

Proteolytic Degradation of SCOP in the Hippocampus Contributes to Activation of MAP Kinase and Memory

Kimiko Shimizu,¹ Trongha Phan,¹ Isabelle M. Mansuy,² and Daniel R. Storm^{1,*}

¹Department of Pharmacology, The University of Washington, Seattle, WA 98195, USA

²Brain Research Institute, University of Zürich, Swiss Federal Institute of Technology Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

*Correspondence: dstorm@u.washington.edu

DOI 10.1016/j.cell.2006.12.047

SUMMARY

Because activation of ERK1/2 MAP kinase (MAPK) is critical for hippocampus-dependent memory, there is considerable interest in mechanisms for regulation of MAPK during memory formation. Here we report that MAPK and CREB-mediated transcription are negatively regulated by SCOP (suprachiasmatic nucleus [SCN] circadian oscillatory protein) and that SCOP is proteolyzed by calpain when hippocampal neurons are stimulated by brain-derived neurotrophic factor (BDNF), KCl depolarization, or NMDA. Moreover, training for novel object memory decreases SCOP in the hippocampus. To determine if hippocampus-dependent memory is influenced by SCOP in vivo, we generated a transgenic mouse strain for the inducible overexpression of SCOP in the forebrain. Overexpression of SCOP completely blocked memory for novel objects. We conclude that degradation of SCOP by calpain contributes to activation of MAPK during memory formation.

INTRODUCTION

MAP Kinase (MAPK) plays a critical role in several forms of neuroplasticity including memory formation (for reviews see Impey et al., 1999; Mazzucchelli and Brambilla, 2000; Sweatt, 2004) and neuronal survival (for reviews see Hetman and Xia, 2000; Heerssen and Segal, 2002). Activation of MAPK may be required for memory consolidation because it mediates activity-dependent, Ca²⁺ stimulation of cAMP response element (CRE)-mediated transcription (Xing et al., 1996; Impey et al., 1998a; Athos et al., 2002) and dendritic translation (Kelleher et al., 2004). For example, MAPK is activated during training for hippocampus-dependent memory (Athos et al., 2002; Atkins et al.,

1998; Blum et al., 1999; Sindreu et al., 2007) and by stimulus paradigms that evoke long-term potentiation (LTP) (English and Sweatt, 1996; Impey et al., 1998b). Inhibitors of MAPK signaling block memory formation (Athos et al., 2002; Atkins et al., 1998; Blum et al., 1999) and increases in CRE-mediated transcription, which are required for contextual memory (Athos et al., 2002; Atkins et al., 1998; Blum et al., 1999). Gene-disruption studies also support the hypothesis that MAPK activity is critical for synaptic plasticity and memory. Disruption of the gene for RasGRF, an activator of Ras, attenuates long-term memory (LTM) for cued fear conditioning and LTP in the basolateral amygdala (Brambilla et al., 1997). In addition, disruption of the ERK1 gene has implicated MAPK-dependent signaling in behavioral plasticity (Mazzucchelli et al., 2002). Consequently, it is important to understand how MAPK is activated in central nervous system (CNS) neurons. Activity-induced increases in intracellular free Ca²⁺ can stimulate MAPK by several mechanisms including calmodulin stimulation of RasGRF (Farnsworth et al., 1995) or indirectly through stimulation of adenylyl cyclase (Wang and Storm, 2003), subsequent activation of protein kinase A (PKA), and stimulation of Rap1 (de Rooij et al., 1998; Vossler et al., 1997).

Negative regulation of MAPK signaling may be just as important for memory as stimulatory mechanisms. Mice deficient in neurofibromatosis type 1, a RAS GTPase-activating protein, have defects in hippocampus-dependent spatial memory, suggesting that a balance between Ras stimulation and inhibition is critical for memory (Silva et al., 1997). Recently, it was discovered that suprachiasmatic nucleus circadian oscillatory protein (SCOP) (Shimizu et al., 1999) is a negative regulator of K-Ras in PC12 cells (Shimizu et al., 2003). SCOP interacts directly with K-Ras through its leucine-rich repeat and inhibits K-Ras function by associating with the nucleotide-free form of K-Ras. This prevents binding of GTP to K-Ras, inhibits its activity, and prevents neurotrophin stimulation of MAPK. In PC12 cells, SCOP overexpression inhibits depolarization stimulation of MAPK by blocking K-Ras activity (Shimizu et al., 2003). SCOP inhibition of K-Ras activity is especially interesting because the K-Ras/MEK/MAPK

pathway is critical for synaptic and behavioral plasticity (Ohno et al., 2001).

Although SCOP attenuation of MAPK signaling has the potential to play an important role in neuroplasticity, mechanisms for regulation of SCOP have not been reported. Furthermore, it has not been established that SCOP regulates MAPK/CRE-mediated transcription in CNS neurons, a transcriptional pathway important for long-term memory (Athos et al., 2002; Atkins et al., 1998; Blum et al., 1999; Bourtchuladze et al., 1994; Pittenger et al., 2002) and neuronal survival (Hetman et al., 1999; Mabuchi et al., 2001; Watt et al., 2004). Here, we report that SCOP negatively regulates CRE-mediated transcription in hippocampal neurons and is rapidly degraded by calpain when neurons are stimulated with brain-derived neurotrophic factor (BDNF), NMDA, or KCl-induced depolarization. Interestingly, SCOP is also rapidly degraded during training for hippocampus-dependent memory, and SCOP-overexpressing transgenic mice have no long-term memory for novel objects. These data suggest that SCOP constrains MAPK activity and that its degradation contributes to the transient activation of MAPK required for some forms of hippocampus-dependent memory.

RESULTS

SCOP Expression in the Hippocampus

SCOP was originally discovered as a protein that undergoes a circadian oscillation in the suprachiasmatic nucleus (SCN) but not in other regions of brain (Shimizu et al., 1999). To explore the role of SCOP in regulation of MAPK signaling in the hippocampus, we examined its distribution in brain by immunohistochemistry (Figure 1A). SCOP immunoreactivity was present predominantly in the somata and processes extending from somata in area CA1 of the rat and mouse hippocampus. SCOP is also expressed in area CA3 but not the dentate gyrus. The SCOP immunostaining in the hippocampus was completely blocked when the SCOP antibody was preabsorbed with SCOP protein. Because of the importance of the hippocampus for memory formation, this study focused on the regulatory role of SCOP in hippocampal neurons *in vitro* and *in vivo*.

SCOP Inhibits CRE-Mediated Transcription in Cultured Hippocampal Neurons

One of the major MAPK-activated transcriptional pathways implicated in hippocampus-dependent memory is CRE-mediated transcription (Athos et al., 2002; Bourtchuladze et al., 1994; Impey et al., 1998b; Xing et al., 1996). To determine if SCOP inhibits stimulation of this transcriptional pathway in hippocampal neurons, neurons were cotransfected with a reporter for CRE-mediated transcription with varying amounts of a SCOP expression vector (Figure 1B). BDNF stimulation of CRE-mediated transcription in hippocampal neurons was progressively inhibited by increasing amounts of SCOP, with almost complete inhibition at the highest levels of SCOP. This indicates that

