-27632 ROCK inhibitor (8.56 μg/0.5 μl/hemisphere) into the LA 30 min before fear conditioning training significantly impaired long-term but not short-term memory as compared to ACSF injected animals ($p < 0.008$; $n = 4$ for ACSF, $n = 6$ for Y-27632). (B) The same rats from (A) that were retrained drug free and showed intact long-term memory. (C) Microinjection of Y-27632 (8.56 μg/0.5 μl/hemisphere) into LA 1.5 hr before test had no effect on memory retrieval ($n = 4$ each group). (D) Cannula placements (white circles: ACSF before training, black circles: Y-27632 before training, white triangles: ACSF before memory test, and black triangles: Y-27632 before memory test).

shown that overexpression of p190 RhoGAP in E18/19 rat cortical cells causes marked reduction in the number of primary dendrites in nonpyramidal neurons and in the number of basal dendrites in pyramidal neurons (Threadgill et al., 1997). Interestingly, Billuart et al. (2001) have shown recently that inactivation of p190 RhoGAP in the *Drosophila* mushroom body (MB) neurons results in axon branch retraction. p190 RhoGAP activity was also found to be negatively regulated by Src and integrin. The authors suggest the possibility that the regulation of p190 RhoGAP in the MB, the *Drosophila* olfactory learning and memory center, may contribute to the physiological plasticity essential for memory formation. They also advance the hypothesis, relevant to our findings, that the reason why only a small number of the *Drosophila* RhoGAP genes exhibited the phenotype during development could come from their pattern of expression, unique subcellular localization, and their ability to form a specific signaling complex.

At the molecular level, evidence is available that p190 RhoGAP is involved in mediating actin reorganization. Specifically, in p190 RhoGAP mutant mice, polymerized actin accumulates extensively in cells of the neural tube floor, suggesting that p190 RhoGAP plays a role in regulating actin assembly (Brouns et al., 2000). p190 RhoGAP has also been implicated in signal transduction downstream to extracellular matrix and cellular adhesion responsive receptors, a molecular pathway important in

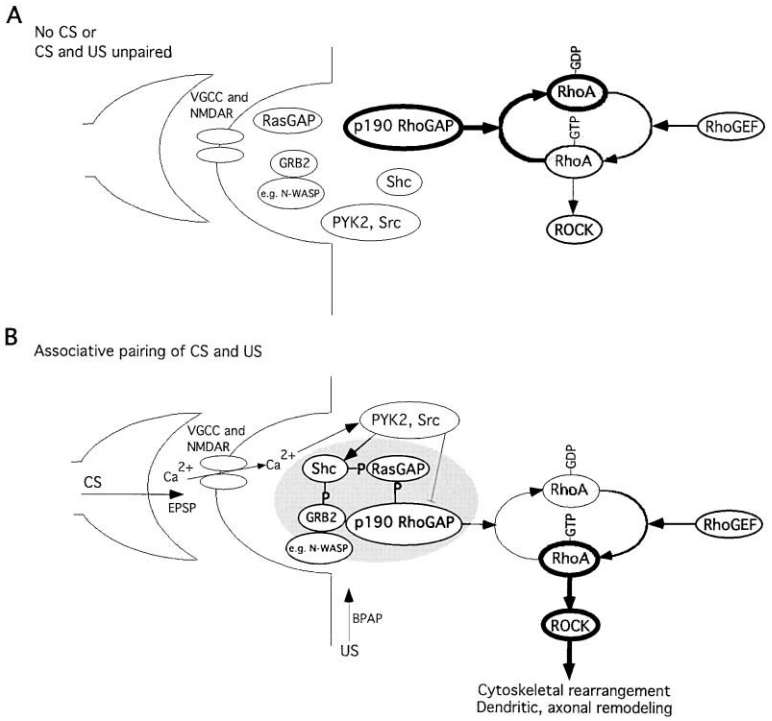

Figure 6. A Model of Synaptic Plasticity in LA Mediated by a GRB2 Protein Complex Formed during Associative Fear Conditioning

