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Inducible Enhancement of Memory Storage and Synaptic Plasticity in Transgenic Mice Expressing an Inhibitor of ATF4 (CREB-2) and C/EBP Proteins

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Summary

To examine the role of C/EBP-related transcription factors in long-term synaptic plasticity and memory storage, we have used the tetracycline-regulated system and expressed in the forebrain of mice a broad dominant-negative inhibitor of C/EBP (EGFP-AZIP), which preferentially interacts with several inhibiting isoforms of C/EBP. EGFP-AZIP also reduces the expression of ATF4, a distant member of the C/EBP family of transcription factors that is homologous to the *Aplysia* memory suppressor gene ApCREB-2. Consistent with the removal of inhibitory constraints on transcription, we find an increase in the pattern of gene transcripts in the hippocampus of EGFP-AZIP transgenic mice and both a reversibly enhanced hippocampal-based spatial memory and LTP. These results suggest that several proteins within the C/EBP family including ATF4 (CREB-2) act to constrain long-term synaptic changes and memory formation. Relief of this inhibition lowers the threshold for hippocampal-dependent long-term synaptic potentiation and memory storage in mice.

Introduction

Work on various organisms has shown that memory and the underlying synaptic plasticity have temporal stages that rely on different molecular events. There is a short-term process lasting minutes that depends on modifications of preexisting proteins and a long-term process lasting hours and days that depends on gene expression and protein synthesis (Goelet et al., 1986; Montarolo et al., 1986; Squire and Barondes, 1970; Agranoff et al., 1967; Flexner et al., 1963). The cyclic-AMP signaling system, which includes the cAMP-dependent protein kinase A (PKA) and the cAMP-responsive element binding protein (CREB), plays important roles in the conversion of short- to long-term memory and short- to long-term synaptic plasticity in both mice (Abel et al., 1997; Bourtchuladze et al., 1994) and invertebrates (Bartsch et al., 1995, 1998; Davis et al., 1995; Yin et al., 1995; Dash et al., 1990). Recent studies revealed that, in addition to activators, there are also memory suppressor genes that act in parallel to the activators in setting the molecular threshold for long-term memory and long-term synaptic plasticity (reviewed in Abel and Kandel, 1998). For example, calcineurin, a cytoplasmic phosphatase, negatively regulates the transition of short- to long-term memory in mice by gating the calcium/PKA signaling cascade (Malleret et al., 2001; Mansuy et al., 1998). In invertebrates, there are also inhibitory constraints to external stimuli at the nuclear level. In *Aplysia*, two basic leucine zipper (bZIP) transcription factors, ApCREB-2 and ApCREB-1b, act as memory suppressor genes for nonassociative learning (Bartsch et al., 1995, 1998). CREB-related transcriptional repressors have also been identified in the olfactory memory of *Drosophila* (Yin et al., 1994).

In mammals, potential nuclear suppressor genes that act in concert with or downstream of CREB in the regulation of long-term memory are not as yet known. The mammalian homolog of the *Aplysia* transcriptional repressor *ApCREB-2* is *ATF4* (activating transcription factor 4), a bZIP transcription factor that interacts with the C/EBP family of transcription factors (Gachon et al., 2001; Gombart et al., 1997; Vallejo et al., 1993). Null mutation of *ATF4* in mice results in abnormal lens formation that precludes behavioral analysis based on vision (Hettmann et al., 2000; Tanaka et al., 1998). In mammals, two of the six C/EBP genes, *C/EBP*-β and *C/EBP*-δ, are induced in hippocampal neurons in response to increased cAMP or calcium (Yukawa et al., 1998) and following aversive inhibitory learning (Taubenfeld et al., 2001b). However, contrary to *Aplysia,* where *ApC/EBP* is recruited downstream from CREB as a memory-enabling gene for long-term facilitation (Alberini et al., 1994), the effects of their upregulation on hippocampal-based synaptic plasticity and memory are not straightforward. Whereas inhibition of *C/EBP*-β expression by a specific antisense oligonucleotide in the hippocampus of rats interfered with memory consolidation of avoidance learning (Taubenfeld et al., 2001a), deletion of *C/EBP*-δ in mice by homologous recombination resulted in a selective enhancement in contextual conditioning response (Sterneck et al., 1998). Thus, at least one isoform of C/EBP proteins is implicated in inhibiting a hippocampal-based memory task.

Null mutations of *C/EBP*-α, *C/EBP*-β, and *C/EBP*-ε have each been generated in mice, but developmental defects or early lethality of these mutants have precluded any neurobehavioral studies (Yamanaka et al., 1997; Screpanti et al., 1995; Wang et al., 1995). To better understand the role of the C/EBP family of transcription factors in mammals, we here use a novel protein-targeting approach aimed at inhibiting the function of C/EBP proteins as a group with a dominant-negative C/EBP (EGFP-AZIP). We use a combination of the fore-

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brain-specific CamKII-α promoter and the tetracycline-regulated system to achieve spatially restricted and temporally reversible expression of the inhibitor in mice (Mayford et al., 1996; Furth et al., 1994). Using this approach, we find that the dominant-negative EGFP-AZIP interacts preferentially with the repressor isoforms within the C/EBP family of proteins and that the expression of ATF4 (CREB-2) is reduced in the forebrains of transgenic mice. As a result of lowering the threshold for C/EBP-regulated transcription, long-term plasticity and memory are more easily attained. This study provides evidence that several members of the C/EBP superfamily of transcription factors act to constrain the conversion of short- to long-term synaptic potentiation and short- to long-term memory storage.

Results

Dominant-Negative C/EBP (EGFP-AZIP) Enhances C/EBP-Mediated Transcription

To target the C/EBP family of proteins, we chose a broad dominant-negative inhibitor of C/EBP called AZIP, which dimerizes with endogenous C/EBP proteins and abrogates DNA binding and gene transcription (Vinson et al., 1993; Krylov et al., 1995). Expression of the AZIP inhibitor in adipocytes of transgenic mice resulted in abnormal development of fat tissue, confirming the in vivo dominant-negative effect of the AZIP protein since C/EBP-α, C/EBP-β, and C/EBP-δ are critical for terminal differentiation of adipocytes (Moitra et al., 1998). In our study, we fused a green fluorescent protein (EGFP) to the N terminus of AZIP to label the broad dominant-negative inhibitor now called EGFP-AZIP.

To demonstrate that EGFP-AZIP retains the ability to interact with C/EBP family proteins, we coexpressed in NIH3T3 cells EGFP-AZIP with individual C/EBP proteins that are implicated in neuronal function. Immunoprecipitation of the whole-cell lysate with a GFP antibody followed by Western blot analyses showed that EGFP-AZIP forms complexes with C/EBP-β, C/EBP-δ, and ATF4 proteins (data not shown). Since it is not known whether the original AZIP inhibitor interacted with ATF4, we specifically confirmed the interaction between EGFP-AZIP and ATF4 using in vitro translated proteins followed by coimmunoprecipitation (Figure 1A).

To explore the effects on transcription of the interactions between EGFP-AZIP and C/EBP proteins, we transfected constructs that encode luciferase reporter gene driven by various promoters in *rtTA/tetO-EGFP-AZIP* double-stable PC12 cells, which express EGFP-AZIP protein upon exposure to doxycycline (see Experimental Procedures). Measurement of luciferase activities in these cells showed that the expression of EGFP-AZIP leads to a 7-fold increase in the transcription of a luciferase reporter gene driven by a promoter containing multiple repeats of C/EBP binding sites (5XERE-luciferase). In contrast, expression of EGFP-AZIP has minimal effects on transcription of the luciferase gene driven by other promoters that contain either the minimal TATA box, repeats of the cAMP-responsive element, or the AP-1 binding sequence (Figure 1B). Thus, expression of EGFP-AZIP that interacts with members of the C/EBP family proteins, including C/EBP-β, C/EBP-δ, and ATF4, leads to a net increase in C/EBP-mediated transcription at basal state.

Forebrain-Specific and Tetracycline-Regulated Expression of EGFP-AZIP in Mice

To study the role of the C/EBP family of transcription factors in hippocampal-based synaptic plasticity and memory storage, we generated double transgenic mice that express EGFP-AZIP inhibitor under the control of a forebrain-restricted tetracycline transactivator (CamKII-tTA). In situ hybridization with a GFP-specific antisense riboprobe detected EGFP-AZIP mRNA in the forebrain of the double transgenic mouse, under a pattern that is specific to the CamKII-α promoter (Figure 1C). Expression of EGFP-AZIP was examined by direct fluorescence imaging and confirmed by immunostaining with an anti-GFP antibody (van den Pol and Ghosh, 1998), showing that EGFP-AZIP protein is most highly expressed in CA1 region followed by dentate gyrus and CA3 region in transgenic mice (Figure 1D). Western blot analysis showed that expression of EGFP-AZIP in the forebrain may be switched off by feeding mice doxycycline (40 mg/kg) (Figure 1E).

EGFP-AZIP Preferentially Interacts with Repressive Isoforms of C/EBP-Related Proteins and Suppresses Expression of ATF4 (CREB-2) in Mice

To examine protein-protein interactions between EGFP-AZIP and the various isoforms of C/EBP in the mouse brain, we immunoprecipitated EGFP-AZIP from forebrain tissues using an anti-GFP antibody followed by immunoblotting with antibodies specific for C/EBP-β, C/EBP-δ, and ATF4. We found that EGFP-AZIP interacts with both isoforms of C/EBP-β: the full-length (33 kDa) activating isoform (also known as liver-enriched activating protein, LAP) and the truncated (18 kDa) repressive isoform lacking a transactivation domain (also known as liver-enriched inhibitory protein, LIP) (Figure 2B, lane 4). The intensities of these bands suggest that the dominant-negative EGFP-AZIP interacts more effectively on a molar basis with the repressive LIP isoform than with the activating LAP isoform. Moreover, the amount of coprecipitated LIP represents a greater proportion of endogenous LIP than that of endogenous LAP being sequestered by EGFP-AZIP, since at basal state LAP is much more abundant than LIP in the forebrain of both wild-type and transgenic mice (Figure 2B, lanes 1 and 3). This preferential binding of EGFP-AZIP for the truncated LIP isoform of C/EBP-β may be imparted by the steric hinderance imposed by the EGFP motif that reduces its affinity for the full-length LAP isoform with an N-terminal transactivation domain.