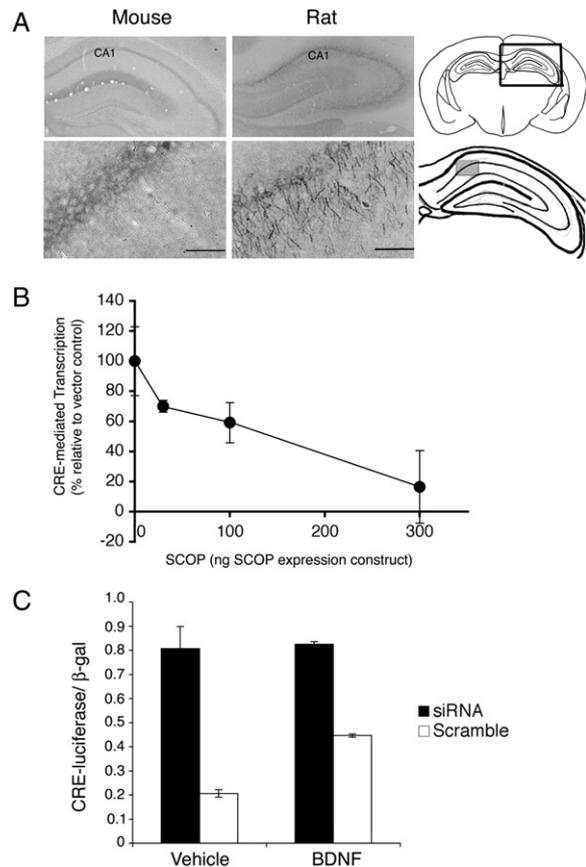


Figure 1. CRE-Mediated Transcription in Hippocampal Neurons Is Inhibited by SCOP

(A) SCOP immunoreactivity was detectable in several areas of the rat and mouse hippocampus, including area CA1. Coronal sections of the hippocampus were stained with SCOP antibody and visualized using 3'-3' diaminobenzidine as described in Experimental Procedures. The diagrams on the right side identify those areas of the hippocampus shown in the images. Scale bars represent 100 μ m.

(B) SCOP overexpression inhibits BDNF stimulation of CRE-mediated transcription. Cultured hippocampal neurons were cotransfected with a CRE-luciferase reporter construct and varying amounts of a SCOP expression plasmid or empty vector (total 300 ng). An expression vector for β -galactosidase was cotransfected for normalization of transfection efficiencies. Luciferase activity was measured 6 hr after BDNF (5 ng/ml) or vehicle treatment. Data are reported as the percent activity relative to the vector control. Error bars, standard error of the mean (SEM) ($n = 3$).

(C) Inhibition of SCOP expression by siRNA stimulates CRE-mediated transcription. Cultured hippocampal neurons were cotransfected with a CRE-luciferase reporter construct with SCOP siRNA, or a scrambled RNA of the same composition. Luciferase activity was measured 24 hr after transfection and was normalized to β -galactosidase activity. Error bars, SEM ($n = 3$).

SCOP can function as a negative regulator of CRE-mediated transcription in hippocampal neurons.

If endogenous SCOP inhibits MAPK and CRE-mediated transcription, inhibition of SCOP expression should increase CRE-mediated transcription. To test this, hippocampal neurons were cotransfected with SCOP siRNA,

or a scrambled RNA sequence of the same composition, and a reporter for CRE-mediated transcription. In unstimulated cells, SCOP siRNA increased CRE-mediated transcription 4-fold, a stimulation twice that obtained with BDNF alone (Figure 1C). These data indicate that endogenous SCOP constrains CRE-mediated transcription in hippocampal neurons. We confirmed that SCOP siRNA attenuates MAPK activation using a lentivirus construct to express the SCOP siRNA. Lentivirus expression of the SCOP siRNA depressed SCOP protein and activated pERK in unstimulated and BDNF-treated hippocampal neurons (Figure S1).

Treatment of Hippocampal Neurons with BDNF, NMDA, or KCl Decreases SCOP Protein

Because SCOP inhibits MAPK signaling and blocks stimulation of CRE-mediated transcription, it was important to determine how SCOP itself is regulated in hippocampal neurons. Since we were unable to detect phosphorylation of SCOP (data not shown), we considered the possibility that SCOP may be degraded when neurons are stimulated with BDNF or other activators of MAPK. We monitored SCOP protein levels by western analysis after hippocampal neurons were treated with BDNF. Within 5 min of BDNF treatment, SCOP protein was reduced by 50% compared to controls and returned to unstimulated levels by 60 min (Figure 2A). We also found that depolarization of cultured hippocampal neurons with KCl or treatment with NMDA reduced SCOP levels 52% and 44%, respectively (Figure 2B). Similar results were obtained when cultured cortical neurons were treated with BDNF; SCOP protein was rapidly degraded and returned to baseline within 3 hr (data not shown). The fact that SCOP protein was never totally degraded suggests that SCOP levels may be maintained by a balance between protein synthesis and proteolysis.

SCOP Is Proteolyzed by Calpain

The rapid reduction in SCOP protein when neurons are treated with BDNF, KCl, or NMDA suggests that it may be degraded by a protease. The fact that SCOP levels return to baseline relatively quickly suggest that transient activation of a protease stimulates SCOP degradation. We focused on Ca^{2+} -stimulated proteases because activation of NMDA receptors (Dingledine, 1983), BDNF (Berninger et al., 1993; Li et al., 1998), and KCl depolarization (Di Virgilio et al., 1987) all cause transient increases in intracellular free Ca^{2+} .

In cell-free, hippocampal extracts, SCOP was rapidly degraded in the presence of free Ca^{2+} but not in its absence, suggesting that SCOP is susceptible to degradation by a Ca^{2+} -activated protease (Figure 3A). To identify the protease responsible for degradation of SCOP we examined inhibitors of several proteases including caspase-3 (Chan and Mattson, 1999; Dash et al., 2000), proteasome (Cline, 2003; Hegde, 2004; Lopez-Salon et al., 2001), and calpain (Chan and Mattson, 1999). These proteases are present at synapses and have been implicated in

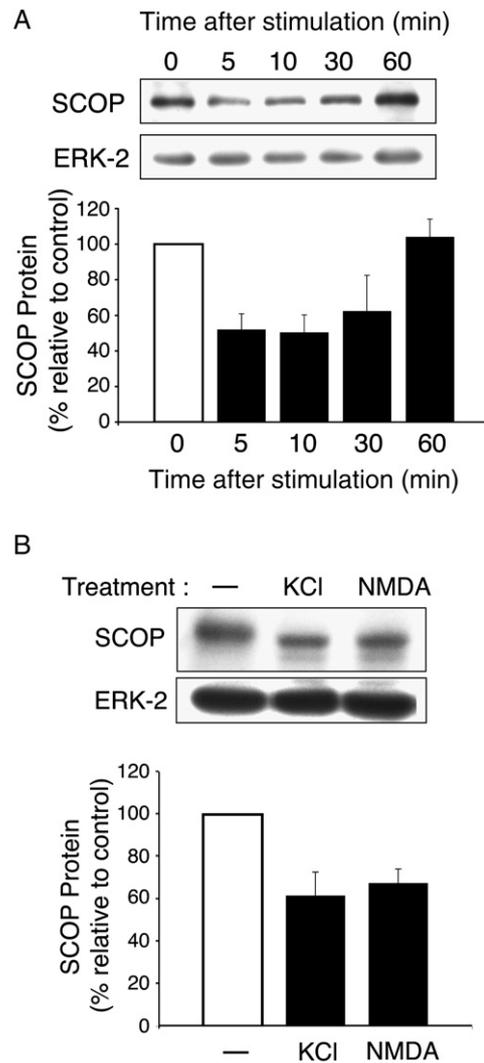


Figure 2. BDNF Stimulates Rapid Degradation of SCOP in Hippocampal Neurons

(A) SCOP protein in cultured hippocampal neurons (day 18 in vitro, DIV 18) was monitored by western analysis various times after treatment with BDNF (50 ng/ml). The bar graph shows SCOP protein normalized to ERK-2. Error bars, SEM (n = 3).

