Research

Activin plays a key role in the maintenance of long-term memory and late-LTP

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A recent study has revealed that fear memory may be vulnerable following retrieval, and is then reconsolidated in a protein synthesis-dependent manner. However, little is known about the molecular mechanisms of these processes. Activin β A, a member of the TGF- β superfamily, is increased in activated neuronal circuits and regulates dendritic spine morphology. To clarify the role of activin in the synaptic plasticity of the adult brain, we examined the effect of inhibiting or enhancing activin function on hippocampal long-term potentiation (LTP). We found that follistatin, a specific inhibitor of activin, blocked the maintenance of late LTP (L-LTP) in the hippocampus. In contrast, administration of activin facilitated the maintenance of early LTP (E-LTP). We generated forebrain-specific activin- or follistatin-transgenic mice in which transgene expression is under the control of the Tet-OFF system. Maintenance of hippocampal L-LTP was blocked in the follistatin-transgenic mice. In the contextual fear-conditioning test, we found that follistatin blocked the formation of long-term memory (LTM) without affecting short-term memory (STM). Furthermore, consolidated memory was selectively weakened by the expression of follistatin during retrieval, but not during the maintenance phase. On the other hand, the maintenance of memory was also influenced by activin overexpression during the retrieval phase. Thus, the level of activin in the brain during the retrieval phase plays a key role in the maintenance of long-term memory.

[Supplemental material is available online at <http://www.learnmem.org>.]

Formation of long-term memory (LTM) consists of several distinct processes: acquisition, consolidation, and reconsolidation, through which memory becomes permanent (Nader et al. 2000; Rodrigues et al. 2004; Tronson and Taylor 2007; Kitamura et al. 2009). The prominent feature of LTM is a requirement for RNA and protein synthesis for consolidation and reconsolidation (Squire and Barondes 1973; Bourchouladze et al. 1994; Silva et al. 1998; Tronson and Taylor 2007; Lee et al. 2008). The reconsolidation process may serve to strengthen or renew the original fear memory (Nader 2003; Tronson and Taylor 2007). Synaptic plasticity is thought to underlie memory formation. Recent studies have shown that the learning process induces long-term potentiation (LTP) (Rogan et al. 1997; Rioult-Pedotti et al. 2000), a form of synaptic plasticity, and, conversely, that LTP is necessary for memory formation (Rodrigues et al. 2004). Similar to LTM,

LTP requires protein and RNA synthesis for its prolonged maintenance, late LTP (L-LTP) (Frey et al. 1988; Abraham et al. 1993; Nguyen et al. 1994; Fukazawa et al. 2003).

In order to understand the molecular mechanism of LTM, we previously isolated a number of neuronal activity-dependent genes, including *activin β A*, *ubiquitin C-terminal hydrolase, vesl-1S/homer-1a*, and *SCRAPPER* (Inokuchi et al. 1996; Hegde et al. 1997; Kato et al. 1997; Yao et al. 2007; Okada et al. 2009). *Activin β A*, a member of the TGF- β superfamily (Massague 1998), is one of the genes whose expression is up-regulated following L-LTP induction (Andreasson and Worley 1995; Inokuchi et al. 1996). Activin is a multifunctional ligand that regulates the proliferation and differentiation of numerous cell types (Mather et al. 1997; Ying et al. 1997). Activin binds to the serine/threonine kinase receptor activin type II (ActRII) that is located on the cell membrane (Pangas and Woodruff 2000). Once ligand is bound, the type II receptor recruits and phosphorylates an activin type I receptor (ActRI). Following stimulation by activin, the transcription factors Smad2 and Smad3 are phosphorylated by ActRI

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(Pangas and Woodruff 2000). Activin receptor ActRII is highly expressed in the forebrain region (Cameron et al. 1994; Funaba et al. 1997), and its scaffold protein ARIP/S-SCAM is also localized to the synaptic region (Hirao et al. 1998; Shoji et al. 2000).

Recent studies reveal that activin regulates the morphology of dendritic spines (Shoji-Kasai et al. 2007) and neurogenesis (Ageta et al. 2008; Abdipranoto-Cowley et al. 2009; Sekiguchi et al. 2009), has a neuroprotective function (Tretter et al. 2000), and plays a role in anxiety-related behavior (Dow et al. 2005; Ageta et al. 2008; Zheng et al. 2008). We previously found that activin increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics (Shoji-Kasai et al. 2007). We hypothesized that activin modulates spine actin dynamics and that this in turn affects LTP persistence. Furthermore, activin induces phosphorylation of the *N*-methyl-D-aspartate receptor (NMDAR), a key component in the formation

of LTP (Nicoll and Malenka 1999), and increases Ca^{2+} influx through NMDAR (Kurisaki et al. 2008). This activin-induced NMDAR activation persists for >24 h. These results suggest that activin has an important role in the formation of L-LTP and LTM.