(A) Under unstimulated baseline conditions, or when animals are subjected to a tone (CS) and a shock (US) in a non-associative manner, the GRB2-mediated complex is absent. Given that previous studies have suggested that Src phosphorylation negatively regulates p190 RhoGAP (e.g., Billuart et al., 2001), an unphosphorylated p190 RhoGAP in these animals is presumed to be active and maintains ROCK in an inactivated state. (B) GRB2-mediated complex is formed when animals undergo associative conditioning in which the CS and US are paired. On the basis of recent studies implicating calcium influx in lateral amygdala-dependent fear conditioning (Weisskopf et al., 1999; Blair et al., 2001; Bauer et al., 2002; Rodrigues et al., 2002) and showing a role of calcium-mediated activation of tyrosine kinases in synaptic plasticity in other brain areas (Huang et al., 2001), we propose the following model. During fear conditioning, influx of calcium through voltage-gated calcium channels and NMDA receptors activates non-receptor tyrosine kinases, such as PYK2, which, in turn, activate Src, and the GRB2-mediated complex is formed. Members of the the Src family of kinases are the main enzymes that phosphorylate also p190 RhoGAP and Shc (Brouns et al., 2001). p190 RhoGAP is negatively regulated by Src phosphorylation, leading to net activation of RhoA and ROCK, by yet an unknown RhoGEF, and to changes in cytoskeleton that regulate synaptic efficacy in LA. Arrow: activate; T-arrow: inhibit. The thickness of lines corresponds to level of activity.

modulation of synaptic contacts and growth of neuronal processes. For example, neurite outgrowth is strongly promoted in p190 RhoGAP-transfected N2A cells within 1 hr following surface coating with the integrin binding extracellular matrix protein laminin (Brouns et al., 2001), and dissociation of cell contacts between blastula ectodermal cells induces the formation of p66Shc-RasGAP-p190 RhoGAP complex (Dupont and Blancq, 1999). Thus, p190 RhoGAP may underlie alteration of synaptic connectivity within the LA by mediating adhesion molecule activities or by regulation of actin rearrangement, which have been shown, in certain cases, to go hand in hand. Adhesion molecules and actin reorganization have also been implicated in neuronal plasticity and memory formation (Grotewiel et al., 1998; Chang et al., 2001; Hatada et al., 2000; Krucker et al., 2000).

We suggest that the effects of p190 RhoGAP on fear conditioning occur via the Rho/ROCK pathway (see Figure 6). Because ROCK is a Rho effector downstream to p190 RhoGAP, we assessed the effects of ROCK inhibition on fear conditioning. Our finding that administration of the ROCK inhibitor prior to conditioning affected long-term but not short-term memory is consistent with a role of the Rho/ROCK pathway in long-term memory formation.

Although this study provides evidence for a role of the Rho/ROCK pathway in memory, previous studies have implicated it in other forms of neural plasticity, especially during development. For example, ROCK has been shown to mediate the effects of RhoA on neurite retraction in neuroblastoma cells (Hirose et al., 1998), dendritic retraction in hippocampal pyramidal neurons (Nakayama et al., 2000), axon outgrowth in cerebellar granule neurons (Bito et al., 2000), axon branch retraction in *Drosophila* mushroom body (Billuart et al., 2001), and dendritic arbor growth in tectal ventricle of *Xenopus* (Li et al., 2002). ROCK phosphorylated substrates (e.g., myosin light chain, LIM kinase, and collapsin response mediator protein-2 [CRMP2]; see Kimura et al., 1996; Arber et al., 1998; Amano et al., 2000) have also been shown to be involved in stress fiber formation and in mediating growth cone morphogenesis (e.g., Totsukawa et al., 2000). Further, inactivation of p190 RhoGAP leads to axon branch retraction, a phenotype mimicked by activation of RhoA or its effector kinase Drok (the *Drosophila* ROCK homolog; Billuart et al., 2001). This latter study also showed that Src negatively regulates p190 RhoGAP. Phosphorylation of p190 RhoGAP by Src has also been shown to abolish its capability to bind GTP, which is necessary for its GAP activity (Roof et al., 2000; Tatsis et al., 1998). The various findings above suggest that phosphorylation of p190 RhoGAP in LA might lead to its inactivation, resulting in net activation of RhoA and ROCK by yet unknown RhoGEF. The regulated activation of ROCK in LA by its upstream regulator Rho, initiated by inactivation of p190 RhoGAP, might then induce morphological changes needed to support long-term memory (See discussion below and the model in Figure 6).