(B) SCOP protein in cultured hippocampal neurons (DIV 18) was monitored by western analysis 10 min after treatment with KCl (50 mM) or NMDA (50 μ M). SCOP protein was normalized relative to ERK-2. Error bars, SEM (n = 3).

neural plasticity and memory formation (for reviews see Cline, 2003; Chan and Mattson, 1999). Neither the caspase-3 inhibitor nor lactacystin, a proteasome inhibitor, blocked Ca^{2+} -induced SCOP degradation (Figure 3B). Only calpeptin inhibited SCOP degradation, indicating that SCOP may be degraded by Ca^{2+} -stimulated calpain. We confirmed that calpain is activated when hippocampal neurons are treated with BDNF (data not shown).

There are two major calpain isoforms, calpain I (μ -form) and calpain II (m-form), which are activated at different

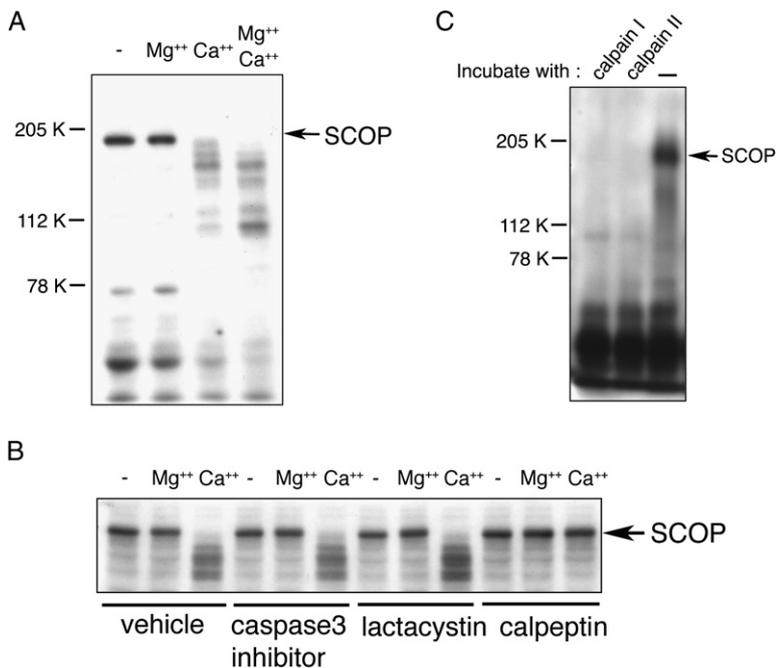


Figure 3. Calpain Catalyzes the Proteolysis of SCOP

(A) SCOP protein was monitored by western analysis after adding Mg²⁺ (10 mM) and/or Ca²⁺ (4 mM) to mouse brain lysates and incubating for 3 hr at 4°C.

(B) Calpeptin inhibited Ca²⁺-stimulated proteolysis of SCOP. The mouse brain extract was pretreated with a caspase-3 inhibitor (10 μM), lactacystin (5 μM), or calpeptin (10 μM) 30 min before adding Ca²⁺. SCOP protein was monitored by western analysis.

(C) Calpain I and II both catalyzed the proteolysis of SCOP in vitro. Calpain I or II was incubated with immunoprecipitated SCOP at 37°C for 1 hr. SCOP protein was monitored by western analysis.

Ca²⁺ concentrations. Within the brain, calpain I is predominantly neuronal, existing at higher levels in the dendrites and cell bodies, whereas relatively higher levels of calpain II have been observed in axons and glia (Nixon, 1986; Onizuka et al., 1995). To determine if SCOP is selectively proteolyzed by calpain I or II, we incubated purified calpain I and II with immunoprecipitated SCOP. Both calpains degraded SCOP protein in vitro (Figure 3C).

To ascertain if calpain proteolyzes SCOP in hippocampal neurons, we treated neurons with calpain inhibitor III and monitored SCOP degradation when BDNF was applied (Figure 4A). The decrease in SCOP levels caused by BDNF was completely blocked by calpain inhibitor III, and furthermore there was a significant increase in SCOP levels after treatment with the protease inhibitor. This suggests that SCOP levels are controlled by a balance between synthesis of the protein and degradation by calpain. This was confirmed by examining SCOP levels when hippocampal neurons were treated with calpain inhibitor III, without BDNF (Figure 4B). Endogenous SCOP protein increases approximately 2-fold by 90 min after treatment with the protease inhibitor. If SCOP negatively regulates MAPK signaling and calpain degrades SCOP, proteolytic degradation of SCOP may contribute to MAPK activation. Indeed, stimulation of MAPK activity in hippocampal neurons by BDNF was decreased at least 2-fold when calpain activity was blocked (Figure 4C).

SCOP Degradation Following Training for Novel Object Memory

Since activation of MAPK is required for object recognition memory (Kelly et al., 2003), it was of considerable interest to determine if the levels of SCOP protein decrease during training for object recognition memory. We measured

SCOP 5 min after training for novel object recognition (Figure 5). In this paradigm, a mouse is allowed to briefly examine two novel objects, A and B. When the mouse is tested at a later time, it is presented with one of the original objects, A, and a new object, C. If the mouse remembers object A, it will spend more time examining object C because of natural curiosity for new objects. Memory for novel objects is reflected by greater preference for the new object C during testing (Figure 5A). We examined SCOP levels following training for novel objects because this is a milder form of training that does not depend on shocking the animal. Untrained controls were handled and placed in the training cage without novel objects. Although the decrease in SCOP seen at 5 min after training for novel objects was only 16%, it was significant ($p < .01$), and was paralleled by an increase in MAPK activity ($p < .05$) (Figure 5B). SCOP protein also decreased when animals were trained for context (Figure S4). Presumably changes in SCOP and MAPK activity are much greater in neurons directly affected during training than that measured by western analysis of extracts from the entire hippocampus.

Overexpression of SCOP in the Hippocampus Blocks Memory for Novel Objects

To determine if hippocampus-dependent memory is sensitive to the amount of SCOP expressed in the hippocampus in vivo, we made a tetracycline-inducible, SCOP-overexpressing mouse strain using the CamKII α promoter to limit overexpression to the forebrain. The integration of transgene was verified by polymerase chain reaction (PCR)-based genotyping using specific primers for tetracycline response element-SCOP (*TRE-SCOP*) and *CamKII-rtTA2* transgene, respectively (Figure 6A). Doxycycline