Results

Activin is indispensable for in vivo L-LTP

We examined the hippocampal dentate gyrus LTP of urethane-anesthetized rats. A strong high-frequency stimulation (HFS, five 400-pulse trains at 400 Hz) produced a long-lasting L-LTP in vivo that persisted for 24 h (Fig. 1A). However, when the activin inhibitor follistatin (0.5 μg) (Nakamura et al. 1990; Sugino et al. 1997) or an anti-activin A antibody (0.6 μg) was pre-injected into the lateral ventricle, the strong HFS induced an LTP that

Figure 1. Activin is required for the maintenance of dentate gyrus L-LTP in vivo. (A) Effect of follistatin, anti-activin A, and anisomycin on LTP persistence. A strong HFS was delivered at time 0 (thick arrow). (B) Effect of activin on LTP persistence. A weak HFS was delivered at time 0 (thin arrow). (C) Basal synaptic transmission was not affected by follistatin, anti-activin A, or activin. The average of the fEPSP slope during the 15 min prior to time 0 served as the baseline (100%) for all trials. (*) $P < 0.05$, (**) $P < 0.005$ between the vehicle and experimental groups as determined by one-way ANOVA followed by Fisher's LSD test. At the end of each experiment, a second HFS (100 pulses at 100 Hz) was delivered (red arrows at 24 h). (Top panels in A,B) Typical fEPSP traces evoked at the times (1, 2, or 3) indicated in each graph. Error bars indicate SEM.

decayed rapidly and returned to basal levels by 9 h (Fig. 1A). The decay time course is similar to that of animals injected with the protein synthesis inhibitor anisomycin (Fig. 1A). In contrast, follistatin or anti-activin A had no significant effect on the initial amplitude and early maintenance of LTP (E-LTP). The effect of follistatin was dose-dependent because the injection of a lower dose (0.05 μ g) did not alter the maintenance of L-LTP (Fig. 1A). To exclude the possibility that the LTP decay caused by follistatin or the anti-activin A antibody was due to irreversible damage to hippocampal neurons, a second HFS (100 pulses at 100 Hz) was applied to the same pathway at the end of each experiment (24 h). We observed an enhancement of the field excitatory postsynaptic potential (fEPSP) slope in all animals (Fig. 1A), which eliminates the possibility that neuronal damage occurred.

In a complementary experiment where activin was pre-injected into the lateral ventricle, we used a weak HFS (a 50-pulse train at 100 Hz). The weak HFS alone elicited E-LTP that returned to the basal level by 6 h (Fig. 1B). However, pre-administration of activin facilitated the maintenance of LTP, since the weak HFS under these conditions produced an LTP that lasted >9 h. Injection of follistatin, anti-activin A, or activin into the lateral ventricle had no significant effect on basal synaptic transmission (Fig. 1C).

When injected 1 h after the delivery of the weak HFS, activin still enhanced E-LTP (Supplemental Fig. S1A). However, activin failed to facilitate LTP maintenance when administered 3 h after the weak HFS. Consistent with this result is the observation that the inhibitory effect of follistatin on L-LTP establishment was observed when it was injected 1 h, but not 3 h, after the delivery of the strong HFS (Supplemental Fig. S1B).

Generation of activin and follistatin transgenic mice using a forebrain-specific Tet-OFF system

To examine the role activin plays in fear memory formation, activin activity was genetically suppressed or increased in the forebrain of transgenic mice carrying a Tet-OFF system. In this system, the tetracycline-controlled transactivator (tTA) is under the control of the CaMKII α promoter to achieve forebrain-specific expression (Fig. 2A; Mayford et al. 1996). We generated two distinct lines of responder mice, ABI and FBI, in which activin and follistatin, respectively, were controlled by the tetracycline response element (TRE) promoter. Double (FBI α TA and ABI α TA)-transgenic mice were obtained by heterozygous crossings. A diet containing DOX (6 mg/g food) was fed to pregnant mice for 7 consecutive days immediately prior to the expected date of confinement to reduce leak expression of follistatin or activin during the late embryonic stage. Double-transgenic ABI α TA and FBI α TA mice were fertile, bred normally, and maintained a normal body weight under the no-DOX treatment.

The tTA binds specifically to the TRE promoter and activates transcription in the absence of DOX (Mayford et al. 1996). In these mice, TRE bidirectionally regulated LacZ (Fig. 2A). Thus, LacZ activity was detected in some brain regions including the hippocampus, striatum, and amygdala in ABI α TA, but not ABI, mice in the absence of DOX treatment. LacZ activity was also detected in some brain regions including the hippocampus and cortex in FBI α TA, but not FBI, mice in the absence of DOX treatment (Fig. 2B; Supplemental Fig. S2). An ELISA analysis revealed that the activin level in the hippocampus of ABI mice (0.06 ng/mg protein, Fig. 2C) was equivalent to that of wild-type mice (Ageta et al. 2008), showing no leaky transgene expression in ABI mice. Moreover, the follistatin level in the hippocampus of FBI α TA mice (0.25–0.5 ng/mg protein) was sufficient to antagonize the endogenous hippocampal activin (0.06 ng/mg protein) in the absence of DOX treatment (Sugino et al. 1997). DOX

administration decreased the ectopic activin and follistatin to basal levels within 3 d in the hippocampus of ABI α TA and FBI α TA mice (Fig. 2C). Suppression by DOX was reversible and almost completely recovered within 3 d for FBI α TA and 14 d for ABI α TA (Fig. 2C). There was no significant difference in the activin level in the hippocampus of ABI and ABI α TA mice on days 1 and 7 (Fig. 2C). No transgene expression was observed in the cerebellum and medulla of ABI α TA and FBI α TA mice in the absence of DOX treatment.