As with cell culture and in vitro translated proteins, we have repeatedly found that EGFP-AZIP also interacts in vivo in the brain with ATF4 protein (Figure 2C). We did not detect an interaction between EGFP-AZIP and C/EBP-δ in the forebrain by the methods of coimmunoprecipitation and Western blot analysis. This may be due to the limited sensitivity of the assay because the interaction between EGFP-AZIP and C/EBP-δ within the forebrain tissue is minimal or unstable.

Since C/EBP proteins can autoregulate their own expressions (Burgess-Beusse et al., 1999), we next examined the consequence of EGFP-AZIP interacting with the C/EBP family of transcription factors. Using pooled samples of forebrain from mice that were matched by age and strain, we found that expression of C/EBP-β

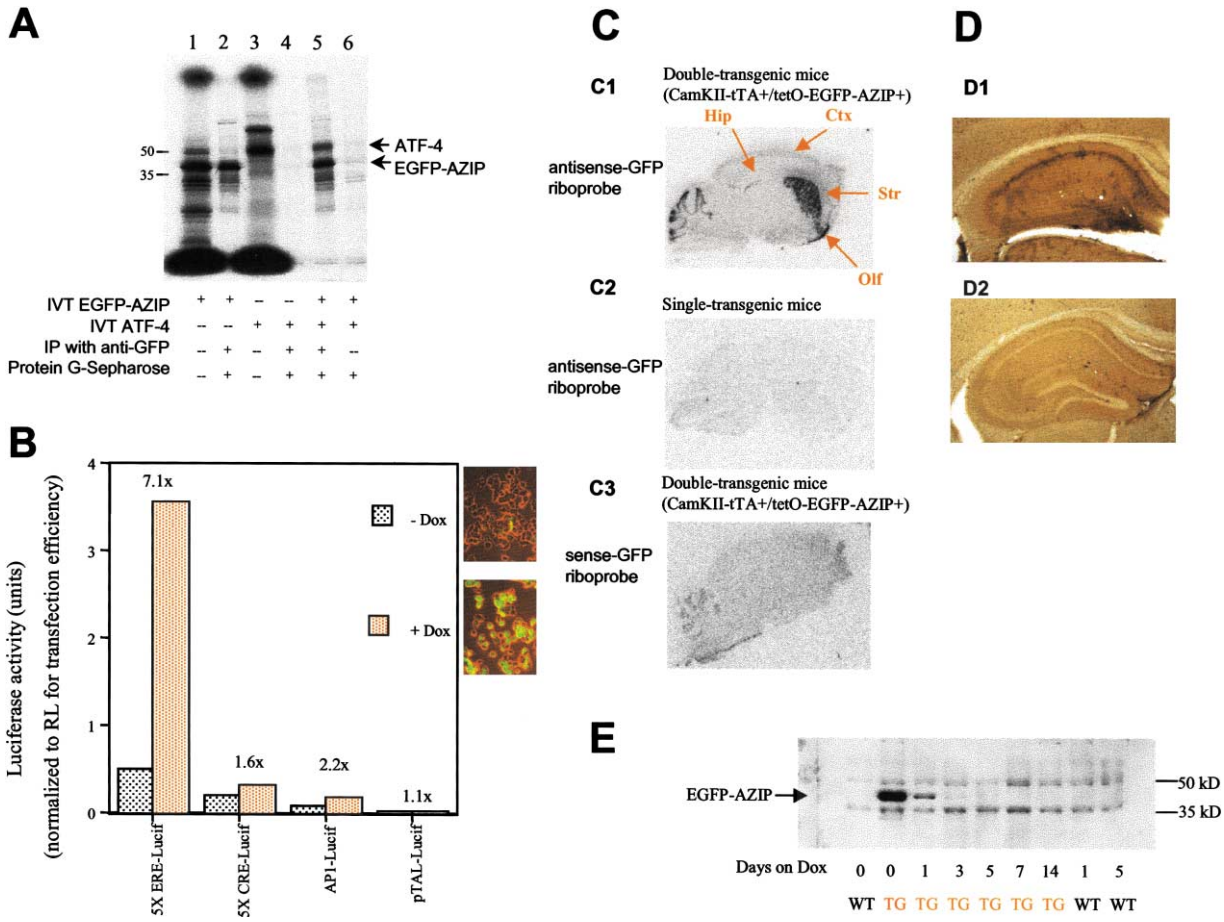

Figure 1. Interaction of EGFP-AZIP with ATF4, Effects on Transcription, and Regulated Expression in Mouse Brains

(A) Reticulocyte in vitro translated EGFP-AZIP protein labeled by [^{35}S]methionine migrates in SDS-PAGE near 40 kDa (lane 1). EGFP-AZIP is immunoprecipitated using anti-GFP antibody (lane 2). In vitro translated ATF4 protein migrates as a 52 kDa protein (lane 3). ATF4 is not immunoprecipitated by anti-GFP antibody (lane 4). Coincubated EGFP-AZIP and ATF4 proteins form heterodimers that are immunoprecipitated by anti-GFP antibody (lane 5) and not by Protein-G Sepharose (lane 6).

(B) Side panels: confocal images of rtTA/tetO-EGFP-AZIP double-stable PC12 cells (clone A7) show that EGFP-AZIP protein is induced upon doxycycline treatment (1 μg/ml) and is stable for 7 days. The graph compares luciferase activities driven by various promoters in the absence and presence of EGFP-AZIP inhibitor. Luciferase activity driven by the C/EBP binding sequence (5XERE-luciferase) is upregulated 7-fold in cells expressing EGFP-AZIP compared to control. Relative luciferase activities driven by CRE (5XCRE-luciferase), AP-1 binding sequence (AP-1-luciferase), and TATA box (pTAL-luciferase) in the presence of EGFP-AZIP are 1.6-fold, 2.2-fold, and 1.1-fold, respectively.

(C) In situ hybridization using GFP antisense riboprobe on sagittal sections shows that EGFP-AZIP mRNA is present in the hippocampus (Hip), striatum (Str), cortex (Ctx), and olfactory tubercle (Olf) of CamKII-tTA/tetO-EGFP-AZIP double transgenic mice (C1) but not in control littermates (C2). Hybridization with GFP sense riboprobe shows that staining in the cerebellum is not specific (C3).

(D) Immunohistochemistry with anti-GFP antibody detects EGFP-AZIP protein in the dentate gyrus, CA3, and CA1 regions of the hippocampus of double transgenic mice (D1) but not the control littermates (D2).

(E) Western blotting using anti-GFP antibody recognizes the 38 kDa EGFP-AZIP protein from the forebrain extracts of double transgenic mice (lane 2). The expression of EGFP-AZIP is gradually reduced upon feeding mice doxycycline diet (lanes 3–5) and it is completely suppressed by day 7 of doxycycline treatment (lane 6). Extracts from the forebrains of wild-type mice were loaded as controls (lanes 1, 8, and 9).

and C/EBP-δ proteins were comparable between wild-type and transgenic mice (data not shown). In contrast, expression of ATF4 protein in transgenic mice was reduced when compared to that of wild-type mice (Figure 2D). The mechanism for a lower steady-state level of ATF4 expression in these mice is not known. It is possible that interference of C/EBP family-mediated transcription by EGFP-AZIP leads to a net reduction in the transcription of ATF4. We think this is unlikely since microarray analyses did not reveal a difference in ATF4 transcripts between wild-type and EGFP-AZIP mice ($p > 0.05$) (Figure 2F). We think it is more likely that dimerization of ATF4 with exogenous EGFP-AZIP leads to an enhanced degradation of ATF4 protein, since ATF4 is a labile protein with a short half-life (Lassot et al., 2001). This increased instability of ATF4 protein upon heterodimerization with EGFP-AZIP would also explain the small quantity of ATF4 protein that is detected following coimmunoprecipitation with anti-GFP antibody (Figure 2C).