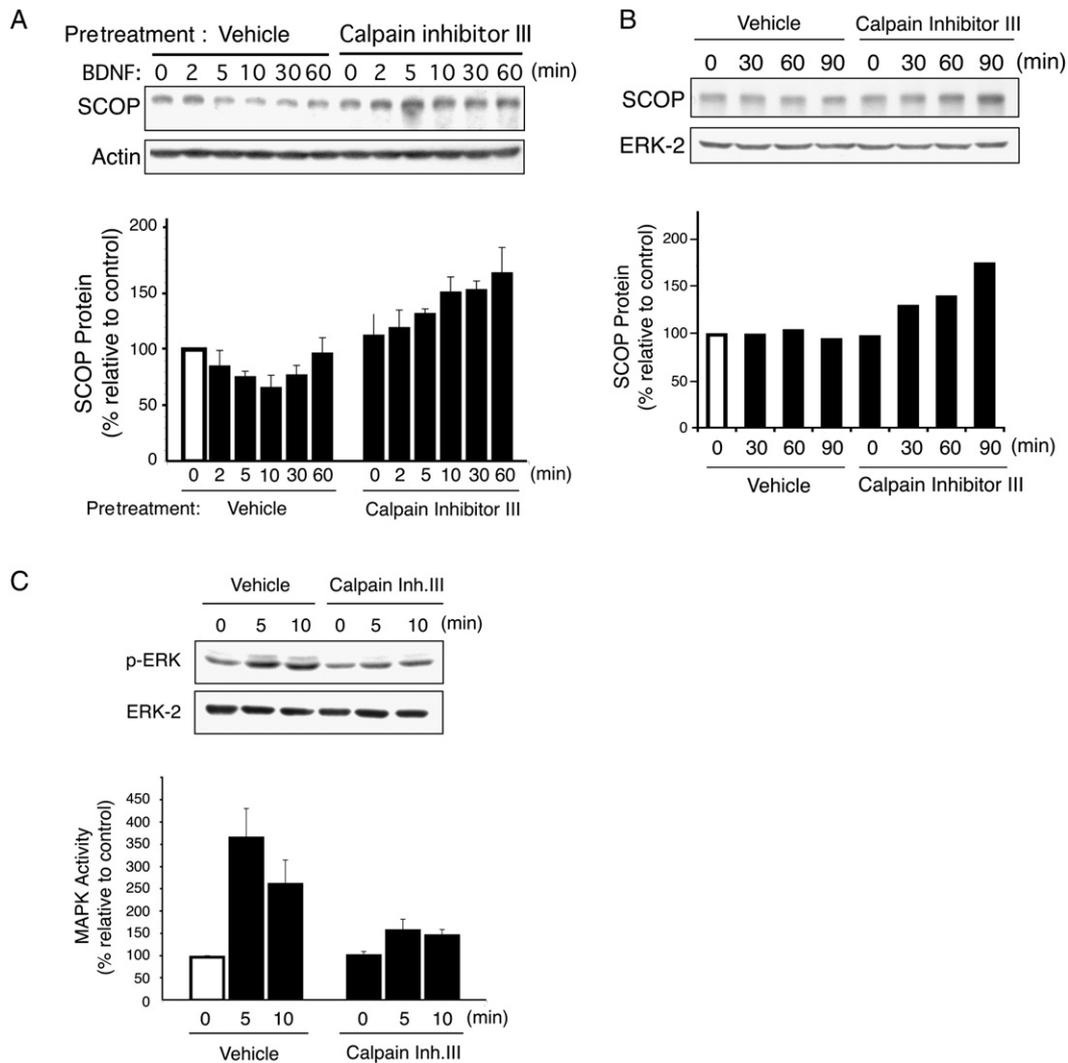


Figure 4. Inhibition of Calpain in Cultured Hippocampal Neurons Blocks SCOP Degradation and Inhibits Activation of MAPK

(A) Calpain inhibitor III inhibited BDNF-induced SCOP degradation in cultured hippocampal neurons. Neurons were pretreated with calpain inhibitor III (25 μ M) or vehicle 30 min before BDNF (5 ng/ml) stimulation and harvested at various times. SCOP protein was monitored by western analysis. SCOP protein was normalized to actin. Error bar, SEM (n=3).

(B) Treatment of hippocampal neurons with calpain inhibitor III increases SCOP. Neurons were pretreated with calpain inhibitor III (25 μ M) or vehicle and harvested at various times after applying calpain inhibitor III (25 μ M) or vehicle. SCOP was quantitated by western analysis. SCOP levels were normalized to ERK-2.

(C) Calpain inhibitor III inhibits BDNF stimulation of MAPK activity measured as pERK in cultured hippocampal neurons. Total ERK-2 measured on the same western blot was used as loading controls. The percentage change in pERK relative to the 0 time point is reported. Error bars, SEM (n = 3).

treated *TRE-SCOP* \times *CamKII-rtTA2* mice showed 1.5- to 2.5-fold higher SCOP in the hippocampus than those not treated with doxycycline (Figure 6A, right panel of western blot and graph); doxycycline-treated *TRE-SCOP* or *CamKII-rtTA2* mice showed no increase in SCOP compared to littermate controls. In control experiments, we verified that doxycycline treatment did not affect the memory of wild-type, *TRE-SCOP*, or *CamKII-rtTA2* mice for novel objects (data not shown). Overexpression of SCOP did not alter open-field activity or contextual memory indicating that general mobility and vision were unaf-

ected (data not shown). Doxycycline treatment of *TRE-SCOP* \times *CamKII-rtTA2* mice, which increases SCOP in the hippocampus, had no effect on memory for novel objects measured 8 min after training (Figure 6B) but completely blocked memory for novel objects measured 24 hr after training (Figure 6C). These data are consistent with other experiments showing that MAPK activity is required for memory consolidation but not acquisition or short-term memory (Blum et al., 1999; Kelleher et al., 2004). We confirmed that overexpression of SCOP in the transgenic mouse strain blocks decreases in SCOP protein and the

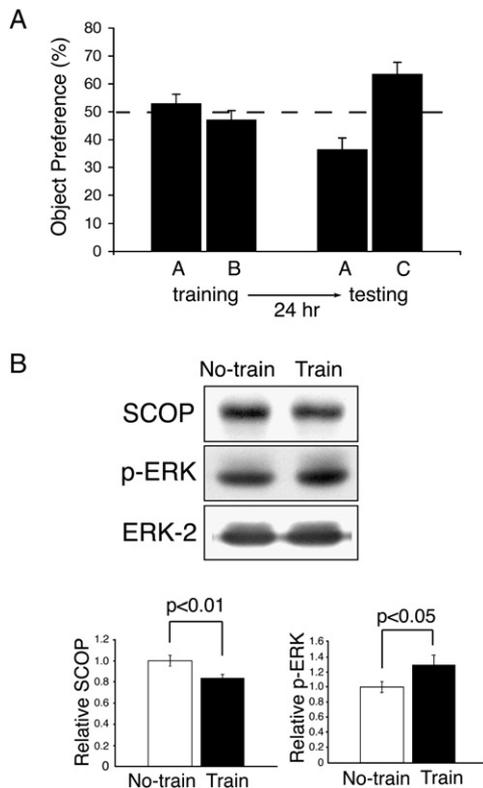


Figure 5. Training for Novel Object Memory Decreases SCOP in the Hippocampus

(A) The time spent exploring two novel objects (A and B) was measured for 5 min during training. Preference for one of the original objects (A) and a new object (C) was quantified 24 hr after training. The dotted line represents no object preference. Error bars, SEM ($n = 8$ mice).

(B) SCOP and p-ERK in the hippocampus from trained and untrained mice were analyzed by western analysis 5 min after training. Untrained mice were handled and placed in the training chamber without novel objects. SCOP and p-ERK were normalized to ERK-2 from the western blot. Error bars, SEM ($n = 9$).

increase in p-ERK associated with training for novel objects (Figure S2). When doxycycline-treated mice were allowed to recover for 3 months, they exhibited normal 24 hr memory for novel objects (Figure 6D). Overexpression of SCOP did not completely block the increases in p-ERK observed during training for context (Figure S5) and did not block contextual memory. We conclude that memory for novel objects is sensitive to the concentration of SCOP in the hippocampus.

The data described above indicates that memory for novel objects may depend on the levels of SCOP protein in the hippocampus. If calpain degradation of SCOP contributes to memory for novel objects, inhibition of calpain, *in vivo*, should block memory. Indeed, when a mixture of calpain inhibitors was bilaterally administered into area CA1 of the hippocampus, memory for novel objects measured 24 hr after training was completely blocked with no effect on short-term memory measured 8 min after training (Figure 7). This indicates that calpain activity is required for

novel object long-term memory, possibly because calpain degrades SCOP *in vivo*. We confirmed that the decrease in SCOP and increase in p-ERK observed 5 min after training for novel objects is blocked by infusion of calpain inhibitors into area CA1 of the hippocampus (Figure S3).