The maintenance of L-LTP is reduced in FBI α TA mice

Hippocampal slices were prepared from 6- to 10-wk-old FBI and FBI α TA mice, and fEPSPs were recorded in the hippocampal CA1 region after the application of single test stimulus every 20 sec (0.05 Hz) to the Schaffer collaterals. We first examined whether the basic property of excitatory synaptic transmission was normal in FBI α TA mice. We measured the initial slope of fEPSP in order to quantify the strength of the synaptic response. The input–output relationship in FBI α TA mice was similar to that of FBI mice (Fig. 3A). In the CA1 region, paired stimulation with short intervals usually causes paired-pulse facilitation (PPF). The extent of PPF with interpulse intervals of 25–500 msec of FBI α TA mice was identical to that of FBI mice (Fig. 3B). These results suggest that there are no physiological differences in the basic properties of excitatory synaptic transmission between FBI α TA and FBI mice.

We then examined whether E-LTP in the CA1 region was altered in FBI α TA mice. After a baseline recording of at least 15 min, LTP was induced by tetanic stimulation. A single train of tetanic stimulation, 100 Hz for 1 sec, elicited identical E-LTP in both strains of mice ($136.2 \pm 14.0\%$ and $144.8 \pm 16.0\%$, 90–120 min after tetanic stimulation in FBI α TA and FBI mice, respectively). Three trains of tetanic stimulation at 20-sec intervals caused much larger E-LTP than that after a single train of tetanus, but E-LTP in FBI α TA mice was similar to that in FBI mice ($161.8 \pm 9.3\%$ and $175.8 \pm 8.0\%$, 90–120 min after tetanic stimulation in FBI α TA and FBI mice, respectively; Fig. 3C). Thus, E-LTP was unaffected in FBI α TA mice.

In contrast, L-LTP in FBI α TA mice was markedly reduced as compared with the robust L-LTP observed in FBI mice (Fig. 3C). The slope of fEPSP 3–3.5 h and 5–5.5 h after tetanic stimulation in FBI α TA mice was $148.6 \pm 11.0\%$ and $139.0 \pm 10.2\%$, whereas that in FBI mice was $174.6 \pm 10.2\%$ and $167.6 \pm 13.2\%$, respectively ($P < 0.05$). The fEPSPs elicited by stimulation of the second pathway to activate an independent set of Schaffer collaterals were unaltered throughout the experiment in both strains of mice. To clarify the biochemical effect of follistatin transgene expression, we analyzed the phosphorylation level of Smad 2/3 in the hippocampus of FBI and FBI α TA. Phosphorylation of Smad 2/3 was significantly decreased in the hippocampus of FBI α TA but not FBI (Supplemental Fig. S3). These results demonstrate that L-LTP was reduced in FBI α TA mice, whose follistatin levels had an ability to suppress endogenous activin activity in the hippocampus.

Anxiety levels of ABI α TA and FBI α TA

Activin has multiple roles in the brain; for example, it influences anxiety-related behavior (Dow et al. 2005; Ageta et al. 2008; Zheng et al. 2008), modulates postnatal neurogenesis (Ageta et al. 2008), and protects neurons from ischemic damage (Tretter et al. 2000). In our previous study, we generated ACM4 and FSM transgenic mice in which activin and follistatin, respectively, were overexpressed in a forebrain-specific manner under the control of the α CaMKII promoter. FSM mice exhibited enhanced anxiety compared with wild-type littermates, while ACM4 mice showed reduced anxiety (Ageta et al. 2008). Therefore, we performed two behavioral analyses, such as a light and dark test and

Figure 2. Generation of activin and follistatin transgenic mice using a forebrain-specific Tet-OFF system. (A) Schematic transgene representations of ABI (upper), FBI (middle), and tTA (lower) constructs. (TRE) Tetracycline response element, (tTA) tetracycline-controlled transactivator, (pA) polyadenylation signal. (B) X-gal staining. (Upper row) Coronal brain sections from ABI, ABItTA, FBI, and FBItTA mice (10–15 wk old). (Lower row) Enlarged images of the hippocampus. Scale bars, 500 μ m. (C) Measurement of activin and follistatin level using an ELISA assay with anti-activin and anti-follistatin antibodies. (Red line) Maximal levels of activin (left panel) or follistatin (right panel) in the hippocampus in the absence of DOX (10–15 wk old). Mice were fed DOX for 3 consecutive days (from noon of day –3 to noon of day 0, orange bars). The animals were then sacrificed and the hippocampus was dissected out in the afternoon of the day indicated and used for the ELISA. (ND) Not detected. The number above each bar indicates the number of animals used. Error bars indicate the SEM. (*) $P < 0.05$, (**) $P < 0.001$ compared with activin in the hippocampus of ABI mice in the absence of DOX, as determined by one-way ANOVA followed by Fisher's LSD test.