Thus, our molecular study of these mice showed a preferential interaction of EGFP-AZIP with the repressive LIP isoform of C/EBP-β rather than with the activating LAP isoform, as well as a selective downregulation of ATF4, the murine homolog of *Aplysia* memory repressor

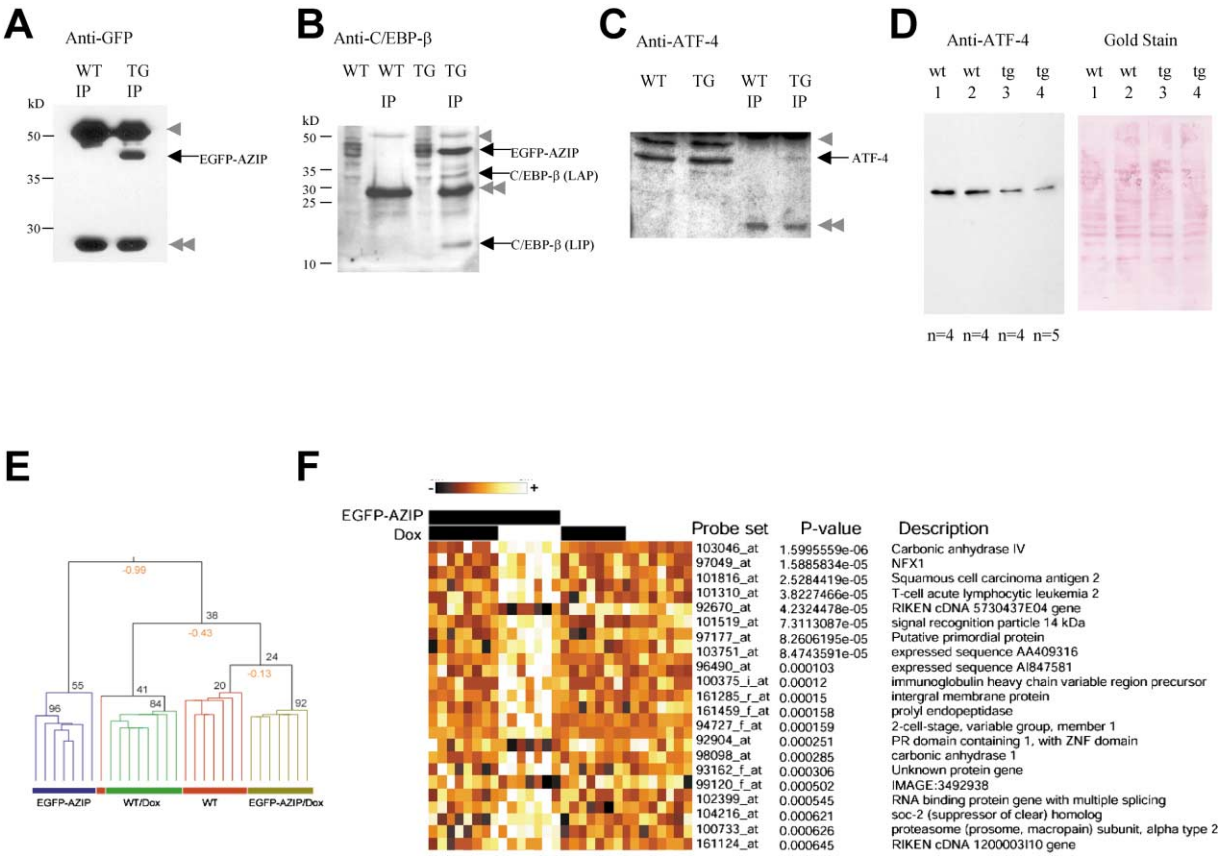

Figure 2. EGFP-AZIP Interacts with C/EBP Repressors and ATF4, Reduces ATF4 (CRE-2) Expression, and Results in a Distinct Transcript Profile

(A–C) Proteins from brain lysates were immunoprecipitated with anti-GFP antibody (Quantum), separated on 10% SDS gel, and probed with specific antibodies.

(A) Western blot with anti-GFP antibody shows that EGFP-AZIP protein is precipitated from the forebrain of transgenic mice (lane 2). The secondary antibody (anti-mouse HRP) crossreacts with IgG_H (single arrowhead) and IgG_L (double arrowheads) of the antibody used in immunoprecipitation.

(B) Immunoblots probed with C/EBP-β antibody shows that both the full-length LAP and the truncated LIP isoforms of C/EBP-β are coimmunoprecipitated with EGFP-AZIP from the forebrain of transgenic mice (lane 4) but not of wild-type mice (lane 2). C/EBP-β antibody recognizes EGFP-AZIP protein due to the leucine zipper domain (lanes 3 and 4). The secondary antibody (anti-rabbit HRP) crossreacts with IgGs of the antibody used in immunoprecipitation (lanes 2 and 4, single and double arrowheads).

(C) Immunoblots probed with ATF4 antibody shows that ATF4 protein is coimmunoprecipitated with EGFP-AZIP protein from the forebrain of transgenic mice (lane 4) but not of wild-type mice (lane 3).

(D) Western blots of pooled forebrain extracts show that expression of ATF4 is reduced in EGFP-AZIP transgenic mice (lanes 3 and 4) compared to that of wild-type (lanes 1 and 2). The same blot was gold-stained, showing that comparable amount of proteins were loaded in each lane.

(E) Results of hierarchical clustering of samples based on 200 genes selected by a one-way ANOVA. The red numbers indicate correlations between the groups at each branch (also indicated graphically by the vertical distance between nodes); the black numbers are bootstrap trial results. All four groups of samples are resolvable (with one error), but this is not surprising since the genes selected are expected to be those that show the strongest differences among the groups. The strongest cluster (showing an average correlation with the remaining samples of −0.99) contains all 7 EGFP-AZIP-active samples. The remaining samples are much more weakly resolved into groups, indicating that the differences between them are smaller.

(F) The top genes selected by t test for differences between the EGFP-AZIP-active group and the controls are shown here. Increasing relative expression values are indicated by brighter colors. p values are uncorrected for multiple testing.

ApCREB-2. These results suggest that EGFP-AZIP transgenic mice harbor an increased ratio of functional C/EBP-related activators to repressors.

Transcript Profile in EGFP-AZIP Transgenic Mice Is Upregulated

To address the degree to which these molecular changes affect gene expression, we explored the overall effect of EGFP-AZIP expression on transcription using GeneChips. We compared EGFP-AZIP transgenic mice with and without doxycycline treatment (n = 7; n = 7) to control mice with and without doxycycline treatment (n = 8; n = 8) (see Experimental Procedures). We first used a gene-by-gene one-way ANOVA to find the genes affected by EGFP-AZIP transgene as well as genes affected by doxycycline. We found that the differences in gene expression among all four groups are dominated by the effect of the active EGFP-AZIP transgene. Analy-

sis by hierarchical clustering showed that EGFP-AZIP-active group has the most distinctive expression profile, forming a cluster separate from the rest of the samples, which are much more poorly resolved into groups (Figure 2E). Principal components analysis of the same data also revealed that the most significant component primarily distinguishes the EGFP-AZIP-active group (data not shown). These results show that expressing EGFP-AZIP had an overall effect on transcription that supersedes any other transcriptional changes present in the data.

As determined by Student's t test comparing the active EGFP-AZIP group to the three control groups combined, most of the genes showing the strongest changes in transcription due to the expression of EGFP-AZIP protein are upregulated (Figure 2F). Using stringent statistical criteria that would predict one expected false positive in the experiment-wide analysis, eight genes show significantly different expression in the EGFP-AZIP transgenic group compared to the rest of the samples ($p < 0.0001$; see Experimental Procedures). These include upregulation of five candidate genes (carbonic anhydrase IV, *SCCA2* [squamous cell carcinoma antigen], *Tal2* [T cell acute lymphocytic leukemia 2], signal recognition particle 14 kDa, and a putative primordial protein), downregulation of a cDNA clone that is homologous to myosin light chain, and two unidentified clones (*NFX1* [a clone that has been withdrawn from GenBank] and an unidentified EST) (Figure 2F). In total, with an unbiased gene expression analysis, we have demonstrated that regulated expression of EGFP-AZIP in transgenic mice is correlated with distinct changes in transcription.

Basal Synaptic Transmission Is Not Altered in EGFP-AZIP Transgenic Mice

We next examined the hippocampal-based synaptic plasticity of these dominant-negative C/EBP mice. To explore plasticity in the CA3-CA1 synapses of hippocampus, we first determined changes in the excitatory postsynaptic potentials (fEPSPs) by extracellular field recordings in the stratum radiatum following electrical stimulation of the Schaffer collateral pathway. Basal synaptic transmission in this pathway was not affected by EGFP-AZIP transgene (Figures 3A and 3B). There was no difference between wild-type (0.80 ± 0.16 mV) and transgenic mice (0.73 ± 0.15 mV) in either the presynaptic fiber volley sizes or the baseline field excitatory postsynaptic potentials (fEPSPs) across a range of stimulus intensities (Figures 3C and 3D).

Since EGFP-AZIP is expressed strongly in CA1 and moderately in CA3, we also assessed presynaptic function. To this end, we measured paired-pulse facilitation (PPF), a short-term enhancement of synaptic efficacy in response to the second of two paired stimuli due to residual Ca^{2+} in the presynaptic terminal following the initial stimulus. PPF was slightly enhanced in EGFP-AZIP transgenic mice, indicating that even though the transgene does not grossly affect synaptic transmission, it produces some alteration in presynaptic functioning. This increased PPF was significant only for the 50 ms interpulse intervals ($p < 0.03$), and it is not statistically significant across different interpulse intervals (Figure 3E). This trend is specific to the expression of EGFP-AZIP transgene as it is obliterated when the transgene is switched off in mice with doxycycline diet (Figure 3F).

EGFP-AZIP Mice Show Enhanced Synaptic Potentiation in Response to a Submaximal Single Train of High-Frequency Stimulation

We next examined the effect of EGFP-AZIP on long-term synaptic potentiation in the Schaffer collateral pathway, first using one train of high-frequency stimulation (HFS) (1 s 100 Hz) at an intensity that evoked 35%–40% of the maximal response. This protocol elicited only a moderate LTP in wild-type mice, whereas it produced a very robust response in EGFP-AZIP mice. The increase in the slopes of fEPSP in the transgenic group compared to that of the wild-type group is evident throughout the recording session (Figure 4A; 1 min, $p < 0.05$; 180 min, $p < 0.035$). Moreover, the enhanced synaptic efficacy is specific to the expression of the transgene (Figure 4B). The reversibility of the enhanced LTP rules out the nonspecific effect of transgene insertion or developmental changes. This result indicates that a submaximal protocol is enough to produce a sustained and enhanced LTP in the EGFP-AZIP group.