DISCUSSION

SCOP was originally identified as a protein whose expression is regulated in a circadian manner within the SCN (Shimizu *et al.*, 1999). The discovery that SCOP inhibits MAPK activity (Shimizu *et al.*, 2003) provides an explanation for the circadian oscillation of MAPK activity and CRE-mediated transcription in the SCN (Obrietan *et al.*, 1998). The presence of SCOP in the hippocampus raised the interesting possibility that it may inhibit MAPK in the hippocampus and play an important role in MAPK signaling during memory formation and other MAPK-regulated events. The objectives of this study were to determine if SCOP regulates CRE-mediated transcription in hippocampal neurons and to identify mechanisms for signaling through SCOP. Furthermore, it was important to determine if SCOP is regulated during training for hippocampus-dependent memory *in vivo* and if varying the levels of SCOP *in vivo* affects memory formation.

We discovered that SCOP inhibits CRE-mediated transcription in hippocampal neurons and that treatment with BDNF, KCl, or NMDA causes rapid degradation of SCOP protein. The decrease in SCOP is dependent upon increased intracellular Ca^{2+} and blocked by calpain inhibitors, suggesting that activity-dependent increases in Ca^{2+} stimulate calpain, which degrades SCOP. When cultured neurons are treated with calpain inhibitors, there is a constant rise in SCOP protein suggesting that the levels of SCOP reflect a balance between newly translated protein and proteolysis. Furthermore, BDNF stimulation of MAPK activity in hippocampus neurons is greatly reduced by calpain inhibitors supporting the hypothesis that calpain-stimulated degradation of SCOP is necessary for full activation of MAPK in neurons.

We believe that SCOP may regulate MAPK signaling *in vivo* because SCOP decreased and MAPK activity increased during training for novel objects or contextual memory. The decrease in SCOP protein, stimulation of MAPK, and memory for novel objects were completely blocked by overexpressing SCOP in the hippocampus. When SCOP was overexpressed, training for contextual fear still gave a measurable decrease in SCOP protein and an increase in MAPK activity. Since the MAPK activation is only partially blocked by SCOP overexpression during contextual training, the mice still exhibit memory for context. This suggests that the training stimulus during contextual training is more robust and leads to greater reduction in SCOP levels. We hypothesize that proteolytic degradation of SCOP releases a constraint on K-Ras activity and contributes to MAPK activation (Figure 8). This hypothesis is supported by data showing that memory for novel objects depends on calpain activity (Figure 7).

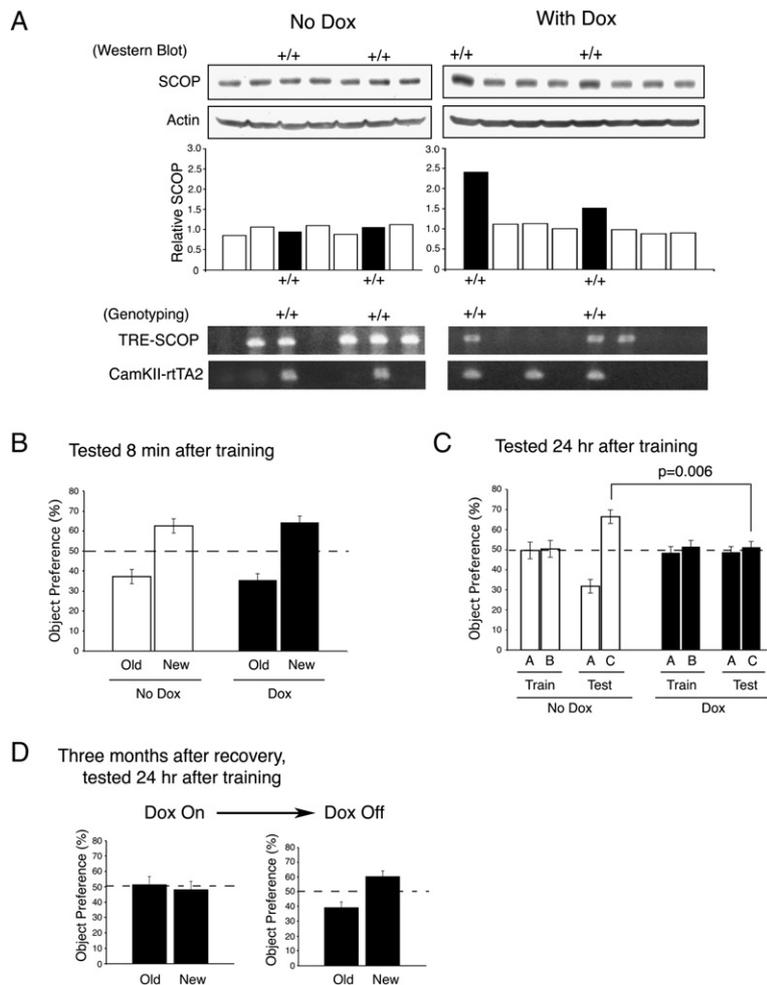


Figure 6. Overexpression of SCOP in the Hippocampus Blocks Long-Term but Not Short-Term Memory for Novel Objects

(A) SCOP in the hippocampus of *TRE-SCOP* × *CamKII-rTA2* transgenic mice was increased by feeding doxycycline (Dox). Upper panels show the SCOP western blot for each mouse, and the bar graphs reports SCOP normalized to actin. Lower panels show the corresponding PCR genotyping of the transgenic mice.

(B) Overexpression of SCOP (Dox) in the fore-brain did not block short-term memory for novel objects measured 8 min after training. Error bars, SEM (no Dox, $n = 8$ mice; Dox, $n = 13$).

(C) Overexpression of SCOP in the forebrain blocked memory for novel objects measured 24 hr after training. Error bars, SEM (no Dox, $n = 12$ mice; Dox, $n = 22$ mice).

(D) Doxycycline-treated mice were allowed to recover for 3 months after removal of doxycycline, trained for novel objects, and tested 24 hr after training. Error bars, SEM ($n = 8$ mice).

Our data does not exclude other mechanisms by which calpain may contribute to activation of MAPK. Although calpain-1 knockout mice show no apparent defects in LTP or fear conditioning (Grammer et al., 2005), SCOP is degraded by both calpain-1 and calpain-2. Consequently, definition of the role of calpains in memory formation through gene disruption may require conditional double knockouts of calpain-1 and calpain-2.

Considering the general importance of MAPK for neuroplasticity, one would expect that its activity in CNS neurons would be tightly regulated (for a review see Agell et al., 2002). There are several mechanisms for activation of MAPK by Ca^{2+} including direct activation through calcium-stimulated Ras GEFs (Farnsworth et al., 1995) and indirect mechanisms mediated through calcium-stimulated adenylyl cyclases and PKA. We propose that in unstimulated neurons, SCOP restrains MAPK activity and that transient decreases in SCOP caused by Ca^{2+} stimulation of calpain is required for activation of MAPK caused by neurotrophins and Ca^{2+} signals. Thus, activation of MAPK in neurons may depend upon a dynamic balance between SCOP degradation and MAPK-activating mechanisms.

Although this study focused on regulation of SCOP in hippocampal neurons and its relationship to memory formation, the data have broad implications because of the central role played by MAPK and CRE-mediated transcription in neuroplasticity (Frankland et al., 2003; Impey et al., 1999; Mazzucchelli and Brambilla, 2000; Sweatt, 2004). It seems likely that other in CNS neurons, including synaptic plasticity (Coogan et al., 1999; English and Sweatt, 1996), protein synthesis (Kelleher et al., 2004), circadian rhythm (Obrietan et al., 1999; Obrietan et al., 1998), modulation of K^+ ion channels (Yuan et al., 2002), and neuronal survival (Hetman et al., 1999), may depend upon SCOP protein and its regulated degradation. For example, the circadian oscillation of SCOP protein in the SCN correlates with the changes in MAPK activity during the circadian cycle (Shimizu et al., 1999). The extent to which regulation of SCOP levels contribute to these processes remains to be established.