risk-taking behavior test, to examine the anxiety levels of FBItTA and ABItTA mice. In a risk-taking behavior test the amount of time spent in the center of an open field strongly correlates with an animal's level of anxiety (Ageta et al. 2008). The double-transgenic FBItTA and ABItTA mice showed normal anxiety-like behavior in the light–dark and risk-taking behavior tests (Supplemental Fig. S4A,B), suggesting that these mice show normal responses compared with the mutant mice used in previous work (ACM and FSM, respectively; Ageta et al. 2008). Furthermore, the performance of the double-transgenic mice

(ABItTA and FBItTA) was comparable to single-transgenic mice (ABI and FBI) in sensitivity to electric footshock (Supplemental Fig. S4C). Neurogenesis in the adult hippocampus was reduced in FBItTA mice, but the reduction was less severe than in FSM mice (Supplemental Fig. S5). Therefore, the observed reduction in neurogenesis in FBItTA mice has no influence on the anxiety level. The differences in anxiety phenotype may be, perhaps, due to different follistatin levels in the brain. The FSM mice exhibited a high level of follistatin expression compared with FBItTA mice (Ageta et al. 2008).

Figure 3. The maintenance of CA1 L-LTP in hippocampal slices from FBlTA mice is reduced as compared with FBI mice. (A) Input–output curve of fEPSP slope (millivolt [mV]/millisecond [msec]) versus stimulus (microampere [μA]) at the Schaffer collateral–CA1 pyramidal cell synapse in FBlTA and FBI mice. Data are presented as means $\pm$ SEM. (B) Paired-pulse ratio of fEPSPs in FBlTA and FBI mice. The initial slope of the fEPSP was measured in order to quantify the strength of the synaptic response. The ratio of the second to first response is plotted against the interstimulus interval. Data are presented as means $\pm$ SEM. (C) E-LTP and L-LTP elicited by three 100-Hz trains spaced at 20-msec intervals in FBlTA and FBI mice. The fEPSP slopes elicited by stimulation of the second independent pathway at 0.017 Hz were stable throughout the experiments. (Insets) Representative fEPSP traces taken at the times indicated on the graphs in C. (*) $P = 0.05$, (NS) not significant.

Once-consolidated fear memory is weakened by follistatin overexpression during the retrieval phase

We performed contextual fear-conditioning tests on FBlTA and FBI mice in the absence of DOX (Fig. 4, Experiment A). A 1-d retention test showed a significant reduction in the freezing response in FBlTA mice in Test-1 (1 d after conditioning) and Test-2 (2 d after conditioning) compared with FBI mice. There was no significant genotype effect on STM formation (Fig. 4, Experiment B). These results are consistent with the requirement for activin in L-LTP (Figs. 1A, 3C). When transgene expression was turned off by administration of DOX (orange bars, Fig. 4) for 3 consecutive days prior to conditioning, the 1-d memory of FBlTA mice was normal (Fig. 4, Experiment C, Test-1). This result indicates that follistatin expression during development of FBlTA mice did not affect the formation of neuronal circuits that are involved in contextual fear conditioning. Thus, LTM consolidation, but not short-term memory, requires activin activity in the forebrain.

One-week memory tests measured a comparable freezing behavior in FBlTA and FBI mice when DOX was administered for 3 consecutive days before conditioning (Fig. 4, Experiment D, Test-1 [indicated as T1]; Test-1 was performed 7 d after conditioning). Thus, the 1-wk memory was normal despite the inhibition of activin signaling during maintenance and retrieval, from day 3 to day 7, when fear conditioning was carried out in the absence of transgene expression. Importantly, when the same animals were retested for freezing behavior 24 h later, the

FBlTA mice showed significantly less freezing response compared with the FBI mice (Fig. 4, Experiment D, Test-2 [T2]; Test-2 was performed 8 d after conditioning). In contrast, there was no difference in freezing between FBlTA and FBI mice in Test-2 of Experiment C. In these two paradigms (Experiments C and D), the interval between conditioning and Test-2 was the same (8 d). One of the major differences in the experimental paradigm was that in Experiment D the forebrain activin signal was inhibited during the retrieval period (Test-1), while in Experiment C it was not. We performed an additional experiment that is based on Experiment D. In this paradigm, mice were continuously fed on DOX to suppress the follistatin expression during the maintenance and retrieval phase (Experiment F). We did not observe a significant genotype effect in either Test-1 or -2 of Experiment F. Thus, inhibition of the activin signal during retrieval may result in significant suppression of the subsequent freezing response.

To examine this hypothesis, conditioned animals were reexposed to the conditioning chamber for 1 min at 4 d after the conditioning, a time at which forebrain activin was blocked (Fig. 4, Experiment E). In this experiment, we used short time reactivation because long time reactivations induce extinction (Suzuki et al. 2004). The other procedures were essentially the same as in Experiment D. We observed that injection of the protein synthesis inhibitor anisomycin 30 min after the reactivation into wild-type mice, which had been subjected to the same experimental paradigm as in Experiment E, resulted in a reduction in the freezing response in Test 24 h later (Supplemental Fig. S6).