The immediate increase in the field potential as well as the enhanced potentiation late after the tetanus suggests that expression of the dominant-negative C/EBP has an effect not only on the later time course but also on early LTP. This enhanced synaptic efficacy was not caused by an increase in the number of presynaptic fibers: the size of the presynaptic fiber volley was similar for both groups of mice (Figures 3C and 3D). The peak amplitudes of depolarization in response to the tetanus were also similar between wild-type and transgenic mice (Figure 4C), suggesting that an equivalent amount of Ca^{2+} was probably entering into the postsynaptic neurons in both groups of mice. Since microarray analyses revealed that the transcript profile is distinct for EGFP-AZIP transgenic mice, it is conceivable that this transcriptional difference has resulted in an altered neuronal readiness of transgenic mice that allows them to immediately respond to stimuli more effectively than control mice.

To dissociate the early and late effects of EGFP-AZIP transgene on long-term synaptic potentiation, we specifically increased the stimulus intensity of the tetanization only in the wild-type group to achieve posttetanic potentiation that is similar to that of the transgenic group. This was achieved using a stimulus intensity that elicits 50% of the maximal response in the wild-type group while maintaining the stimulus intensity that corresponds to 35% of the maximal response in the transgenic group (Figure 4D). LTP appeared similar between these mice for approximately 90 min. However, at the end of recording session, synaptic potentiation was sustained only in the transgenic group (Figure 4D; 4 hr, $p < 0.02$). These data indicate that when the differences in the induction of LTP between transgenic and control mice were equalized by evoking a similar level of early LTP, there still exists an enhanced late-phase long-term synaptic potentiation (L-LTP) in transgenic mice.

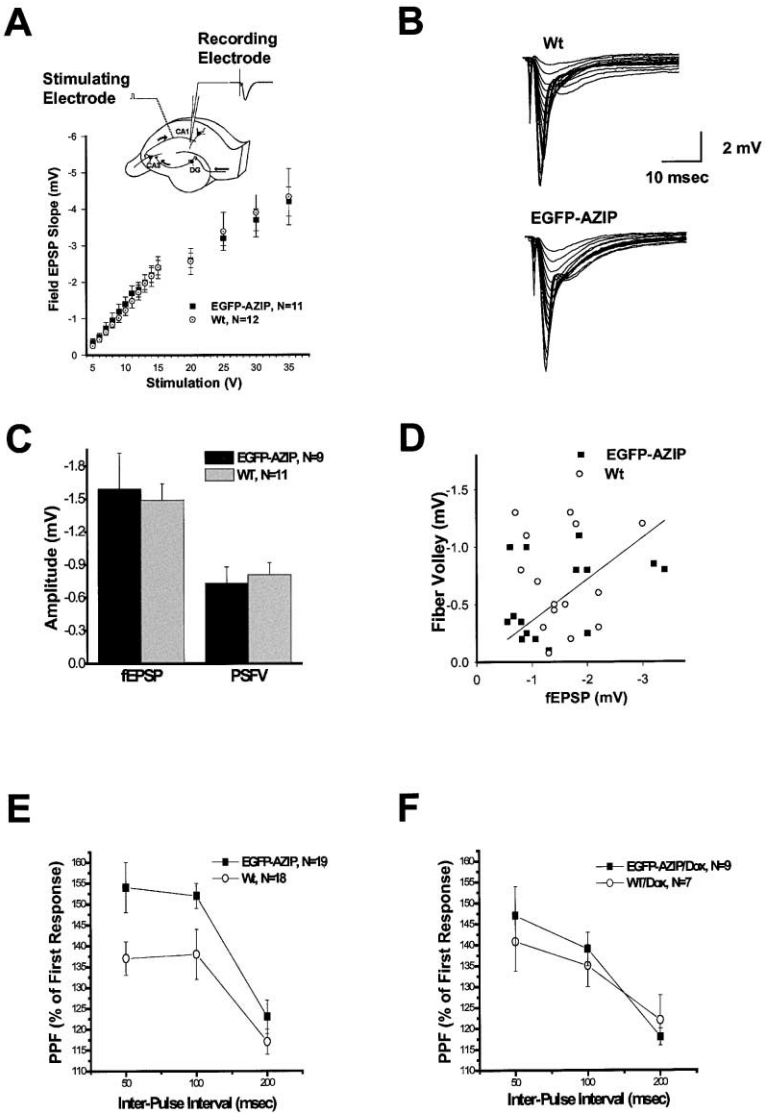

Figure 3. Basal Synaptic Transmission and Short-Term Synaptic Plasticity

(A) Scheme of electrophysiological recordings from the hippocampal slices and the input-output curves over a range of stimulus voltages showing similar response for both groups of mice.

(B) Representative traces during the recordings were similar for both groups of animals.

(C) Amplitudes of field EPSP (fEPSP) and presynaptic fiber volleys (PSFV) were similar for both groups of mice.

(D) A regression fit of fEPSP versus presynaptic fiber volley at a stimulating intensity that produces 35% of the maximum response shows a similar pattern of response between the two groups.

(E) Paired-pulse facilitation (PPF) is increased in EGFP-AZIP transgenic mice compared to wild-type mice at short interstimulus intervals (50 and 100 ms) but not at longer ones.

(F) The trend of increased PPF is reversed when the transgene is turned off with doxycycline (40 mg/kg) for 2 weeks before the experiment.

One Train of High-Frequency Stimulation Evokes Transcription- and Translation-Dependent L-LTP in EGFP-AZIP Transgenic Mice

To what extent is the enhanced LTP in EGFP-AZIP mice, following single tetanic stimulation, due to facilitated transcription? We addressed this question by examining the dependence of LTP on macromolecular synthesis. We found that the LTP produced in wild-type mice by one 100 Hz train was not affected by anisomycin (30 μm), an inhibitor of translation (Figure 5B), or actinomycin-D (40 μm), an inhibitor of transcription (Figure 5D). By contrast, the LTP thus produced in EGFP-AZIP mice was reduced in the presence of either anisomycin (Figure 5A, $p < 0.005$) or actinomycin-D (Figure 5C, $p < 0.001$). This finding strongly supports the idea that the mutant mice have a lower threshold for transcriptional activation, presumably because they contain a greater ratio of free transcriptional activators to repressors, and thus a relatively weak stimulus elicits the nuclear-dependent processes only in EGFP-AZIP mice.

To further explore the molecular nature of the LTP in these mice, we investigated the involvement of PKA activity. Perfusion of hippocampal slices with PKA inhibitor (KT5720, 1 μM) blocked LTP in both wild-type (Figure 5F, $p < 0.003$) and EGFP-AZIP transgenic mice following 1 train HFS (Figure 5E, $p < 0.002$). Thus, a single 100 Hz tetanus produces in wild-type mice a PKA-dependent form of LTP and in EGFP-AZIP mice a transcription- and translation-dependent form of LTP that also depends on PKA at all time points.

It is interesting to note that 1 train HFS produces a PKA-dependent form of LTP in wild-type mice lasting for 3 hr without recruiting the transcription- and translation-dependent processes. This form of LTP has properties that are similar to the intermediate form of LTP (I-LTP; Winder et al., 1998). The robust LTP seen with these wild-type mice in this experiment is most likely due to their increased vigor as a result of a hybrid genetic background. It is in stark contrast to inbred strains of mice that are known to have a less robust induction and

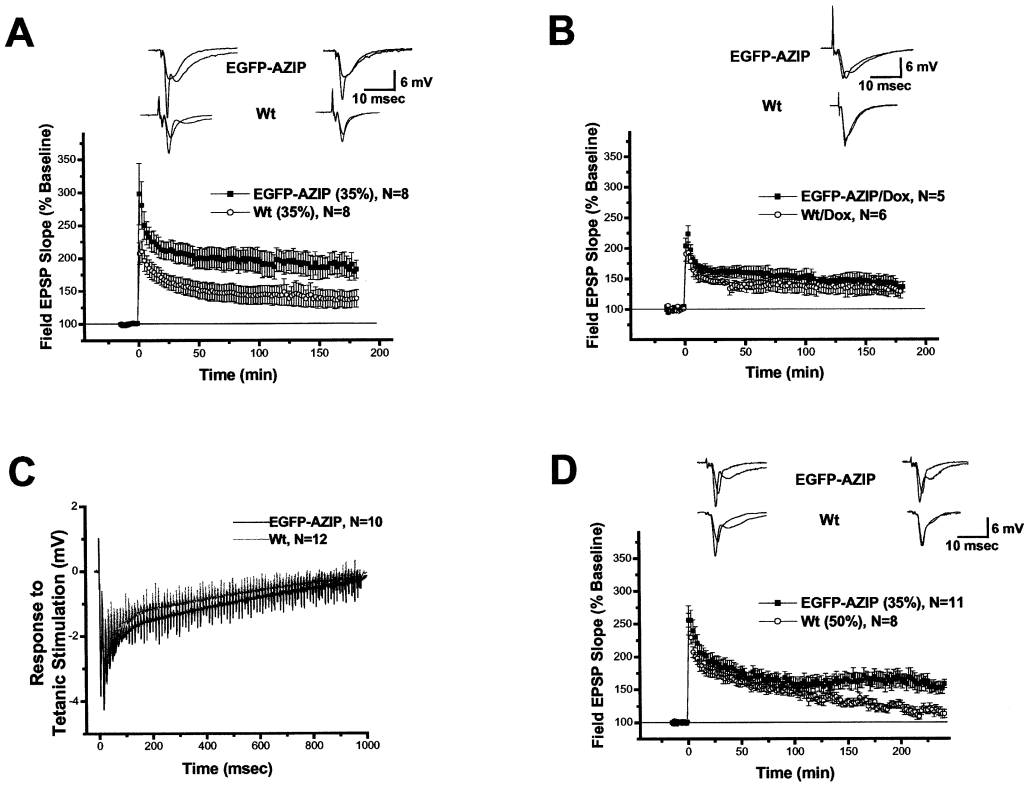

Figure 4. One Train of High-Frequency Stimulation Produces Enhanced LTP in EGFP-AZIP Transgenic Mice

(A) Using a stimulus that evokes 35% of maximal response, EGFP-AZIP mice display enhanced LTP that lasts at least 3 hr. Insets display representative traces immediately after the tetanus and at the end of the experiment.