In summary, maximal activation of MAPK activity in hippocampal neurons depends on degradation of SCOP by calpain. Training for novel object memory lowers SCOP in brain, and memory for novel objects was blocked when SCOP was increased 2-fold in the hippocampus.

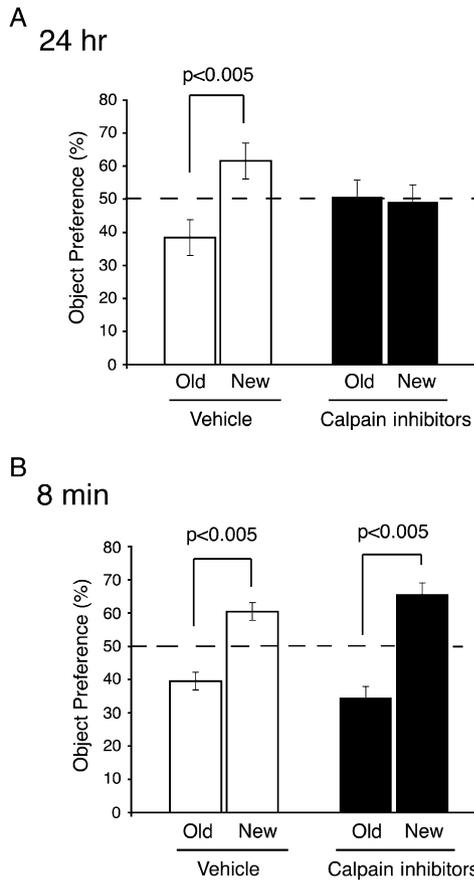


Figure 7. Inhibition of Calpain In Vivo Blocks Memory for Novel Objects

A calpain inhibitor cocktail containing a mixture of calpeptin, calpain inhibitor I and calpain inhibitor III or vehicle was bilaterally infused in area CA1 in the hippocampus. Mice were trained 45 min after infusion. Object preference was measured 24 hr after training (A) or 8 min after training (B). Error bars, SEM (n=10 (24 hr), n=8 (8 min) mice in each group). The dotted line represents performance by chance 50%.

We hypothesize that calpain-catalyzed degradation of SCOP in response to activity-dependent Ca^{2+} increases is required for activation of MAPK during formation of hippocampus-dependent memory.

EXPERIMENTAL PROCEDURES

Cell Culture

Neurons were cultured as previously described with some modifications (Chan et al., 1998). Primary hippocampal neurons were cultured from newborn C57BL6 pups and maintained in Neurobasal-A (GIBCO) with minimal supplements. Pups were sacrificed by decapitation, and the hippocampi were digested in 5 ml of 10 units/ml papain at 37°C for 30 min. After two rinses, the tissue was triturated three times in dissociation medium (27 mM K_2SO_4 , 15 mM $MgCl_2$, 74 mM Na_2SO_4 , 18 mM glucose, 225 μM $CaCl_2$, 0.0012% phenol red and 2 mM HEPES, pH 7.4) using a 5 ml disposable plastic pipette. Cells were plated into 24-well plates or 3 cm dishes coated with poly-D-lysine (50 $\mu g/ml$) at 5×10^4 cells/cm² in Neurobasal-A medium containing B-27 supplement with 10 units/ml penicillin, 10 $\mu g/ml$ streptomycin, 0.5 $\mu g/ml$

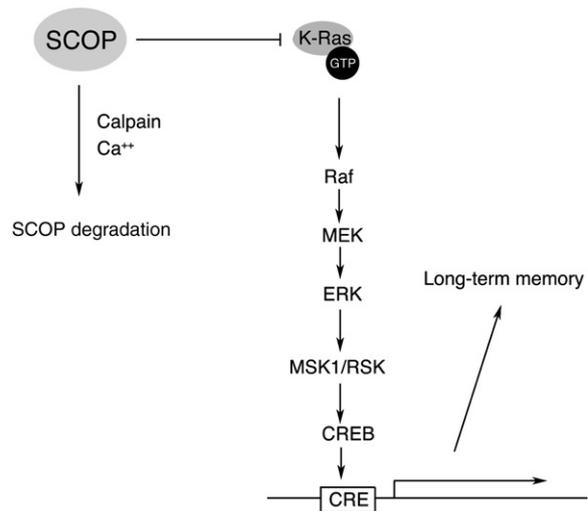


Figure 8. Model for Role of SCOP in MAPK Activation

It is hypothesized that SCOP attenuates MAPK activity in hippocampal neurons by binding to the nucleotide-free form of K-Ras, thereby inhibiting stimulation of the MAPK pathway. Calpain may contribute to ERK activation by degrading SCOP.

glutamine, and 1% fetal bovine serum. The medium was changed to Neurobasal-A with B-27 containing 0.5 $\mu g/ml$ glutamine and 2 μM AraC 24 hr after plating. Neurons were maintained in Neurobasal-A with B-27 and 0.5 $\mu g/ml$ glutamine, and half of the media was changed every 2 days. Plates were incubated in a CO₂ incubator at 37°C.

Preparation of siRNA

The siRNA targeted against SCOP mRNA was designed with a two-base 3' overhang. The target site was nucleotides 292–312 from the start codon (AACTCGTGCTGCTGAGGAGA). The single-strand siRNA nucleotides were chemically synthesized with dTdT 3' overhangs (CUCGCGUCGUCGUCGAGGAGAtt, UCUCUCAGCAGCAGCGGA Gtt) (Ambion). A scrambled sequence of the same nucleotide composition as the SCOP siRNA with no significant sequence homology to the genome was used as a negative control (UAUGCCGGCUGGA GUACCGtt, CGGUACUCCAGCCGGCAUAtt). Complementary pairs of siRNA oligonucleotides were annealed in annealing buffer (6 mM HEPES, pH 7.4, 20 mM potassium acetate, 0.4 mM magnesium acetate). The annealed double-stranded RNAs were confirmed by acrylamide electrophoresis. For transfection of neurons, 16 nM of siRNA was used.

Expression Plasmids

The full-length SCOP overexpression plasmid and CRE ($\alpha 168$)-luciferase have been described previously (Matthews et al., 1994; Shimizu et al., 2003).

Reporter Assays for CRE-Mediated Transcription

Cultured hippocampal neurons in 24-well plates at day 7 in vitro were transfected with a complex of 0.15 μg of CRE-luciferase, 0.15 μg of the internal control plasmid EF1 α -lacZ, 0.3 μg of the described plasmid (in each well of a 24-well plate), and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, 5 ng/ml BDNF in 0.2% BSA or vehicle (0.2% BSA) was applied. Reporter assays were conducted 6 hr post-BDNF stimulation. Luciferase activity was measured as previously described (Matthews et al., 1994) and normalized to β -galactosidase activity (Impey et al., 1996).

Immunohistochemistry

Animals were deeply anesthetized with Ketamine/Xylazine cocktail and intracardially perfused with saline containing 0.1% NaNO₂ and 2.0 mM EDTA. They were then continuously perfused with ice-cold 2% paraformaldehyde in PBS. Brains were removed, postfixed for 3 hr, and dehydrated in 30% sucrose in PBS overnight. The dehydrated brains were frozen in dry ice and sliced into 25- μ m-thick sections using a microtome. Sections were washed three times with 0.5% Triton X-100 in PBS and incubated in 0.5% Triton X-100, 0.5% BSA, and 1% normal goat serum for 30 min at 37°C. They were then incubated with anti-SCOP antibody (α EC) (1:50) overnight at 4°C (Shimizu et al., 1999). Immunoreactivity was detected by the conventional avidin-biotin complex method (Vectastain ABC Kit; Vector Lab) using 3-3' diaminobenzidine as chromogen. Brain sections were mounted using DPX mountant (Fluka).