In experiment E, we observed a significant reduction in freezing level in Test-1 (7 d after conditioning) of FBlTA mice compared with FBI mice, strongly suggesting that the reactivation of fear memory in the absence of forebrain activin activity caused the decreased freezing response.

Fear memory is influenced by activin overexpression during the retrieval phase

A complementary experiment with ABITTA and ABI mice strengthens the idea that forebrain activin is important for proper processing of fear memory following memory reactivation (retrieval) (Fig. 5, Experiment G). In this experiment, we used a relatively weak conditioning protocol to avoid saturation of freezing response. A 3-wk memory test showed a normal freezing response in ABITTA mice compared with ABI mice (Test-1 in Experiment G, 21 d after conditioning), when the activin level was reduced to the basal level at conditioning by DOX administration. However, when the freezing response of the animals was tested 24 h later, ABITTA mice showed significantly more freezing than ABI mice (Test-2 in Experiment G, 22 d after conditioning). Furthermore, we observed a significant increase in freezing level in Test (21 d after conditioning) of ABITTA compared with their

Figure 4. Once-consolidated fear memory is weakened by follistatin overexpression during the retrieval phase. (A) Experimental schedule. The horizontal axis indicates the time line. Mice were fed DOX for 3 consecutive days before conditioning (from noon of day -3 to noon of day 0), as indicated (orange bars). The density of blue color indicates the predicted level of follistatin in the forebrain. The numbers of mice used were: Experiment A (FBItTA, $n = 8$; FBI, $n = 9$); Experiment B (FBItTA, $n = 10$; FBI, $n = 10$); Experiment C (FBItTA, $n = 8$; FBI, $n = 11$); Experiment D (FBItTA, $n = 10$; FBI, $n = 10$); Experiment E (FBItTA, $n = 17$; FBI, $n = 13$); Experiment F (FBItTA, $n = 11$; FBI, $n = 11$). In all the experimental paradigms, conditioning was performed in the afternoon of day 0 with the same footshock protocol. (T1) Test-1, (T2) Test-2. (B) Freezing response during the test period. (FZ%) Average freezing percentage during the 6-min test period. (*) $P < 0.05$, (**) $P < 0.001$, statistically significant differences between FBItTA and FBI mice, as determined by one-way ANOVA followed by Fisher's LSD test. Error bars indicate SEM. Experiment C: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,17)} = 0.39$, $P = 0.539$; Test effect, $F_{(1,17)} = 2.32$, $P = 0.146$; genotype $\times$ Test, $F_{(1,17)} = 0.03$, $P = 0.8629$. Experiment D: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,17)} = 0.71$, $P = 0.4$; Test effect, $F_{(1,17)} = 10.16$, $P = 0.0054$; genotype $\times$ Test, $F_{(1,17)} = 6.58$, $P = 0.02$. Experiment F: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,20)} = 0.57$, $P = 0.46$; Test effect, $F_{(1,20)} = 0.10$, $P = 0.75$; genotype $\times$ Test, $F_{(1,20)} = 2.92$, $P = 0.103$.

littermates (ABI and wild-type mice) when conditioned animals were reexposed to the conditioning chamber for 1 min at 17 d after conditioning, a time at which forebrain activin was increased in ABItTA (Fig. 5, Experiment H). Taken together, these results indicate that the functional activin level in the forebrain during fear memory retrieval (in this case, Test-1 in Experiment G and reactivation in Experiment H) determines the later freezing response (Test-2 in Experiment G and Test in Experiment H).

Discussion

In this study we showed that activin is indispensable for the late maintenance of hippocampal dentate gyrus L-LTP in vivo and CA1 L-LTP in slice preparation. In the marine snail *Aplysia*, TGF- β induces long-term, but not short-term facilitation at the synapses between the sensory and motor neurons (Zhang et al. 1997). Furthermore, in rat cultured hippocampal neurons, treatment with TGF- β 2, another isoform of TGF- β , affected synaptic strength and induced phosphorylation of CREB (Fukushima et al. 2007). Thus, the TGF- β family of proteins, namely, activin and TGF- β 1/2, participate not only in development but also in the neuronal plasticity of the mature CNS. In addition, we revealed the existence of prolonged E-LTP in the dentate gyrus, which on the one hand differs from E-LTP in its longer persistence and activin dependency; and on the other differs from L-LTP in its shorter persistence and lack of requirement for protein synthesis. This prolonged E-LTP has been previously described as an intermediate phase LTP (I-LTP) to occur in area CA1 of the hippocampus (Winder et al. 1998). It was found to differ from E-LTP and L-LTP in its molecular mechanisms since it is dependent on protein kinase A, does not require protein synthesis, and is suppressed by calcineurin overexpression. Although it is not clear whether dentate gyrus prolonged E-LTP and CA1 I-LTP share the same molecular mechanisms, it appears that temporally distinct tri-phase LTP is a common characteristic of hippocampal LTPs.