We also found that in LA, GRB2 coimmunoprecipitated with Shc and RasGAP significantly more in paired animals than in naïve or unpaired rats (Figures 4A1 and 4B1). The formation of this complex is probably mediated by tyrosine phosphorylation of the proteins since

other studies have shown that tyrosine phosphorylation of Shc promotes its interaction with SH2-containing proteins such as GRB2 (Cattaneo and Pelicci, 1998). Furthermore, p190 RhoGAP phosphorylation by the Src proteins induces its interaction with SH2-containing proteins such as p120 RasGAP (Hu and Settleman, 1997; Roof et al., 1998). A GRB2-p66Shc-RasGAP-p190 RhoGAP complex was observed during early morphogenic events of gastrulation and is modulated by changes in cell contacts (Dupont and Blancq, 1999). The formation of this molecular complex, in LA, may promote the translocation of p190 RhoGAP by Shc or GRB2 to a distinct neuronal area in close proximity to its site of action. For example, GRB2 and members of the Rho GTPase family bind to N-WASP, a protein involved in actin polymerization (Snapper and Rosen, 1999). In addition, extracellular stimulation, such as activation of integrin, induces rapid translocation of p190 RhoGAP to cytoskeleton and membrane ruffling where it colocalized with polymerized actin (Nakahara et al., 1998; Sharma, 1998). It is therefore plausible that fear conditioning couples p190 RhoGAP to the cytoskeleton via GRB2 and induces modulation of actin dynamics as well as changes in dendritic and spine morphology in LA. Actin dynamics are involved in remodeling the morphology of dendrites and spines (Matus, 2000), and these structures have been strongly implicated in synaptic plasticity (Nimchinsky et al., 2002). For example, induction of LTP is associated with the production of dendritic spines and filopodia (Nimchinsky et al., 2002), and inhibition of actin dynamics suppresses LTP (Krucker et al., 2000). The presence of both p190 RhoGAP and GRB2 in dendrites and spines (Figures 3D and 3E) suggests that these molecules might work in concert to induce the structural changes that confer synaptic specificity to memories.

In Figure 6, we propose a model to account for the results reported here. In brief, during associative fear conditioning, influx of calcium through voltage-gated calcium channels and NMDA receptors (Blair et al., 2001; Bauer et al., 2002; Rodrigues et al., 2002) activates nonreceptor tyrosine kinases, such as PYK2, which, in turn, activate Src (Huang et al., 2001). As a result, the GRB2-mediated complex is formed. The phosphorylated p190 RhoGAP becomes inactivated (e.g., Billuart et al., 2001), leading to a net ROCK activation. Consequently, structural changes in synaptic connectivity, which are believed to underlie long-term memory (see Weiler et al., 1995; Kandel, 2001), occur. The exact manner in which a yet unknown RhoGEF leads to net activation of RhoA and ROCK, and to changes in cytoskeleton organization that regulate synaptic efficacy in LA, is a topic for further investigation.

Experimental Procedures

Animals

All studies involved male Sprague Dawley rats (Hilltop Labs, Scottdale, Pennsylvania), weighing 250–300 g. The animals were housed separately in plastic Nalgene cages and placed on a 12 hr light/dark cycle with ad libitum food and water. All procedures were in accordance with the National Institutes of Health guide and were approved by the New York University Animal Care and Use Committee.

Reagents

Anti-p190 (G19020), -GRB2 (G16720), -RasGAP (G12920), and -Shc (S68020) antibodies were from Transduction Laboratories (Lexington, Kentucky) and anti-phosphotyrosine antibody (61-5800) was purchased from Zymed (San Francisco, California). For immunoprecipitation, agarose-conjugated anti-GRB2 (sc-255 AC) was a product of Santa Cruz Biotechnology (Santa Cruz, California). For immunohistochemistry, anti-GRB2 was from Santa Cruz (sc-255). HRP-protein A (NA9120) and an enhanced chemoluminiscence (ECL) kit (RPN 2106) were from Amersham Pharmacia Biotech (Piscataway, New Jersey). HRP-goat anti-mouse (115-035-146) was from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). ROCK inhibitor, Y-27632, was from Biomol (Plymouth Meeting, Pennsylvania). All chemicals were of analytical grade or the highest grade available.

Fear Conditioning Procedure

Fear conditioning took place in a Plexiglas rodent conditioning chamber with a metal grid floor (model E10-10; Coulbourn Instruments, Lehigh Valley, Pennsylvania), dimly illuminated by a single house light and enclosed within a sound-attenuating chamber (model E10-20). Rats were habituated to the training chamber for 15 min for 3 days. On the fourth day the rats were divided into three groups: (1) naïve group exposed to the chamber for 14 min; (2) paired group presented with five pairings of tone for 20 s (CS: conditioned stimulus; 5 kHz, 75 dB) that co-terminated with a foot shock (US: unconditioned stimulus; 0.5 s, 1.3 mA); and (3) unpaired group which received non-overlapping presentations of the CS and US. For the ROCK inhibition studies, the animals were habituated for 15 min a day prior to training. Animals were trained with two CS-US pairing (CS: 30 s tone, 5 kHz, 75 dB; US: 1 s of 1.5 mA footshock that co-terminated with the tone).