Immunoprecipitation of SCOP

Whole brains from adult male mice were homogenized in 10-fold excess (v/v) of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA). The resulting homogenate was centrifuged at 10,000 \times g for 15 min. The supernatant was used for immunoprecipitation and pre-cleared on protein A agarose beads (Oncogene) at 4°C for 2 hr incubation on a rotating wheel. The precleared cell lysate was incubated overnight at 4°C with a C-terminal SCOP antibody (Shimizu et al., 1999). New protein agarose beads were added and incubated for 2 hr at 4°C. The beads were washed four times with buffer A.

Western Analysis

Mice were sacrificed by cervical dislocation and the hippocampi were rapidly dissected and immediately frozen in dry ice. Two hippocampi were sonicated for 10 s in the sonication buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 5 mM EGTA, 5 mM β -mercaptoethanol, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride, pH 7.4) with a protease inhibitor cocktail (complete mini, EDTA-free, Roche) and phosphatase inhibitor cocktail I and II (Sigma). Neurons from 3 cm culture dishes were treated with 150 μ l of 2 \times SDS sample buffer and cells were scraped from the dish. The resulting homogenates were treated with SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE and western analysis.

Housing and Handling of Mice

Male C57BL6 mice at 8–11 weeks of age were used. Food and water were provided ad libitum, and animals were kept on a 12 hr light/dark cycle. To reduce handling-induced stress, mice were housed individually and handled daily for one week prior to experiments.

Behavioral Assays

Context-trained mice were placed into a conditioning chamber (Solid State Shocker/Distributor; Coulbourn Instruments, Allentown, PA) and allowed to explore. After 2 min, they were shocked one time (0.7 mA, 2 s). Mice were then allowed to recover for 1 min and returned to home cages. Unpaired controls were shocked once for 2 s immediately after being placed in context. Novel object-trained mice were first habituated in a training rat cage for 3–4 hr before training. After this, two different objects A and B were presented for 5 min, after which they were returned to their home cage. They were tested in the training cage with a new object C and one of the original objects A or B. During training and testing, the time spent examining each object was recorded for 5 min. Memory for familiar objects is manifested as a preference for the new object C during testing. Control mice were placed into the training cage with no novel objects.

Cannulation and Calpain Inhibitor Infusion

Mice (8 weeks old) were cannulated as described previously (Athos et al., 2002). Briefly, mice were anesthetized with an intraperitoneal injection (20 μ l/g body weight) of a mixture of ketamine (7.0 mg/ml) and xylazine (0.44 mg/ml) dissolved in bacteriostatic saline. Anesthetized

mice were mounted on a stereotaxic frame (10 micron model, Cartesian Research), and 30-gauge dummy cannulae were implanted just above the pyramidal cell layer of CA1 region of dorsal hippocampus. Cannulae resided 1.5 mm lateral, 1.25 mm ventral, and 1.5 mm posterior from bregma. The cannulae were affixed with dental acrylic and fitted with 24-gauge guide cannulae to maintain cannula position. Mice were housed individually and allowed at least 1 week of postoperative recovery before handling. Calpain inhibitor I, calpain inhibitor III, and calpeptin were dissolved in DMSO and further diluted in 10% Cremophor EL (SIGMA) in saline so that the final concentrations of inhibitors were 2 mM each. Drug and vehicle solutions had a final concentration of 18% DMSO and 8.2% Cremophor EL. One-half microliters of drug or vehicle was infused bilaterally into the cannulae through a 30-gauge injection needle 45 min before object recognition training. The final concentrations of the inhibitors in the hippocampus were 20 μ M. The infusions were carried out at a rate of 0.25 μ l/min using a motorized syringe pump (kdScientific).

Generation of Tet-on SCOP Overexpression Mouse

Complementary DNA encoding rat SCOP was inserted into the multicloning site of pTRE2 (BD Biosciences) so that the expression of SCOP was placed under the control of Tet-responsive promoter (TRE). A TRE-SCOP-poly A cassette from the subcloned plasmid was injected (pronuclear) into C57BL6/C3H X C57BL6 embryos. The genotype was determined by PCR using transgene-specific primers. Transgenic mice were bred into the C57BL6 background for at least six generations. Manipulations were approved and in accordance with the animal care committee's guidelines at the University of Washington.

SCOP Overexpression in the Forebrain

CamKII α promoter-rtTA2 (*CamKII-rtTA2*) mouse was generously provided by Dr. Isabelle Mansuy's laboratory at the Swiss Federal Institute of Technology. *CamKII-rtTA2* mice were mated with *TRE-SCOP* mice to obtain double transgenic mice, which allowed SCOP overexpression in the forebrain to be mice controlled by tetracycline. Genotyping was determined by PCR. Control mice were littermates carrying no transgene or either one of the transgenes. Before testing, mice were fed doxycycline (SIGMA) at 6 mg per g food, as indicated. For all experiments, we used male mice 2–5 months old for behavioral studies.

Supplemental Data

Supplemental Data include five figures and can be found with this article online at <http://www.cell.com/cgi/content/full/128/6/1219/DC1/>.

ACKNOWLEDGMENTS

We thank Dr. Katsuya Nagai at Y.M.P. International Corporation, Dr. Masato Okada at the Research Institute for Microbial Diseases of Osaka University, and Dr. Yoshitaka Fukuda at the University of Tokyo for laying the groundwork for SCOP protein. And we thank members of the Storm lab for critical reading of the manuscript. This research was supported by NIH grant NS 20498.

Received: April 4, 2006

Revised: November 28, 2006

Accepted: December 29, 2006

Published: March 22, 2007

REFERENCES

Agell, N., Bachs, O., Rocamora, N., and Villalonga, P. (2002). Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. *Cell. Signal.* 14, 649–654.