We demonstrated that activin in the brain is required for formation of L-LTP and consolidation of LTM (Figs. 1, 3, and 4). Follistatin failed to suppress L-LTP maintenance when it was administered 3 h after a strong HFS (Supplemental Fig. S1), and this result is consistent with the behavioral analysis of FBItTA mice. After the acquisition phase, the ectopic expression of follistatin in the maintenance phase did not affect LTM formation (Fig. 4, Experiment D, Test-1), indicating that the presence of follistatin in the maintenance phase has no effect on either L-LTP or LTM. Thus, our results strengthen the correlation between L-LTP and LTM.

There are several mechanisms by which activin may participate in L-LTP and LTM. Activin modulates dendritic spine morphology and increases the number of synaptic contacts (Shoji-Kasai et al. 2007). Activin potentiates NMDA receptor-mediated signaling cascades for long periods of time (Muller et al. 2006; Kurisaki et al. 2008). In

Figure 5. Fear memory is influenced by activin overexpression during the retrieval phase. (A) Experimental schedule. Horizontal axis indicates the time line. Mice were fed DOX for 3 consecutive days before conditioning (from noon of day -3 to noon of day 0, orange bar). The density of the red color indicates the predicted level of activin in the forebrain. Conditioning was performed in the afternoon of day 0. (B) Freezing response during test period. (FZ%) Average freezing percentage during the first 3 min in the 6-min test period. The numbers of mice used were: Experiment G (ABIItTA, $n = 15$; ABI, $n = 16$); Experiment H (ABIItTA, $n = 9$; ABI, $n = 5$; wild-type littermates, $n = 4$). (*) $P = 0.05$, (**) $P = 0.001$, statistically significant difference between ABIItTA and ABI mice (Experiment G) or mixed genotypes ABI and wild group (Experiment H), as determined by one-way ANOVA followed by Fisher's LSD test. Error bars indicate SEM. (WT) Wild-type littermates. Experiment G: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,23)} = 4.18$, $P = 0.0526$; Test effect, $F_{(1,23)} = 0.70$, $P = 0.410$; genotype $\times$ Test, $F_{(1,23)} = 1.63$, $P = 0.214$.

addition, activin tunes GABAergic neurotransmission (Zheng et al. 2008), which affects learning and memory (Collinson et al. 2002). All of these activin functions may contribute to the prolonged synaptic plasticity that may underlie LTM formation.

Inhibition of the activin signal during retrieval resulted in a significant suppression of subsequent expression of fear memory (Fig. 4, Experiment D, Test-2). Memory retrieval triggers two opposing processes, reconsolidation or extinction (Tronson and Taylor 2007; Quirk and Mueller 2008). Whether reconsolidation or extinction follows memory retrieval depends on the experimental conditions, such as training and retrieval conditions (Suzuki et al. 2004; Tronson and Taylor 2007). When reconsolidation dominates following memory retrieval, amnesic reagents reduce subsequent memory expression (Nader et al. 2000; Tronson and Taylor 2007). We injected the protein synthesis inhibitor anisomycin 30 min after reactivation (retrieval) into wild-type mice that had been subjected to the same experimental paradigm as in Experiment E of Figure 4. We observed a reduction in the fear response in Test (Supplemental Fig. S6). This suggests that the experimental condition used in Experiment E triggers reconsolidation. In addition, we observed that there was no significant extinction effect in control mice in Experiment D ($P = 0.7839$, one-way analysis of variance [ANOVA] with Fisher's test). On the other hand, in Experiment H in control mice, the freezing level was significantly lower than that of Test-1 in

Experiment G ($P = 0.008$, one-way ANOVA with Fisher's test). As the experimental condition used in Experiment H may trigger extinction by the short-time reactivation, control mice showed a lower level of freezing in Experiment H when compared with Test-1 in Experiment G. Unfortunately, we could not record the freezing level during the reactivation phase in Experiment H because of the short period of time (reactivation took a total of 1 min, which included transportation time from the home cage to the test chamber located in a soundproof room [15 sec], exposure time to the chamber [30 sec], and another transportation time from the chamber to the home cage [15 sec]). Taken together, the suppression of freezing observed in Experiments D and E could be caused by inhibition of reconsolidation. On the other hand, the constant level of freezing in ABIItTA mice observed in Experiments G and H could be attributed to the inhibition of extinction. In any case, the level of activin in the brain during the retrieval phase plays a key role in the maintenance of LTM.

Recent studies show that fear memory can be weakened by inhibiting CREB (Kida et al. 2002), *zif268* (Lee et al. 2004), C/EBP β (Tronel et al. 2005), ERK (Kelly et al. 2003), or PKA (Tronson et al. 2006) during memory retrieval. The present study shows that activin inhibition during memory retrieval suppresses previously consolidated fear memories. Thus, activin signaling could be a promising target for treatment of disorders that are based upon strong traumatic memories, such as post traumatic stress disorder and phobias.

Materials and Methods

Animals

Male Wistar rats (5–6 mo old) were used for the LTP experiments. All procedures involving the use of animals complied with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of the Mitsubishi Kagaku Institute of Life Sciences.

All behavior experiments were conducted in a blind fashion on male mice. Two weeks before behavioral analysis, animals were housed individually in plastic cages and maintained on a 12:12 h light:dark cycle. Food and water were provided ad libitum. The mice were handled daily for 7 d before behavioral analysis.