Testing of Conditioned Fear Memory

Rats were tested 1 hr (short-term memory) and/or 24 hr (long-term memory) after conditioning for tone memory in a different chamber than the conditioning chamber. The chamber was brightly illuminated with three house lights and contained a flat black Formica floor that had been washed with peppermint soap. Animals were videotaped during testing for later scoring. After a 3–5 min acclimation period to the test chamber, rats were presented with five 30 s tones (20 s for the five pairing animals; 5 kHz, 75 dB; intertrial interval: 100 s). After tone testing, rats returned to their home cages and to the colony. Freezing of animals during tone presentation was scored. Differences among groups were evaluated using ANOVA (Scheffe contrast tests were used with an α level of 0.05).

Tissue Dissecting, Immunoprecipitation, and Immunoblotting

Rats were sacrificed at indicated time points (see the Results section) following fear conditioning training and their brains were rapidly removed and frozen on dry ice. The lateral amygdala nucleus (and the adjacent region) was punched from frozen brain with a blunted 0.5 mm diameter sample corer (Fine science tools, Foster City, California) and kept at -80°C. Tissue was then homogenized, using a glass-Teflon homogenizer, in homogenizing buffer (25 mM Tris-HCl [pH = 7.4], 2 mM sodium orthovanadate, 0.5 mM EGTA, 2 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 25 mM NaCl, 1% Tx-100, and protease inhibitor cocktail 1:1000 [Sigma]). Equal amounts of protein (25–50 μg; measured by Lowry) for all groups were subjected to immunoprecipitation. The homogenates were incubated with gentle agitation at 4°C with 6 μg agarose-GRB2 antibodies for 2 hr. The immunoprecipitates were washed thrice with homogenizing buffer, boiled in Laemli SDS-sample buffer for 3 min, and subjected to SDS-PAGE and immunoblotting. Blots were blocked with blocking buffer (5% nonfat dry milk in wash buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20]) or, for anti-phosphotyrosine antibodies, 1% BSA in wash buffer (0.9% NaCl, 0.05% Tween 20, and 10 mM Tris [pH 7.6]) for 1 hr at room temperature. Blots were subjected to the primary antibody (p190 1:500, GRB2 1:5000, RasGAP 1:1000, Shc 1:1000, or anti-phosphotyrosine 1:1500) in wash buffer containing 5% nonfat dry milk (wash buffer without BSA for anti-phosphotyrosine) for 1 hr at

room temperature. Blots were washed with wash buffer for 30 min. The blots were then subjected to HRP-conjugated goat anti-mouse (1:2000) or HRP-linked protein A (1:20,000 for anti-phosphotyrosine) in wash buffer containing 5% nonfat dry milk (wash buffer without BSA for anti-phosphotyrosine) for 1 hr at room temperature. Blots were washed with wash buffer for 30 min. The blots were subjected to ECL for 1 min and exposed to X-ray film. The films were scanned (Snapscan1212, AGFA) and the intensity of the protein bands on the film was analyzed using the Intelligent Quantifier (V.3.0) software (Genomic Solutions, Ann Arbor, Michigan). Differences among groups were evaluated using ANOVA (Scheffe contrast tests were used with an α level of 0.05).

Immunohistochemistry

Rats were anaesthetized with pentobarbital (100 mg/kg i.p.) and perfused intracardially with fixative. The brains were removed, post-fixed, and sectioned on a vibratome. Sections were blocked in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) for 30 min followed by overnight incubation at room temperature with rabbit anti-GRB2 antibodies (1:50) or mouse anti-p190 antibodies (1:50) in blocking solution. The tissue was washed thrice, 5 min each, with PBS. The slices were incubated with anti-mouse (for p190) or anti-rabbit biotinylated IgG (Vector) in PBS containing 1% BSA for 30 min. The slices were washed thrice, 5 min each. Slices were then incubated with ABC solution (Vector) for 30 min followed by incubation with DAB and hydrogen peroxidase (Sigma) in PBS. In controls, primary antibody was omitted to evaluate cross reactivity. In these controls, no staining was detected in amygdala. Images were acquired using a Zeiss Axiophot microscope attached to a Coolsnap digital camera (Roper Scientific) and processed with Adobe Photoshop.