(B) LTP is similarly elicited as in (A), but mice were fed with doxycycline (40 mg/kg) for 2 weeks before experiment. The enhancement in EGFP-AZIP mice disappears to the level of the control when the transgene is switched off.

(C) Averaged traces of fEPSP during the 1 s 100 Hz stimulation show a slightly greater response for the transgenic than the wild-type samples. Estimation of depolarization by averaged integrated area under fEPSP and averaged minimal peak were both not significantly different ($p = 0.387$, $p = 0.333$).

(D) Using a stimulus that evokes 50% of maximal response, wild-type mice achieve the same level of posttetanic potentiation as in EGFP-AZIP mice using a stimulus that evokes 35% of maximal response. There is a significantly enhanced late-phase long-term potentiation in transgenic mice that is apparent 90 min after tetanus.

expression of LTP at hippocampal synapses (Nguyen et al., 2000a, 2000b).

Forskolin-Induced L-LTP that Is Dependent on Macromolecular Synthesis Is Enhanced in EGFP-AZIP Transgenic Mice

To better explore the role of PKA in the enhanced late-phase LTP in EGFP-AZIP transgenic mice, we used forskolin, a pharmacological activator of adenylate cyclase, to elicit cAMP-dependent LTP in the Schaffer collateral pathways of these mice (Huang and Kandel, 1995; Pockett et al., 1993). This protocol has the advantage of specifically activating cAMP-signaling cascade while bypassing the electrical stimulation and the subsequently increased PTP in EGFP-AZIP mice. We observed that the pharmacologically induced LTP has a slower kinetic compared to that of electrically induced LTP, consistent with reports in the literature. The forskolin-induced LTP was enhanced in EGFP-AZIP mice relative to wild-type controls (Figure 6A, $p < 0.02$), and it is specific to the expression of the transgene (Figure 6B).

Perfusion of hippocampal slices with anisomycin leads to decaying of synaptic potentiation at approximately 30 min after forskolin stimulation that nearly reaches baseline levels 3 hr later (Figure 6C, $p < 0.0005$; Figure 6D, $p < 0.05$). In the presence of actinomycin-D, synaptic potentiation started to decay 45–50 min after forskolin stimulation and approximated baseline levels at the end of the recording period (Figure 6E, $p < 0.00005$; Figure 6F, $p < 0.04$). Since these inhibitors blocked forskolin-induced L-LTP with a similar efficacy in both groups of mice (Figures 6D and 6F, insets), it can be inferred that the enhancement in EGFP-AZIP mice is due to a differentially enhanced cAMP-dependent transcription and translation compared to wild-type mice.

Saturating High-Frequency Stimulation Does Not Produce Further Enhancement in EGFP-AZIP Transgenic Mice

We also compared the response of hippocampal slices from wild-type and transgenic mice to repeated trains of high-frequency stimulation that is known to elicit nuclear-dependent processes even in wild-type mice. We found that short-term potentiation (STP) induced by the

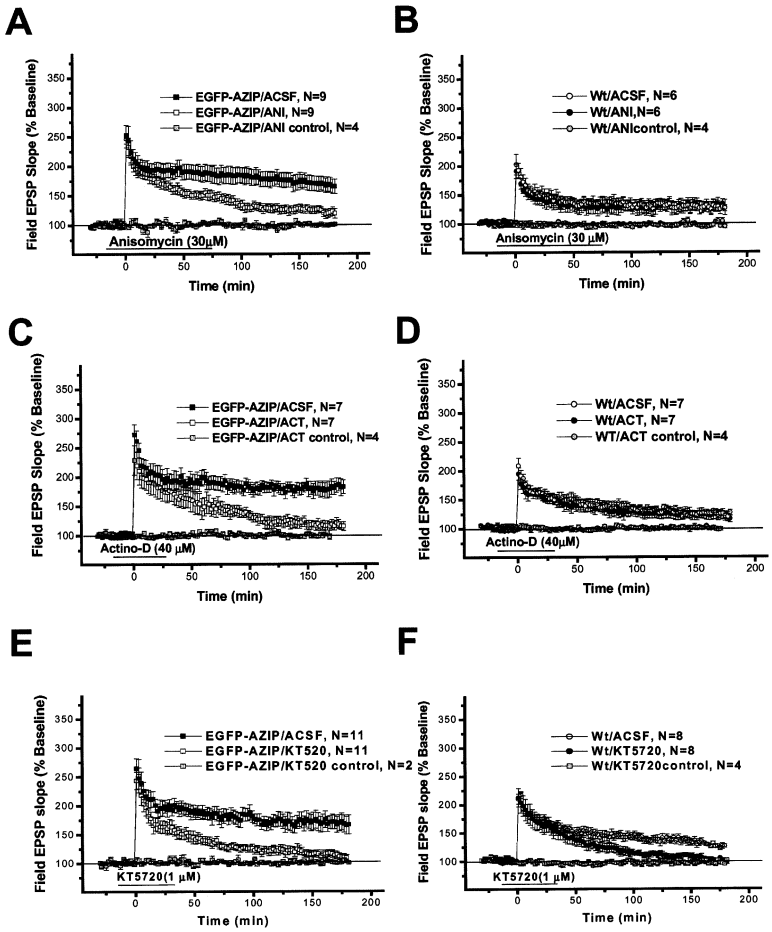

Figure 5. The Enhanced LTP in EGFP-AZIP Transgenic Mice Following 1 Train HFS Is Sensitive to Anisomycin and Actinomycin-D and Is Dependent on PKA Activity

(A) The enhanced LTP in the transgenic animals produced by the 1 train high-frequency protocol is reduced in the presence of protein synthesis inhibitor anisomycin.

(B) LTP produced by the 1 train high-frequency protocol in wild-type animals is not affected by anisomycin.

(C) The enhanced LTP in transgenic mice is reduced by the transcription inhibitor, actinomycin-D, at later time points (>50 min).

(D) LTP produced in wild-type mice is not affected by actinomycin-D.

(E) The enhanced LTP in transgenic animals is reduced in the presence of PKA inhibitor KT5720.

(F) Application of KT5720 also reduces LTP in wild-type animals.

first three trains was enhanced in the transgenic group (Figure 7A, inset), consistent with the enhanced STP observed with one train. However, the overall LTP produced in mutant mice by four trains of 100 Hz was no larger than that produced by one train, and it was similar to the 4 train LTP elicited in wild-type mice (Figure 7A). These results suggest that in wild-type mice 4 tetanus trains are necessary to elicit robust L-LTP, whereas in EGFP-AZIP mice 1 train is saturating, and 4 tetanus trains do not produce further enhancement.

To determine whether this enhancement is limited specifically to single train of 100 Hz stimulation, we examined synaptic modifications in these mice following lower-frequency stimulation. Using 30 s 5 Hz stimulation to elicit LTP, we found that synaptic potentiation was slightly enhanced in EGFP-AZIP mice compared to wild-type mice, but the difference was not statistically significant (Figure 7B). Finally, we examined long-term depression (LTD), another form of activity-dependent synaptic modification elicited by prolonged low-frequency stimulation. As expected, 15 min of 1 Hz stimulation produced LTD in the CA1 areas of wild-type mice. By contrast, the same stimulus elicited only a transient synaptic depression in EGFP-AZIP mice that gradually converted into synaptic potentiation (Figure 7C, $p < 0.0003$). This enhanced synaptic response was again specific to the expression of the transgene (Figure 7D). Altogether, our results indicate that EGFP-AZIP enhances the transcription-dependent late-phase LTP as well as short-term synaptic efficacy while occluding LTD, thereby affecting the transcript profile and the "molecular readiness" of hippocampal neurons in transgenic mice.

EGFP-AZIP Transgenic and Wild-Type Mice Have the Same Initial Performance and Subsequent Spatial Memory Following 4 Trials per Day Training in Morris Water Maze

To examine whether the enhanced synaptic efficacy in the hippocampal neurons of EGFP-AZIP mice is manifested at the behavioral level, we tested the hippocampal-based spatial memory formation of these mice using the Morris water maze (Morris et al., 1982; see Experimental Procedures). Because this task relies heavily on the visual acuity and motor ability of mice—performances that do not depend on hippocampal functions—we first measured the time it takes for these mice to swim onto a platform that is indicated by a flagpole. We found similarly reduced escape latencies across 2 days of training for all groups of mice, indicating that all animals learned to locate the visible platform equally well (Figure 8A). The expression of EGFP-AZIP in structures other than hippocampus such as striatum, therefore, did not alter visual, motor, or motivational processes.