Dentate gyrus LTP in vivo

Dentate gyrus LTP experiments with urethane-anesthetized rats were carried out as described previously (Ikegami et al. 1996; Inokuchi et al. 1996; Ikegami and Inokuchi 2000; Fukazawa et al. 2003) with the following modifications: To avoid subjecting the animals to unnecessary pain, the wound margins were locally infiltrated and anesthetized with 2% xylocaine. The monopolar recording electrode was then inserted into the hilus of the dentate gyrus (4.0 mm posterior, 2.8 mm lateral, 3.0 mm ventral to the dura). The bipolar stimulating electrode was positioned ipsilaterally to the medial perforant pathway (8.0 mm posterior, 4.0 mm lateral, 3.0 mm ventral to the dura). The stimulus intensities

were set at the current that evoked 50% of the maximum population spike amplitude and were kept constant throughout the experiment. Test stimuli were delivered at 30-sec intervals to record the fEPSP. After recording a stable basal transmission for 30 min, an HFS was delivered and the fEPSPs were recorded for 24 h. In the follistatin infusion experiments, a strong HFS was used, which consisted of five trains of 400 pulses at 400 Hz at 2-min intervals. In the activin infusion experiments, we used a weak HFS, which consisted of 50 pulses at 100 Hz. In most experiments, an HFS (100 pulses at 100 Hz) was given after the 24-h recording period and responses were recorded for a further 60 min. The initial EPSP slopes are shown as a percentage of the mean value obtained in the 15 min immediately prior to delivery of the HFS. To determine whether the magnitude of LTP differed among the groups, responses from the last 15 min block of recording for each 60 min period were compared statistically. The data are expressed as means $\pm$ standard error of the mean (SEM) and were analyzed by one-way or two-way ANOVA followed by Fisher's least significant difference (LSD) test.

Drug infusions

Follistatin and activin were prepared from bovine follicular fluid as described previously (Nakamura et al. 1992). Follistatin and activin were dissolved in 50 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.125% bovine serum albumin (BSA). Anisomycin (Sigma-Aldrich) was dissolved in equimolar HCl, diluted with saline, and adjusted to pH 7.4 with NaOH.

In the experiments shown in Figure 1, activin, follistatin, anti-activin A, or the vehicle solution (PBS containing 0.125% BSA) were infused 45 min prior to the first HFS delivery. Anisomycin was infused 60 min prior to the first HFS delivery. The drugs were infused into the left lateral ventricle (1.0 mm posterior to the bregma, 1.8 mm lateral, 3.5 mm ventral to the dura) ipsilateral to the recording site with a 27-gauge microsyringe. The drug solution (total volume of 2.0 μ L) was infused at a rate of 0.5 μ L/min and the microsyringe was left in place for 5 min after the injection.

In the experiments shown in Figure 2, activin, follistatin, or vehicle solution (PBS containing 0.125% BSA) were injected at 1 or 3 h after the first HFS delivery using a cannula device (brain infusion kit, Alzet) that had been implanted into the lateral ventricle and connected to a microsyringe. The drug solution (total volume of 2.0 μ L) was infused at a rate of 0.5 μ L/min.

Transgenic mice

To generate the transgene vectors, we used the pBI-G plasmid (Clontech), which has a multiple cloning site, TRE promoter, a LacZ gene, and β -globin poly A and SV40 poly A sequences (Fig. 2A). We introduced a *XhoI-FseI-PacI-HindIII-AscI* site to the *NotI* site of pBI-G and refer to this plasmid as pBI-G2. Coding sequences for *activin* and *follistatin* were isolated by *AscI-XhoI* digestion from the pCaM-activin-Myc and pCaM-follistatin-Myc plasmids (Ageta et al. 2008), respectively, and inserted into the *AscI-XhoI*-digested pBI-G2 to generate the pBI-G2-activin-Myc and pBI-G2-follistatin-Myc plasmids. *AscI* fragments were isolated from pBI-G2-activin-Myc or pBI-G2-follistatin-Myc and microinjected into the pronuclei of one-cell embryos of C57BL/6J mice to produce transgenic mice (Hogan et al. 1994). Microinjected embryos were transferred to the oviducts of pseudopregnant females. We purchased tTA mice (B6; CBA-TgN [CamK2tTA]-1Mmay) from the Jackson Laboratory (Maine), and these mice were backcrossed for six generations to the C57BL/6J background mice before crossing with FBI mice. Littermate single transgenic mice, FBI and ABI, were used as controls against FBI-tTA and ABI-tTA mice, respectively. Male mice 70–100 d old were used for behavioral analysis. Mice were fed DOX (Sigma, D-9891, 6 mg/g food) for 3 consecutive days where indicated. The founder mice and offspring were identified by Southern blot analysis using the LacZ gene as a probe and PCR analysis using two independent transgene-specific primer pairs. Forward (f) and reverse (r) PCR primers for genotyping were as follows: tTA, f-5'-TGCCGCCA TTATTACGACAA-3' and r-5'-TCCTCGCGTCTAAGTGGAG-3',

f-5'-TTGCGTATTGGAAGATCAAG-3' and r-5'-GATGGTAGACCC GTAATTGT-3'; FBI, f-5'-TATTCGCGTAAGGAAATCCA-3' and r-5'-GCCGAGTTTGTTCAGAAAGCA-3', f-5'-TCCTTGCTCAGTTCG GTCTT-3' and r-5'-CCCCACTTTGCGTTTCTTCT-3'; ABI, f-5'-TA TTCGCGTAAGGAAATCCA-3' and r-5'-GCCGAGTTTGTTCAGAA AGCA-3', f-5'-ATCTCCACATACCCGTTCTC-3' and r-5'-CTCCC CACTTTCGCTTCTTCT-3'.