For double-labeling, slices were sectioned from brain as described above and incubated for 30 min in PBS containing 1% BSA followed by overnight co-incubation with rabbit anti-GRB2 antibody (1:50) and mouse anti-p190 (1:50). Slices were then rinsed thrice with PBS and incubated for 1 hr with Alexa Fluor® 488 goat anti-rabbit (1:200) and Alexa Fluor® 594 goat anti-mouse (1:200; Molecular Probes, Inc.). Slices were rinsed thrice with PBS. Tissue sections were analyzed using a Zeiss 310 laser confocal microscope equipped with an argon-krypton laser. Immunofluorescent signals were captured sequentially using the 488 (green) and 543 (red) channels and processed using Adobe Photoshop. Control experiments included omitting the primary antibodies. When primary antibody was omitted to evaluate cross-reactivity to IgGs, no staining was detected in amygdala or other brain regions. Additional controls were performed by mismatching the secondary antibody in single-label experiments. Finally, single-labeled material was scanned into the correct and incorrect channels. Control studies showed that the secondary antibodies were specific for mouse or rabbit IgG's and that "bleed-through" of labels into the inappropriate channels did not occur.

Tissue sections designated for EM were processed as previously described (Farb and LeDoux, 1997). In brief, immunolabeled amygdala tissue sections were incubated in 1% osmium tetraoxide/PB for 60 min, dehydrated in a graded series of alcohols, stained en bloc in uranyl acetate, further dehydrated in acetone, and subsequently flat-embedded in Epon. Portions of the tissue containing the amygdala were embedded in Beem capsules and placed at 60°C for 18–24 hr. A camera lucida drawing was made of the amygdala, and ultrathin sections (85 nm) from the amygdala were cut and collected on 8–12 nickel grids. The tissue was examined using a JEOL 1200EX electron microscope at magnifications of 10–15,000. Neuronal and glial elements were classified using the definitions as previously described in the amygdala (Farb and LeDoux, 1997).

Surgical Procedure

Microinjection into the lateral amygdala was performed via chronically implanted cannulas. For surgery, rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.; Ketaset; Phoenix, St. Joseph, Missouri), xylazine (6.0 mg/kg, i.p.; Xyla-Jet; Phoenix, Arizona), and medetomidine (0.5 mg/kg, i.p.; Domitor; Pfizer, New York, New York), restrained in a stereotaxic apparatus (Kopf), and implanted bilaterally with guide cannulas (22 gauge; Plastics One, Roanoke, Virginia), equipped with internal cannulas that extended out 1.5 mm from the base of the guide cannulae, aimed to the lateral amygdaloid nucleus (LA; coordinates in reference to bregma: anteroposterior (AP), [−] 3.0; lateral (L), ± 5.3; and dorsoventral (DV), [−] 8.0] (Paxinos and Watson, 1986). The cannulas were fixed in place with acrylic dental cement and secured by three skull screws, and internal cannulas removed. Dummy cannula that was 0.5 mm longer than the chronic cannula was placed in the guide cannula to prevent clogging. After surgery, rats were administered butorphanol tartrate (2.0 mg/kg, i.p.; Torbugesic; Fort Dodge Laboratories, Fort Dodge, Iowa) and atipamezole (1.0 mg/kg, i.p.; Antisedan; Pfizer) for analgesia and reversal of the anesthetic. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations.

Microinjection

The dummy cannula was removed from the guide cannula, and a 28 gauge injection cannula was carefully inserted and lowered 1.5 mm below the tip of the guide cannula. The injection cannula was connected via polyurethane tubing, back-filled with sesame oil with a small air bubble separating the oil from the drug solution, to a 1.0 μl Hamilton syringe. Microinjection was driven by an infusion pump at a rate of 0.25 μl/min. ROCK inhibitor Y-27632, dissolved in ACSF, was microinjected bilaterally (8.56 μg/0.5 μl/hemisphere) at the times indicated (see Results). After microinjection, the injection cannula was left for an additional 1 min before withdrawal to reduce efflux of injection liquid along the injection tract.

Histology

To verify the injection cannula tip location, rats were anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused transcardially with 10% buffered formalin. Brains were postfixed in 30% sucrose in 10% buffered formalin. Postfixed brains were sectioned on a cryostat at 50 μm, and stained for Nissl with thionin. Sections were coverslipped with Permount and examined by light microscopy for injector tip location.

Acknowledgments

We thank Keren Paz for excellent advice and discussions. This work was supported by NIH grants MH58911, MH46516, MH38774, MH00956, and a Human Frontier Science Program postdoctoral fellowship.

Received: April 4, 2002
Revised: September 16, 2002

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