We then trained these mice for 5 days using the stan-

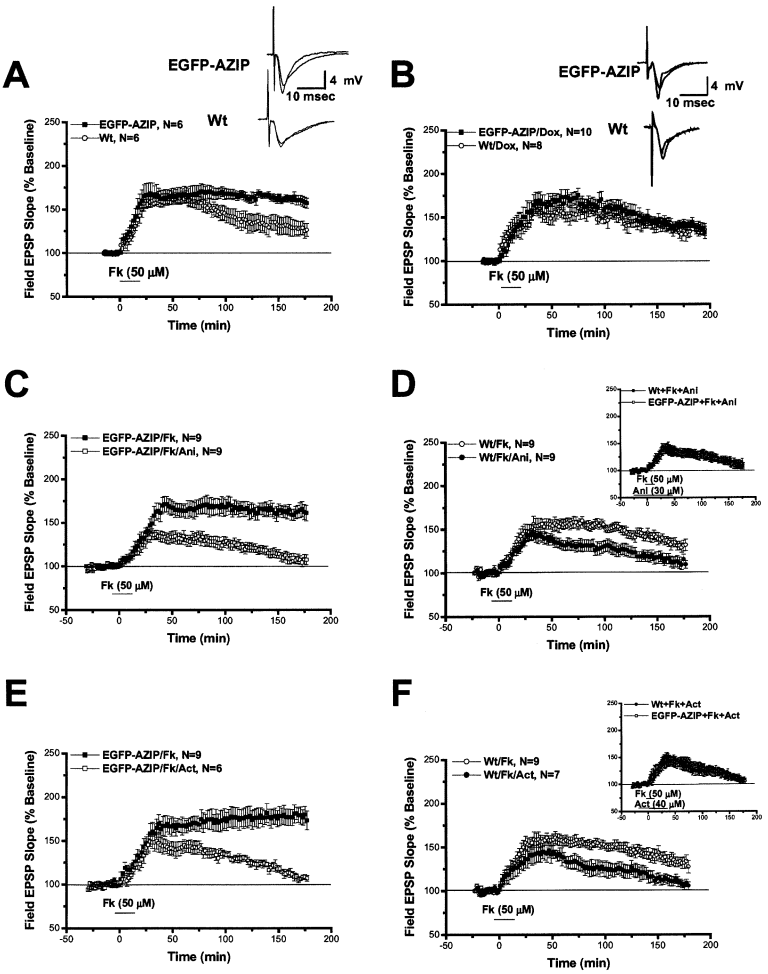

Figure 6. Late-Phase LTP Induced by Forskolin Is Enhanced in EGFP-AZIP Transgenic Mice

(A) EGFP-AZIP transgenic mice show an enhanced L-LTP induced by forskolin compared to the response of wild-type mice. Insets display representative traces recorded at the end of the experiment.

(B) The enhanced LTP in response to forskolin in EGFP-AZIP transgenic mice disappears when mice were fed with doxycycline. Insets display representative traces recorded at the end of the experiment.

(C) Forskolin-induced L-LTP in EGFP-AZIP mice is sensitive to anisomycin inhibition of translation.

(D) Forskolin-induced LTP in wild-type mice is similarly inhibited by anisomycin. Inset shows that anisomycin has a similar efficacy in suppressing both LTP in wild-type mice and the enhanced L-LTP in EGFP-AZIP transgenic mice.

(E) Forskolin-induced L-LTP in EGFP-AZIP mice is sensitive to actinomycin-D, a transcription inhibitor.

(F) Forskolin-induced LTP in wild-type mice is similarly inhibited by actinomycin-D. Inset shows that actinomycin-D suppresses both LTP in wild-type mice and the enhanced L-LTP in EGFP-AZIP to a similar degree.

dard 4 trials per day (4 T/d) protocol to locate a hidden platform using the hippocampal-dependent spatial strategy. Using this protocol, both transgenic and control mice learned to locate the hidden platform with improved escape latencies and decreased path lengths across successive days (Figures 8B and 8C). Statistical analysis revealed no difference between controls and transgenics in swim speed or thigmotaxis, an indicator of anxiety (Wolfer et al., 1998; data not shown). As might be expected from the enhanced PKA-dependent LTP even in wild-type mice of this hybrid strain, these mice learned the maze task more readily than other strains of mice (Montkowski et al., 1997). With only 5 days of training, these mice reached an optimal performance level similar to that acquired by mice of C57 background with 10 days of training under the same experimental conditions (Malleret et al., 2001).

One week following the acquisition, we found that all groups of mice learned and remembered the platform location equally well following the intensive training during a probe trial showing a similar preference for target quadrant 3 where the platform used to be located (Figure 8D). The similar performance exhibited by controls and transgenics fed with doxycycline implied that there were no observable deleterious side effects or toxicity with the administration of doxycycline.

EGFP-AZIP Transgenic Mice Show an Enhanced Hippocampal-Dependent Spatial Memory when Challenged with a More Demanding Training Protocol

Since the enhanced synaptic efficacy in EGFP-AZIP mice was only seen in response to submaximal electrical stimulation, including both 1 train of high-frequency stimulation and low-frequency stimulation, we tested these mice in the spatial memory task using a more cognitively demanding protocol, where mice were trained only once per day (1 T/d) with a new platform location. The observed decrease in escape latencies and path lengths across 5 days of training indicated that all groups of mice learned to locate the new goal location with improved efficiency (Figures 8E and 8F; trials effect, all $p < 0.001$). There were a few differences in their performance under this mild protocol (1 T/d) compared to that of the intense protocol (4 T/d). As expected, there was a greater variability in the daily performance of animals as well as a longer escape latency at the end of the 1 T/d training compared to those of the 4 T/d

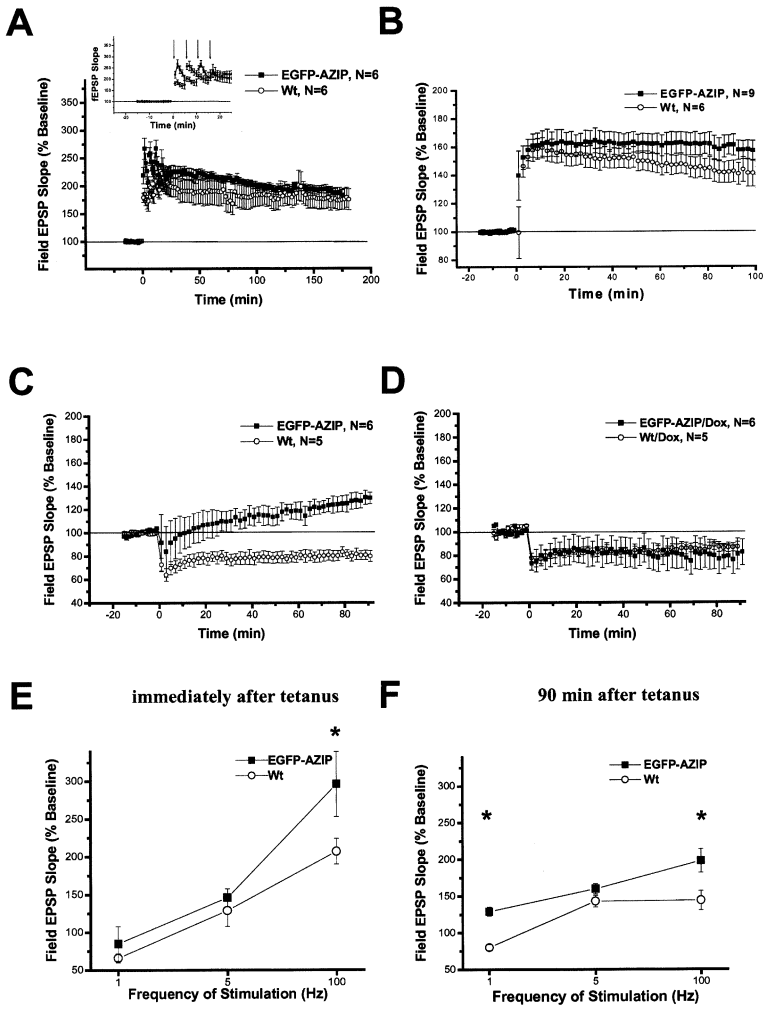

Figure 7. EGFP-AZIP Mice Do Not Show Further Enhancement in Response to 4 Trains of High-Frequency Stimulation but Respond to Low-Frequency Stimulation in Favor of Potentiation

(A) L-LTP induced by 4 trains of 100 Hz stimulation was the same in wild-type and transgenic animals. Inset shows an enhanced potentiation in transgenic mice following 1, 2, and 3 train stimulations.

(B) LTP induced by theta frequency stimulation (30 s, 5 Hz) is slightly enhanced in EGFP-AZIP animals; however, the trend is not significant.

(C) Synaptic depression induced with low-frequency stimulation (15 min, 1 Hz) is transient and is subsequently converted into mild potentiation in EGFP-AZIP transgenic mice.

(D) LTD phenotype disappears when EGFP-AZIP transgene is turned off with 2 week doxycycline (40 mg/kg) diet.

(E) Frequency-response curve immediately after tetanization shows a slightly enhanced potentiation in EGFP-AZIP mice only following high-frequency stimulation compared to that of the control mice.

(F) Frequency-response curve at 90 min following tetanization shows significantly enhanced response in EGFP-AZIP mice with either 100 Hz or 1 Hz stimulation.

intensive training protocol. Using the mild-training protocol, we found that the escape latency for EGFP-AZIP mice was shorter than that of other groups of mice (controls and transgenics on dox), which was, however, not apparent under the intensive training protocol (Figures 8E versus 8B). The ANOVA revealed a significant effect of genotype (latency, $p < 0.02$; path length, $p < 0.02$). Post hoc comparisons showed significant differences between control and transgenic mice and between transgenic mice and transgenic mice on dox, but not between transgenic mice on dox and control mice, indicating that this enhancement in performance is specific to the expression of EGFP-AZIP protein in the adult animals.