X-gal staining

Animals were sacrificed using an overdose of anesthesia, and the brains were dissected and immediately frozen on dry ice. Cryostat sections (20- μ m thick) were cut and mounted onto polylysine-coated glass slides. Sections were air-dried and stored at 80°C until use in X-gal staining. X-gal staining was carried out as described (Takeuchi et al. 1995).

CA1 LTP in slice preparation

Transversely cut hippocampal slices (300 μ m) were prepared from 6- to 10-wk-old transgenic FBI and FBI-tTA mice, and were immersed in ice-cold artificial cerebrospinal fluid (ACSF). ACSF was saturated with 95% O₂/5% CO₂, and contained: 124 mM NaCl, 3 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgCl₂, 26 mM NaHCO₃, 1 mM NaH₂PO₄, and 10 mM D-glucose. Slices were maintained in ACSF at room temperature for at least 1 h before recording. They were then transferred to a submersion chamber and perfused continuously (2 mL/min) with ACSF at 30°C. Schaffer collaterals were stimulated with 0.1-msec pulses using bipolar tungsten electrodes. fEPSPs were recorded extracellularly in the stratum radiatum of the CA1 region using glass microelectrodes filled with ACSF (tip resistance ca. 3–6 M Ω). Stimulus intensities were set to evoke 30%–50% of the maximal fEPSP slope. LTP was induced after recording a stable 15- to 30-min baseline fEPSP. The test pulse frequency was 0.05 Hz. For some of the experiments, two stimulating electrodes were positioned in the stratum radiatum layer to activate two independent sets of Schaffer collaterals. Pathway independence was assessed by applying two pulses with 100-msec interpulse intervals and confirming the absence of PPF between the pathways (collision test). After baseline stimulation the pathway stimulated at 0.05 Hz received one or three trains of tetanic stimulation at a frequency of 100 Hz for 1 sec at 20-sec intervals. The second pathways were stimulated at 0.017 Hz and served as a control.

Electrophysiological data were collected from two strains of transgenic mice on alternate days using PowerLab (ADInstruments) and three EPC series amplifiers (HEKA Elektronik). Data were low-pass filtered (1 kHz) and sampled at 10 kHz. As a measure of synaptic strength, the initial slope of the evoked fEPSPs was calculated and expressed as percent change from the baseline mean. Error bars denote SEM values. All data were used for analysis unless the pipette resistance deviated more than 30% from baseline values or the stimulus artifact, or the shape of fiber volleys changed significantly. To test for group differences between LTP values across conditions, a Student's *t*-test was performed.

Contextual fear conditioning

After the risk-taking behavior test and light/dark test, we performed the contextual fear conditioning test on each experimental group. For the experiments in Figure 4, 2 min after putting the mice into a chamber, mice received electrical footshocks three times (0.5 mA, 1 sec, intershock interval of 1 min). After the shock they remained in the chamber for an additional 2 min. For experiments in Figure 5, 2 min after putting the mice into a chamber, mice received electrical footshocks four times (0.2 mA, 0.5 sec, intershock interval of 3 sec). After the shock they remained in the chamber for an additional 4 min. To monitor the freezing response in the test, mice were again put into the same chamber for 6 min, except for Test-1 of experiment A (duration, 1 min) in Figure 4. Freezing behavior, which is defined as no movement during consecutive 2-sec intervals, was analyzed by an automated imaging system (Muromachi Kagaku). In Experiments E and H, reactivation took a total of 1 min, which included transportation

time from the home cage to the test chamber located in a sound-proof room (15 sec), exposure time to the chamber (30 sec), and another transportation time from the chamber to the home cage (15 sec).

Sensitivity to electrical stimulation

After the contextual fear-conditioning test, we measured the sensitivity of mice to footshock (Supplemental Fig. S4C). In this test, each mouse is placed in a conditioning chamber and receives 1-sec shocks of increasing intensity (Inoue et al. 2009). The interval between shocks is 10 sec. The sequence of the current used was as follows: 0.05 mA, 0.08 mA, 0.1 mA, 0.2 mA, 0.3 mA, 0.4 mA, 0.5 mA, 0.6 mA, and 0.8 mA. The minimal level of current required to elicit the stereotypical responses of running, vocalization, and jumping was determined.

Miscellaneous methods

ELISA and analysis of neurogenesis were carried out essentially as described previously (Ageta et al. 2008). Behavioral analyses, including the risk-taking behavior and light/dark tests, were carried out as described (Ageta et al. 2008).

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