- Athos, J., Impey, S., Pineda, V.V., Chen, X., and Storm, D.R. (2002). Hippocampal CRE-mediated gene expression is required for contextual memory formation. *Nat. Neurosci.* *5*, 1119–1120.
- Atkins, C.M., Selcher, J.C., Petraitis, J.J., Trzaskos, J.M., and Sweatt, J.D. (1998). The MAPK cascade is required for mammalian associative learning. *Nat. Neurosci.* *1*, 602–609.
- Berninger, B., Garcia, D.E., Inagaki, N., Hahnel, C., and Lindholm, D. (1993). BDNF and NT-3 induce intracellular Ca²⁺ elevation in hippocampal neurones. *Neuroreport* *4*, 1303–1306.
- Blum, S., Moore, A.N., Adams, F., and Dash, P.K. (1999). A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J. Neurosci.* *19*, 3535–3544.
- Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* *79*, 59–68.
- Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C.E., Ramsey, M., Wolfer, D.P., Cestari, V., RossiArnaud, C., et al. (1997). A role for the Ras signalling pathway in synaptic transmission and long-term memory. *Nature* *390*, 281–286.
- Chan, S.L., and Mattson, M.P. (1999). Caspase and calpain substrates: roles in synaptic plasticity and cell death. *J. Neurosci. Res.* *58*, 167–190.
- Chan, G.C., Hinds, T.R., Impey, S., and Storm, D.R. (1998). Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. *J. Neurosci.* *18*, 5322–5332.
- Cline, H. (2003). Synaptic plasticity: importance of proteasome-mediated protein turnover. *Curr. Biol.* *13*, R514–R516.
- Coogan, A.N., O'Leary, D.M., and O'Connor, J.J. (1999). P42/44 MAP kinase inhibitor PD98059 attenuates multiple forms of synaptic plasticity in rat dentate gyrus in vitro. *J. Neurophysiol.* *81*, 103–110.
- Dash, P.K., Blum, S., and Moore, A.N. (2000). Caspase activity plays an essential role in long-term memory. *Neuroreport* *11*, 2811–2816.
- de Rooij, J., Zwartkruis, F.J.T., Verheijen, M.H.G., Cool, R.H., Hijman, S.M.B., Wittinghofer, A., and Bos, J.L. (1998). Epac is a Rap1 guanine-nucleotide exchange factor directly activated by cAMP. *Nature* *396*, 474–477.
- Di Virgilio, F., Milani, D., Leon, A., Meldolesi, J., and Pozzan, T. (1987). Voltage-dependent activation and inactivation of calcium channels in PC12 cells. Correlation with neurotransmitter release. *J. Biol. Chem.* *262*, 9189–9195.
- Dingledine, R. (1983). Excitatory amino acids: modes of action on hippocampal pyramidal cells. *Fed. Proc.* *42*, 2881–2885.
- English, J.D., and Sweatt, D.J. (1996). Activation of p42 MAP kinase in hippocampal long term potentiation. *J. Biol. Chem.* *271*, 24329–24332.
- Farnsworth, C.L., Freshney, N.W., Rosen, L.B., Ghosh, A., Greenberg, M.E., and Feig, L.A. (1995). Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature* *376*, 524–527.
- Frankland, P.W., Ohno, M., Takahashi, E., Chen, A.R., Costa, R.M., Kushner, S.A., and Silva, A.J. (2003). Pharmacologically regulated induction of silent mutations (PRISM): combined pharmacological and genetic approaches for learning and memory. *Neuroscientist* *9*, 104–109.
- Grammer, M., Kuchay, S., and Baudry, M. (2005). Lack of phenotype for LTP and fear conditioning learning in calpain 1 knock-out mice. *Neurobiol. Learn. Mem.* *84*, 222–227.
- Heerssen, H.M., and Segal, R.A. (2002). Location, location, location: a spatial view of neurotrophin signal transduction. *Trends Neurosci.* *25*, 160–165.
- Hegde, A.N. (2004). Ubiquitin-proteasome-mediated local protein degradation and synaptic plasticity. *Prog. Neurobiol.* *73*, 311–357.
- Hetman, M., and Xia, Z. (2000). Signaling pathways mediating anti-apoptotic action of neurotrophins. *Acta Neurobiol. Exp.(Wars)* *60*, 531–545.
- Hetman, M., Kanning, K., Cavanaugh, J.E., and Xia, Z. (1999). Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J. Biol. Chem.* *274*, 22569–22580.
- Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C., and Storm, D.R. (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. *Neuron* *16*, 973–982.
- Impey, S., Smith, D.M., Obrietan, K., Donahue, R., Wade, C., and Storm, D.R. (1998a). Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat. Neurosci.* *1*, 595–601.
- Impey, S., Obrietan, K., Wong, S.T., Poser, S., Yano, S., Wayman, G., Delouille, J.C., Chan, G., and Storm, D.R. (1998b). Cross talk between ERK and PKA is required for Ca²⁺ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* *21*, 869–883.
- Impey, S., Obrietan, K., and Storm, D.R. (1999). Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron* *23*, 11–14.
- Kelleher, R.J., 3rd, Govindarajan, A., Jung, H.Y., Kang, H., and Tonegawa, S. (2004). Translational control by MAPK signaling in long-term synaptic plasticity and memory. *Cell* *116*, 467–479.
- Kelly, A., Laroche, S., and Davis, S. (2003). Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory. *J. Neurosci.* *23*, 5354–5360.
- Li, Y.X., Zhang, Y., Lester, H.A., Schuman, E.M., and Davidson, N. (1998). Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. *J. Neurosci.* *18*, 10231–10240.
- Lopez-Salon, M., Alonso, M., Vianna, M.R., Viola, H., Mello e Souza, T., Izquierdo, I., Pasquini, J.M., and Medina, J.H. (2001). The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *Eur. J. Neurosci.* *14*, 1820–1826.
- Mabuchi, T., Kitagawa, K., Kuwabara, K., Takasawa, K., Ohtsuki, T., Xia, Z., Storm, D., Yanagihara, T., Hori, M., and Matsumoto, M. (2001). Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate in vitro and ischemia in vivo. *J. Neurosci.* *21*, 9204–9213.
- Matthews, R.P., Guthrie, C.R., Wailes, L.M., Zhao, X., Means, A.R., and McKnight, G.S. (1994). Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.* *14*, 6107–6116.
- Mazzucchelli, C., and Brambilla, R. (2000). Ras-related and MAPK signalling in neuronal plasticity and memory formation. *Cell. Mol. Life Sci.* *57*, 604–611.
- Mazzucchelli, C., Vantaggiato, C., Ciamei, A., Fasano, S., Pakhotin, P., Krezel, W., Welzl, H., Wolfer, D.P., Pages, G., Valverde, O., et al. (2002). Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron* *34*, 807–820.
- Nixon, R.A. (1986). Fodrin degradation by calcium-activated neutral proteinase (CANP) in retinal ganglion cell neurons and optic glia: preferential localization of CANP activities in neurons. *J. Neurosci.* *6*, 1264–1271.
- Obrietan, K., Impey, S., Smith, D., Athos, J., and Storm, D.R. (1999). Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J. Biol. Chem.* *274*, 17748–17756.

- Obrietan, K., Impey, S., and Storm, D.R. (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nat. Neurosci.* *1*, 693–700.
- Ohno, M., Frankland, P.W., Chen, A.P., Costa, R.M., and Silva, A.J. (2001). Inducible, pharmacogenetic approaches to the study of learning and memory. *Nat. Neurosci.* *4*, 1238–1243.
- Onizuka, K., Kunimatsu, M., Ozaki, Y., Muramatsu, K., Sasaki, M., and Nishino, H. (1995). Distribution of mu-calpain proenzyme in the brain and other neural tissues in the rat. *Brain Res.* *697*, 179–186.
- Pittenger, C., Huang, Y.Y., Paletzki, R.F., Bourtchouladze, R., Scanlin, H., Vronskaya, S., and Kandel, E.R. (2002). Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. *Neuron* *34*, 447–462.
- Shimizu, K., Okada, M., Takano, A., and Nagai, K. (1999). SCOP a novel gene product expressed in a circadian manner in rat suprachiasmatic nucleus. *FEBS Lett.* *458*, 363–369.
- Shimizu, K., Okada, M., Nagai, K., and Fukada, Y. (2003). Suprachiasmatic nucleus circadian oscillatory protein, a novel binding partner of K-Ras in the membrane rafts, negatively regulates MAPK pathway. *J. Biol. Chem.* *278*, 14920–14925.
- Silva, A.J., Frankland, P.W., Marowitz, Z., Friedman, E., Lazlo, G., Cioffi, D., Jacks, T., and Bourtchouladze, R. (1997). A mouse model for the learning and memory deficits associated with neurofibromatosis type I. *Nat. Genet.* *15*, 281–284.
- Sindreu, C.B., Schiner, Z.S., and Storm, D.R. (2007). Ca^{2+} -stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. *Neuron* *53*, 79–89.
- Sweatt, J.D. (2004). Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr. Opin. Neurobiol.* *14*, 311–317.
- Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S., and Stork, P.J. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* *89*, 73–82.
- Wang, H., and Storm, D.R. (2003). Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. *Mol. Pharmacol.* *63*, 463–468.
- Watt, W., Sakano, H., Lee, Z., Reusch, J., Trinh, K., and Storm, D. (2004). Odorant stimulation enhances survival of olfactory sensory neurons via MAPK and CREB. *Neuron* *41*, 955–967.
- Xing, J., Ginty, D.D., and Greenberg, M.E. (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* *273*, 959–963.
- Yuan, L.L., Adams, J.P., Swank, M., Sweatt, J.D., and Johnston, D. (2002). Protein kinase modulation of dendritic K^+ channels in hippocampus involves a mitogen-activated protein kinase pathway. *J. Neurosci.* *22*, 4860–4868.