To test whether the enhanced learning exhibited by EGFP-AZIP mice also translated into a better memory, we tested these mice in another probe trial 1 week following the end of the transfer stage. Determination of the percentage time mice spent in each of the quadrants showed that only mice expressing EGFP-AZIP protein exhibited a preference for the new target zone in quadrant 1 exceeding the 25% chance level (Figure 8G, quadrant effect for transgenics, $p = 0.002$; quadrant effect for controls and transgenics on dox, both $p > 0.05$). Our results, therefore, suggest that relief of the C/EBP-related transcriptional repressors allows the animals to more readily acquire the hippocampal-dependent spatial memory. As was observed with synaptic plasticity, this enhanced memory is revealed only with a relatively weak training protocol.

Discussion

Interference of Transcription Mediated by C/EBP Repressors and ATF4 Enhances Plasticity and Memory

C/EBP has diverged with evolution so that, unlike *Aplysia* where only a single isoform of C/EBP exists and is recruited for long-term facilitation (Alberini et al., 1994), several isoforms of C/EBP exist in mice and are recruited under different conditions to either facilitate or inhibit hippocampal-dependent memory formation (Sterneck et al., 1998; Taubenfeld et al., 2001a). Studies of mutant mice with single deletions of either *C/EBP*-β or *ATF4* did not reveal a role in LTP, presumably due to compensations by remaining C/EBP isoforms (Y.Y. Huang, personal communication). We therefore examined LTP and long-term memory using a regulated dominant-negative C/EBP (EGFP-AZIP) designed to target

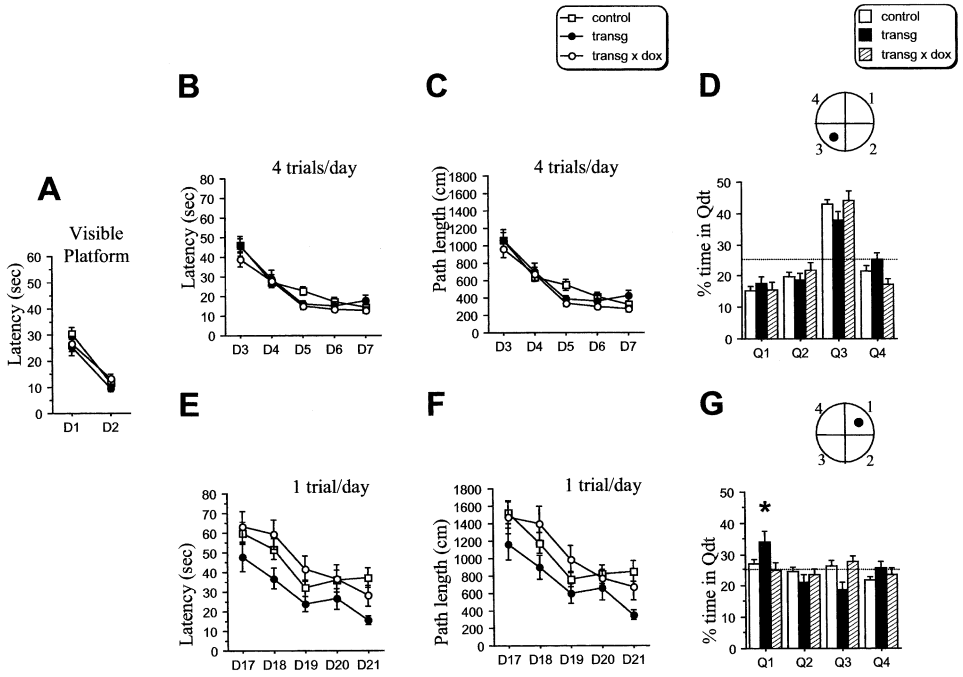

Figure 8. EGFP-AZIP Transgenic Mice Show an Enhanced Long-Term Spatial Memory with a Weak Training Protocol in the Hippocampal-Dependent Morris Water Maze

(A) Latency (s; mean ± SE) across 2 days of visible cued platform task was similar for all groups of mice (see Experimental Procedures), indicating that the expression of the transgene does not affect hippocampus-independent motor skills and emotional states of mice.

(B and C) Latency (s; mean ± SE) and path length (cm; mean ± SE) across days 3–7 of acquisition shows that EGFP-AZIP transgenic mice acquired the spatial hidden platform task similarly to the control groups, using the 4 trials per day training protocol.

(D) The time (%; mean ± SE) mice spent in the different quadrants during probe trial 1 on day 14 (1 week after acquisition) shows that all the animals remembered the hidden platform location by spending at least 40% of the test time in target quadrant 3. The schemas (top) represent the pool with the platform location (black dot). The dashed horizontal line indicates the chance level at 25%.

(E and F) Latency (s; mean ± SE) and path length (cm; mean ± SE) across days 17–21 during the transfer phase shows that transgenic mice were better than the wild-type at locating the new hidden platform position, using the 1 trial per day training protocol.

(G) During probe trial 2 on day 28, 1 week after the 1 trial per day transfer phase, only EGFP-AZIP transgenic mice showed a significant preference for the new target quadrant 1.

the whole family of C/EBP transcription factors. We find that EGFP-AZIP affects repressors within the C/EBP superfamily of transcription factors more than activators, resulting in reduced expression of ATF4 (CREB-2). This relief of C/EBP-related repressors shifts the nuclear balance in favor of C/EBP-related transcriptional activators and yields a distinct profile of gene expression. Following one 100 Hz tetanus, a protocol that produces in this line of wild-type mice PKA-dependent I-LTP, EGFP-AZIP mice attain more readily a transcriptional-dependent L-LTP. Similarly, a mild training protocol that is insufficient for wild-type mice to form long-term memory of the Morris maze allows for the attainment of long-term memory of the maze by EGFP-AZIP mice.

Possible Candidate Genes Downstream of C/EBP Involved in Synaptic Plasticity

In addition to finding evidence for enhanced long-term plasticity and memory, we also find in transgenic mice enhanced paired-pulse facilitation, enhanced posttetanic potentiation, and a shift in the frequency-dependent responses toward facilitation. These data suggest, as one possibility, that EGFP-AZIP might interact with other unidentified molecules and evoke additional mechanisms besides those affecting transcription (Buck et al., 2001; Wang et al., 2001). Although we cannot exclude this possibility, we think the changes in short-term synaptic efficacy and the frequency-dependent response in transgenic mice are attributable to the altered basal transcription following the lifetime expression of EGFP-AZIP protein that in turn enables these mice to respond more effectively to experimental stimulation. Microarray analyses reveal that *SCCA2*, a serpin that is potentially regulated by C/EBP-β (Sakaguchi et al., 1999), and Tal2, a gene encoding a bHLH transcription factor, are upregulated in transgenic mice. Both genes have been implicated in neuronal plasticity (Hastings et al., 1997; Luthi et al., 1997; Nishibori et al., 1995; Bucher et al., 2000; Mori et al., 1999). Conversely, the downregulation of myosin light chain transcript in EGFP-AZIP mice might affect paired-pulse facilitation, a presynaptic form of short-term synaptic plasticity, since myosin light chain and its kinase are involved in the mobilization of synaptic vesicles (Ryan, 1999). Of particular interest is carbonic anhydrase, which is thought to transform the GABAergic modulation of pyramidal neurons from inhibition to excitation. This GABA-mediated switch in neuronal response has been proposed to amplify excitatory inputs to CA1 neurons (Sun and Alkon, 2001, 2002) and

may contribute to the shift in the frequency-dependent response toward synaptic potentiation in EGFP-AZIP mice. Alternatively, the reduced LTD in EGFP-AZIP mice might reflect an altered localization or function of $GABA_B$ receptors resulting from a decrease in ATF4 expression (Vernon et al., 2001; White et al., 2000), thereby affecting the induction of LTD (Wagner and Alger, 1995). Further work will be required to examine the direct involvement of these candidate genes in affecting short-term synaptic changes and the frequency-dependent response.

Although it is possible that some of the differentially expressed candidate genes picked up in our Affymetrix screen could contribute to the enhanced LTP and memory in transgenic mice, we have no evidence that they are critically responsible for the phenotype. Moreover, we have certainly not identified all the genes that may be affected by EGFP-AZIP, since the arrays contain only 10,000 of the approximately 30,000 genes in the mouse genome. The sensitivity of the microarray method may be too low to detect many important genes expressed at low levels or in only a small number of cells. Indeed, we used the microarray analysis primarily as an assay designed to demonstrate that expression of EGFP-AZIP in transgenic mice alters the nuclear balance in favor of transcriptional activation.

Members of the C/EBP Family of Transcription Factors Act as Nuclear Constraints of Long-Term Synaptic Plasticity and Memory in Mammals

We have several reasons to think that besides the changes in basal transcription, additional mechanisms such as a lowered nuclear threshold for transcription are also at play in EGFP-AZIP mice and contribute to the enhanced LTP and long-term memory. (1) In experiments where we used forskolin to activate more directly the cAMP-dependent signaling downstream of receptor signaling, we observed in EGFP-AZIP mice an enhancement of late-phase LTP that is entirely attributable to facilitated transcriptional events. (2) When the stimulus intensity was adjusted to achieve a similar magnitude of early LTP for both wild-type and transgenic mice, we saw an enhanced late-phase LTP only in transgenic mice. (3) In the presence of inhibitors of macromolecular synthesis, we found that 1 train 100 Hz stimulation elicited transcription and translation only in EGFP-AZIP mice but not in wild-type mice. Together these three results strongly support the idea that there exists in EGFP-AZIP mice a lowered threshold that facilitates transcriptional events.

