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 βA, 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; Bourchuladze 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 βA, ubiquitin C-terminal hydrolase, vest-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 βA, a member of the TGF-β superfamily (Massague 1998), is one of the genes whose expression is up-regulated following L-LTP induction (Andreason and Worley 1995; Inokuchi et al. 1996). 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.
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](image_url)

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 μg) 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 CaMKIIa 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 (FBItTA and ABItTA)-transgenic mice were obtained by heterozygous crossings. A diet containing DOX (6 mg/g food) was fed to pregnant mice for 7 days to suppress endogenous activin activity in the hippocampus. Phosphorylation of Smad 2 (3 was significantly decreased in the hippocampus of FBItTA but not FBI (Supplemental Fig. S3). These results demonstrate that L-LTP was reduced in FBItTA mice, whose follistatin levels had an ability to suppress endogenous activin activity in the hippocampus.

**Anxiety levels of ABItTA and FBItTA**

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 aCaMKII 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
risk-taking behavior test, to examine the anxiety levels of FBltTA and ABltTA 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 FBltTA and ABltTA 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 (ABltTA and FBltTA) 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 FBltTA mice, but the reduction was less severe than in FSM mice (Supplemental Fig. S5). Therefore, the observed reduction in neurogenesis in FBltTA 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 FBltTA mice (Ageta et al. 2008).

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, ABltTA, FBI, and FBltTA mice (10–15 wk old). (Lower row) Enlarged images of the hippocampus. Scale bars, 500 μm. (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.
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 3. The maintenance of CA1 L-LTP in hippocampal slices from FBItTA 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 FBItTA and FBI mice. Data are presented as means ± SEM. (B) Paired-pulse ratio of fEPSPs in FBItTA 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 ± SEM. (C) E-LTP and L-LTP elicited by three 100-Hz trains spaced at 20-msec intervals in FBItTA 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) 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 FBItTA 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 FBItTA 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 FBItTA mice was normal (Fig. 4, Experiment C, Test-1). This result indicates that follistatin expression during development of FBItTA 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 FBItTA 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
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 preparations. 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-β2, 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
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 ABItTA 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/EBPβ (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

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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 (ABItTA, n = 15; ABI, n = 16); Experiment H (ABItTA, n = 9; ABI, n = 5; wild-type littermates, n = 4). (*) P = 0.05, (**) P = 0.001, statistically significant difference between ABItTA 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, $F_{1,23} = 1.63, P = 0.214$. 

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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 fEPSP 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 ± 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% BSA (Sigma). Anisomycin (Sigma-Aldrich) was dissolved in equimolar saline (PBS, pH 7.4) containing 0.125% BSA. 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) ipsilaterally to the recording site with a 27-gauge microsyringe. The drug solution (total volume of 2.0 μL) was infused at a rate of 0.5 μL/min and the microsyringe was left in place for 5 min after the injection.

In the experiments shown in Figure 2, activin, follistatin, or 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) ipsilaterally to the recording site with a 27-gauge microsyringe. The drug solution (total volume of 2.0 μL) was infused at a rate of 0.5 μL/min.

Transgenic mice

To generate the transgene vectors, we used the pBl-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 Xhol-FseI-PacI-HindIII-Ascl site to the Nof site of pBl-G and refer to this plasmid as pBl-G2. Coding sequences for activin and follistatin were isolated by Ascl-Xhol digestion from the pCam-activin-Myc and pCam-follistatin-Myc plasmids (Agata et al. 2008), respectively, and inserted into the Ascl-Xhol-digested pBl-G2 to generate the pBl-G2-activin-Myc and pBl-G2-follistatin-Myc plasmids. Asel fragments were isolated from pBl-G2-activin-Myc or pBl-G2-follistatin-Myc and microinjected into the pronuclei of one-cell embryos of C57Bl/6 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/6 background mice before crossing with FBL mice. Littermate single transgenic mice, FBL and ABL, were used as controls against FBT TA and ABlTA 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 primers. Forward (f) and reverse (r) primers for genotyping were as follows: TTA, f-5′-TGGCGGCCCA TTATACGCAGAAA-3′ and r-5′-TCTTGGCCCGTCATTGAATGG-3′;
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-s 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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