There are relatively few examples of mutant mice showing an enhancement in hippocampal synaptic plasticity in conjunction with an improved hippocampal-based memory. Attempts to improve memory by manipulations of memory-enabling genes in flies and mice often lead to constitutively activated signaling cascades that results in enhanced in vitro synaptic plasticity with reduced learning. This is most likely due to the undesirable effect of abrogating the dependence of neuronal responses on incoming signals (Tully et al., 1994; Jia et al., 1996; Mayford et al., 1996; Gerlai et al., 1998; Migaud et al., 1998). Mice overexpressing the immature form of the NMDA receptor subunit (NR2B) (Tang et al., 1999) or those expressing an inhibitor of the phosphatase calcineurin (Malleret et al., 2001) remain the two best examples of activity-dependent increases in synaptic responses correlated with improved learning and memory. Our use of forebrain-restricted and temporally regulated expression of a broad dominant-negative inhibitor against ATF4 and C/EBP in transgenic mice provides the first example where selective suppression of transcriptional factors leads to coordinated enhancement in both hippocampal-based synaptic plasticity and memory storage.

Thus, this study has two important implications: first, we extend the concept of memory suppressor genes in mice to the level of nuclear transcription factors, downstream of membrane receptors (Tang et al., 1999) and cytoplasmic phosphatases (Malleret et al., 2001). Relief of repressors within the C/EBP superfamily of transcription factors allows more readily the attainment of long-term hippocampal-dependent synaptic potentiation and memory storage in mice. This is consistent with the suggested role of C/EBP-δ in compromising hippocampal-dependent contextual fear conditioning in mice (Sterneck et al., 1998). At the same time, our results do not refute the finding that C/EBP-β is required for the consolidation of new inhibitory avoidance memory in rats (Taubenfeld et al., 2001a), since we have preferentially interfered with repressive isoforms of C/EBP proteins and examined a different type of hippocampal-dependent spatial memory. Second, we implicate ATF4 (CREB-2) in repressing LTP and long-term memory in mice, suggesting that the role for CREB-2 in neuronal plasticity may be partially conserved from invertebrates to mammals. The enhanced synaptic plasticity and memory in transgenic mice expressing a reduced amount of ATF4 echoes the enhanced synaptic facilitation in *Aplysia* neurons upon relief of ApCREB-2 repression (Bartsch et al., 1995). Thus, our results demonstrate that plasticity and memory enhancement can occur through a transcriptional mechanism that is preserved phylogenetically. In this context, we predict that the effect of EGFP-AZIP on lowering the nuclear threshold for an enhanced L-LTP phenotype will be more pronounced in other strains of mice, since mice used in this study have a more robust activity at baseline.

Long-term spatial memory and synaptic plasticity require gene expression that is mediated by a large number of proteins interacting in a complex and varied way in response to multiple stimuli. The ability of the C/EBP-related proteins to form a wide array of dimers that then bind to a broad range of promoters containing a loose consensus CCAAT box makes them ideal nuclear modulators of gene expression (Osada et al., 1996). Since different isoforms have different DNA binding affinities in vivo and varied abilities to activate or inhibit gene transcription, the overall transcriptional activity mediated by the CCAAT box will depend on which C/EBP proteins are present and activated at particular times in the cells studied. Although we have not identified the proteins responsible for the enhanced complex phenotype in EGFP-AZIP transgenic mice, by combining molecular, physiological, and behavioral experiments, we have been able to demonstrate that perturbation of the C/EBP family of transcription factors so as to favor transcriptional activation allows for an easier attainment of long-term synaptic potentiation and memory storage.

Therefore, these mice may provide tools for future work in furthering insights into the molecular events downstream from C/EBP transcription that contribute to enhanced synaptic plasticity and memory storage in mammals.

Experimental Procedures

Plasmids and Cloning

The tet-responsive C/EBP dominant-negative plasmid pKS4i400EGFP-AZIP was constructed using standard cloning procedures (Sambrook et al., 1989). The 294 bp AZIP fragment was PCR amplified from ptetTKFlagDPD4H-F, a generous gift from Charles Vinson, and cloned into pEGFP-C3 vector (Clontech). The construct was rendered tetracycline responsive by cloning into EcoRV site of plasmid pKS4i400 from D.B.

Generation and Maintenance of Transgenic Mice

Generation of transgenic mice is as described in Hogan et al. (1994). A 7 kb fragment (tetO-EGFP-AZIP-polyA flanked by the insulator sequences) was microinjected into BL6/CBA oocytes. Founder mice were identified by Southern blotting of tail DNA with a 400 bp-tetO probe and subsequently backcrossed to C57BL6 F1TAC mice ($>4\times$) to generate offspring with C57BL6 background. They were then mated with CamKII-tTA mice (line B) (Mayford et al., 1996) and backcrossed to 129SvEv to generate C57/129Sv F1 hybrid mice, following the published guidelines (1997). We designate the double transgenic mice as EGFP-AZIP transgenic group and the littermates carrying either single or no transgene as wild-type/controls. For transgene reversal, mice were fed doxycycline (40 mg/kg) for at least 2 weeks prior to experiments. All animals were group housed according to standard IACUC protocol in the animal facility with 12 hr light/dark cycle. Experiments were performed with experimenters blinded to the genotype of the mice.

In Situ Hybridization, Immunohistochemistry, and Western Blot

In situ hybridization was performed following Current Protocols in Neuroscience, using 15 μm cryosections and [^{33}P]UTP-labeled EGFP riboprobes, made by in vitro transcription (Roche) and purified by spin column (Roche) and Trizol (Life Technologies). For immunohistochemistry, anti-GFP antibody (Quantum, AFP, 1:2000) was used on free-floating 50 μm vibratome-sections, followed with goat anti-rabbit IgG-biotin and avidin-biotin-peroxidase immunostaining using the M.O.M. kit and Vectastatin ABC Elite kit (Vector). For Western blot, protein concentration was determined by BCA microassay (Pierce). Antibodies used were GFP (Chemicon MAB2510, 1:1000), C/EBP-β (Santa Cruz, 1:1000), and ATF4 (generated by us against the full-length protein, 1:2500). Antibody-antigen complexes were detected with appropriate secondary antibody that is conjugated to horseradish peroxidase (BioRad) and visualized by ECL (Pierce).

Coimmunoprecipitation and In Vitro Protein Binding Assay

NIH3T3 cells were cotransfected with plasmids by lipofectamine (Life Technologies). Extracts were homogenized in lysis buffer (10% glycerol, 50 mM HEPES [pH 7.25], 100 mM NaCl, 1 mM DTT, 6 mM Mg acetate, 1 mM EGTA, 1% NP40, 0.5 mg/ml BSA) with protease inhibitors (Roche). Sample were preadsorbed with Protein-G Sepharose (Amersham), then incubated with anti-GFP antibody (Quantum, AFP) $\times$ 1 hr followed by Protein-G Sepharose overnight. The coimmunoprecipitated proteins were identified by Western blot using anti-C/EBP antibodies (Santa Cruz) or anti-ATF4 antibody followed by ECL (Pierce). ^{35}S-methionine-labeled EGFP-AZIP and ATF4 were translated in the TNT rabbit reticulocyte lysate (Promega), followed by immunoprecipitation and Western blot as above.

Stable PC12 Cells, Transfections, and Reporter Gene Assays

PC12-tetOn cells (Clontech) containing reverse tTA (rtTA) were cotransfected with pKS4i400EGFP-AZIP and pPUR plasmids using lipofectamine Pfx4 (Invitrogen). Puromycin selected several stable clones with clone A7 showing the lowest baseline and a highly inducible expression of EGFP-AZIP upon doxycycline treatment (1 μg/ml). For reporter gene assays, cells were seeded into 6-well plates (VWR) with only half of them treated with doxycycline. Pfx2 (Invitrogen) transfected cells with reporter constructs. Luciferase activities were quantitated by chemiluminescence (Promega) in a Turner 20e luminometer.

GeneChip Array Assay

Adult mouse hippocampi were dissected out and immediately frozen in liquid nitrogen. Hippocampi were homogenized for 30 s and total RNA extracted using TRIzol (Invitrogen Life Technologies). RNA amplification was performed according to the GeneChip Expression Analysis Technical Manual (Affymetrix). Fragmented amplified RNA from each mouse was hybridized to a separate U74Av2 GeneChip array. All arrays were screened for quality and those with high background were excluded from the analysis.

Microarray Data Analysis

We used standard ANOVAs and t tests for each gene in the data set to identify genes showing group-dependent expression changes. Statistical significance thresholds assumed independence of each gene and are thus likely to be somewhat conservative. The most stringent criterion used ($p < 0.0001$) would result in an expected approximately 1 false positive experiment-wide. (There are about 10,000 different genes represented on the arrays we used, yielding a required p value of 1/10,000 = 0.0001.)

Clustering and Principal Components Analysis

Average-linkage hierarchical clustering was applied to the expression profiles for each sample (Eisen et al., 1998). Bootstrap resampling of the data was used to establish confidence intervals for the clustering hierarchy at each node (Felsenstein, 1985). A standard principal components analysis was used to explore the primary sources of variance in the data.

Hippocampal Electrophysiology

Hippocampal slices were prepared as described previously (Winder et al., 1998). Bipolar stimulating electrodes were placed in the stratum radiatum to stimulate the Schaffer collateral fibers, and fEPSP were recorded in area CA1 with ACSF-filled electrodes. Test stimuli were applied at stimulus intensity that elicits a fEPSP slope that was 35% or 50% of the maximum at a rate of 1 per min (0.02 Hz). Statistical analysis was performed using t tests and ANOVAs.

Morris Water Maze Task

97 mice were used for this experiment: 52 controls (23 wt = no or single transgene, 29 wt on Dox), 21 TG, and 24 TG on Dox. This task has been extensively described elsewhere (Morris et al., 1982; Malleret et al., 2001). This particular experiment consists of three training phases: visible platform, hidden platform 4 trials per day, and hidden platform 1 trial a day.

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